Inhibition of Glutathione S-Transferases
Studies with quinones and ethacrynic acid

Remming van Glutathion S-Transferases
Studies met chinonen en etacrynezuur
(met een samenvatting in het Nederlands)

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Contents page

voor Jacqueline

Chapter 1

General Introduction

1.1 Glutathione S-transferases: properties, function, expression, and catalytic aspects

In the course of evolution, organisms have developed enzyme-systems that protect against toxic effects of xenobiotics. In general, these enzymes increase the hydrophilic properties of compounds, thus transforming them into products capable of being transported and excreted in the urine or bile. The chemical reactions xenobiotics undergo, can be conveniently divided into two major classes, viz. phase I-reactions in which oxidation, reduction, and hydrolysis reactions often serve to provide a chemical handle on the xenobiotic molecule and phase II-reaction in which conjugation reactions further append hydrophilic groups to the xenobiotics (Armstrong 1987).

The glutathione S-transferases (GST) (EC 2.5.1.18) catalyze the conjugation reaction of the tripeptide glutathione (γ-Glu-Cys-Gly) to numerous electrophilic substrates, the first step in the mercapturic acid pathway (Jakoby 1978). This conjugation reaction has been widely recognized to be crucial for the detoxification of several agents of both endogenous and exogenous origin. Several classes of substrates are known, such as epoxides, quinones, α,β-unsaturated aldehydes and ketones, alkyl- and aryl halides (Mannervik 1985). The glutathione conjugate formed is usually less toxic than the parent compound, although in several cases bioactivation occurs (Van Bladeren 1988). In addition to the enzyme function, it is proposed that the GST serve as binding protein by both covalent and non-covalent interaction (Vos and Van Bladeren 1990). Both the abundance of the enzyme, comprising about 4% of the soluble protein in human liver (Van Ommen *et al.* 1990) and the high concentrations of glutathione (up to 5-10 mM) indicate the importance of glutathione and GST in the maintenance of health (Armstrong 1987). For a historical perspective and as well for more recent reviews

the reader is referred to Jakoby (1978), Mannervik (1985), Mannervik and Danielson (1988), Coles and Ketterer (1990), and Armstrong (1991).

GST have been isolated and characterized from various organs in a variety of species, including mammals and vertebrates (Mannervik 1985). Most of the enzymes are present in the cytosol, although a microsomal form has been characterized (Morgenstern and DePierre 1988). In particular, rat and human cytosolic GST have been extensively studied, and this introduction will further concentrate on these cytosolic GST. The cytosolic enzymes found in mammalian species exist as homodimeric and heterodimeric proteins, with molecular masses of the subunits varying from about 24.5 to 27 kDa. Sofar, 15 rat subunits and 10 human subunits have been identified (Table 1). The existence of multiple isoenzymes of GST probably signifies their biological importance. As a group of enzymes the GST catalyse a broader spectrum of substrates than could be achieved with a single enzyme form. Although individual enzymes tolerate a wide range of substrates, each enzyme displays an unique preference for certain electrophilic groups and exhibits a high degree of stereoselectivity for some compounds. The GST appear to be organized in four distinct gene families designated alpha, mu, pi (Mannervik 1985) and theta (Meyer *et al.* 1991). Sequence homology among subunits within the same gene class is quite high, normally in the range of 60-80%, while inter-gene class homology displays a relatively low degree of sequence identity (about 30%). Heterodimers have only been isolated from subunits of the same gene class, suggesting that there are specific structural requirements for familial subunit-subunit interactions (Ji *et al.* 1992).

The expression of GST is cell- and tissue-specific, in which the liver has the highest activities and number of molecular forms (Tsuchida and Sato 1992). Other organs share some, but not all forms are expressed. For instance the human liver contains only trace amounts of pi-class GST, which on the other hand is found in relatively high amounts in several other organs, in particular in placenta, kidneys and intestine. The notable phenomenon of increased levels of GST expression in certain drug resistant cell lines and tumors will be discussed separately in section 1.3. Developmental changes in GST expression have been noted, e.g. the fetal liver expresses the normally almost absent pi-class GST. The regulation of

Table 1. Classification of cytosolic * homodimeric glutathione S-transferases from rat and human*.

Class	Rat	Human
Alpha	1-1	A1-1
	2-2	A2-2
	8-8	A3-3
	10-10	skin '9.9'
Mu	3-3	M1a-1a
	4-4	M1b-1b
	6-6	M2-2
	9-9	M3-3
	11-11	
	Yb_2-Yb_2	
Pi	7-7	P1-1
Theta	5-5	T1-1
	12-12	
	13-13	
	Yrs-Yrs	

*adapted from Tsuchida and Sato 1992, Mannervik 1992, and Harris *et al.* 1991.

the GST expression is dependent on both hormonal and environmental factors (Mannervik 1985). Besides a large variability in tissue distribution, GST isoenzymes may demonstrate a differential localization within certain tissues or even within cells (Vos and Van Bladeren 1990). Genetic variation of GST expression has been observed, in particular the genetic polymorphism of human GST M1a-1a, which is due to deletion of the GST M1a-1a gene. About 50% of all individuals lack the expression of GST M1a-1a (Van Ommen *et al.* 1990).

Until recently, little direct information on the structure of the active site was available. Indirect

evidence was used to postulate that the active site is composed of both a glutathione binding site (G-site) and adjacent to this site a second substrate binding site (H-site) (Mannervik 1985). The G-site has a very high specificity for glutathione. On the basis of studies with a series of glutathione analogues, it has been postulated that the γ-glutamyl part of glutathione is the main binding determinant of glutathione to the G-site, while the glycyl part of glutathione is the least essential for the binding (Adang *et al.* 1990). However, this could not be confirmed in X-ray diffraction studies (Ji *et al.* 1992).

The analysis of the kinetic mechanism of GST has led to a generally accepted model, in which GST has two distinct subunits each bearing a catalytically independent site (Mannervik 1985). The isolated monomers are completely inactive (Dirr and Reinemer 1991; Aceto *et al.* 1992), although there is no obvious interaction between the sites. The enzyme kinetics of GST display a slight deviation from Michaelis-Menten rate behaviour when the concentration of glutathione is varied (Mannervik 1985). Most studies support the steady-state random mechanism in which substrates bind sequentially (Mannervik 1985). Nevertheless, in several cases the kinetics may be conveniently described as a unireactant system by the use of one substrate at saturating conditions (Mannervik and Danielson 1988).

In early studies, chemical modification was used to elucidate the possible involvement of various amino acid residues in the catalytic activity. From these studies, it has been suggested that cysteine residues (Askelöf *et al.* 1975, Carne *et al.* 1978) and histidine (Awasthi *et al.* 1987) were present either in the active site or in the vicinity of the active site and that these amino acid residues may play an essential role in the catalysis. The use of site-directed mutagenesis unambiguously ruled out the involvement of both cysteine (Chen *et al.* 1992; Widersten *et al.* 1991) and histidine (Zhang *et al.* 1991; Kong *et al.* 1991; Wang *et al.* 1992) in the catalysis. The chemical modification of arginine residues also inactivated GST (Schäffer *et al.* 1988; Asaoka 1989), while replacement of certain individual arginine residues by site-directed mutagenesis diminished the enzymatic activity, indicating that these arginines might play an essential role in the catalysis (Kong *et al.* 1992).

More recently, an essential role of a tyrosine residu in the catalytic mechanism was recognized (Stenberg *et al.*

1991; Liu *et al.* 1992; Kong *et al.* 1992; Kolm *et al.* 1992). A tyrosine residue near the N-terminus of GST is conserved in the sequences of all of the cytosolic enzymes found. It was shown that the sulfhydryl proton of enzyme bound glutathione has a pK_a of 6.6 which is about 2-3 pK units less than in aqueous solution (Graminski *et al.* 1989). Since the pK_a of the glutathione complexes of a GST mutant in which tyrosine was replaced by a phenylalanine residue (E^{Y6F}) is 7.8, it was suggested that the hydrogen bond between this tyrosine residue and the enzyme-bound glutathione helps to lower the pK_a of glutathione in the binary enzyme substrate complex (Liu *et al.* 1992).

Recently, significant progress in the knowledge of GST has been made, since suitable crystals for X-ray diffraction studies have become available (Reinemer *et al.* 1991; Ji *et al.* 1992). The three-dimensional structure of the GST from pig class pi and rat class have very similar folding topology. Both the subunits of the proteins have a smaller α/β domain (domain I, residues 1-82 and 1-74, for GST 3-3 and GST P1-1 respectively) which can be considered to be the glutathione binding site, while the larger all-helical domain II (residues 90-217 and 81-207 for GST 3-3 and GST P1-1, respectively) is predominantly involved in the xenobiotic substrate binding (Ji *et al.* 1992). Moreover, the overall feature of the subunit contacts in both dimers are quite similar. The active site of the mu-class GST is located in a deep (19-Å) cavity which is composed of three mobile structural elements (Ji *et al.* 1992). The three-dimensional structure reveals the hydrogen bond of the hydroxyl group of tyrosine 6 with the sulphur of glutathione, which is the only direct hydrogen bond, confirming its essential role (Ji *et al.* 1992).

1.2 Glutathione S-transferases and drug resistance.

The effectiveness of many clinically useful anticancer drugs is severely limited by drug resistance. Drug resistance can be either intrinsic, in which the resistance is present even during the start of the therapy, or acquired, in which resistance arises during the course of the therapy (Waxman 1990). Multidrug resistance in human cancer occurs when malignant tumors display cross-resistance to a broad spectrum of structurally unrelated drugs (Murren and Hait 1992). The classical and most intensely studied form of multidrug resistance (MDR-I phenotype) is mediated by overexpression of an

approximately 170 kDa membrane glycoprotein, an energy-dependent drug efflux pump, which has been called P-glycoprotein (Tirikainen and Krusius 1991). In many cases, however, the etiology of drug resistance is clearly multicausal, and many biochemical mechanisms are involved (for reviews see: Hayes and Wolf 1990; Borst 1991; Black and Wolf 1991). Increased levels of GST, especially the pi-class, have been identified in some P-glycoprotein positive MDR cell lines, however transfection experiments have not demonstrated a role for GST in these cell lines (Townsend *et al.* 1992; Murren and Hait 1992).

On the other hand, several findings suggest the involvement of GST, besides other mechanisms, in a different phenotype of drug resistance, viz resistance to alkylating agents such as chlorambucil, melphalan, and nitrosoureas (Morrow and Cowan 1990). These findings include GST overexpression of especially the pi-class in tumors (Wang and Tew' 1985; Sato 1989), the direct conjugation of alkylating agents by GST (Dulik *et al.* 1986; Ciacco *et al.* 1991), and the expression of GST in yeast and mammalian cell lines by genetic engineering which confers to these organism resistance to alkylating agents (Lewis *et al.* 1988; Black *et al.* 1990). Especially the alpha-class GST has been correlated to this alkylating resistance phenotype (Ciaccio *et al.* 1991), although also the mu- and pi-classes of GST have been implicated (Black and Wolf 1991).

A role of GST in the resistance to alkylating agents has also been shown by the use of GST inhibitors. These GST inhibitors increased the cytotoxic action of alkylating agents (Tew *et al.* 1988; Hall *et al.* 1989). A promising strategy to overcome this alkylator-resistance phenotype may be based on inhibition of GST.

1.3 Inhibition of glutathione S-transferases.

Any substance that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an enzyme-inhibitor. The inhibition of enzymes may be divided in two types: reversible and irreversible inhibition.

1.3.1 Reversible inhibition of glutathione S-transferases.

Reversible inhibition requires that the enzyme and inhibitor form a reversible noncovalent complex. As stated in section 1.1 the enzyme kinetics of GST and thus the reversible inhibition of GST may be adequately described by a simple unireactant system (with one substrate saturating). In this simple case, there are three main types of reversible inhibition: competitive (inhibitor combines with free enzyme in a manner that prevents substrate binding), non-competitive (inhibitor binds reversibly, randomly on a different site than the substrate), and uncompetitive inhibition (inhibitor binds not to the free enzyme, but to the enzyme-substrate complex). For more complicated systems the reader is referred to Segel (1974) in which e.g. the partial (non)-competitive and mixed type inhibition are discussed.

All the above mentioned types of reversible inhibition of GST have been observed (Clark *et al.* 1967; Tipping *et al.* 1979; Diericks 1983). The reversible inhibition can be expressed by an inhibition constant (K_i) for all of these types of inhibition mechanism (Segel 1974). However, most studies express the reversible inhibition as a I_{50}-value; which is the concentration of inhibitor resulting in 50% inhibition in a defined experiment (unless otherwise stated: 1 mM glutathione and 1 mM CDNB). This value does not need a mechanistic interpretation, and thus needs relatively few measurements. The relation between I_{50} and K_i for 3 types of inhibition is given in Figure 1.

Competitive Inhibition

$$I_{50} = (1 + [S]/K_m) * K_i$$

Uncompetitive Inhibition

$$I_{50} = (1 + K_m/[S]) * K_i$$

Noncompetitive Inhibition

$$I_{50} = K_i$$

Figure. 1 The Relation between I_{50} and K_i (Segel 1974).

An extensive list of inhibitors of GST (with I_{50}-values between <0.1 to 1000 µM) has been published (Mannervik and Danielson 1988), and this list is still growing (Vos and Van Bladeren 1990; Van Bladeren and Van Ommen 1991). Several types of inhibitors are known, like e.g. the glutathione derivatives, the glutathione analogs, the chlorophenoxy alkyl acid herbicides, diuretic drugs, bile acids, plant phenols, dyes, metal compounds, isothiocyanates, hypolipidemic drugs, halogenated anaesthetics, steroid hormone derivatives, anti-inflammatory drugs, chalcones and others. The inhibitory potency ranking of these compounds is difficult, and depends e.g. on the species, class and even the GST isoenzyme used. In general, especially the mu GST are very susceptible to reversible inhibition. The glutathione derivatives and analogues are amoung the most potent inhibitors of many GST.

Recently, an interesting and efficient competitive inhibitor (towards glutathione) of the rat mu-class GST, γ-L-glutamyl-D-α-aminoadipic acid, has been synthesized (Adang *et al.* 1991). This dipeptide meets many requirements for a good in vivo applicable inhibitor of GST. The hydrolyzable peptide bond between cysteine and glycine is deleted, the thiol group is replaced and the critical γ-glutamine is made resistant against γ-glutamyl transpeptidase degradation.

The flavonoids, a class of plant-derived phenolic substances, have a considerable inhibitory effect on rat GST (Merlos *et al.* 1991). Among the most potent flavonoids as reversible inhibitors were quercetin, kaempferol, morin and luteolin (with I_{50} ranging from 0.03-0.07 µM). Since the flavonoids are present in the diet in relatively large amounts, further studies about the in vivo effect of flavonoids are desirable.

Several studies were addressed to the well-known binding of nonsubstrate ligands like bilirubin and hemin to GST (Mannervik 1985). Fatty acids and fatty acid esters, classes of compounds which are relatively non-toxic, caused a marked inhibition of rat and mouse GST (Mitra *et al.* 1991). Especially arachidonic acid was very effective (I_{50} of 17 µM). The fatty acid binding site involved in the inhibition of GST P1-1 was identified, as the residues 141-188 (Nishihira *et al.* 1992). This inhibition of GST by the fatty acids needs further exploration in vivo.

1.3.2 Irreversible inhibition of glutathione S-transferases.

Irreversible inhibition is characterized by a covalent modification of an enzyme which leads to an irreversible loss of activity. Inactivation may be the result of modification of an essential residue involved in catalysis, by sterically impeding the substrate binding, by distorting the protein, or by impairing its mobility (Ferhnst 1984).

Several irreversible inhibitors of GST are known. Askelöf *et al.* (1975) was the first to show that cysteine modification of GST may result in irreversible inhibition. Table 2 summarizes several studies in which the target amino acid was identified directly. In particular the pi-class GST is very susceptible to SH-reagents.

An interesting class of irreversible inhibitors are the active site-directed or affinity labels, which are designed to resemble a substrate of GST to "target" the inhibitor specifically to the active site and to form covalent bonds with GST.

E.g. the affinity labeling of rat GST isoenzymes by S-(4-bromo-2,3-dioxobutyl)glutathione reveals some interesting features (Katusz and Colman 1991; Katusz *et al.* 1992). In the mu-class this compound reacted with tyrosines, while in the rat alpha-class a cysteine was the target amino acid.

Our lab has addressed several studies to the glutathione conjugates of halogenated quinones, since these conjugates are very potent active-site directed inhibitors (Van Ommen *et al.* 1988; Van Ommen *et al.* 1989; Vos *et al.* 1989).

Quinones react as a rule efficiently with sulfhydryl groups, either by arylation or oxidation of thiols to a disulfide. Tetrachloro-1,4-benzoquinone (TCBQ), has been shown to react with and inactivate rat GST very efficiently (Van Ommen *et al.* 1989). The readily formed glutathione conjugate of TCBQ (GSTCBQ), reacted even more efficiently with rat GST (Van Ommen *et al.* 1988). The involvement of the active site was suggested by using S-hexylglutathione, a compound with known affinity to the GST active site. The targeting effect of GSTCBQ seems to be based on the affinity of the glutathione moiety for the active site.

Table 2. Irreversible inhibition[1] of rat of human GST

isoenzyme	target site	reference list
S-(4-bromo-2,3-dioxobutyl)glutathione		
GST 4-4	Tyr (Cys[2])	Katusz 1991
GST 3-3	Tyr (Cys[2])	Katusz 1992a
GST 1-1	Cys	Katusz 1992b
Maleimide derivatives		
GST P1-1	Cys	Desideri 1991; Lo Bello 1990; Tamai 1990
GST 7-7	Cys	Tamai 1990
1-chloro-2,4-dinitrobenzene		
GST P1-1	Cys	Caccuri 1992
GST 7-7	Cys	Adams 1992
H_2O_2		
GST P1-1	Cys	Shen 1991, 1993
GST 7-7	Cys	Shen 1991
Disulfides		
GST P1-1	Cys	Terada 1993
GST 3-3	Cys	Adang 1991

[1]) at least 50% inhibition with concentration inhibitor less than 0.5 mM, at 25°C within 1 hour. [2]) regarded as not essential.

1.4 Aim of the study

Inhibition of GST has been studied for a variety of reasons. From a mechanistic point of view, the kinetic mechanism of the reaction, the architecture of the active site, and the discrimination between isoenzymes of GST have all been studied using more or less selective inhibitors. From section 1.2 it is clear that inhibition of GST may also be a subject of relevance in relation to the drug resistant alkylator-phenotype. Especially for this latter reason, several groups have examined potential GST inhibitors.

Some years ago, we concluded that TCBQ and in particular GSTCBQ might be used as a starting point to develop in vivo applicable irreversible inhibitors. The effectiveness of GSTCBQ was based on the combination of a reactive moiety (TCBQ) with a group with affinity for the enzyme (glutathione). The investigations described in this thesis were designed to further elucidate this concept: Firstly, several classes of compounds were studied looking for the capacity to selectively irreversibly inhibit GST in vitro. Next, on the longer term, we set out to assess whether these types of inhibitors of GST might be applicable in vivo.

The first study was primarily designed to investigate, whether these TCBQ and related quinone-based inhibitors are capable to inhibit *human* GST in vitro. The in vitro GST-inhibitory potential of several naturally occurring catechols and catecholamines was subsequently investigated. These compounds might provide a "pro-drug" concept, since their oxidation generates quinones. The glutathione conjugates formed upon conjugation with glutathione, were included in these studies, since the glutathione moiety might provide a targetting effect.

Ethacrynic acid, an α,β-unsaturated ketone, is another interesting inhibitor, since it is a known potent inhibitor of rat GST isoenzymes (Ahokas *et al.* 1985). Moreover, ethacrynic acid readily reacts with sulfhydryl groups. Potentially, covalent interaction with GST might inactivate the enzyme. Studies were designed to assess the mechanism and extent of inhibition of the individual isoenzymes of both rat and human GST in vitro.

Finally, in the past many studies on the inhibition of GST in cell lines have been hampered by the lack of a suitable tool to assess the total inhibition. We developed a system to assess the total amount of GST inhibition in cell lines. TCBQ, and ethacrynic acid and derivatives were studied using this system.

This thesis consists of two parts, to enable a rapid overview of the studies with the structurally rather unrelated quinones, and ethacrynic acid.

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PART I

Chapter 2

Irreversible inhibition of human glutathione S-transferase isoenzymes by tetrachloro-1,4-benzoquinone and its glutathione conjugate

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Abstract

The quinones tetrachloro-1,4-benzoquinone (TCBQ) and its glutathione conjugate (GS-TCBQ) are potent irreversible inhibitors of most human glutathione S-transferase (GST) isoenzymes. Human P1-1, M1b-1b, and M1a-1a are almost completely inhibited at a molar ratio TCBQ/GST = 2/1. The isoenzyme A1-1 was inhibited up to 75%, and higher concentrations (TCBQ/GST = 6/1) were needed to reach this maximum effect. For these isoenzymes 75-85% of the maximal amount of inhibition was already reached on incubation of equimolar ratios of TCBQ and subunit GST, while approximately 1 nmol (0.82-0.95) 1,4-[U-^{14}C]TCBQ per nmol subunit GST could be covalently bound. These results suggest that these GST isoenzymes posses only one cysteine in or near the active site of GST, which is completely responsible for the inhibition. In agreement, human isoenzyme A2-2 which possesses no cysteine, was not inhibited and no TCBQ was bound to it. The rate of inhibition was studied at 0°: TCBQ, trichloro-1,4-benzoquinone and GSTCBQ all inhibit GST very fast. Especially for A1-1, the inhibition by the glutathione conjugate is significantly faster than inhibition by TCBQ: the glutathione moiety seems to target the quinone to the enzyme. For the other isoenzymes only minor differences are observed between TCBQ and its glutathione conjugate under the conditions used.

Introduction

The glutathione S-transferases (GSTs) are a group of isoenzymes that catalyze the reaction of electrophilic compounds with glutathione [1]. Among the broad substrate spectrum are α,β-unsaturated carbonyl derivates, epoxides, quinones and a range of other alkylating agents [1]. In addition, GSTs also detoxify electrophilic metabolites by serving as targets for alkylation or arylation [2-5]. However, the effect of this reaction on the activity of GSTs has not been studied in detail.

The GSTs of rat, mouse and man are divided in three distinct classes: the α-, μ-, and π-class. They vary widely in tissue distribution. The highest concentrations of GSTs are found in the liver, with the exception of the π-class which is most abundant in the kidney and placenta. The μ-class GSTs are expressed in only 50% of human individuals [6].

Inhibitors of GSTs are of considerable interest: firstly, it has been proposed that inhibition of GSTs could overcome the resistance to some antineoplastic drugs that certain tumor cells display [7, 8]. Secondly, GSTs are involved in the biosynthesis of leukotrienes and prostaglandins [9]. Modulation of the biosynthesis of these compounds by inhibition of GSTs could be of potential therapeutic benefit in the treatment of related disorders [9].

A large number of inhibitors of GSTs are known [6]. Most of these however act in a competitive manner, i.e. their effects are reversible. Recently, tetrachloro-1,4-benzoquinone (TCBQ) has been shown to inhibit rat glutathione S-transferase very strongly in an irreversible fashion [10]. This compound has been shown to react with cysteine residues in the vicinity of the active site [11]. Using rat isoenzymes, several characteristics of the reaction were studied. Most importantly, the glutathione conjugate of TCBQ (GSTCBQ) has been shown to inhibit even more strongly: the glutathione moiety seems to target the conjugate to the active site of the enzyme. The corresponding β-mercaptoethanol conjugate, showed a much slower rate of inhibition [10]. Lastly, in contrast with other reagents [11], the reaction of the quinone as well as the quinone conjugate with the first cysteine of GST, has the major effect on the inhibition [12].

The present study has been designed to determine the inhibition of the *human* isoenzymes by TCBQ, the rate of inhibition by TCBQ, trichloro-1,4-benzoquinone, and GSTCBQ, as well to determine the number of cysteine residues that react with TCBQ.

Materials and Methods

Chemicals and radiochemicals. Pentachloro-[U-^{14}C]phenol was from CEA (Gif sur Yvette, France, 36.9 µCi/µmol). TCBQ was from Merck (Darmstadt, F.R.G.), its glutathione conjugate was synthesized as described elsewhere [10]. Trichloro-1,4-benzoquinone (1,4-TriClBQ) was a generous gift of B. Spenkelink (Agricultural University Wageningen, The Netherlands). 1,4-[U-^{14}C]TCBQ was prepared from pentachloro-[U-^{14}C]phenol by microsomes from dexamethasone-induced male rats, as described previously [13]. The conditions used were: incubation for 1 hr at 37° with 1 mg/mL microsomal protein, 1 mM NADPH, with after 30 min and extra 1 mM addition, 2 µmol pentachloro-[U-^{14}C]phenol (36.9 µCi/µmol) in 1.5 mL of acetone, 0.1 M potassium phosphate buffer (pH 7.4), 3 mM $MgCl_2$ and 2 mM ascorbic acid. The total volume was 100 mL. The reaction was stopped with 1 mL of 6 N HCl and extra ascorbic acid was added to 10 mM. 1,4-[U-^{14}C]TCHQ and pentachloro-[U-^{14}C]phenol were extracted [three times with 140 mL of acetone: ethylacetate (1:2), and H_2O was removed with Na_2SO_4. The solvent was removed by evaporation. 1,4-TCHQ was purified by preparative HPLC (Zorbax ODS, Dupont, 21.2 mm x 25 cm) using a gradient of 50-100% methanol against 0.05% formic acid in 45 min, followed for 45 min at 100% methanol, with a flow of 3 mL/min, and UV detection at 296 nm. The conversion was approximately 37%. 1,4-[U-^{14}C]TCBQ was freshly prepared for each experiment by quantitative oxidation for 5 min at 25° with an excess of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in methanol and purified by HPLC as described above, and collected on ice (k': 1.9, 2.4, and 3.5, respectively, for 1,4-TCHQ, TCBQ and pentachlorophenol).

Purification. GST isoenzymes were purified from liver and placenta using S-hexylglutathione affinity chromatography. Separation of the GST isoenzymes was achieved with chromatofocusing with a mono P-column (Pharmacia, The Netherlands), as previously described [14]. The purity was confirmed by HPLC analysis [15] and isoelectric focusing [14]. Specific activities with CDNB as

second substrate (see below) were: 37.4, 23.8, 89.8, 85.4 and 69.9, respectively, for human A1-1, A2-2, M1a-1a, M1b-1b and P1-1. All enzyme concentrations are expressed as the concentration of the subunit.

Enzyme assays. To determine the amount of inhibition at different molar ratios of TCBQ/GST, incubations of 25 pmol enzyme (M_r: 25,900, 25,900, 26,700, 26,600 and 24,800, respectively, for human A1-1, A2-2, M1a-1a, M1b-1b and P1-1 [16]) with 6.25 to 250 pmol TCBQ were performed for 15 min at 25°, in 0.1 M potassium phosphate buffer (pH 6.5), supplemented with 1 mM EDTA, after which GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25° (pH 6.5), according to Habig *et al*. [17].

In order to detect a time-dependent inhibition of GST by TCBQ, 1,4-TriClBQ and GSTCBQ, 25 pmol of enzyme was incubated with 75 pmol of quinone in a cuvette in 110 µL at 0°. At various time intervals 850 µL, containing glutathione (1 µmol) and potassium phosphate buffer (final concentration: 0.1 M, with 1 mM EDTA, pH 6.5) at 25° was added, whereafter 40 µL CDNB (1 µmol) was added and the inhibition of enzymatic CDNB conjugation was measured at 340 nm at 25°.

All enzyme assays were performed in duplicate, while controls were treated in the same way.

Measurement of covalent binding of TCBQ *to human GST*. The specific activity of freshly prepared 1,4-[U-^{14}C]TCBQ was decreased to 10,000 dpm/nmol. One nanomole of enzyme was incubated in a microconcentration tube (centricon TM 10, Amicon, U.S.A.) with 10 nmol 1,4-[U-^{14}C]TCBQ for 30 min at 25° in 1 mL of 0.1 M potassium phosphate (pH 6.5) with 1 mM EDTA, followed by incubation for 5 min with 2 mM ascorbic acid. Solvent containing unreacted (hydro)quinone was removed by centrifugation for 30 min at 5000 g (at 10°), followed by three washing steps: addition of 0.5 mL of methanol/H_2O (1:1) and centrifugation for 40 min at 5000 g. The dry filter was dissolved overnight in 1 mL of soluene 350 (Packard, U.S.A.) at 37° and the sample was screened for radioactivity in 15 mL of scintillation liquid (Hionic fluor, Packard).

Statistical methods. Nonlinear regression analysis was performed with the statistical package Genstat5. Parallel curve analysis was performed using the equation of the curve: $Y = a_i + b_i e^{-k_i t}$.

Results

Extent of inhibition. The inhibition of human GSTs by TCBQ is shown in Fig. 1. Almost complete inhibition (up to 98%) for the isoenzymes of the μ- and π-classes was observed at a molar ratio TCBQ/GST = 2/1. Inhibition of the representatives of the α-class was not complete: the isoenzyme A1-1 was inhibited up to 75%, and a higher concentration was needed to reach the maximum effect (TCBQ/GST = 6/1). Human A2-2 was not inhibited at all. Interestingly, for all these isoenzymes (with the exception of A2-2), the major part of the inhibition (about 75 to 85%) has been reached at TCBQ/GST = 1/1.

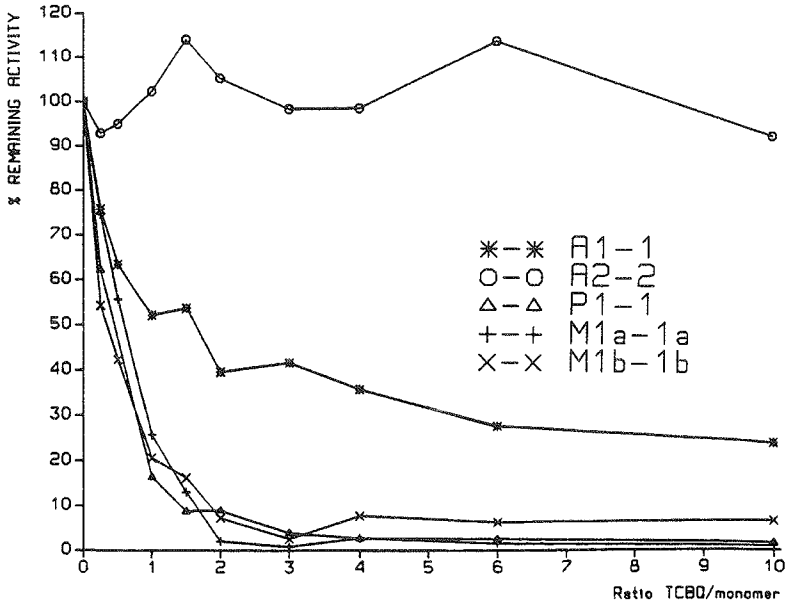

Fig. 1 Remaining Activity at different molar ratios of TCBQ/enzyme, after incubation of 25 nM subunit GST A1-1, A2-2, M1a-1a, M1b-1b, and P1-1, with 6.25-250 nM TCBQ for 15 min at 25°, after which GST activity towards CDNB was measured at 25°. The results are the average of two incubations.

Timecourse of inhibition. The timecourse of inhibition with TCBQ and GSTCBQ for all isoenzymes is shown in Fig. 2. In order to slow down the reaction, the incubations were performed at 0°. Both TCBQ and GSTCBQ inhibited the GST isoenzymes A1-1, P1-1, M1a-1a, and P1-1 very quickly. Some interesting differences were observed: the rate of

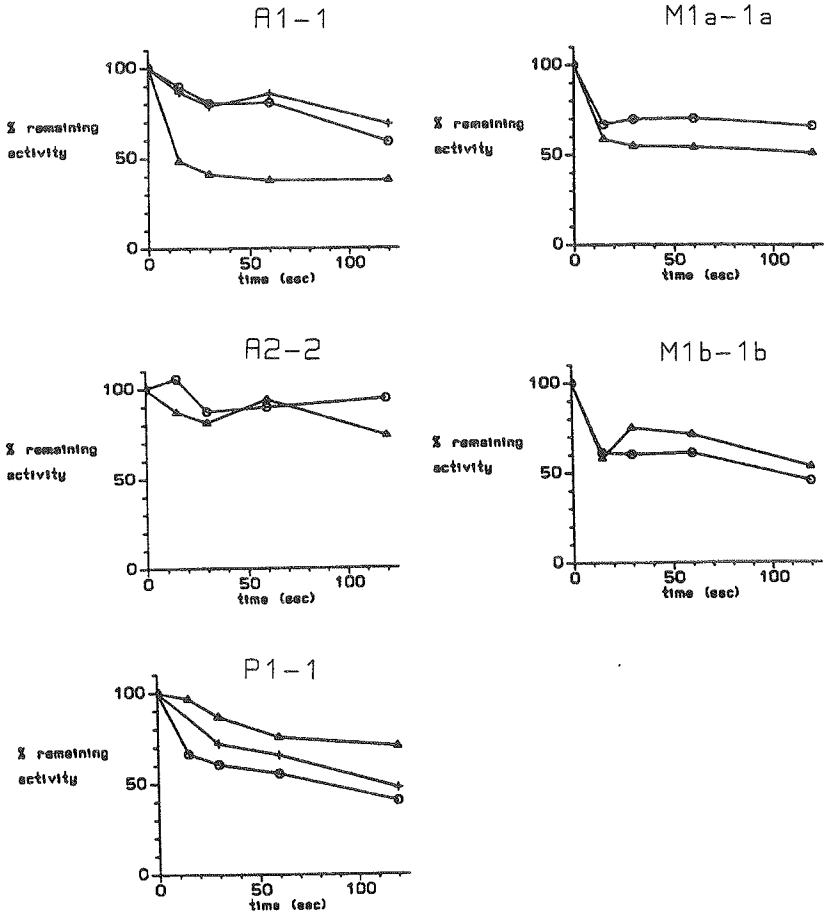

Fig. 2. Time-dependent inhibition of human GST by 1,4-TCBQ (○), 1,4-TriClBQ (+), and its glutathione conjugate (△), 25 pmol enzyme was incubated with 75 pmol quinone in a cuvette in 110 μL at 0°. At various time intervals 850 μL, containing glutathione (1 μmol) and potassium phosphate buffer (final concentration: 0.1 M, with 1 mM EDTA, pH 6.5) at 25° was added, whereafter 40 μL CDNB (1 μmol) was added and the inhibition of enzymatic CDNB conjugation was measured at 340 nm. The results are the average of two incubations.

inhibition by GSTCBQ was significantly higher than the rate of inhibition by TCBQ, only for the human A1-1. Under the conditions used only minor differences between TCBQ and the glutathione conjugate were observed for M1a-1a and M1b-1b, while for human P1-1 the parent quinone without the glutathione moiety inhibited faster. A2-2 was again not inhibited (Fig. 2). The inhibitory effect of 1,4-TriClBQ was also investigated for the isoenzymes A1-1 and P1-1. 1,4-TriClBQ still inhibited GST A1-1 and P1-1 very quickly, but somewhat slower than TCBQ. The targeting effect of the glutathione moiety for the isoenzyme A1-1 was somewhat more obvious, while 1,4-TriClBQ still inhibits GST P1-1 somewhat faster than the glutathione conjugate.

Covalent binding. GST isoenzymes were incubated with radiolabelled TCBQ to study the extent it covalently binds. The maximal binding is presented in Table 1. All GST isoenzymes bind about 1 nmol (0.82-0.95) per nmol GST, with the exception of A2-2, where no binding was observed.

Table 1 - Covalent binding of TCBQ to human glutathione S-transferases

Isoenzyme	Covalent binding (nmol TCBQ/nmol GST subunit)
A1-1	0.83 ± 0.07
A2-2	0.08 ± 0.00
P1-1	0.89 ± 0.27
M1a-1a	0.95 ± 0.12
M1b-1b	0.82 ± 0.12

One nanomole (subunit) enzyme was incubated with 10 nmol 1,4-[U-^{14}C]TCBQ for 30 min at 25°, for experimental details see Materials and Methods. Values are the average ± SD of duplicate incubations.

Discussion

In the present study it has been shown that human GST, A1-1, M1a-1a, M1b-1b and P1-1 are inhibited strongly as a result of covalent modification. Quinones are known to react very rapidly with sulfhydryl groups [10]. As expected, the human A2-2 which possesses no cysteine residues [18], is thus not inhibited by TCBQ, and only 0.08 nmol TCBQ could be bound per nmol subunit A2-2. The low amount of binding observed presumably reflects the relatively slow reaction of quinones with amino groups [19]. The isoenzymes A1-1, M1a-1a, M1b-1b and P1-1 have been shown to be inhibited to about 75-85% of the maximum amount at a molar ratio, TCBQ/GST = 1/1, while approximately one cysteine could be modified by 1,4-[U-^{14}C]TCBQ. This suggests that human GST A1-1, M1a-1a, M1b-1b and P1-1 possess one cysteine residue in or near the vicinity of the active site, which is completely responsible for the inhibition. These phenomena have already been reported for some isoenzymes of rat and human GST: the modification of only one cysteine of rat isoenzyme 4-4 and 7-7 by respectively TCBQ or *N*-ethylmaleimide, and of only one cysteine of human π by *N*-(4-anilino-1-naphthyl) maleimide or CDNB, resulted in complete inhibition [2, 12, 20, 21]. However, the quinones differ in their inhibitory characteristics from these compounds: lower concentrations are needed and in contrast to *N*-ethylmaleimide, they also inhibit the human α- and μ-classes [2]. The total amount of cysteine residues per GST subunit ranges from 1 (A1) to 4 (M1a, M1b, P1) [22-24]. Thus, the binding of only one nmol TCBQ per nmol GST further indicates that complete modification of all cysteine residues does not occur for human M1a-1a and P1-1. In accordance with the result, it has been shown that *N*-(4-anilino-1-naphthyl) maleimide reacts only with one cysteine residue per human P1-1 subunit [20], the other three cysteines are probably located inside the hydrophobic core, and/or form disulphide bonds. Using rat isoenzyme 4-4, all three available cysteine residues could be modified by TCBQ [12], thus the cysteine residues of rat isoenzyme 4-4 (μ-class) are better accessible than those of human M1a-1a.

The glutathione conjugate of TCBQ, which retains its oxidized structure [10], inhibited a mixture of rat GST isoenzymes at a much higher rate than the corresponding β-mercaptoethanol conjugate. The glutathione moiety seems to

target the quinone to the enzyme. In the present study, the rate of inhibition by TCBQ and its glutathione conjugate were mutually compared. In general only minor differences were observed. Thus, intracellullarly formed GSTCBQ does not slow the rate of inhibition. In the case of the isoenzyme A1-1, this glutathione conjugate even accelerated the inhibition.

In conclusion, *in vivo* the cysteine residues of human GST can become a target for quinones, as well as for other alkylating and arylating agents such as ethacrynic acid, acrolein, maleimide derivates and metabolites of bromobenzene [2-5], thereby providing a second detoxification pathway by GST. However, with the exception of A2-2, all these subunited possess one cysteine residue that is important for the conjugating activity. If that residue is modified, the enzyme will be inhibited.

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Chapter 3

In vitro and _in vivo_ reversible and irreversible inhibition of rat glutathione S-transferase isoenzymes by caffeic acid and its 2-S-glutathionyl conjugate

J.H.T.M. Ploemen, B. van Ommen, A.M. de Haan, J.G. Schefferlie and P.J. van Bladeren.

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Abstract

The reversible and irreversible inhibition of glutathione S-transferases (GST) by caffeic acid [3-(3,4-dihydroxyphenyl)-2-propenoic acid] was studied *in vitro* using purified rat isoenzymes, and *in vivo* in male Wistar (WU) rats. The concentrations of caffeic acid that inhibited reversibly 50% of the activity of different GST isoenzymes towards 1-chloro-2,4-dinitrobenzene (CDNB) (I_{50} values) were 58 (GST 4-4), 360 (GST 3-3) and 470 µM (GST 7-7), and higher than 640 µM for GST isoenzymes of the α class (GST 1-1 and 2-2). The major glutathione conjugate of caffeic acid, 2-S-glutathionylcaffeic acid (2-GSCA), was a much more potent reversible inhibitor of GST, with I_{50} values of 7.1 (GST 3-3), 13 (GST 1-1), 26 (GST 4-4), 36 (GST 7-7) and more than 125 µM (GST 2-2). On the other hand, caffeic acid was a much more efficient irreversible inhibitor of GST than 2- GSCA. In this respect, GST 7-7 was by far the most sensitive enzyme. The remaining activity towards CDNB (expressed as percentage of control) after incubating 1.25 µM-GST with 100 µM caffeic acid for 6 hr at 37°C was 34 (GST 2-2), 24 (GST 1-1), 23 (GST 4-4), 10 (GST 3-3) and 5% (GST 7-7). Almost no irreversible inhibition of GST 1-1 and 3-3 occurred during incubation with 2-GSCA. Incubation of caffeic acid with liver microsomes from dexamethasone-induced rats catalyzed the oxidation of caffeic acid about 18 times more effectively as compared with the spontaneous oxidation, as determined by the formation of GSH conjugates from caffeic acid. *In vivo*, the

effect of single oral doses of caffeic acid (50-500 mg/kg body weight) on the cytosolic GST activity towards CDNB was studied 18 hr after dosing in the liver, kidney and intestinal mucosa. A marginal but significant linear relationship was found between the amount of caffeic acid dosed and the irreversible inhibition of GST activity in the liver, with a maximum of about 14% inhibition in the highest dose group. This inhibition coincided with a small decrease in the μ-class GST subunits, which was only significant for GST subunit 4.

Introduction

Caffeic acid, a natural phenolic antioxidant, is widely distributed in the plant kingdom (Herrmann, 1956). Chlorogenic acid, the ester of caffeic acid and quinic acid, is thought to be the most widespread caffeic acid-containing compound (Herrmann, 1956). As an example, coffee beans contain up to 6% of this compound (Herrmann, 1956). Caftaric acid, another major ester of caffeic acid, is found in grapes as the major phenol (up to 100 mg/litre in fresh juice from white grapes; Cheynier *et al.*, 1986). Much attention is given to the auto- and enzymic oxidation of caffeic acid derivatives because these undesirable reactions result in the loss of nutritional and aesthetic values of food products from plants (Cillier and Singleton, 1991). The rate of oxidation is dependent on many factors such as pH and oxygen concentration (Cheynier and Van Hulst, 1988; Cillier and Singleton, 1989). The enzyme polyphenoloxidase can considerably increase the rate of oxidation (Cheynier *et al.*, 1986). As a consequence of both auto- and enzyme-catalyzed reactions, a reactive *o*-quinone metabolite is formed (Cillier and Singleton, 1990). This *o*-quinone can either be reduced or react with nucleophiles by way of a Michaelis-type addition. In the case of glutathione (GSH), both reduction with the concomitant formation of oxidized GSH and formation of a GSH adduct of caffeic acid occur (Cheynier and Van Hulst, 1988; Cillier and Singleton, 1990). The relative importance of these two reactions is dependent on the concentrations of GSH and caffeic acid (Cillier and Singleton, 1990).

Quinones, especially halogenated quinones, are also known for their strong irreversible inhibition of glutathione S-transferases (GST) (Van Ommen *et al.*, 1988). GST exist as a family of dimeric isoenzymes, of which the

most important function is the conjugation of various electrophilic xenobiotics with the co-substrate GSH (Armstrong, 1991; Mannervik, 1985; Mannervik and Danielson, 1988). Most GST isoenzymes possess a nucleophilic amino acid which is rapidly modified by quinones, a reaction which inactivates the enzyme. This amino acid is presumably a cysteine residue (Ploemen *et al.*, 1991), which is located in or near the active site of GST (Van Ommen *et al.*, 1989).

Interestingly, the GSH conjugates of several quinones react more rapidly with GST (thereby inactivating the enzyme), presumably as a result of the higher affinity of the GSH part of the molecule for the active site and/or the reaction with a different amino acid (Van Ommen *et al.*, 1989). In that respect, attention has recently been draw to tyrosine since this residue is the target site for a closely related compound, S-4-bromo-2,3-dioxobutyl) glutathione (Katusz and Colman, 1991; Katusz *et al.*, 1992). In addition to the discussed irreversible inhibitory potential, these GSH conjugates also inhibit GST reversibly because of the affinity of the GSH moiety for the active site.

Inhibition of GST may have serious consequences, because the enzyme plays a key role in many detoxification reactions as well as a number of endogenous processes. On the other hand, inhibitors of GST can also be a useful device in the investigation of the role of GST in specific reactions such as the undesirable inactivation of some drugs (Black and Wolf, 1991). Caffeic acid and related plant phenols are known as reversible inhibitors of rat GST mixtures, with concentrations that inhibit 50% of GST activity (I_{50} values) of 140 µM (Das *et al.*, 1984). However, the potential irreversible inhibition of individual GST isoenzymes by caffeic acid-derived quinones has not been studied. The present study was designated to study both the reversible and irreversible inhibition of GST isoenzymes *in vitro* and *in vivo* by caffeic acid and its major glutathione conjugate.

Materials and Methods

Chemicals. Caffeic acid [3-(3,4-dihydroxyphenyl)-2-propenoic acid], S-hexylglutathione, NADPH, GSH, tragacanth and tyrosinase (3,130 U/mg solid) were from Sigma Chemical Co. (St Louis, MO, USA). HPLC-grade trifluoroacetic acid

was obtained from Baker (Deventer, The Netherlands), HPLC-grade methanol was from Rathburn Chemicals Ltd (Walkerburn, Scotland, UK). HPLC-grade acetonitrile was from Westburg (Leusden, The Netherlands). Epoxy-activated Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden).

Synthesis of 2-S-glutathionylcaffeic acid. 2-S-glutathionylcaffeic acid (2-GSCA) was prepared using tyrosinase in analogy to the synthesis of 2-cysteinylcaffeic acid (Cillier and Singleton, 1990). 0.1 mmol caffeic acid was incubated with 10 mmol GSH in the presence of 2000 U tyrosinase/ml in 0.1 M-sodium citrate (pH 5.3) for 30 min at 15°C (total vol. 100 ml). The reaction was followed by HPLC, using a Zorbax ODS column (4.6 x 250 mm). Elution was performed at a flow rate of 1 ml/min, with 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B), and with a linear gradient of 2-20% B in 13 min followed by a gradient to 100% B in 7 min (k' = 6.9 and 7.5 for the major product and caffeic acid, respectively). The incubate was lyophilized, purified repeatedly by preparative HPLC, using a Zorbax ODS column (21.2 x 250 mm), and eluted isocratically at a flow rate of 4 ml/min with 12% acetonitrile in a 0.05% aqueous acetic acid solution. A product of more than 95% purity, as judged by [^{1}H] nuclear magnetic resonance (400 mhz), was obtained and was identified as 2-GSCA with the following information: for the caffeic acid part, δ 8.3 (d, J = 16.4 Hz), 7.3 (d, J = 8.4 Hz), 7.0 (d, J = 8.8 Hz) and 6.4 (d, J = 16.0 Hz), as published for 2-cysteinylcaffeic acid (Cillier, 1990); and for the GSH part δ 2.1 (m, 2H, Glu β), 2.4 (t, 2H, J = 7.6, Glu γ), 3.1 (dd, 1H, J = 14.6, 9.8, Cys β H_a), 3.3 (dd, 1H, J = 14.6, 4.2, Cys β H_b), 3.70 (1H Glu α), 3.73 (2H Gly α), 4.2 (dd, 1H, J = 9.6, 4.0, Cys α). The UV spectrum of 2-GSCA (λ_{max} 252 and 320 nm; maximum ratio 252:320 = 0.85) was identical to 2-cysteinylcaffeic acid (Cillier and Singleton, 1990). A 5% impurity, eluting as a shoulder of 2-GSCA, was tentatively identified as 5-GSCA because of the spectral resemblance to 5-cysteinylcaffeic acid [λ_{max} 257 and 322 nm; maximum ratio 257:322 = 1; (Cillier and Singleton, 1990)].

GST purification and assay. GST isoenzymes were purified from the liver and kidney (GST 7-7) using affinity chromatography (S-hexylglutathione-Sepharose 6B), and separation of the various isoenzymes was achieved by chromatofocusing or ionexchange chromatography as described

previously (Ploemen *et al.*, 1993; Vos *et al.*, 1988). Purity was confirmed by sodium dodecyl sulphate gel electrophoresis, iso-electric focusing and HPLC analysis as described previously (Bogaards *et al.*, 1989; Vos *et al.*, 1988). All enzyme concentrations were expressed as the concentration of the subunit [M_r: 25,500, 27,500, 26,300, 26,300, and 24,800, respectively, for GST 1, 2, 3,4 and 7 (Hayes *et al.*, 1990)]. Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Inhibition studies. Determinations of reversible inhibition were carried out in triplicate by mixing in cuvettes used for the enzyme assay (in final concentrations) 25 nM-GST with either caffeic acid (5, 10, 20, 40, 80, 160, 320 or 640 µM) or 2-GSCA (2, 5, 25, 50 or 125 µM), after which the enzymatic activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured directly. Time-dependent irreversible inhibition was measured in duplicate at a minimum of six time points by incubating 1.25 µM-GST (1-1, 2-2, 3-3, 4-4 and 7-7) with 0.1 mM-caffeic acid or 2-GSCA in 25 mM-potassium phosphate buffer (pH 7.4, vol. 0.3 ml) at 37°C, after which the activity towards CDNB was determined; in the case of 2-GSCA only GST 1-1 and 3-3 were studied. The same experiments were also performed in the presence of 0.1 mM-GSH. The remaining activity was expressed as percentage of blank incubation (enzyme without inhibitor). Although the dilution without loss of inhibition already shows the irreversibility of the reaction, the irreversible nature of the chemical reaction of enzyme and inhibitor was also established by storage of time-dependent inhibited enzyme (as described above) with GSH (1 mM GSH, to remove the reactive metabolite derived from caffeic acid; storage up to 6 hr), which did not affect the amount of inhibition.

Reaction of caffeic acid and GST. Caffeic acid (0.1 mM) was incubated at 37°C in 25 mM-potassium phosphate buffer (pH 7.4) with a mixture of GST isoenzymes (about 25-40 µg GST 1-1, 2-2, 3-3, 4-4 and 7-7; vol. 1 ml). The reaction was followed by HPLC by injecting 0.1 ml on a Vydac TP5 column (3 x 200 mm; 0.6 ml/min; using solvent A and B; for gradient see below).

Binding of caffeic acid-modified GST to bioaffinity matrix. GST 1-2 (75 µM), 3-4 (76 µM) and 4-4 (19 µM) were treated with 800 U tyrosinase (5 min at 25°C) in the absence (controls) or presence of 1 mM-caffeic acid (vol.

0.4 ml). The incubate was loaded on a S-hexylglutathione bioaffinity matrix, whereafter the GST was eluted as described (Vos *et al.*, 1988). Quantification of GST in the eluate was performed by injecting 0.1 ml on a Vydac TP5 wide-pore reversed-phase column (4.6 x 250 mm), which was eluted at a flow rate of 1 ml/min with solvents A and B (see above) with a linear gradient of 38-47% B in 18 min followed by a gradient to 60% B in 5 min and finally by a gradient to 62% B in 7 min.

Microsomal incubations. 0.1 mM-caffeic acid was incubated at 37°C with 0.25 mM-GSH in the presence of liver microsomes, derived from dexamethasone-induced rats at a concentration of 1 mg protein/ml and prepared as described previously (Van Ommen *et al.*, 1985), and 1 mM-NADPH (extra addition of 1 mM-NADPH after 15 min). The final vol. was 500 µl (buffer: 0.1 M-potassium phosphate, pH 7.4, with 3 mM-$MgCl_2$). After 30 min, 25 µl HCl (6 M) was added to stop the reaction. The incubation was centrifuged for 4 min at 10,000 g, after which 0.1 ml of the supernatant was injected on HPLC [Zorbax ODS column (4.6 x 250 mm), flow rate 0.9 ml/min; 2% acetic acid solution and methanol, with a linear gradient of 35-76% methanol in 10 min]. Under these conditions the conjugates (2-GSCA and 5-GSCA) were separated from caffeic acid (k' = 1.2 and 1.7, for the conjugates and caffeic acid, respectively). The conversion of caffeic acid to glutathione conjugates was measured by peak-area integration at 252 nm. Incubations were performed in triplicate.

Animals and doses. The effect of a single oral dose of caffeic acid on the activity of GST isoenzymes was studied with Wistar rats (WU). 40 male Wistar rats (8-9 wk old) were housed individually 3 days before the start of the experiment in wire-bottomed stainless-steel cages in a room at 20-24°C, 40-70% relative humidity, with a ventilation rate of 10 air changes/hr and a light/dark cycle of 12 hr. They were fed *ad lib.* with a TNO open-formula basal diet, and water was also provided *ad lib.* Allocation into five groups was performed at random. The mean body weight (± SEM) for the groups was 332 ± 15, 328 ± 11, 356 ± 13, 334 ± 11 and 347 ± 10 for groups A, B, C, D and E, respectively (see below). These groups were orally dosed with respectively 0, 50, 100, 250 and 500 mg caffeic acid/mg body weight. Caffeic acid was administered as a 0.5% tragacanth suspension (10 ml/kg body weight). No clinical symptoms during the experiment were observed in any of the

treatment groups. After 18 hr the animals were killed by decapitation under ether anaesthesia. From all rats the liver, kidney, and the mucosa of the small intestine were collected. The small intestine was washed with homogenizing buffer (0.1 M-Tris-HCl, 0.14 M-KCl, pH 7.4), after which the mucosa was collected.

Preparation of cytosols. Tissues were homogenized with a Potter-Elvehjem tissue homogenizer in ice-cold homogenizing buffer (see above). All following procedures were performed at 0-6°C. Cytosol was prepared by centrifugation for 75 min at 105,000 g. The fat layer was removed, and the cytosol was stored at -80°C in 1-ml portions. Isolation, separation and quantification of GST were performed as described previously (Bogaards *et al.*, 1989 and 1990).

Glutathione S-transferase assay. The activity of cytosolic or individual GST isoenzymes was determined with CDNB as second substrate, using the spectrophotometric method of Habig *et al.* (1974).

Statistical analysis. Levels of significance were tested by one-way analysis of variance (ANOVA). The relationship between dose and effect was determined by linear-regression analysis. I_{50} values were determined using the probit function: $p = b_0 + b_1 \ln (C)$ (with C = concentration) (Finney, 1971).

Results

Reversible inhibition. Reversible inhibition, expressed as I_{50} (μM), is shown in Table 1. GST 4-4 is the isoenzyme which was most strongly inhibited by caffeic acid. The reversible inhibition decreased in the order of GST 3-3, GST 7-7, GST 1-1 and GST 2-2, with the I_{50} for the α-class enzymes outside the concentration range used. An exponential curve was fitted to predict individual values: for 140 μM-caffeic acid (I_{50} for a mixture of GST; Das *et al.*, 1984), the calculated remaining activity ranged from 25, 65, 70, 90 and 95%, for GST 4-4, GST 3-3, GST 7-7, GST 1-1 and GST 2-2, respectively. As expected, because of the GSH moiety 2-GSCA was a much more potent reversible inhibitor of rat GST. For all but the α-class GST 2-2, the I_{50} was below 50 μM. GST 2-2 showed 38% inhibition at 125 μM.

Table 1. Concnetrations of caffeic acid and 2-S-glutathionylcaffeic acid (2-GSCA) resulting in 50% inhibition of the catalytic activity of different classes of rat glutathione S-transferase isoenzymes towards CDNB.

Enzyme	Caffeic acid	2-GSCA
α-class		
GST 1-1	> 640	13 (11-15)
GST 2-2	> 640	> 125
μ-class		
GST 3-3	360 (295-455)*	7.1 (6.2-8.0)
GST 4-4	58 (51-66)	26 (23-31)
π-class		
GST 7-7	470 (380-640)	36 (32-42)

* 95% confidence interval given in parentheses.
25 nM-GST was incubated with different concentrations of inhibitor, after which the enzymatic activity was determined towards CDNB (as described in Materials and Methods). Assays were carried out in triplicate with at least five concentrations of caffeic acid or 2-GSCA. The concentration of inhibitor resulting in 50% inhibition of activity towards CDNB (I_{50}) was calculated with the probit function: $P = b_0 + b_1 \ln(C)$.

Reaction of caffeic acid with GSH. Tyrosinase efficiency catalyzed the oxidation of caffeic to an o-quinone intermediate, which react with thiols (Cillier and Singleton, 1990). The addition of GSH occurs preferentially at the 2-position of the benzene ring. Addition at the 5-position also occurs, however, at a lower rate in analogy to the addition of cysteine (Ciller and Singleton, 1990).

Microsomal incubation of 0.1 mM-caffeic acid for 30 min at 37°C with 0.25 mM-GSH increased the rate of oxidation of caffeic acid about 18 times compared with the spontaneous oxidation, as measured by conjugate formation (% conversion by integration of peak area at 252 nm (± SD): 1.3 ± 0.2 and 23.6 ± 1.0 for blank incubations and incubations with microsomes, respectively).

Irreversible inhibition. The irreversible inhibition of the individual GST isoenzymes by incubating with caffeic acid and 2-GSCA is shown in Fig. 1. GST 7-7 was by far the most sensitive isoenzyme, followed by GST 3-3. Slower and, in general, similar rates of inactivation could be observed for the other GST isoenzymes (Fig. 1). This inactivation could be completely prevented by incubation of GST with caffeic acid in the presence of 0.1 mM-GSH. No significant irreversible inhibition of GST 1-1 and 3-3 with 2-GSCA was observed (Fig. 1).

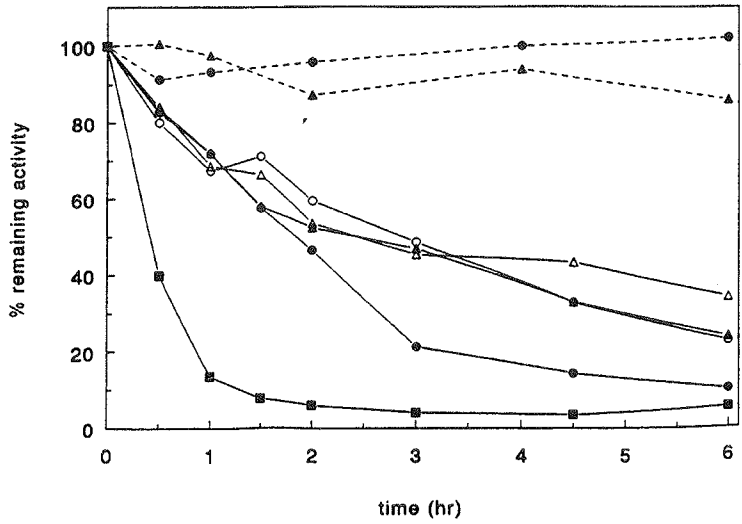

Fig. 1. Time-dependent irreversible inhibition of glutathione *S*-transferase (GST). 1.25 μM-GST was incubated with 0.1 mM-caffeic acid (solid lines) or 2-glutathionylcaffeic acid (dashed lines) at 37°C, after which the activity towards 1-chloro-2,4-dinitrobenzene was determined: GST 1-1 (▲); GST 2-2 (△); GST 3-3 (●); GST 4-4 (○); GST 7-7 (■). The remaining activity was expressed as percentage of blank incubation. The maximal loss of activity in the blank was less than 10%, with the exception of a 16 and 24% loss for GST 3-3 and 7-7, respectively. Each time point is the average of two incubations (for individual points, the coefficient of variation was less than 20%). For experimental details see Materials and Methods.

Reaction of caffeic acid and GST. The covalent interaction of caffeic acid with GST was also studied using HPLC separation of the individual subunits after incubation of a mixture of GST isoenzymes with caffeic acid. GST 7-7 was the most reactive isoenzyme (Fig. 2). The decrease of GST (λ = 214 nm) was accompanied by an increase of enzyme-bound caffeic acid (as detected at λ = 320 nm). After prolonged incubation peak-broadening was observed, suggesting a change in the structural properties of the subunits. In a control incubation without caffeic acid, ony a small loss of the peak area (at 214 nm) of the GST subunit 7 was observed (10-15% loss after 180 min).

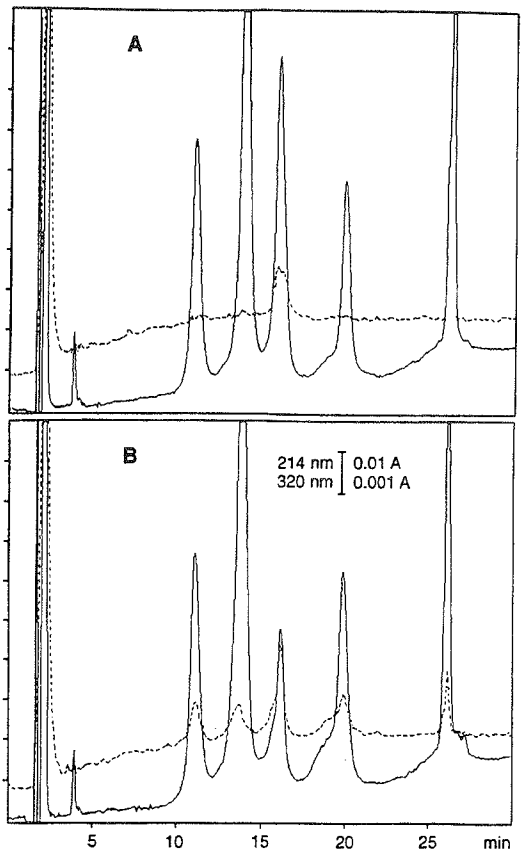

Fig. 2. Typical HPLC chromatograms of GST incubated at 37°C with 0.1 mM-caffeic acid. The GST subunits were eluted in the following order: subunit 3, subunit 4, subunit 7, subunit 2 and subunit 1. Caffeic acid was incubated for 30 min (A) or 120 min (B) with approx. 25–40 μg GST 1-1, 2-2, 3-3, 4-4 and 7-7 in a final volume of 1 ml, after which 0.1 ml was injected on Vydac TP5 (3 mm × 20 cm) [214 nm (solid lines) and 320 nm (dashed lines)]. For experimental details see Materials and Methods.

The affinity of the S-hexylglutathione bioaffinity maxtrix towards caffeic acid-modified GST was studied using GST 1-2, 3-4 and 4-4. In this study, oxidation of caffeic acid was achieved with tyrosinase according to Cillier and Singleton (1990). For this purpose, GST was incubated with caffeic acid in the presence of tyrosinase, which resulted in about 60-80% inhibition of the catalytic activity towards CDNB (n = 3, data not shown), whereafter the incubate was purified on the bioaffinity column. The eluate was analysed using a Vydac column. The normal GST subunit peaks almost completely disappeared from the chromatograms, while virtually no new peaks were present (data not shown).

GST activity and subunit composition of the liver, kidney and small intestinal mucosa. The effect of a single dose of caffeic acid on total GST activity measured with CDNB as substrate is presented in Fig. 3. With CDNB as second substrate, a small but significant linear relationship between the dose of caffeic acid and the level of inhibition of GST was observed in the rat liver. Administration of caffeic acid

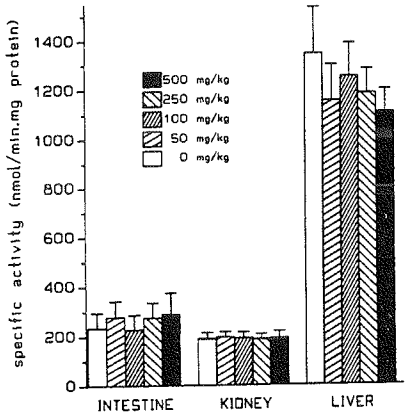

Fig. 3. Cytosolic GST activity in the liver, kidney and small intestinal mucosa of rats treated with a single oral dose of caffeic acid. Groups of eight male Wistar (WU) rats received 0, 50, 100, 250 or 500 mg caffeic acid/kg body weight. After 18 hr, GST activity was determined in the cytosol, using CDNB as the second substrate. Significant differences in means by one-way analysis of variance were observed only in the liver ($P < 0.025$). The relationship between GST activity (nmol/min/mg) and dose of caffeic acid (mg/kg) in the liver were determined by linear-regression analysis [estimated parameters: regression coefficient of caffeic acid -0.351 ($P = 0.012$), with a constant term of 1269 (nmol/min/mg)]. For experimental details see Materials and Methods.

resulted in a decrease in GST activity of approximately 14% in the highest dose group (as estimated by regression analysis). This coincided with a linear decrease in μ-class subunit concentration, which was however only significant for GST subunit 4 (Table 2). In the highest dose group, a decrease in GST subunit 4 of approximately 17% was observed (as estimated by regression analysis). No significant inhibition of the catalytic activity towards CDNB was observed in the kidney or small intestine mucosa.

Table 2. Effect of a single dose of caffeic acid on rat liver glutathione S-transferase (GST) subunit content

Dose of caffeic acid (mg/k body weight)	Subunit concentration (μg/mg cytosolic protein)			
	GST 1-1	GST 2-2	GST 3-3	GST 4-4*#
0 (control)	10.8±2.4	9.0±2.6	12.0±2.6	11.6±2.0
50	9.7±2.8	9.5±1.1	11.3±1.9	11.1±1.7
100	10.4±2.0	9.7±1.8	12.1±1.9	11.7±1.8
250	9.7±1.7	8.5±1.7	11.0±1.6	9.9±1.5
500	10.8±1.3	8.8±1.6	11.2±0.8	9.8±1.0

* Significant by one-way analysis of variance ($P < 0.075$).
\# The relationship between GST subunit 4 (μg/mg) and dose of caffeic acid (mg/kg) was determined by linear-regression analysis [estimated parameters: regression coefficient of caffied acid -0.00392 ($P = 0.013$), *with a constant term of* 11.51 (μg/mg)].
GST subunits were separated and quantified according to the procedures described in Materials and Methods.

Discussion

So far, most studies on the effects of various inhibitors of GST have concentrated on inhibition of the

reversible type. Especially *in vivo*, changes in GST activity by irreversible binding of compounds have been the subject of only few investigations. The aim of our study was to investigate both types of inhibition of GST isoenzymes by caffeic acid and its major GSH conjugate (2-GSCA), both *in vitro* and *in vivo*.

In vitro, 2-GSCA was clearly a more effective reversible inhibitor than the parent compound, in analogy with other GSH conjugates (Mannervik and Danielson, 1988; Ploemen *et al.*, 1990). Strinkingly, GST 2-2 was insensitive to both compounds. With caffeic acid, Das *et al.* (1984) found an I_{50} value of 140 µM using a mixture of rat GST. From our data, it is clear that with this concentration only GST 4-4 is inhibited strongly, indicating the necessity of the use of individual GST isoenzymes.

On the other hand, caffeic acid is a much more potent irreversible inhibitor than 2-GSCA. For its time-dependent inhibition, caffeic acid presumably has to be oxidized to an *o*-quinone structure, which inactivates the GST isoenzymes, in analogy with tetrachloro-1,4-benzoquinone which inhibits GST by covalent modification of the cysteine residues of the enzyme (Ploemen *et al.*, 1991; Van Ommen *et al.*, 1989). In accordance with this hypothesis, an excess of GSH protects against inactivation of GST by caffeic acid, since GSH efficiently traps the unstable *o*-quinone metabolite and either reduces it back to the parent compound or generates 2-GSCA (Cillier and Singleton, 1990). It is tempting to conclude that a slower rate of spontaneous oxidation of 2-GSCA to an *o*-quinone structure may explain the observed lack of irreversible inhibition of GST 1-1 and 3-3 by 2-GSCA, since the structurally related conjugate 2-gluthionylcaftaric acid is more resistant to oxidation than its parent compound (Cheynier *et al.*, 1988). Moreover, the addition of cysteine at the 5-position of the quinone derived from 2-GSCA presumably occurs at a slower rate, in analogy with the structurally related 2-S-cysteinylcaffeic acid (Cillier and Singleton, 1990). On the other hand, the binding of 2-GSCA to the target amino acid of the enzyme may be sterically impeded.

GST 7-7 is by far the most sensitive isoenzyme to inactivation by incubation with caffeic acid, as shown by the enzymatic activity towards CDNB. This coincides with the formation of adducts between GST 7-7 and caffeic acid derivatives, as shown by HPLC. Recently, it has become clear that this *π*-class isoenzyme possesses a highly

reactive cysteine residue, the modification of which leads to inactivation (Ricci *et al.*, 1991; Tamai *et al.*, 1990). In addition, GST 7-7 is known for its unique susceptibility to oxidation, which also inactivates the enzyme (Shen *et al.*, 1991). The rate of inactivation of GST 7-7 strongly correlates with the published rate of autoxidation of caffeic acid (Cillier and Singleton, 1989). This susceptibility to oxidation of GST 7-7 and the strong correlation between its inactivation and caffeic acid oxidation suggest that, besides the observed covalent modification of GST 7-7 by caffeic acid derivatives, oxidation of the reactive cysteine of GST 7-7 by caffeic acid might contribute to inactivation.

Caffeic acid is efficiently absorbed in rats after oral administration (Camarasa *et al.*, 1988). *In vivo*, the reversible inhibition of GST by caffeic acid is dependent on the concentration of the inhibitor in the cell. This reversible inhibition cannot be determined using the catalytic activity towards CDNB because of the dilution factors of about 10,000 times (as a result of tissue homogenization and dilution in the enzymic assay). On the other hand, irreversible inhibition can be measured directly using the activity towards CDNB or by determining subunit content because the caffeic acid-modified enzyme subunits are only very poorly purified on the S-hexylglutathione bioaffinity column. Only marginal irreversible inhibition could be seen in rat liver cytosols (up to approx. 14%) concomitantly with a slight decrease in the μ-class subunits (only significant for GST 4-4). GST subunit 4 is unique for its sensitivity to both reversible (*in vitro*) and irreversible inhibition (*in vivo* and *vitro*). It is likely that because of the relatively high content of oxidizing enzymes (e.g. mixed-function oxidases) in the liver, relatively high concentrations of quinone intermediates can be formed. This was supported by the fact that microsomal incubations catalysed the oxidation of caffeic acid. However, GSH is an effective scavenger of the quinone. Because of the lack of strong irreversible inhibition *in vivo* and the low I_{50} value (especially for 2-GSCA), it is reasonable to conclude that further *in vivo* studies have to concentrate on the reversible inhibition of GST by caffeic acid and its derivatives (e.g. by measuring their actual concentrations in the cell).

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Chapter 4

Inhibition of human glutathione S-transferases by dopamine, α-methyldopa and their 5-S-glutathionyl conjugates

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Abstract

The reversible and irreversible inhibition of human glutathione S-transferases (GST) by dopamine, α-methyldopa and their 5-S-glutathionylconjugates (termed 5-GSDA and 5-GSMDOPA, respectively) was studied using purified isoenzymes. The *reversible* inhibition, using CDNB as substrate and expressed as I_{50}, ranged from 0.18-0.24 (GST M1a-1a), 0.19-0.24 (GST M1b-1b) to 0.5-0.54 mM (GST A1-1) for 5-GSDA and 5-GSMDOPA, respectively. About 20% inhibition was observed for GST A2-2 and P1-1, using 0.5 mM of both 5-GSDA and 5-GSMDOPA. No significant reversible inhibition was observed with dopamine and α-methyldopa.

Tyrosinase was used to generate *ortho*-quinones from dopamine and α-methyldopa which may bind covalently to GST and thereupon irreversibly inhibit GST. In this respect, GST P1-1 was by far the most sensitive enzyme. The inhibition (expressed as % of control) after incubating 0.5 μM GST in the presence of 100 units/ml tyrosinase with 5 μM of the catecholamines for 10 min at 25°C, was 99% and 67% for dopamine and α-methyldopa, respectively. Moderate irreversible inhibition of GST A1-1 by both dopamine and α-methyldopa (33% and 25%, respectively), and of GST M1b-1b by dopamine (45%) was also observed. GST P1-1 is also the only isoenzyme susceptible to irreversible inhibition by 5-GSDA (33% inhibition), while no significant inhibition was observed with 5-GSMDOPA.

A minor part of the inhibition by dopamine (23%), and the complete inhibition by 5-GSDA was restored by reduction

with dithiotreitol. This suggests that GST P1-1 is inhibited by disulfide formation in the case of 5-GSDA, while this oxidative pathway also substantially contributes to the inactivation by dopamine. This was supported by the HPLC-profile of the GST P1-1 subunit which was strongly affected by dopamine, while for 5-GSDA after reduction with dithiotreitol the original elution profile of the subunit returned.

Introduction

The usual metabolism of catecholamines in catecholaminergic neurons of the central and peripheral nervous system proceeds through two major enzymatic pathways, involving monoamine oxidase and catechol-*o*-methyltransferase [1]. In addition, especially in non-nervous tissue, oxidative pathways play a significant role, since the catechol moiety is readily oxidized [1]. It is well known that e.g. the oxidation products of 3,4-dihydroxyphenylalanine (dopa) are involved in melanogenesis [2]. The oxidation of catecholamines is a complex process which proceeds by one-electron oxidation, in which the catecholamines are first converted to *ortho*-semiquinones, which after disproportionation give rise to the corresponding *ortho*-quinones and the parent compounds [1]. In addition, the quinones of the catecholamines are also formed by two-electron oxidation catalyzed e.g. by tyrosinase [2]. The unstable quinone may rapidly be reduced, or form covalent adducts with sulfhydryl groups of either small molecules like the abundant tripeptide glutathione, or proteins [3]. In (eu)melanine biosynthesis, this nucleophilic addition reaction competes with an intramolecular cyclization of the *ortho*-quinones with an amino group in the side chain, which is followed by a complex cascade of intramolecular rearrangements in which redox reactions are involved [2]. The covalent interaction of quinones with accessible and essential protein sulfhydrils may result in (un)desirable enzyme inhibition [4]. The family of the glutathione S-transferases (GST) is very susceptible to this type of inhibition [5,6].

The predominantly cytosolic GST catalyze the conjugation of glutathione with electrophilic compounds of both endogenous and xenobiotic origin such as e.g. epoxides, α,β-unsaturated aldehydes and ketones, alkyl and aryl halides [7,8]. The catalytic rate enhancement is in general

relatively low compared with other enzymes, although this might be compensated by their abundance [9]. Besides the function as catalyst, GST also acts as binding protein [7]. In higher organisms the cytosolic GST have been divided in 4 gene families, termed alpha, mu, pi, and theta [9,10].

A large variety of reversible inhibitors of the GST is known [9]. In general, these inhibitors can be divided in glutathione derivatives, second substrate analogs, and nonsubstrate binding ligands [9]. As stated above, GST is also sensitive to an irreversible inactivation by quinones as result of covalent modification of cysteine residues [6]. This type of inactivation was enhanced for the glutathione conjugates of a range of structurally related quinones [6,11]. Evidence is accumulating that the glutathione moiety binds non-covalently to the active site, thereby "targeting" the quinone moiety to the enzyme to form covalent bonds presumably with a cysteine residue of the protein. Moreover, the rate of inactivation of the mu-class GST by the glutathione conjugates of quinones may also be enhanced by reaction with a different target amino acid, since the structurally related compound S-(4-Bromo-2,3-dioxobutyl)glutathione, has been shown to react rapidly and specifically with a tyrosine of rat GST 4-4 [12].

Recently, there has been a growing interest for in vivo applicable inhibitors of GST, since evidence indicates that the GST are involved in cellular drug resistance [13-15]. The present study has been designed to assess the individual reversible and irreversible inhibitory capacity for human GST by the neurotransmitter dopamine and the structurally related α-methyldopa and their 5-S-glutathionyl conjugates (Fig. 1). α-Methyldopa (also known as Aldomet) was chosen because its relative large side chain, and since this drug is commonly used as an antihypertensive agent.

Materials and Methods.

Chemicals. α-methyldopa (3-(3,4-dihydroxyphenyl)-2-methyl-L-alanine), dopamine hydrochloride (3-hydroxy-tyramine hydrochloride; 99% pure) were purchased from Janssen Chimica (Geel, Belgium). Glutathione and dithiotreitol were from Boehringer (Mannheim, Germany). S-hexylglutathione and tyrosinase (from mushroom; 3,130 units/mg solid) were from Sigma Chemical Co. (St. Louis, USA). HPLC-grade TFA was obtained from J.T. Baker Inc.

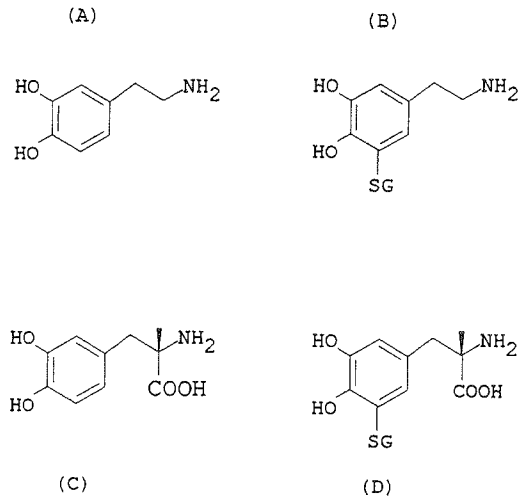

Fig. 1. The molecular structure of dopamine (A), α-methyldopa (B), 5-S-glutathionyldopamine (C) and 5-S-glutathionyl-α-methyldopa (D). GS = glutathione (γ-GLU-CYS-GLY)

(Phillipsburg, NJ, USA), HPLC-grade methanol was from Rathburn Chemicals Limited (Walkerburn, Scotland). Epoxy-activated Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden).

Synthesis of 5-S-glutathionyl conjugates of dopamine and a-methyldopa (5-GSDA and 5-GSMDOPA). The glutathione conjugates were prepared using tyrosinase. 0.2 mmol dopamine or α-methyldopa was incubated with 5 mmol glutathione in the presence of 100 units of tyrosinase/ml in 0.1 M ammonium acetate buffer pH 5.8 for 4 hours at 25°C (total volume of 100 ml). The reaction was followed on RP-HPLC, using Zorbax ODS (4.6 * 250 mm). Elution was performed at a flow of 1 ml/min with 0.1% TFA (solvent A) and 0.1% TFA in methanol (solvent B), 1 min isocratically at 1% B, followed with a linear gradient of 1-75% B in 11 min, finally followed by a gradient to 100% B in 2.5 min; (k'= 2.3, 2.8, 3.2, 3.4) for dopamine, 5-GSDA, α-methyldopa, and 5-GSMDOPA, respectively). The incubates were lyophilized, dissolved in 10 ml of 40 mM sodium

acetate buffer pH 4.7, filtered through a 0.45 µM Millex^{r}-HA filter (Millipore, Molsheim, France), and 2 ml fractions were purified by preparative RP-HPLC, using zorbax ODS (21.2 * 250 mm), eluted isocratically at a flow of 4 ml/min with 6% methanol in 10 mM ammonium acetate buffer pH 4.7 (k'= 1.7, and 0.8, for 5-GSDA, and 5-GSMDOPA, respectively). Fractions containing the glutathione conjugates were lyophilized, dissolved in 10 ml of the sodium acetate buffer and repeatedly purified using the preparative RP-HPLC method (see above). Glutathione conjugates of >99% purity, as judged by HPLC-analysis (see above) and ^{1}H-NMR (400 MHz, D_2O, δ=4.7 ppm) were obtained. The ^{1}H-NMR spectrum of 5-GSDA was assigned as follows: signals for the catecholamine part δ 6.90 (d, 1H, J=1.7 Hz), 6.80 (d, 1H, J=1.7 Hz), 3.20 (t, 2H, J= 7.2 Hz), 2.83 (t, 2H, J=7.2 Hz), and for the glutathione part δ 2.15 (m, 2H, Glu β), 2.49 (t, 2H, J=7.5 Hz, Glu γ), 3.20 (m, 1H, Cys β (H_a)), 3.32 (dd, 1H, J= 14.5 and 5.0 Hz, Cys β (H_b)), 3.83 (s, 2H, Gly α), 4.00 (t, 1H, J= 6.6 Hz, Glu α), 4.42 (dd, 1H, J= 8.3 and 5.0 Hz, Cys α). The ^{1}H-NMR spectrum of 5-GSMDOPA gave the following resonances: for the catecholamine part δ 6.86 (d, 1H, J=2.0 Hz), 6.75 (d, 1H, J=2.0 Hz), 3.22 (d, 1H, J= 14.5 Hz, CH_2 (H_b)), 2.88 (d, 1H, J= 14.5 Hz, CH_2 (H_a)), 1.58 (s, 3H, CH_3), and for the glutathione part δ 2.13 (m, 2H, Glu β), 2.47 (t, 2H, J= 7.7 Hz, Glu γ), 3.18 (dd, 1H, J= 14.3 and 7.9 Hz, Cys β (H_a)), 3.36 (dd, 1H, J= 14.3 and 7.9 Hz, Cys β (H_b)), 3.85 (s, 2H, Gly α), 3.89 (t, 1H, J= 6.5 Hz, Glu α), 4.40 (dd, 1H, J= 7.9 and 4.8 Hz, Cys α). The coupling of 1.7-2.0 Hz between the protons in the aromatic region corresponds to meta positions of these protons. Assignments have been confirmed by a 2D-COSY experiment. The UV-spectrum of both glutathione conjugates were identical, with λ_{max} 256 and 291 nm; ϵ_{291}= 1850 M-1.cm-1.

Stability of 5-S-glutathionyl conjugates of dopamine and α-methyldopa. The stability of 5-GSDA and 5-GSMDOPA, under conditions likely giving autoxidation, was studied by incubation of 100 µM of 5-GSDA and 5-GSMDOPA at 37°C in 50 mM potassium phosphate buffer pH 7.4 (with 10% methanol; final volume 4 ml), by injecting 12 samples of 100 µl of a time-series (0 to 12 h) on RP-HPLC (see above). The stability was compared with the parent compounds incubated in the same way. The peak areas were integrated with Nelson Analytical Model 2600 Chromatography Software at 291 nm and 279 nm, for the glutathione conjugates and the parent

compounds, respectively, and expressed as % of t=0. $T_{\frac{1}{2}}$ (h) values were determined using logarithm probit analysis [16].

The formation of glutathione adducts of 5-GSDA and 5-GSMDOPA was studied by incubation of 0.5 mM of the catechols with or without 3.5 mM glutathione (as trapping agent) in the presence of 100 units/ml tyrosinase in 0.1 M potassium phosphate buffer pH 7.4 for 10 min at 25°C (final volume 0.25 ml). 50 µl samples were injected on RP-HPLC (Zorbax ODS, 250 * 4.6 mm, for elution see above). Blanc incubations without tyrosinase were performed in the same way. The conversion was measured by peak area integration at 291 nm (see above) and expressed as % of the blanc incubations. These experiments were performed in triplicate. No significant conversion was seen in the absence of tyrosinase.

The stability of 5-GSDA and 5-GSMDOPA was also studied in microsomal incubations. 0.5 mM conjugate was incubated at 37°C with 3.5 mM glutathione in the presence of rat liver microsomes at a concentration of 1 mg protein/ml, prepared as described previously [17], and 1 mM NADPH. The final volume was 1 ml (buffer: 0.1 M potassium phosphate pH 7.4 with 3 mM $MgCl_2$). After 30 min, 200 µl of 20% acetic acid was added to stop the reaction. The incubates were filtered using a 0.22 µM filter (Millex^r-GS, Millipore), after which 30 µl of the filtrate was injected on HPLC (Zorbax ODS column, see above). The conversion of the conjugates was measured by peak area integration at 291 nm. Incubations were performed in triplicate.

GST purification and assay. GST isoenzymes were purified from liver and placenta (GST P1-1) using affinity chromatography (S-hexylglutathione-Sepharose 6B), and separation of the various isoenzymes was achieved by chromatofocusing as described previously [18]. Purity was confirmed by SDS gel electrophoresis, isoelectric focussing and HPLC-analysis as described. All enzyme concentrations are expressed as the concentration of the subunit (M_r: 25,900, 25,900, 26,700, 26,600, and 24800, respectively for GST subunit A1, A2, M1a, M1b, P1 [19]). Protein was determined by the method of Lowry [20], using bovine serum albumin as standard.

Inhibition studies. The reversible inhibition was determined by mixing in the cuvettes of the enzymic assay (in final concentrations) of 10 nM GST (in triplicate) with 50, 100, 200, and 500 µM of 5-GSDA and 5-GSMDOPA, and 2.5

mM of the parent compounds, after which the enzymatic activity towards CDNB was measured immediately (see below).

The time-dependent irreversible inhibition was measured by incubating 0.5 µM GST with 5 µM of the catecholamines and their glutathione conjugates in the presence of 100 units/ml tyrosinase for 10 min at 25°C in 0.1 M potassium phosphate buffer pH 7.4 (triplicate; final volume 50 µl). No significant loss of catalytic activity was observed with tyrosinase in the absence of catecholamines (blanc incubations). The reaction was terminated with an excess of glutathione (50 µL of 2 mM glutathione), after which the samples were stored on ice. Blanc incubations were performed in the same way, with the omission of the catecholamines. After storage for 2 hours (to assess the irreversibility by trapping the potentially released catecholamines with an excess of glutathione), the catalytic activity was determined towards CDNB (see below) by transferring 10 µl samples into a 250 µl cuvettes. Remaining activity was expressed as % of blanc incubation.

The inhibition of GST P1-1 by dopamine and 5-GSDA. To study the effect of the strong reducing agent dithiotreitol on the extent of time-dependent inhibition of GST P1-1 by dopamine and 5-GSDA, 0.5 µM GST P1-1 was inactivated with the catecholamines in the presence of tyrosinase as described above (in duplicate, final volume 100 µl). The reaction was terminated with 100 µl of 2 mM glutathione (blanc incubations) or 100 µl of 0.1 M dithiotreitol (both in 0.1 M potassium phosphate pH 8.4), whereafter the samples were incubated for 10 min at 37°C. The catalytic activity was determined towards CDNB (see below). The enzymatic activity was also measured after prolonged incubation with either glutathione or dithiotreitol (18 h at 6°C).

To study the adduct formation of dopamine with GST P1-1, 2 µM of GST P1-1 was incubated with 5 µM dopamine for 10 min at 25°C in 110 µl of 0.1 M potassium phosphate pH 7.4 in the presence or absence of tyrosinase (100 units/ml), followed by incubation for 10 min at 37°C with 11 µl of 0.5 M dithiotreitol. Quantification of native and modified GST P1-1 was performed by injecting 0.1 ml on a Vydac Protein & Peptide C_{18} column (4.6 * 250 mm), eluted at a flow of 1 ml/min, with 0.1% TFA in deionized water (solvent A) and 0.1% TFA in acetonitrile (solvent B), with a linear gradient of 35-55% B in 20 min, followed for 5 min at 55%

B. Peak area was integrated at 214 nm. Incubations were performed in duplicate.

Glutathione S-transferase assay. The activity of individual GST isoenzymes was determined with 1 mM glutathione and 1 mM CDNB as second substrate at 25°C in 0.1 M potassium phosphate buffer pH 6.5, using the spectrophotometric method of Habig [21].

Statistical analysis. Levels of significance were tested by one-way ANOVA ($P < 0.05$), while comparison between groups were made using Dunnett control group comparison test ($P < 0.01$).

Results

The stability of the 5-GSDA and 5-GSMDOPA and their parent compounds were studied at 37°C. In general, the glutathione conjugates were less stable than the parent compounds, while the α-methyldopa moiety was less stable than dopamine. The $t_{\frac{1}{2}}$ (h) values (with between brackets the 95% confidence interval) were 10.5 (9.2...12.5), 5.6 (5.1...6.3), 5.3 (4.8...5.9), and 1.8 h (1.6...2.1), for dopamine, α-methyldopa, 5-GSDA, and 5-GSMDOPA, respectively. Since no trapping (thiol) agent was added to the incubations, the breakdown of the compounds is most likely due to cyclization of the quinones formed upon autoxidation. These result may indicate that either the rate of autoxidation or the rate of intramolecular cyclization of the glutathione conjugates was faster than for the parent catecholamines.

The 5-S-glutathionylconjugates were incubated with tyrosinase and glutathione, to study whether tyrosinase is able to oxidize the 5-S-glutathionylconjugates and whether subsequent nucleophilic addition of thiol may occur. A small but significant ($P < 0.01$) part of 5-GSDA was turned over in the presence of glutathione (Table 1). No conversion of 5-GSDA occurred in the absence of glutathione, suggesting that under the conditions used nucleophilic addition occurred, without significant cyclization. Interestingly, tyrosinase catalyzed conversion of 5-GSMDOPA was not observed. Finally, the tyrosinase incubations (with glutathione) resulted in more than 90% conversion of α-methyldopa and dopamine within 10 min (data not shown).

Using microsomes (which contain mixed function oxidases) to oxidize the 5-S-glutathionylconjugates, the same trend

as for the tyrosinase (+ glutathione) incubations was observed: only relatively poor but significant ($P < 0.01$) conversion of 5-GSDA.

Table 1. Substrate consumption of 5-GSDA and 5-GSMDOPA (expressed as % recovery of blanc incubations) after incubation with tyrosinase and microsomes.

compound	tyrosinase without glutathione	tyrosinase	microsomal incubation
5-GSDA	94 ± 13.4	80 ± 3.9*	73 ± 2.2*
5-GSMDOPA	98 ± 0.6	98 ± 0.8	94 ± 4.9

0.5 mM of 5-GSDA and 5-GSMDOPA was incubated in the presence of 3.5 mM glutathione for 10 min at 25°C with 100 units/ml tyrosinase or for 30 min at 37°C with 1 mg/ml rat liver microsomes. Incubations without glutathione were also performed with tyrosinase. The recovery (expressed as % of blanc incubations without tyrosinase or microsomes, respectively) was measured by peak area integration at 291 nm. Values are the average of 3 incubations (± SD).

* Statistical difference ($P < 0.01$) between control group and experimental group with Dunnett comparison test, after significant ANOVA ($P < 0.05$).

Reversible inhibition. Dopamine and α-methyldopa were insubstantial reversible inhibitors of the human GST. The catalytic activity towards CDNB in the presence of 2.5 mM of both compounds was inhibited less than 15% for GST A1-1, A2-2, M1a-1a, M1b-1b, and P1-1 (result not shown). As expected, the reversible inhibition was enhanced by the glutathione moiety (Table 2). In general, only small differences between 5-GSDA and 5-GSMDOPA existed. The mu-class isoenzymes M1a-1a and M1b-1b were the most strongly inhibited GST isoenzymes by both conjugates. Within the mu-class virtually no difference between M1a-1a and M1b-1b was noticeable. On the other hand, the alpha-class GST A1-1 was

significantly more inhibited than A2-2. GST A2-2 and P1-1 were the least inhibited isoenzymes.

Table 2. I_{50} (mM)* -values towards CDNB of rat glutathione S-transferase incubated with 5-GSDA or 5-GSMDOPA.

enzym	5-GSDA	5-GSMDOPA
alpha-class		
GST A1-1	0.50	0.54
GST A2-2	>0.50 (18%)	>0.50 (22%)
mu-class		
GST M1a-1a	0.24	0.18
GST M1b-1b	0.24	0.19
pi-class		
GST P1-1	>0.50 (15%)	>0.50 (22%)

10 nM enzyme was incubated with different concentrations of inhibitor, after which the enzymatic activity was determined towards CDNB. At least 4 concentrations in triplicate were used. Between brackets the % inhibition with 0.5 mM inhibitor.

* The concentration of inhibitor resulting in 50% inhibition of activity towards CDNB (I_{50}).

Irreversible inhibition. The experimental conditions that were shown to convert the parent catecholamines and 5-GSDA, were used to generate quinones of the catecholamines *in situ*, to study the irreversible inhibition of the individual GST isoenzymes. The concentration of catecholamine was reduced to 5 µM, to prevent reversible inhibition (the concentration of catecholamine will subsequently be reduced in the CDNB-assay to a final concentration of 0.1 µM). GST P1-1 was by far the most sensitive isoenzyme for the quinones derived of dopamine and α-methyldopa, and was the only GST which was significantly inhibited by incubation with 5-GSDA ($P<0.01$) (Fig. 2). Furthermore, a noticeable sensitivity of GST M1b-1b for dopamine was present, while GST A1-1 was sensitive to inhibition by both α-methyldopa and dopamine. GST A2-2 and M1a-1a were not inhibited by any of the compounds used.

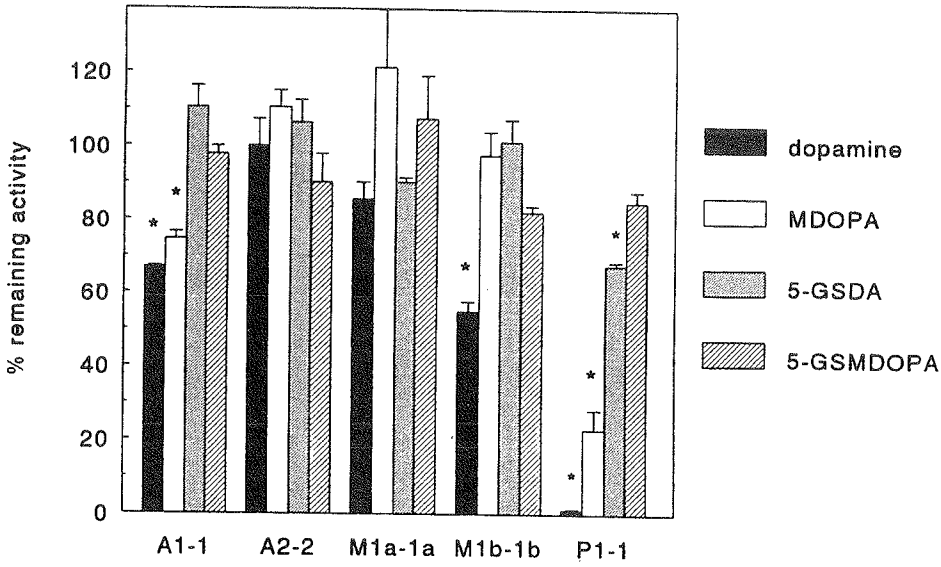

Fig. 2. Irreversible inhibition of human GST by dopamine, α-methyldopa (MDOPA), 5-GSDA, and 5-GSMDOPA in the presence of tyrosinase. 0.5 µM GST was incubated with 5 µM of inhibitor in the presence of 100 units/ml tyrosinase for 10 min at 25°C. The reaction was terminated with 1 mM glutathione. Blanc incubations were performed in the same way, with the omission of the catecholamines. The catalytic activity was determined towards CDNB. Values are the average of three incubations and expressed as % remaining activity of blanc incubation (± SEM). For experimental details see Materials and Methods.

* Statistical difference ($P < 0.01$) between control group and experimental group with Dunnett comparison test, after significant ANOVA ($P < 0.05$).

With the strong reducing agent dithiotreitol, the inhibition of GST P1-1 by 5-GSDA could be completely abolished (Table 3). A considerable extent of the inhibition of GST P1-1 by dopamine could also be restored by dithiotreitol (about 23% recovery of the inhibition). With prolonged (overnight) incubation, no further restoration of activity was achieved (results not shown),

Table 3. The effect of the reducing agent dithiotreitol (DTT) on the % remaining activity of GST P1-1 inactivated by dopamine and 5-GSDA

enzyme	- DTT	+ DTT
dopamine	0.4 ± 1.0	23.4 ± 3.0
5-GSDA	76.4 ± 4.6	102.8 ± 2.2

0.5 µM GST P1-1 was inhibited with 5 µM of the catecholamines in the presence of 100 units/ml tyrosinase for 10 min at 25°C, followed by incubation for 10 min at 37°C with either 1 mM glutathione (- DTT) or 50 mM DTT. The catalytic activity was determined towards CDNB, and expressed as % remaining activity of blanc incubation incubated in the same way without the catecholamines. Values are the average of two incubations (±SD).

indicating that a consistent part of the inhibition is not reversible by reduction.

The covalent interaction of dopamine and 5-GSDA with GST P1-1 was also studied using an HPLC-separation of the individual subunits of GST P1-1, incubated with the catecholamines in the presence of tyrosinase and subsequently treated with dithiotreitol to reduce disulfides (Fig. 3). After incubation with dopamine, peak broadening and a new peak was detected, suggesting a major change in the the structural properties of the subunits. On the other hand, incubation with 5-GSDA did not reveal this phenomenon.

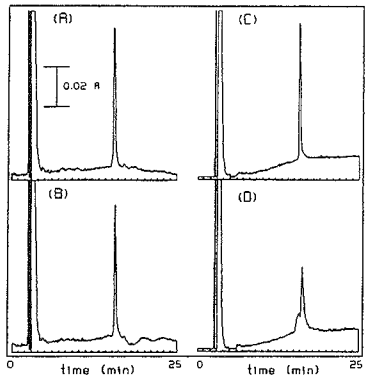

Fig. 3. *Typical HPLC-chromatograms of GST P1-1 subunit incubated with dopamine and 5-GSDA after incubation with tyrosinase and subsequently treated with dithiotreitol to reduce disulfides.* 2 µM of GST P1-1 was incubated with tyrosinase (100 units/ml) and 5 µM of the catecholamines, followed by incubation with 0.05 M dithiotreitol. The GST P1-1 subunit was separated using RP-HPLC. UV-detection at at 214 nm. Incubations were performed in duplicate. For experimental details see Materials and Methods. *(A)* GST P1-1 + 5-GSDA +tyrosinase; *(B)* GST P1-1 + tyrosinase; *(C)* GST + dopamine; *(D)* GST P1-1 + dopamine + tyrosinase.

Discussion

The potential for covalent binding to essential sulfhydryl groups of enzymes with subsequent inactivation of these enzymes by quinones derived from catecholamines has been noted before [2,4]. Previously, it has been reported that quinones derived from catecholamines inhibit housefly GST efficiently [22]. In the present study it has been shown that the human pi-class GST P1-1 is a selective and sensitive target site for quinones derived from dopamine and α-methyldopa, which thereupon inhibit the enzyme. The GST P1-1 is also, albeit to a lesser extent, susceptible to inactivation by the 5-S-glutathionyl conjugate of dopamine. The GST A2-2, which does not possess any cysteine residues [23], was not inactivated by the catecholamines used, again indicating that a cysteine residue is presumably the target site.

It is well established that the GST of pi-class possess a highly reactive cysteine residue, modification of which

results in enzyme inactivation [24,25]. The cysteine residue involved in this inactivation has been located at the 47th position [24,25], close to the G-site of GST [26]. The three-dimensional structure revealed however that this cysteine is not a structural part of the active site [26]. The non-essential role of cysteines in the catalytic activity has also been shown with site-directed mutagenesis [27,28]. The modification of this cysteine presumably inactivates the enzyme either by conformation changes, or by blocking the substrate binding [26,29]. The cysteine residues of the GST pi-class also may undergo a (reversible) oxidative inactivation, by the formation of an intersubunit disulfide between the cysteines at the 47th and 101th-position [30,31]. Since a notable part of the activity of GST P1-1 inactivated with dopamine-quinone was restored after reduction with dithiotreitol, it can be concluded that the quinones derived from dopamine interact with GST P1-1 by both the reversible oxidation reaction (minor pathway) and the nucleophilic addition (major pathway). This was confirmed by the HPLC-profile of the subunit of GST P1-1, which was strongly affected by dopamine even after reduction with dithiotreitol.

On the other hand, the quinones derived from 5-GSDA inactivate GST P1-1 completely via disulfide formation, since the inactivation was fully abolished by reduction with dithiotreitol. This was also confirmed by the HPLC-chromatogram of the GST subunit of P1-1, which was unaffected after reduction. It is unclear why the extent of inactivation is relatively low and why only disulfide formation occurred. Most likely, the relatively low rate of quinone formation from 5-GSDA, as well as the low chemical reactivity of the nucleophilic addition of a second thiol to 5-GSDA both contribute to this phenomenon. This postulate was supported by the present study in which only poor conversion of 5-GSDA with both microsomal incubations and tyrosinase was observed, as well as by earlier studies in which was shown that the nucleophilic addition of thiol to quinones (derived with relative large concentrations of tyrosinase from structurally related cysteinylconjugates of catecholamines) occurred at a low rate [2,3,32]. Thus in the case of 5-GSDA, most likely the oxidation of cysteine 47 of GST P1-1 occurred more rapidly than nucleophilic addition to this cysteine. The oxidation of cysteine 47 is accompanied by disulfide formation, presumably with cysteine 101.

The significance of the reversible inhibition of GST by glutathione derivatives has been noted before [9]. E.g. the potent reversible inhibitors S-hexylglutathione, S-(*p*-bromobenzyl)glutathione and the glutathione conjugate of ethacrynic acid have I_{50}-values ranging from <0.1 to 20 µM for the human alpha-, mu-, and pi-class [9,33]. Virtually no reversible inhibition of the human GST was observed with the catecholamines themselves, nevertheless the glutathione conjugates of the catecholamines reversibly inhibited all of the studied human GST. However, compared with the above mentioned compounds, these conjugates are relatively moderate inhibitors of GST. Interestingly, in the case of the glutathione conjugates of ethacrynic acid, S-(*p*-bromobenzyl) and now the catecholamines, the human mu-class is much more sensitive to reversible product (analog) inhibition than the alpha- and pi-class, indicating that especially this GST class might be very susceptible to product-binding.

The in vivo occurrence of catecholamine oxidation products has been demonstrated by the recovery of 5-S-cysteinyldopamine and 5-S-cysteinyldopa in the human brain [34], and in the tyrosinase catalyzed synthesis of red brown melanin pigment in normal and malignant melanocytes [35]. Under normal physiological conditions it seems unlikely that significant inactivation of GST P1-1 occurs, since the protective agent glutathione is abundant throughout the body in 1-10 mM concentration [8,9], and e.g. in the human brain at 1 mM [36]. Whenever the protective mechanism is disrupted [37] or whenever the catecholamines are administered in relative large doses, the possibility exists however that the quinones derived from catecholamines might significantly react with GST P1-1. These enzymes are present in human brain and melanoma cells [9,38]. Dopamine and analogs were shown to inhibit melanoma growth by inhibition of DNA polymerase α, probably by interaction with a sulfhydryl group [39]. In this respect, it will be interesting to assess the role of GST inhibition by these compounds in the observed growth inhibition of melanoma cells, since GST inhibition may be involved in the inhibition of the cell proliferation [40].

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Chapter 5

S-(2,4-dinitrophenyl)glutathione excretion into the medium by rat hepatoma cells. Effects of ethacrynic acid and tetrachloro-1,4-benzoquinone and derivatives

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Submitted

Abstract

An assay is presented to study glutathione conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) in rat H-35 Reuber hepatoma cells, by the quantification of the excretion of S-(2,4-dinitrophenyl)glutathione (DNPSG) on HPLC. Cells exposed to 10 µM CDNB in PBS, displayed linear excretion of DNPSG up to 10-20 min. The amount of conjugate left intracellularly was minimal (at the end of the assay 5.0 to 8.1 pmol/nmol protein), compared to the amount excreted (about 19 nmol).

The effects of two types of inhibitors of purified glutathione S-transferases (GST) isoenzymes were studied with this assay, using a preincubation of 1 hr. A significant ($P<0.025$) linear relationship between the concentration (0-50 µM) of both ethacrynic acid (EA) and its dibromo dihydro derivative (diBrEA) and the DNPSG excretion (after 10 min exposure to CDNB) was observed, with a maximum of 30% and 50% reduction of DNPSG excretion for EA and diBrEA, respectively. The intracellular concentration of DNPSG at the end of the experiment was not affected, thus excluding an effect of EA on the glutathione conjugate transport. Intracellular glutathione levels were also similar to control values. From the fact that GST activity in cells lysed at the end of the experiment was also similar to controls, it can be concluded that EA inhibits intracellular GST in a reversible manner. With the other type of inhibitors, tetrachloro-1,4-benzoquinone (TCBQ) and its glutathione conjugate, of which the glycine

carboxyl group was esterified to improve the absorption, no such concentration dependent inhibition of the DNPSG excretion was found, indicating that these compounds are not usable as GST inhibitors in cells systems.

Introduction

Extensive lists of reversible and irreversible inhibitors of glutathione S-transferases (GST) activity have been published (Mannervik and Danielson, 1988; Van Bladeren and Van Ommen, 1991). In general, inhibitors of enzymes can be used to study the mechanism of the catalysis or the architecture of the active site. In the case of the GST, use has also been made of inhibitors to distinguish various isoenzymes (Mannervik 1985). However, the role of the cytosolic GST in the alkylating drug resistant phenotype, observed in the treatment of tumors (Waxman, 1990; Black and Wolf, 1991) potentially makes inhibition of GST clinically useful.

The reversible inhibition of GST is usually reported in I_{50}-values. Fortunately, this I_{50}-value has predominantly been determined with one co-substrate (1-chloro-2,4-dinitrobenzene, CDNB) under established experimental condition (Habig *et al.*, 1974), which enables comparison of I_{50}-values obtained from structurally unrelated inhibitors. On the other hand, the comparison of the irreversible inhibition data of even structurally related compounds is strongly hampered since in general little similarity between these studies exists (Van Ommen *et al.*, 1988; Berhane and Mannervik, 1990, Tamai *et al.*, 1990; Desideri *et al.*, 1991).

The extrapolation of inhibition data, obtained with pure enzyme preparations, to the *in vitro* cell or *in vivo* situation is relatively complicated. The pharmacokinetics and metabolism of the inhibitor determine its intracellular concentration and this concentration may change continuously. Moreover, the GST concentration *in situ* is much higher than the enzyme concentration used in the usual enzyme assays (Mannervik, 1985).

Although GST inhibition in biological systems can easily be determined in cytosolic preparations, using this method, the extent of reversible inhibition is always very much underestimated, since the non-covalently linked inhibitor is extremely diluted in both the cytosol preparation and in the enzymic assay.

The direct measurement of the glutathione conjugation with a model substrate in intact cell cultures has the advantage that conjugation and inhibition are studied, without dilution of enzymes or inhibitors. Both types of inhibition (irreversible and reversible) are determined, and moreover the potential contribution of glutathione depletion by the inhibitor is included. The glutathione conjugate excretion by isolated cells has been determined directly with e.g. bromosulphthalein and α-bromoisovalerate (for review see: Mulder and Te Koppele, 1988). A system to determine the inhibition of the glutathione conjugation in cell lines may be based on any of these substrates.

The present study describes a rapid method to determine the glutathione conjugation with CDNB, by the measurement of excretion of the glutathione conjugate of CDNB (S-(2,4-dinitrophenyl)glutathione, DNPSG) in the medium, in the (Reuber H-35) rat hepatoma cell line. CDNB was chosen as model substrate since it is the commonly used co-substrate of GST in enzyme assays. The effects of potent inhibitors of GST isoenzymes (halogenated quinones and ethacrynic acid) on the DNPSG excretion was studied.

Materials and Methods

Materials and syntheses. Glutathione and dithiotreitol were obtained from Boehringer, Mannheim, Germany. Tetrachloro-1,4-benzoquinone (TCBQ) was from Merck, Darmstadt, Germany. 1-Chloro-2,4-dinitrobenzene (CDNB), Ethacrynic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), S-hexylglutathione, N-acetyl-L-cysteine, and 2,5-dimethoxycinnamic acid were obtained from Sigma Chemical Co., St. Louis, MO, USA. Dibromo, dihydro ethacrynic acid was synthesized as previously described (Ploemen *et al.*, 1993). HPLC-grade trifluoroacetic acid was obtained from Baker (Deventer, The Netherlands), HPLC-grade methanol was from Rathburn Chemicals Limited (Walkerburn, Scotland). HPLC-grade acetonitrile was from Westburg (Leusden, The Netherlands). Epoxy-activated Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). DNPSG was synthesized according to Lindwall and Boyer (1987). The monoethyl ester of glutathione [(γ-L-glutamyl-L-cysteinyl(ethyl glycinate)] was synthesized according to Anderson and Meister (1989). The glutathione adduct of the monoethyl ester of glutathione and TCBQ [GS(ethyl)TCBQ] was prepared in

analogy to the synthesis of the 2-S-glutathionyl-3,4,5-trichloro-1,4-benzoquinone (GSTCBQ) (Van Ommen *et al.*, 1988). Briefly, 0.26 mmol of the monoethyl ester of glutathione in 20 ml methanol was added dropwise to 24 mmol of TCBQ in 500 ml of methanol during one minute, and stirred for one minute. After evaporation and repeated extractions with ethyl acetate to remove unreated TCBQ, the resulting conjugate was purified by preparative RP-HPLC (Zorbax ODS, 250*21.2 mm), eluted isocratically (flow 4 ml/min) with 60% methanol and 40% of a 0.1% (v/v) aqueous formic acid solution (k'= 3.0). The identity and purity of the product was established by the UV-spectrum, which was identical to GSTCBQ (UV-maxima at 249 and 288 nm cf. Van Ommen *et al.* 1990), FAB mass spectrometry (*m/z* = 544.9), and analytical RP-HPLC (purity >95%) using the system described in Van Ommen *et al.* (1988).

Drug exposure and cytotoxicity assays. Rat Reuber H-35 hepatoma cells were cultured in Ham's F12 medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal calf serum, 50 IU/l penicillin, 50 mg/l streptomycin, 50 mg/l gentamicin, at 37°C in a humid atmosphere containing 5% CO_2. For each experiment, approximately $8*10^5$ cells were plated onto a petri dish and cultured until a semiconfluent monolayer was obtained (in about 2 days). Cells were exposed to agents in triplicate for one hour at 37°C in the culture medium (final volume 10 ml). All test compounds were freshly prepared in DMSO (final concentration in the medium always 0.5%).

Ethacrynic acid (50 µM) and TCBQ (25 µM) were incubated for one hour in the culture medium or PBS to study the stability. Samples (0.1 ml) were injected on HPLC to quantify the amount of parent compounds, using the HPLC-separation as described (Van Ommen *et al.*, 1988; Ploemen *et al.*, 1990). Ethacrynic acid was far more stable in culture medium than TCBQ, since more than 90% and less than 5% of the unchanged parent compound was detected after one hour of incubation, of respectively ethacrynic acid and TCBQ (data not shown).

In order to exclude cytotoxic effects during the assay for glutathione conjugation, only concentrations of the test compounds giving at least 90% viable cells were used in the experiments. Drug effects on cell proliferation and cytotoxic effects were determined by the microculture MTT assay, and expressed as % viable cells (Mickisch *et al.*, 1990). At least 10 concentrations in octuplicate were used

to determine the 90% survival value (Mickisch *et al.*, 1990). The 90% cell survival concentrations determined were 25, 12.5, >80, 80, and 11.1 µM; for TCBQ, GS(ethyl)TCBQ, ethacrynic acid, dibromo dihydro ethacrynic acid, and CDNB, respectively).

The intracellular glutathione conjugation was estimated by the DNPSG excretion in the medium. Cells were cultured and exposed to the test compounds (see above). Next, the cells were washed with PBS (to remove the test compounds from the medium), whereafter 10 µM of CDNB in PBS (10 ml) was added. One ml aliquots were taken from the medium at 10, 20, and 30 min, N-acetyl-L-cysteine (in 25 µl of water) was added immediately to these samples (final concentration 1 mM) to remove unreacted CDNB. 100 µg of 2,5-dimethoxycinnamic acid (in 100 µl of ethanol) was added as internal standard. The samples were stored on ice. DNPSG was separated from the N-acetyl-L-cysteine conjugate of CDNB by injecting 0.1 ml on a Zorbax ODS reversed phase column (4.6 * 250 mm), eluted at a flow rate of 1 ml/min, with 0.1% v/v trifluoro acetic acid (solvent A) and 0.1% v/v trifluoro acetic acid in methanol (solvent B) with a linear gradient of 30-90% B in 10 min, followed by a gradient to 100% in 7 min (k'= 2.8, and 3.8, for respectively DNPSG, and the N-acetyl-L-cysteine conjugate of CDNB). Quantification of DNPSG was performed by peak area integration at 340 nm, using concentration/absorbance curves of the DNPSG standard. The new peak visible in the HPLC-chromatogram (peak II, Fig.1), is likely the conjugate of N-acetyl-L-cysteine and CDNB, since it has a similar UV-spectrum as DNPSG (λ_{max} = 340 nm) and its retention time is identically to the product formed upon incubations of only glutathione and N-acetyl-L-cysteine. DNPSG in the samples (stored at 0-6°C) was stable for at least 2 weeks.

The intracellular DNPSG and the glutathione concentration were determined in control cells and following exposure to the test compounds and subsequent CDNB exposure, as described (Lindwall and Boyer, 1987). Briefly, after trypsinization the cells were centrifuged (5 min 800 rpm) in ice-cold PBS, the pellet was carefully rinsed with ice-cold PBS and resuspended in PBS. Aliquots were used to determine the protein concentration (Lowry *et al.*, 1951). The cells were disrupted with 5% phoshoric acid, and denatured protein was removed by filtration. The HPLC analysis was used to quantify DNPSG, while glutathione was determined as described (Reed *et al.*, 1980).

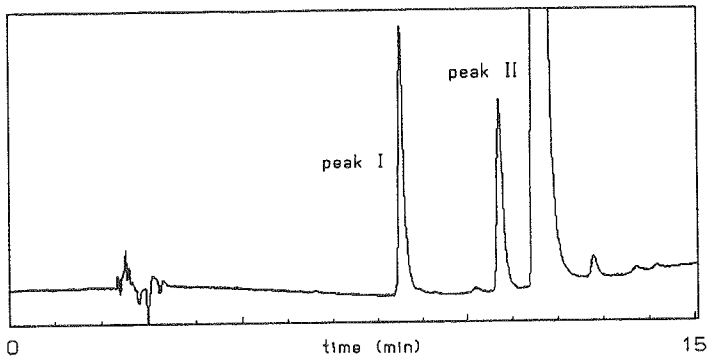

Figure 1. *Typical HPLC-chromatogram of medium of rat H-35 hepatoma cells exposed to CDNB*. The large peak present (at 11.8 min) is 2,5-dimethoxycinnamic acid which was added as internal standard. Peak I= DNPSG; Peak II= N-acetyl-L-conjugate of CDNB. *Y-axis:* full scale 0.02 A at 340 nm.

GST subunit composition of rat H-35 hepatoma. 19 dishes (75 cm^2) were cultured with rat H-35 hepatoma cells until a semiconfluent monolayer was obtained. After trypsinization the cells were centrifuged (5 min 800 rpm) in PBS (containing 1 mM dithiotreitol), and disrupted by sonification. The cell homogenates were centrifuged (30 min 15000 g, at 6°C) and the supernatants were used to determine the GST subunit composition by S-hexylglutathione Sepharose 6B affinity chromatography followed by wide pore RP-HPLC separation as described (Bogaards *et al*. 1989).

GST assay in supernatant. After exposure (3 hr) to the test compounds (see above), the cells were washed (with about 10-15 ml PBS) and harvested by scraping with a rubber policeman in 1 ml PBS, whereafter the cells were disrupted by sonification and the supernatant was prepared (see above). The GST activity was measured in the supernatant with CDNB according to Habig *et al*. (1974).

Statistical Methods. The relationship between concentration and effect was determined by linear-regression analysis. Levels of significance were tested by one-way ANOVA ($P < 0.05$), while comparison between groups were made using Dunnett control group comparison test ($P < 0.01$).

Results

GST subunit composition of rat H-35 hepatoma. The GST subunit composition was determined with bioaffinity chromatography, followed by RP-HPLC separation of the GST subunits. Rat H-35 hepatoma expressed almost exclusively the mu-class GST subunits 3 and 4, in a ratio of approximately 1:4. A small unknown peak (about 6% of the total GST subunits, assuming a similar molar extinction coefficient) was observed, eluting between the peaks of GST subunit 1 and 2, which might be subunit 6 (Coles and Ketterer, 1990).

Measurement of glutathione conjugation with CDNB. The model substrate CDNB was used to determine the glutathione conjugation in rat H-35 hepatoma cells, by following the excretion of its glutathione conjugate in the medium by HPLC analysis. The excretion of DNPSG was in general linear over a time span of 10-20 min (see control, Fig.2-4). However, since a deviation of the linearity after 15 min was observed in some cases, only the values at 10 min were used to determine the relationship between concentration

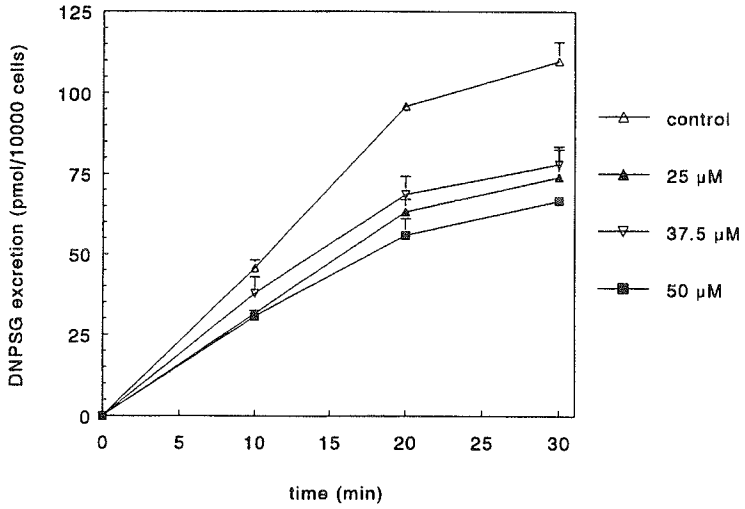

Figure 2. *The DNPSG excretion of rat H-35 hepatoma cells exposed to ethacrynic acid.* Cells were exposed to drugs for one hour, washed with PBS and subsequent exposed to 10 µM CDNB. The glutathione conjugation was determined by the excretion of DNPSG into the medium. Values are the average (N=3) ± SEM.

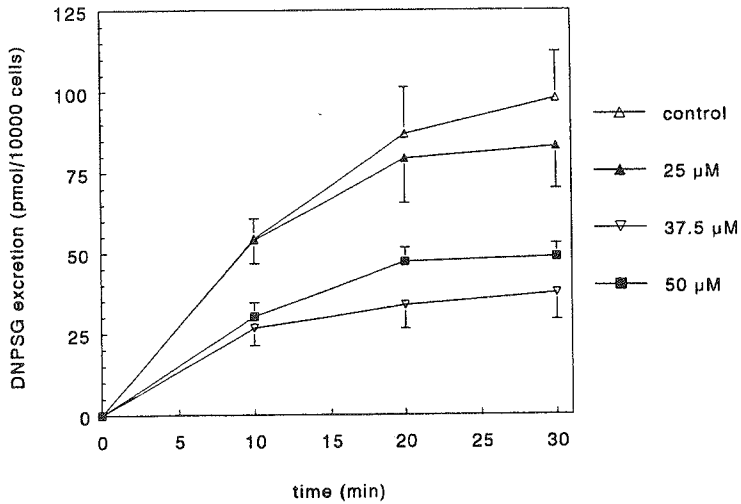

Figure 3. *The DNPSG excretion of rat H-35 hepatoma cells exposed to dibromo dihydro ethacrynic acid.* Cells were exposed to drugs for one hour, washed with PBS and subsequent exposed to 10 µM CDNB. The glutathione conjugation was determined by the excretion of DNPSG into the medium. Values are the average (N=3) ± SEM.

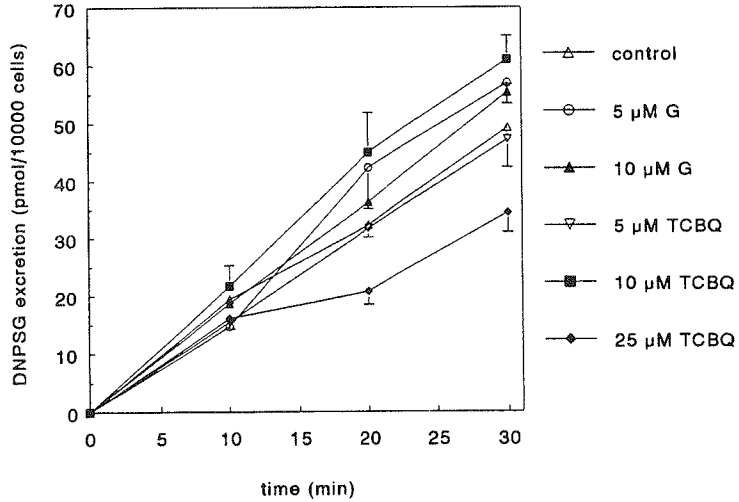

Figure 4. *The DNPSG excretion of rat H-35 hepatoma cells exposed to TCBQ and GS(ethyl)TCBQ.* Cells were exposed to drugs for one hour, washed with PBS and subsequent exposed to 10 µM CDNB. The glutathione conjugation was determined by the excretion of DNPSG into the medium. Values are the average (N=3) ± SEM. *Abbreviation*: G= GS(ethyl)TCBQ

and effect (see below). At this time-point, the excretion of DNPSG in the control incubations varied between the various assays from 19 to 54 pmol/10^4 cells (Fig.4 and Fig.2, respectively). Because of this variation the expression as % of control incubations is indicated. The maximal consumption of CDNB (calculated from the excretion after 30 min (Fig.2) and the number of cells per dish (about $1.7*10^6$ cells) was about 19 nmol, which is 19% of the total amount of CDNB present per dish.

Inhibition studies. The effect of several GST inhibitors on the DNPSG excretion in H-35 hepatoma cells was studied. The relationship between concentration of inhibitor (µM) and excretion of DNPSG was determined by linear regression analysis at 10 min (see above). For both ethacrynic acid and its dibromo dihydro derivative a significant linear relationship was found [estimated parameters: regression coefficient of ethacrynic acid -0.264 ($P=0.022$) with constant term of 43.9 (pmol/10^4 cells), and regression coefficient for dibromo dihydro ethacrynic acid -0.578 ($P=0.013$) with constant term of 57.7 (pmol/10^4 cells)]. At the highest concentration, a decrease of approximately 30% and 50% was observed (as estimated by regression analysis), for ethacrynic acid and dibromo dihydro ethacrynic acid, respectively. GS(ethyl)TCBQ was used instead of the GST inhibitor GSTCBQ, since the monoethylester of glutathione has improved absorption characteristics (Anderson and Meister, 1989). For TCBQ and GS(ethyl)TCBQ no significant relationship between concentration of inhibitor and DNPSG excretion was observed (Fig.4).

For comparison, the GST activity was determined in the supernatant of simular numbers of rat hepatoma cells (Table 1). An increased exposure time (3 versus 1 hr) and an increased concentration of ethacrynic acid (80 versus 40 µM) was used as compared with the DNPSG method. Even under these conditions, no significant decrease in GST activity was observed for either ethacrynic acid or TCBQ and their derivatives. Thus irreversible inhibition can be excluded.

Table 1. Effect of exposure to test compounds on the GST activity towards CDNB in the supernatant of rat H-35 hepatoma cells.

Compound	GST activity (nmol/min.mg)
blank	105 ± 4
25 µM TCBQ	130 ± 9
12.5 µM GS(ethyl)TCBQ	134 ± 16
blank	112 ± 4
80 µM ethacrynic acid	110 ± 12
80 µM dibromo dihydro ethacrynic acid	102 ± 26

$8*10^5$ rat H-35 hepatoma cells were plated onto a petri dish (56 cm^2) and cultured until a semiconfluent monolayer was obtained (in about 2 days), followed by incubation with the test compounds for 3 h at 37°C in culture medium, whereafter supernatant was prepared. Values are the average of 3 measurements (± SEM).

To study the observed inhibition of the DNPSG excretion in more detail we studied the effect of the addition of the two parent inhibitors (Ethacrynic acid or TCBQ) on either the glutathione concentration or the intracellular concentration of DNPSG. Those two parameters were determined at the end of the assay (Table 2). No (significant) effects were observed. However, the variation in the DNPSG levels was high, presumably as a result of the low intracellular concentration of DNPSG (close to the detection limit of the UV-detection).

Table 2. Intracellular DNPSG and glutathione concentration in rat H-35-hepatoma cells exposed to test compounds and subsequent exposure to CDNB.

Compound	DNPSG (pmol/mg protein)	glutathione (nmol/mg protein)
blank	6.9 ± 1.52	3.9 ± 0.57
25 µM TCBQ	8.1 ± 5.00	4.3 ± 0.40
50 µM ethacrynic acid	5.0 ± 1.59	4.8 ± 0.68

Rat H-35 hepatoma was incubated with test compounds in culture medium. After 1 hr at 37°C the cells were washed with PBS and exposed to 10 µM CDNB (in PBS) for 30 min at 37°C. Cell extracts were made and DNPSG and glutathione were determined. Values are the average ± SEM with N=3.

Discussion

Despite the large number of inhibitors of pure GST isoenzymes known, only few attempts have been made to study GST inhibition in biological systems. The aim of our present study was to develop a simple assay to determine the glutathione conjugation in rat hepatoma cells, and to study the effects of two promising classes of GST inhibitors (ethacrynic acid and TCBQ) with this assay.

In the assay presented in this paper (HPLC separation of DNPSG from serum-free medium, after preincubation with GST inhibitors), the concentration of the substrates may vary. The preincubation with the GST inhibitor was performed in complete culture medium. In this way the glutathione content can be augmented by the presence of the sulfur-containing precursors cysteine and methionine (according to the manufacturer respectively 0.2 mM and 30 µM) to maximize the glutathione biosynthesis (Reed, 1990). The co-substrate CDNB is a hydrophobic compound that very rapidly enters the cell probably via simple diffusion (Oude Elferink *et al.*, 1989). Using hepatocytes, the absence of (major) glutathione depletion using a higher CDNB concentration (40 µM) had been reported (Lindwall and Boyer, 1987). The

linearity of the DNPSG excretion suggests that no glutathione depletion occurred. On the other hand, using the analysis at time-point 10 min at which the excretion of DNPSG is linear, effects of product inhibition of the GST (by the glutathione conjugate formed) may be minimized.

In many different organs and cell types, DNPSG is efficiently excreted by an ATP-dependent glutathione S-conjugate export pump (Ishikawa, 1992). Several glutathione conjugates have been shown to competitively inhibit this pump, while the efflux was a saturable process (Lindwall and Boyer, 1987; Kobayashi *et al.*, 1990; Akerboom *et al.*, 1991; Oude Elferink *et al.*, 1989). The reported K_i's in general range from 50 µM to to 1 mM (Lindwall and Boyer, 1987; Akerboom *et al.*, 1991). From the fact that the vast majority of the CDNB conjugates was excreted from the hepatoma cells, it might be concluded that these also possess the conjugate pump. A small increase in the intracellular concentration of DNPSG was observed after treatment with ethacrynic acid, however this difference does not reach statistical significance. Even with 50 µM ethacrynic acid, the intracellular accumulation of DNPSG (viz. 8.1 pmol/mg protein) is still much smaller than the accumulated excretion (viz. 11300 pmol, calculated after 30 min exposure with number of cells is $1.7*10^6$). However, since considerable underestimation of the extent of DNPSG formation might occur when strong inhibition of the export pump occurs, this phenomenon may not to be overlooked.

Ethacrynic acid has been shown to inhibit pure GST isoenzymes very efficiently in a reversible manner (Ahokas *et al.*, 1985; Ploemen *et al.*, 1990). In the present study, a concentration dependent inhibition of the glutathione conjugation by ethacrynic acid was observed. This coincides with no (major) depletion of glutathione (with up to 50 µM ethacrynic acid), as reported for other cell lines (Tew *et al.*, 1988; Rhodes and Twentyman, 1992; Singh *et al.*, 1992). GST of the pi-class was shown to be sensitive to irreversible inhibition by ethacrynic acid (Ploemen *et al.*, 1993). In rat H-35 hepatoma cells no such irreversible inhibition was observed, as expected since this cell line almost exclusively expresses the GST subunit 3 and 4 of the mu-class. Using pure enzymes, I_{50}-values of about 1 µM were observed for the mu-class GST (Ploemen *et al.*, 1990; Hansson *et al.*, 1991), while in the rat hepatoma cell line about 30% inhibition is observed by incubation with 50 µM ethacrynic acid. This comparison again indicates the

difficulty in extrapolating data obtained from enzyme assays to cell systems.

Dibromo dihydro ethacrynic acid has an enhanced irreversible inhibitory potential of pure GST enzymes, while the reversible inhibitory capacity was comparable to ethacrynic acid itself (Ploemen *et al.*, 1993). Again, no irreversible inhibition was observed. An increased cellular absorption and/or the occurrence of glutathione depletion might cause the decreased glutathione conjugation as compared with ethacrynic acid itself.

The halogenated quinones irreversibly inhibited almost every GST isoenzyme studied (Van Ommen *et al.*, 1988; Ploemen *et al.*, 1991). The GST, of especially the alpha-class, were inactivated even faster by the glutathione conjugate of TCBQ (Van Ommen *et al.*, 1991; Ploemen *et al.*, 1991). It is assumed that the glutathione moiety targets the compound to the the active site of GST. Many cell types will however not readily take up glutathione, thus the absorption properties need to be improved. To this end a new derivative was synthesized, GS(ethyl)TCBQ, in which the glycine carboxyl group of the glutathione moiety was esterified. This glutathione monoethyl ester is readily transported into cells (Anderson and Meister, 1989), while the glycyl carboxylate group modification does not affect the affinity for GST (Adang *et al.*, 1990). Nevertheless, neither TCBQ nor GS(ethyl)TCBQ displayed inhibition of the conjugation in rat hepatoma cells. Using complete medium, TCBQ readily reacted with medium compounds, presumably e.g. with the above mentioned cysteine, moreover at 37°C TCBQ will undergo also significant hydrolysis (Kutyrev, 1991). For *in vitro* application, halogenated quinones as such are not suitable. A prodrug concept, in which non-toxic and chemically unreactive precursors are synthesized, seems the indicated route of investigation.

In conclusion, an assay is presented which was used to estimate the intracellular glutathione conjugating activity in rat H-35 hepatoma cells. Using the assay it was shown that ethacrynic acid and the dibromo dihydro derivative are good intracellular inhibitors, and the halogenated quinones are not.

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Chapter 6

Summary part I

The first part of this thesis describes studies on the inhibitory effect of quinones and some catechols on human and rat GST. An in vitro system was developed to study the inhibition of the glutathione conjugation in intact cells.

In chapter 2, the irreversible inhibition of human GST of the alpha-, mu-, and pi-class by TCBQ and GSTCBQ is described. A considerable irreversible inhibition (>70% with tenfold excess of inhibitor over enzyme) of GST was observed. Using these conditions, incorporation of about one nmol TCBQ per nmol subunit GST was observed. GST A2-2, which is the only GST which does not possess cysteine, was not inhibited and did not bind TCBQ. It is tempting to conclude that those human GST which were irreversibly inhibited, contain a cysteine in or near the active site, which is completely responsible for the inactivation by TCBQ.

GSTCBQ inactivated human GST isoenzymes very fast. However, only in the case of GST A1-1 the inactivation proceeded significantly faster than for TCBQ: in this case the glutathione moiety seemed to target the quinone to the enzyme.

Chapter 3 describes the reversible and irreversible inhibition of rat GST by the catechol caffeic acid. Moderate reversible inhibition could be reached with caffeic acid (I_{50}-values ranging from 58 to >640 µM). The major glutathione conjugate of caffeic acid, 2-S-glutathionylcaffeic acid (2-GSCA) was a much more potent reversible inhibitor of GST (with I_{50}-values ranging from 7 to >125 µM). On the other hand, significant time-dependent irreversible inhibition of rat GST, especially of the pi-class GST 7-7, was observed in incubations with caffeic acid, while no significant irreversible inactivation with 2-GSCA was found.

Chapter 3 also describes the *in vivo* effect of single oral doses of caffeic acid (50-500 mg/kg body weight) on the irreversible inhibition of the GST activity in rats 18 hr after dosing in liver, kidney and intestinal mucosa. Only in liver, a marginal but significant irreversible inhibition was observed (about 14% in the highest dose group), which coincides with a decrease of subunit GST 4. Further studies should concentrate on the reversible inhibition by caffeic acid and its glutathione conjugate.

Chapter 4 deals with the reversible and irreversible inhibition of human GST by the catecholamines dopamine, α-methyldopa and their 5-S-glutathionylconjugates. As compared with caffeic acid, only moderate reversible inhibition by the glutathione conjugates was observed (I_{50}-values ranging from 0.18-0.54 mM), while the parent catecholamines did not significantly inhibit GST reversibly. Using tyrosinase to generate quinones *in situ* the pi-class GST P1-1 was by far the most susceptible enzyme towards irreversible inhibition (67-99% inhibition under the conditions used) by both the parent catecholamines, while only GST P1-1 is irreversibly inhibited (33% inhibition) by the glutathione conjugate of dopamine.

The nature of the time-dependent inactivation of GST P1-1 by dopamine and its glutathione conjugate was studied in more detail. It was shown that a minor part of the inactivation was due to disulfide formation in the enzyme in the case of dopamine, while the inactivation by its glutathione conjugate was fully the result of disulfide formation.

Chapter 5 describes the effect of a non-toxic concentration of TCBQ and its glutathione conjugate, of which the glycine carboxyl group was esterified to improve the absorption, on the DNPSG excretion in rat hepatoma cells. In these cells which contain mainly the mu-class GST 3 and 4, no significant reduction of this excretion was observed, concomitant with the absence of an effect on the GST activity towards CDNB in the supernatant, and the concentration of glutathione in cells. Thus TCBQ and its derived conjugate do not seem to inhibit GST in cellular systems.

PART II

Chapter 7

Inhibition of rat and human glutathione S-transferase isoenzymes by ethacrynic acid and its glutathione conjugate

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Biochemical Pharmacology **40**: 1631-1635, 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.

Introduction

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^{r} [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-catalyzed [14]. The nature of the inhibition of GST by ethacrynic acid is not well documented: 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 Methos

Chemicals. Ethacrynic acid (Sigma Co., St Louis, MO, USA) was dissolved in absolute ethanol; its glutathione conjugate in 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA (Merck, FRG), both stored (not longer than 3 h) 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%~~ 10% 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 [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 (A1-2, M1a-1a), human placenta (P1-1) 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 A1-2, M1a-1a and P1-1.

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 dialyzing 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 dialyzed 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 spectrophotometrically 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). Secondly, incubations of GST with ethacrynic acid were performed, after which unreacted ethacrynic acid was removed by dialysis.

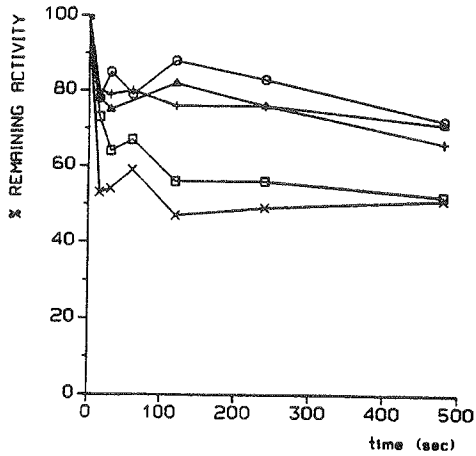

Fig. 1. Time course of inhibition of rat GST isoenzymes 1-1 (□), 2-2 (○), 3-3 (△), 3-4 (+) or 4-4 (×). Five μM ethacrynic acid and 0.25 μM enzyme were mixed at 0°, and a 25-pmol enzyme sample (50 pmol for 1-1) was transferred into a cuvette containing 1 mM CDNB, and 1 mM glutathione was added and conjugation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

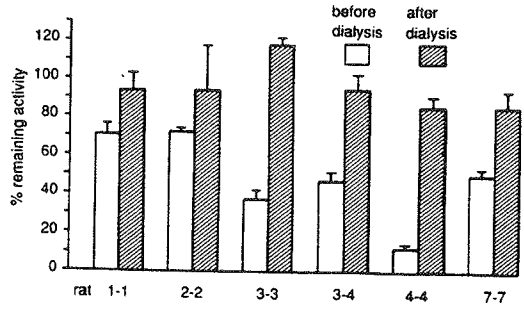

Fig. 2. Effect of dialysis on the remaining activity of rat GST isoenzymes. Five μM enzyme was incubated with 500 μM ethacrynic acid at room temperature, and a 50-pmol (for 1-1, 2-2, 3-3 and 3-4) or a 100-pmol (for 4-4 and 7-7) enzyme sample was transferred into a cuvette with 1 mM CDNB and 1 mM glutathione 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%.

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.

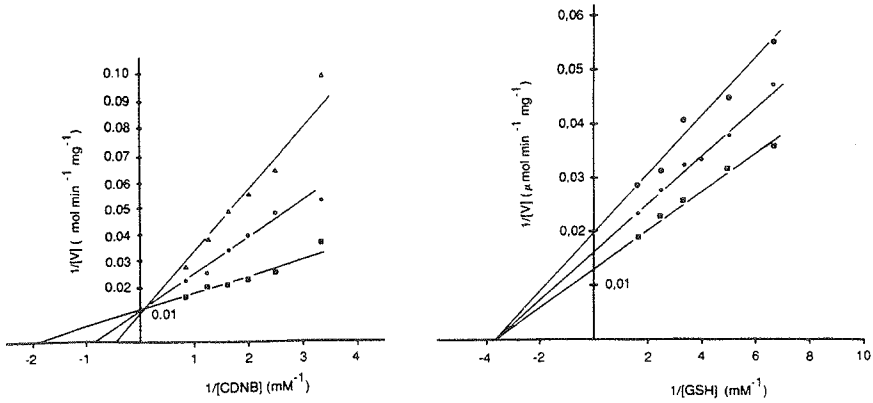

Fig. 3. Lineweaver–Burk plot showing competitive inhibition of rat GST isoenzyme 1-1 towards CDNB by 5 (*) or 10 μM ($\triangle$) ethacrynic acid (left) or non-competitive inhibition towards glutathione (right) by 1.0 (*), 2.5 ($\bigcirc$) μM ethacrynic acid. Control ($\square$). The values are the average 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%.

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.

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.

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 tumours. The present study indicates 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 incubating 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 information 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 enhanced 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 turn-over.

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.

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Chapter 8

Isoenzyme selective irreversible inhibition of rat and human glutathione S-transferase isoenzymes by ethacrynic acid and two brominated derivatives

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Biochemical Pharmacology **45**: 633-639, 1993.

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 allisoenzymes, 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 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.

Introduction

The glutathione S-transferases (GST) are a multigene family of isoenzymes which catalyze 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].

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 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 x 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

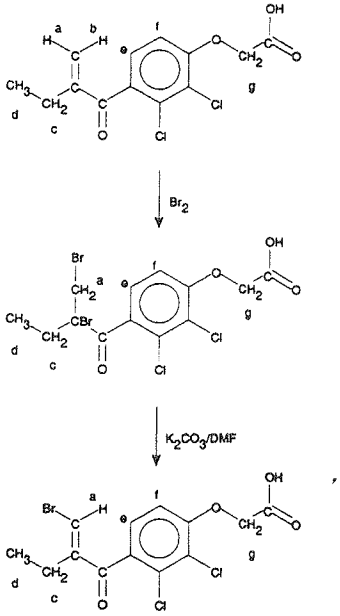

Fig. 1 - *The structure of ethacrynic acid and its dibromo dihydro and monobromo derivative*. The letters at the protons correspond with the NMR data.

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 ^1H NMR (see below) and HPLC, using Hypersil ODS (100 x 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 ^1H 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).

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 polybuffered 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 quilibrated 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 nitrogen.

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.

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.

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).

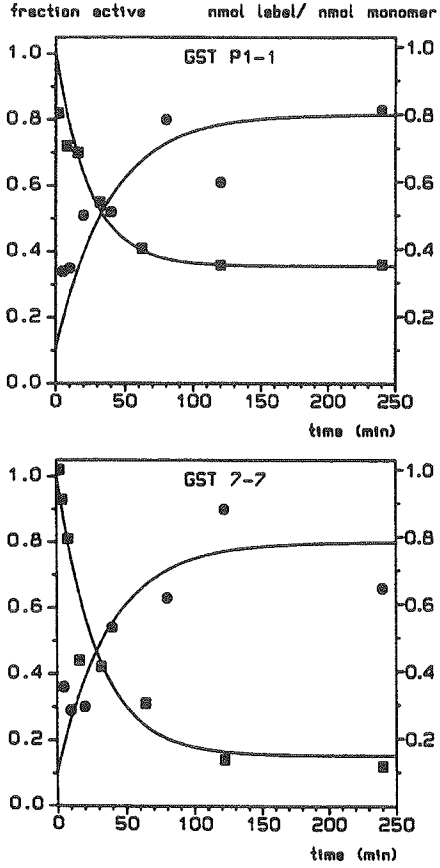

Fig. 2. The time course of the labeling of the GST isoenzymes human P1-1 and rat GST 7-7 was investigated at 20° by incubating 1.25 μM enzyme with 6.25 μM ethacrynic acid, after which the enzymatic activity towards CDNB and the amount of radioactivity incorporated were determined. (■) Remaining fraction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

The time course of labeling versus inhibition was determined for the pi-class GST, of both human and rat, under mild conditions (Fig. 2). Under these conditions, both human and rat GST isoenzymes of pi-class were inhibited in about 100-300 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).

Table 2. Effects 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.

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 experiment has shown that the alpha-class is either not affected by the dibromo dihydro derivative (GST

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.

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 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) disulphides. 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 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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Chapter 9

Reversible conjugation of ethacrynic acid with glutathione and human glutathione S-transferase P1-1

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Submitted

Abstract

The reversibility of the conjugation reaction of the diuretic drug ethacrynic acid (EA), an α,β-unsaturated ketone, with glutathione and glutathione S-transferase P1-1 (GST P1-1) has been studied. When the glutathione conjugate of EA was incubated with a fivefold molar excess of N-acetyl-L-cysteine *or* GST P1-1, a time-dependent transfer of EA to N-acetyl-L-cysteine or GST P1-1 was observed. With increasing pH, the pseudo first order rate constants of transfer of EA to N-acetyl-L-cysteine increased from 0.010 h^{-1} (pH 6.4), to 0.040 h^{-1} (pH 7.4), and 0.076 h^{-1} (pH 8.4).

From the fact that preincubation of GST P1-1 with 1-chloro-2,4-dinitrobenzene reduced the incorporation of [^{14}C]EA from 0.94 ± 0.21 to 0.16 ± 0.02 mol EA per mol subunit, and from automated Edman degradation of the major radioactive peptide isolated after pepsin digestion of the [^{14}C]EA-labeled enzyme, it was concluded that the reaction of EA takes place with cysteine 47 of GST P1-1.

When, GST P1-1 was inactivated with a fivefold molar excess of EA, adding an excess of glutathione resulted in full restoration of the catalytic activity in about 120 h.

These findings may have several implications: although, under normal physiological conditions the inhibition of GST P1-1 by covalent binding of EA would be reversed by glutathione, leaving reversible inhibition by the the glutathione conjugate of EA and EA itself as the main mechanism of inhibition. When glutathion levels are low the

covalent inhibition might be predominant resulting in a completely different time course of inhibition.

Introduction

Conjugation with the tripeptide glutathione is considered to be an important detoxification reaction for electrophilic xenobiotics, including numerous cytostatic agents. In general this reaction is catalysed by GST (1-3). Most GST occur in cytosol and belong to one of four multigene families, termed alpha, mu, pi, and theta (1,4). Evidence has accumulated that increased GST activity may be one of the causes of drug resistance, especially with respect to alkylating agents (5).

The diuretic drug ethacrynic acid, an α,β-unsaturated ketone, is a potent *reversible* inhibitor of GST isoenzymes (6,7), and has been used to study the role of GST in drug resistance in vitro, using cell lines (8), and in a phase I clinical study with the cytostatic agent thiotepa (9). Moreover, a concentration-dependent inhibition by ethacrynic acid of the enzyme-catalyzed conjugation of glutathione with the clinically important alkylating agent chlorambucil, has been reported (10). The *reversible* inhibition would further be enhanced by the formation of the glutathione conjugate of ethacrynic acid, which is an even stronger inhibitor for all GST but the pi-class (7). For both human and rat GST of the pi-class, covalent modification of GST concomitant with an *irreversible* loss of activity, could be achieved using slightly more drastic incubation conditions (11).

Conjugation with glutathione does not always leads to the detoxification of electrophilic xenobiotics (12). In addition to glutathione conjugates that are reactive by themselves, other types of glutathione conjugates may undergo further metabolism to a reactive species (12). A special case involves glutathione conjugates that exert their toxic effects through release of reactive species: the glutathione conjugates serve as transport and targeting agents. This situation occurs when the glutathione conjugation reaction is reversible, as found e.g. for some methyl isocyanates (13) and isothiocyanates (14). The Michael addition of glutathione with α,β-unsaturated aldehydes and ketones is another well-known reversible reaction (15). A reversible Michael reaction has e.g. been

shown to be involved in the covalent binding of the veterinary drug furazolidone (16).

Since ethacrynic acid also contains an α,β-unsaturated ketone moiety, the present study was designed to investigate the reversible covalent interaction of ethacrynic acid with glutathione as well as with GST P1-1. The interaction of ethacrynic acid with this enzyme was included, since the inactivation of the GST of the pi-class in several cases has been shown to be the result of the modification of a highly reactive cysteine residue (17,18), and since pi-class, next to alpha, is one of the primary GST classes that are involved in drug resistance (5).

Materials and Methods

Chemicals and enzymes. Ethacrynic acid (2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy)acetic acid, S-hexyl-glutathione, N-acetyl-L-cysteine, and Tris[hydroxymethyl]-aminomethane were from Sigma Chemical Co., St. Louis, MO. Epoxy-activated Sepharose 6B was purchased from Pharmacia, Uppsala, Sweden. [^{14}C]Ethacrynic acid was purchased from Amersham, Buckinghamshire, U.K. (15 mCi/mmol). Trifluoroacetic acid was from Baker Inc., Philipsburg, NJ. Pepsin (from porcine gastric mucosa) was obtained from Boehringer, Mannheim, Germany.

The radioactive conjugate of ethacrynic acid was prepared by adding 6 µmoles of glutathione in 180 µl of 0.1 M potassium phosphate buffer pH 8 with 50% ethanol, to 1.3 µmoles of [^{14}C]ethacrynic acid. After overnight incubation, the glutathione conjugate of ethacrynic acid was purified by preparative RP-HPLC using zorbax ODS (21.2 * 250 mm), eluted at a flow rate of 4 ml/min with 0.01% formic acid (solvent I) and methanol (solvent II), with a linear gradient of 40-100% II in 60 min (k'= 2.0 and 3.1 for the conjugate and ethacrynic acid respectively). About 70% conversion of ethacrynic acid to the glutathione conjugate was obtained. Methanol was removed under N_2, after which a stock-solution (of 136 µM) of the glutathione conjugate was stored at -30°C. A product of 95+ % purity was obtained as judged with RP-HPLC analysis, with an identical retention time to the non-radioactive conjugate (7).

The N-acetyl-L-cysteine conjugate of ethacrynic acid was prepared, in analogy to the synthesis of the glutathione conjugate (7). The ^{1}H-NMR (400 MHz, D_2O) spectrum of the N-acetyl-L-cysteine conjugate are consistent with the

expected structure (11); with the following information for the ethacrynic acid part: δ 7.81/7.79 (dd, 1H, *J*= 8.8 Hz), δ 7.23 (d, 1H, *J*= 8.8 Hz), 4.99 (s, 2H), δ 3.8 (m, 1H), δ 1.95/1.81 (dm, 2H), δ 1.1 (m, 3H); and for the N-acetyl-L-cysteine part δ 2.19 (s, 3H, $-CH_3$). The proton signals of cys α overlap with D_2O (δ= 4.7), while the signals of cys β and the protons next to the sulphur-atom (of the ethacrynic acid moiety) were found in the region of δ 2.9-3.1, as a complicated multiplet pattern.

GST P1-1 was purified as described (11). Protein was determined by the method of Lowry, using bovine serum albumin as standard (19).

Incubations. The glutathione conjugate of ethacrynic acid (0.5 mM) was incubated at 20°C with N-acetyl-L-cysteine (2.5 mM), in 0.4 ml of 0.1 M potassium phosphate buffer with 0.1 mM EDTA at three pH-levels (6.4, 7.4, and 8.4). For each pH, 22 independent samples were prepared. At each time point, 20 µl was injected on RP-HPLC, using a Zorbax ODS (250 * 4.6 mm) column, eluted at a flow rate of 1 ml/min with 0.1% TFA in deionized water (solvent A) and in methanol (solvent B), with a linear gradient of 30-95% B in 18 min, followed by 2 min at 95% B (k'= 4.5, 5.1, and 6.0 for the glutathione conjugate, N-acetyl-L-cysteine conjugate, and ethacrynic acid respectively). Peak areas at 270 nm were integrated with Nelson Analytical Model 2600 Chromatography Software. EDTA was added to the incubations to prevent the trapping agent N-acetyl-L-cysteine and the free glutathione from oxidation to their disulfides. Since no free ethacrynic acid could be detected, it is concluded that EDTA protected sufficiently against oxidation.

Covalent binding of [^{14}C]ethacrynic acid was studied in a volume of 75 µl 0.1 M potassium phosphate buffer pH 7.4 with 0.1 mM EDTA (buffer A), after preincubation of 25 µM GST P1-1 for 75 min at room temperature with (n=3) or without (n=2) 1 mM CDNB, whereafter the enzyme was incubated for 110 min with [^{14}C]ethacrynic acid (final concentration, 100 µM). Enzyme-bound ethacrynic acid was separated from ethacrynic acid by RP-HPLC (Vydac TP5 column, 200*3 mm). Elution was performed with a flow of 0.6 ml/min, with solvent A (see above) and 0.1% TFA in acetonitrile (solvent C), with a linear gradient of 30-60% C in 30 min (k'= 4.0, and 6.3 for ethacrynic acid and enzyme with bound ethacrynic acid respectively). UV-detection (at 214 nm) was used to identify the enzyme peak,

while simultaneously the radioactivity was measured using an on line radiochemical detector.

The [^{14}C]ethacrynic acid labeled GST P1-1 (0.25 mg) was digested with pepsin [enzyme to protein ratio 1/20 (w/w)] in 0.05 M Tris/H_3PO_4 (pH 1.8) for 18 hr at 37°C. The pepsin peptide mixture was purified on a RP column (Vydac Protein & peptide C18, 250 * 4.6 mm), eluted with solvent A and C (see above), 5 min isocratically at 100% A, followed by a linear gradient from 0-60% C in 70 min (flow rate 1 ml/min). The main radioactive peak was repeatedly purified on the same column. The peptide was degraded using automated Edman degradation on an Applied Biosystems Model 475 peptide sequencer on-line connected to Model 120A PTH-analyzer.

The catalytic activity of GST P1-1, inactivated with ethacrynic acid, was monitored after the addition of glutathione. 1 µM GST P1-1 was preincubated in buffer A (see above) with or without 10 µM of ethacrynic acid (final volume, 200 µl), after which glutathione was added (final concentration: 0, 10, 100, and 1000 µM). These incubations were performed in triplicate, at room temperature. At various time points, 20 pmol enzyme samples were transferred to cuvettes, after which the catalytic activity towards CDNB was measured (20). A time series was stopped, when the remaining catalytic activity in the corresponding blank incubation was less than 70%.

To study the interaction of GST P1-1 with the glutathione conjugate of [^{14}C]ethacrynic acid, seven independent samples of 10 µM GST P1-1 were incubated at room temperature with 2 µM of the radioactive glutathione conjugate in buffer A (see above), (final volume: 50 µl). To separate the glutathione conjugate and free ethacrynic acid from the enzyme with bound ethacrynic acid, 30 µl was injected on the Vydac TP5 column (see above).

Results

The occurrence of the retro Michael cleavage of the glutathione conjugate of ethacrynic acid was studied by incubation of the glutathione conjugate of ethacrynic acid with an excess of N-acetyl-L-cysteine (Fig. 1). The transfer of the ethacrynic acid moiety to N-acetyl-L-cysteine was followed with time by quantification of the glutathione and N-acetyl-L-cysteine conjugates of ethacrynic acid on RP-HPLC. The rate of transfer increased

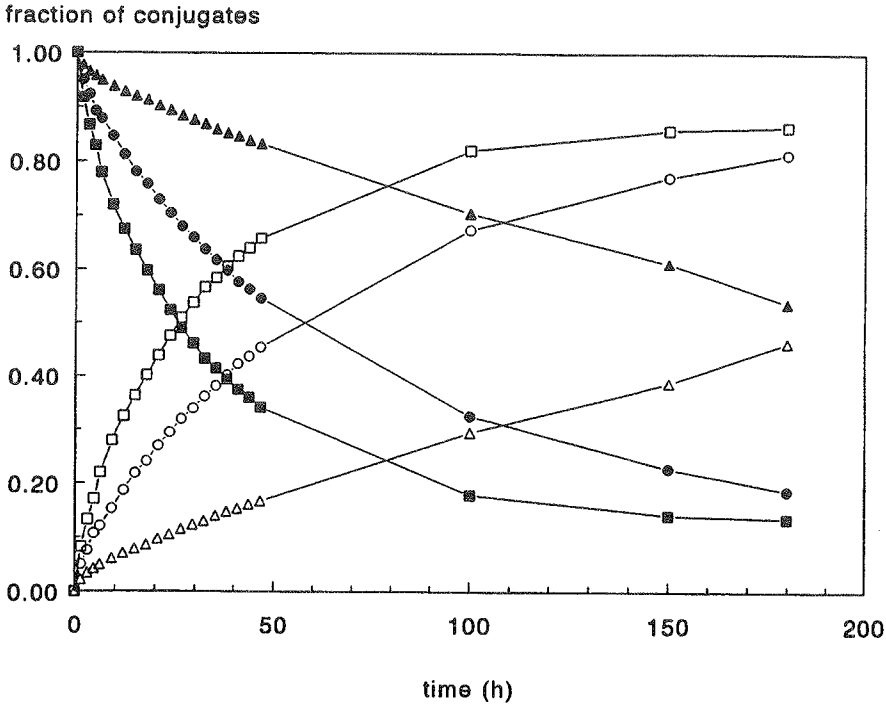

FIG. 1 *Transfer of ethacrynic acid from its glutathione conjugate to N-acetyl-L-cysteine (NAC).* The glutathione conjugate of ethacrynic acid (0.5 mM) was incubated at 20°C with a fivefold excess of NAC. The transfer of the ethacrynic acid moiety to NAC was followed with time by quantification of the conjugates on RP-HPLC by integration of the peak areas at 270 nm. The reaction was studied at three pH-levels; pH 6.4 (▲), 7.4 (●), and 8.4 (■). Open symbols reflect the newly formed NAC conjugates, while the closed symbols reflect the glutathione conjugate of ethacrynic acid.

with increasing pH, with pseudo first order rates of 0.010 h^{-1}, 0.040 h^{-1}, and 0.076 h^{-1}, for pH 6.4, pH 7.4, and 8.4, respectively. After 180 h of incubation at pH 8.4, an equilibrium was reached between the glutathione and the N-acetyl-L-cysteine conjugate, suggesting that the distribution of ethacrynic acid over glutathione and N-

acetyl-L-cysteine is mainly determined by their relative concentrations.

Retro Michael cleavage can also occur with GST P1-1 bound ethacrynic acid, if the assumption is right that ethacrynic acid reacts with a cysteine residue of GST P1-1 (11). In order to check this assumption, the incorporation of [^{14}C]ethacrynic acid in GST P1-1 after preincubation with CDNB was studied, which is known to inactivate GST P1-1 by modification of cysteine 47 (17). 0.94 ± 0.21 nmol label per nmol GST P1-1 could be incorporated in blanc incubations, identical to an earlier study (11). As expected, CDNB protects against incorporation of ethacrynic acid: 0.16 ± 0.02 nmol label per nmol GST P1-1 could be incorporated after preincubation with CDNB, supporting the hypothesis that ethacrynic acid reacts with cysteine 47 of GST P1-1. In order to identify the amino acid involved in the reaction, the GST P1-1 with bound ethacrynic acid was digested with pepsin and the resulting peptides were separated on HPLC. A main radioactive peak was identified, which contained >80% of the radioactivity, eluting at 45 min (Fig 2). The amino acid sequence indicated that it spans residues 44-46 in the primary amino acid sequence of GST P1-1 (Lys-Ala-Ser) (21-23), while an unknown residue was observed in the 4th-cycle (presumably the Cys-ethacrynic acid adduct). Thus it was again concluded that Cys 47 is the main target site.

Then, GST P1-1 (1 µM) was incubated with ethacrynic acid (10 µM), resulting in 90% loss of activity towards CDNB, and glutathione was added. The catalytic activity towards CDNB was measured over a 120 hr period (Fig. 3). Full restoration of the catalytic activity occurs with 0.1 mM and 1 mM glutathione (insert Fig. 3). The 10 µM incubation initially shows partial restoration of catalytic activity, but after prolonged incubation loss of catalytic activity is observed, probably due to oxidation. This is also observed in the corresponding control incubation (a 30% loss of activity in about 30 h; result not shown). Without a trapping agent for free ethacrynic acid, no restoration of activity was observed (Fig. 3).

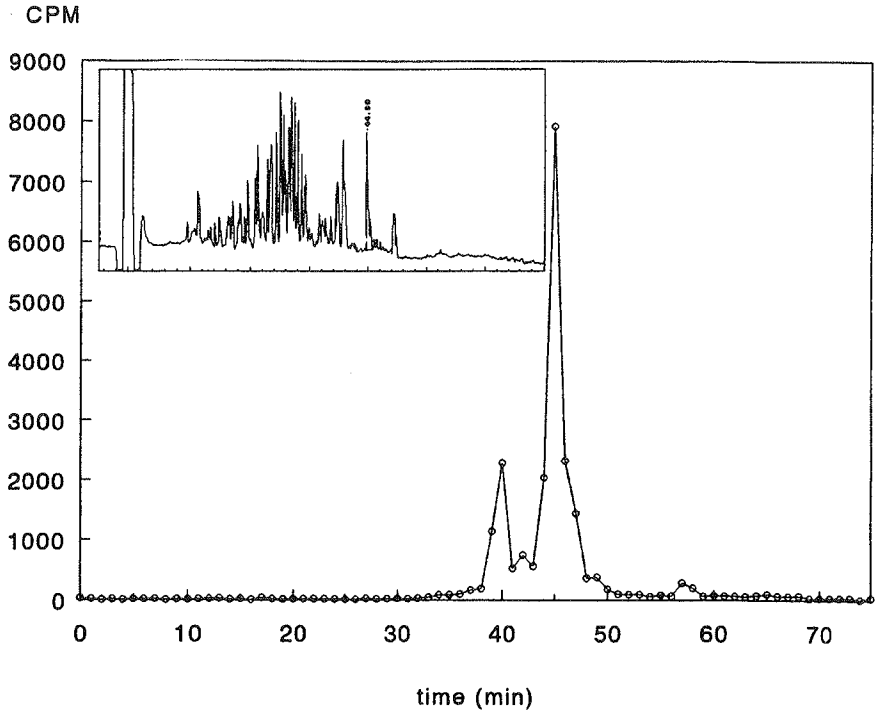

FIG. 2 *HPLC analysis of the pepsin digest of ethacrynic acid-labeled GSTP1-1*. From one minute fractions, samples (40 µl) were screened for radioactivity. *Insert:* peptides monitored from 0-75 min at 214 nm, peak at 44.8 min indicated (full scale 0.3 aufs).

In order to investigate whether retro Michael cleavage of the glutathione conjugate of ethacrynic acid concomitant with incorporation of ethacrynic acid in GST P1-1 occurs, a 5-fold molar excess of the enzyme was incubated with the glutathione conjugate of [^{14}C]ethacrynic acid. A time dependent increase of enzyme bound label was observed (Fig. 4), in accordance with the reversible nature of the reactions.

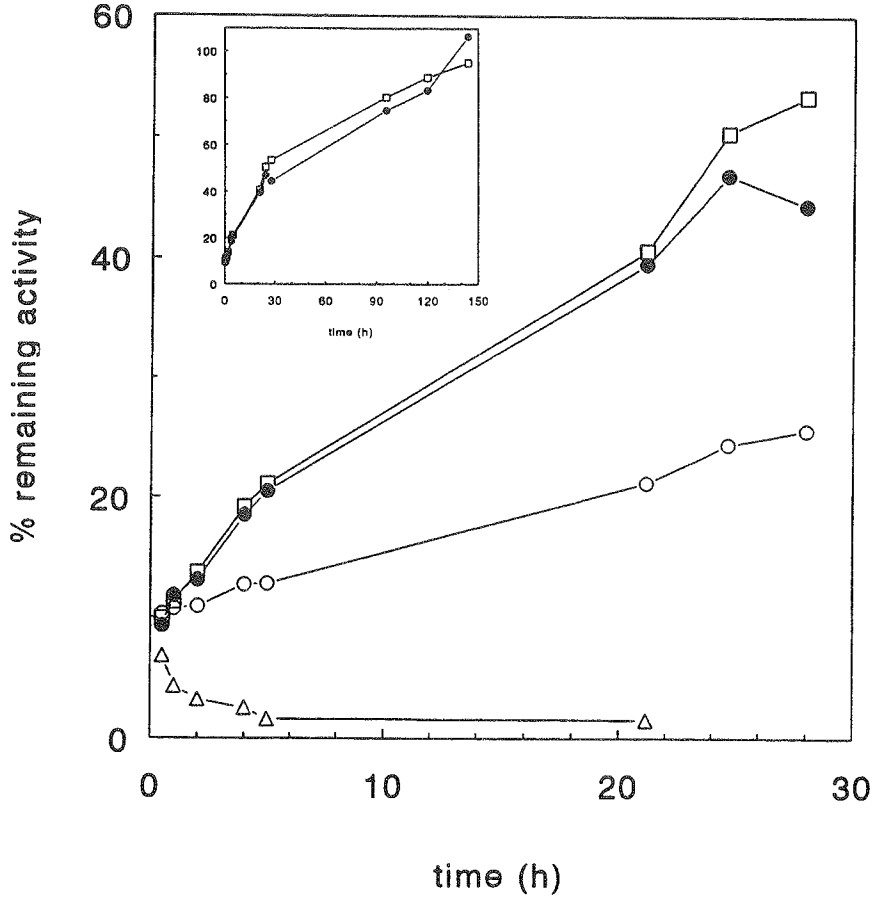

FIG. 3 *Restoration of the catalytic activity of ethacrynic acid-inactivated human GST P1-1, by incubation with glutathione.* GST P1-1 (1 μM) was incubated with ethacrynic acid (10 μM), resulting in 90% loss of catalytic activity towards CDNB. Glutathione (GSH) was added [0 (△), 0.01 (○), 0.1 (□), and 1 mM (●)]. The catalytic activity towards CDNB was measured over a 120 h period, and expressed as per cent of control incubations with non ethacrynic acid modified enzyme (note: incubations were stopped when the remaining activity in the corresponding control was less than 70%). *Insert:* effect of prolonged incubation.

Individual points are the average of three measurements, with coefficients of variation less than 15%.

Discussion

α,β-unsaturated aldehydes and ketones have long been known to form conjugates with glutathione, both spontaneously and enzyme-catalyzed (24). The extent to which the enzyme plays a role differs widely amoung members of this class of compounds (25). The chemical reaction involved in the conjugation of ethacrynic acid and structurally related compounds, a Michael addition, is reversible. In the present study, it was shown that this retro Michael cleavage of ethacrynic acid and glutathione indeed occurs. Thus, ethacrynic acid may be transferred from one low molecular weight compound to another, or to reactive and accessible cysteines in proteins, e.g. cysteine 47 of GST P1-1 as observed. This phenomenom is probably a common feature of α,β-unsaturated aldehydes and ketones: transport via a thiol conjugate and subsequent regeneration of the reactive agent thus may be involved in the biological activity of such adducts (15).

The nature of the inhibition of GST by ethacrynic acid has been studied in detail, since it was reported that ethacrynic acid in vivo bound covalently to rat GST 3-4 (26).

Previously we showed that ethacrynic acid and its glutathione conjugate were potent *reversible* inhibitors of GST isoenzymes, with I_{50}-values in the range of 1-10 μM (7). This reversible inhibition was suggested to be the predominant inhibitory mechanism in vivo, since the incorporation of ethacrynic acid in GST 3-4 did not appear to inactivate the enzyme (7,11). In the case of GST P1-1, it was shown recently that the mechanisms of reversible inhibition by ethacrynic acid and its glutathione conjugate were distinct (competitive and non-competitive, respectively) (27). For the alpha- and mu-class the conjugate of ethacrynic acid was an even more potent reversible inhibitor, while for the pi-class some conflicting results have been reported (7,27).

GST of the pi-class are inhibited by covalent binding of α,β-unsaturated aldehydes and ketones: acrolein, a toxic aldehyde that occurs as environmental pollutant (28), and also ethacrynic acid (11), specifically inactivated GST of the pi-class. It is now clear that the inhibition is only transitory, since the chemical reaction is reversible: full restoration of the catalytic activity can be achieved by prolonged incubation with an excess of glutathione. In an

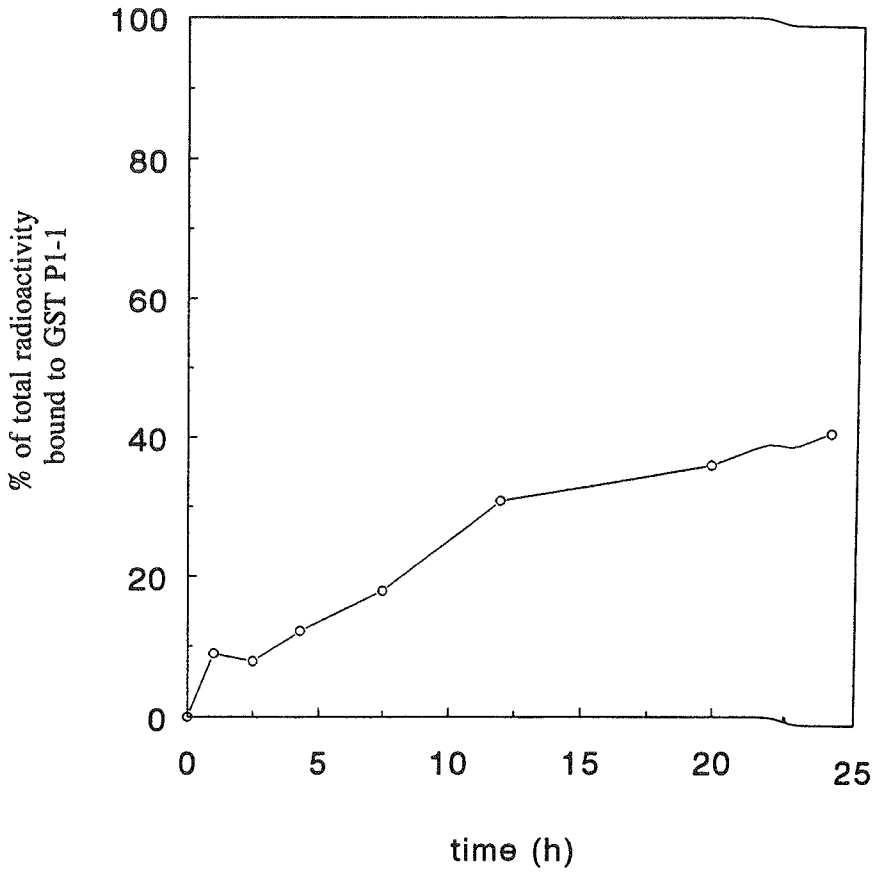

FIG. 4 *The interaction of GST P1-1 with the glutathione conjugate of [^{14}C]ethacrynic acid.* 10 µM GST P1-1 was incubated with 2 µM of the glutathione conjugate, [^{14}C]-labeled in the ethacrynic acid moiety. To separate the glutathione conjugate and free ethacrynic acid from the enzyme-bound fraction, a RP-HPLC method was used as described under "Materials and Methods". The transfer of the ethacrynic acid moiety is expressed as per cent of total [^{14}C]-label.

earlier study, only marginal inactivation of GST 7-7 by ethacrynic acid was observed using overnight dialysis experiments (7). Presumably the reversibility of the chemical reaction contributed to the failure of this dialysis experiment to detect time-dependent inhibition of GST P1-1 by ethacrynic acid.

The implications of the present findings may be several. Firstly, this mode of action, reversible covalent binding to GST P1-1 and glutathione, may have some significance for the use of ethacrynic acid as in vivo inhibitor for GST P1-1 in drug resistance. Under normal psysiological conditions (glutathione concentration 1-10 mM, (1)), glutathione may be expected to reverse any covalent binding of ethacrynic acid to GST P1-1, and the inhibition of GST would only occur reversibly, through the glutathione conjugate of ethacrynic acid and ethacrynic acid itself. However, in those cells with either high levels of GST P1-1 and/or low levels of glutathione, covalent inhibition of GST P1-1 might be predominant. The time course of inhibition would be completely different in these two cases.

Recently, it was shown that chronic exposure of human colon carcinoma cells to ethacrynic acid led to a 2-3-fold increase of GST P1-1 activity, by an induction of the enzyme at the transcriptional level (29). This phenomenon has been proposed by Talalay and co-workers to be a general one (30): compounds that contain a Michael acceptor or from which a Michael acceptor can be formed during metabolism are usually inducing agents for GST. The contrary effects observed for Michael acceptors, i.e. inhibition of GST by covalent modification, and induction of GST merit more attention.

Furthermore, some α,β-unsaturated aldehydes are established inhibitors of growth. In the case of 4-hydroxynonenal and related compounds it was shown that inhibition of DNA synthesis was involved, presumably as a result of a reaction with a functional sulfhydryl group of DNA polymerase (15). More recently, another type of growth inhibition has been reported, which involves the α,β-unsaturated ketone Δ^{12}-PGJ_2, a cyclopentenone prostaglandin which readily forms glutathione conjugates (31). These conjugations should in principle also be able to undergo retro Michael cleavage to reform the parent compounds. Interestingly, it has been shown that ethacrynic acid, along with other inhibitors of GST, also has

antiproliferative effects on cell lines (32), which seem to be reversible.

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Chapter 10

Summary part II

In part II of this thesis, the reversible and irreversible inhibition of GST by ethacrynic acid and derivatives is described. Moreover, an attempt was made to estimate the relevance of the reverse Michael cleavage of ethacrynic acid bound to GST. Finally, the effects on the DNPSG excretion were estimated in intact cells.

Chapter 7 deals with the reversible inhibition of rat and human GST by ethacrynic acid. Ethacrynic acid is a very potent reversible inhibitor with I_{50}-values in the range of <0.1-6 µM. In particular the human mu-class GST is very susceptible to reversible inhibition. The reversible inhibitory capacity is retained after conjugation with glutathione. In the case of the alpha- and mu-class the reversible inhibition is even stronger with the conjugate. Chapter 7 also demonstrates that the susceptibility of GST for the irreversible inhibition by ethacrynic acid is relatively low. No significant irreversible inhibition was observed, using conditions similar to the TCBQ experiments described in chapter 2.

Chapter 8 describes that incubation of GST of the pi-class with ethacrynic acid using slightly more drastic conditions (one hour at 37°C) results in a time-dependent inhibition. About 0.8 nmol ethacrynic acid per nmol human P1-1 and 0.8 nmol per nmol rat GST 7-7 could be incorporated, resulting in 65-93% inhibition. Isoenzymes of the alpha- and mu-class also bound ethacrynic acid (ranging from 0.2-0.6 nmol/nmol enzyme), however without loss of catalytic activity.

Chapter 8 also deals with the enhanced irreversible inhibitory capacity of a newly synthesized dibromo dihydro derivative of ethacrynic acid. This compound inhibits the pi-class very efficiently, resulting in 90-96% inhibition. Interestingly, this compound is also a powerful

irreversible inhibitor of the mu-class GST isoenzymes, resulting in 52 to 70% inhibition.

Chapter 9 describes the reversibility of the conjugation reaction of ethacrynic acid with glutathione and GST P1-1. Prolonged incubation of the glutathione conjugate of ethacrynic acid (up to 120-150 hr) with a fivefold molar excess of N-acetyl-L-cysteine or GST P1-1, indeed showed a time-dependent transfer of ethacrynic acid to N-acetyl-L-cysteine or GST P1-1. Moreover, ethacrynic acid bound to GST P1-1, transferred to an excess of glutathione (within 120 hr), with concomitant full restoration of the catalytic activity. Thus, depending on e.g. the relative concentration of the reactants, transfer reactions may occur in either direction. From the fact that preincubation of GST P1-1 with CDNB reduced the incorporation of [^{14}C]-ethacrynic acid, and from automated Edman degradation of the major radioactive peptide isolated after pepsine digestion of the [^{14}C]-ethacrynic acid labeled enzyme, it was concluded that the reaction of ethacrynic acid takes place with cysteine 47 of GST P1-1.

The effects of ethacrynic acid and its dibromo dihydro derivative on the DNPSG excretion in rat hepatoma cells was presented in chapter 5. A significant linear relationship between the concentration (0-50 μM) of ethacrynic acid and its dibromo dihydro derivative and the DNPSG excretion was observed, with a maximum of 30% and 50% reduction of DNPSG excretion for ethacrynic acid and its dibromo dihydro derivative, respectively. At the end of the experiment the intracellular DNDSG and glutathione levels were similar to control values. From the fact that GST activity in cells lysed at the end of exposure period was also similar to control values, it can be concluded that ethacrynic acid inhibits intracellular GST in a reversible manner.

PART III

Chapter 11

General discussion and Summary

The studies described in this thesis focused on inhibitors of human and rat GST isoenzymes. Several strategies can be envisaged for the discovery and design of such agents. The GST have two sites for substrates binding, one for glutathione, one for the second substrate. Several groups have concentrated on glutathione analogues, which selectively bind GST isoenzymes in vitro (Graminski *et al.* 1989; Adang *et al.* 1991; Castro *et al.* 1993). We have concentrated on compounds binding to the second substrate binding site, which inactivate or irreversibly modify the enzymes. Two groups of compounds were studied, TCBQ/GSTCBQ and a series of other quinones and related catechols, and ethacrynic acid and derivatives.

11.1 Quinones

Our studies with TCBQ originated from our investigations of the microsomal metabolism of hexachlorobenzene. Hexachlorobenzene is mainly converted to pentachlorophenol, while a small amount of tetrachloro-1,4-hydroquinone was also detected (Ommen *et al.* 1986; Ommen *et al.* 1988a). From the latter a reactive compound was identified which reacted very efficiently with glutathione. This compound, viz. TCBQ and in particular GSTCBQ, inactivated all studied rat GST isoenzymes to a considerable extent (Ommen *et al.* 1988b). Several experiments suggested that the quinone reacted with a cysteine residue of GST (Van Ommen *et al.* 1989). In an earlier study, in vitro inhibition of GST mixtures by 3,4,5,6-tetrachloro-1,2-benzoquinone (*ortho*-TCBQ) had already been observed (Dierickx, 1983). Most strikingly, however, the glutathione conjugate inactivated the GST with an increased rate as compared with the parent quinones. This might indicate that these glutathione conjugates have

affinity for the active site, thereby increasing the selectivity of the compound (the so-called "targetting" effect). Indeed, data were obtained which suggested that GSTCBQ reacted active site-directed with rat GST (Van Ommen *et al.* 1988b).

The effect of several structurally related 1,4-benzoquinones and 1,4-naphthoquinones was subsequently studied (Vos *et al.* 1989). For the benzoquinones and the naphthoquinones the extent of inhibition increased with an increasing number of halogen substituents. Neither the type of halogen nor the position of the chlorine-atoms was of major importance. The GST 3-3 was found to be the most sensitive towards a whole series of inhibitors, while the activity of GST 2-2 was least affected (Vos *et al.* 1989). Finally, a series of halogenated glutathione conjugates of 1,4-benzoquinones was studied using GST 1-1. Again with increasing numbers of chlorine substituents, the rate of inhibition greatly increased (Van Ommen *et al.* 1991a). The targetting effect, described as the ratio between the rates of inhibition for a given quinone with and without the glutathione substituent, was largest for the dichlorobenzoquinones. It was suggested that GST 1-1, which possesses two cysteine residues, was inactivated by the modification of one of these cysteine residues, which is probably located near the active site. Compounds with affinity for the active site, like the glutathione conjugates, modify the one located near the active site. This was supported by the observation that complete inactivation of GST 1-1 by 2,5-dichlorobenzoquinone was achieved only after modification of two residues, whereas the corresponding glutathione conjugate already inhibited after the modification of one (Van Ommen *et al.* 1991a). The target amino acid of the conjugate might be cysteine 111, since another irreversible GST inhibitor, S-(4-bromo-2,3-dioxobutyl)glutathione inactivated GST 1-1 by the modification of this cysteine (Katusz *et al.* 1992a).

In this thesis it was shown that TCBQ can be used to inactivate *human* GST in vitro. Further indirect evidence was provided that TCBQ reacted with a cysteine residue, since the GST A2-2 which does not contain cysteines, was the only isoenzyme which did not incorporate TCBQ and thus remained catalytically active. GSTCBQ only unambiguously displayed the targetting effect with GST A1-1. In contrast, GST P1-1 was less effectively inactivated by GSTCBQ. Similar results were obtained using the structurally

related compounds, trichloro-benzoquinone and the glutathione conjugate of 2,5-dichloro-1,4-benzoquinone (Van Ommen *et al.* 1991b). The absence of a targetting effect in the pi-class might be explained by the fact that the GST of the pi-class contains an accessible and reactive cysteine residue (Reinemer *et al.* 1991; Caccuri *et al.* 1992), which reacts more rapidly with the chemically more reactive TCBQ (the pure amino acid cysteine reacts more rapidly with TCBQ than with GSTCBQ). In this thesis it was also demonstrated that despite the effective GST inhibition in vitro, no significant inhibition of the excretion of the glutathione conjugate (DNPSG) by rat hepatoma cells was observed. However, it should be recognized that GSTCBQ and TCBQ are, despite their high reactivity with GST, still very distinct from the ideal so-called suicide inhibitor or trojan-horse (Fersht 1984), which should be chemically unreactive in the absence of the target GST. Nevertheless, because of their high potency of inactivation of GST isoenzymes, future attempts to synthesize irreversible inhibitors may be based on quinone structures. Recently, the three dimensional structure of some GST isoenzymes became available (Reinemer *et al.* 1991; Ji *et al.* 1992), which might facilitate a more rational inhibitor design. In cooperation with Prof.Dr. RN Armstrong (University of Maryland, USA) studies are currently in progress to identify the target site of GSTCBQ on GST 3-3. From the three dimensional structure, target amino acid were identified which might be involved in the binding of GSTCBQ to GST 3-3. These amino acids (Tyr6, Cys86, Cys114, Tyr115, C173) have been replaced by serine or phenylalanine with site-directed mutagenesis. These GST mutants may be helpful in identifying the target site. The studies of Katusz and co-workers (Katusz *et al.* 1991, 1992a, 1992b) with the structurally related compound S-(4-bromo-2,3-dioxobutyl)glutathione clearly demonstrate that the target amino acid might vary (a cysteine, tyrosine and/or tyrosine and cysteine, for GST 1-1, GST 4-4, and GST 3-3, respectively). Thus, in an effort to prepare isoenzyme selective inhibitors, all the isoenzymes may have to be studied individually.

In this thesis, the in vitro *irreversibly* inhibitory potential for GST by catechol derived quinones and the *reversible* inhibitory potential of the parent structures were determined. These compounds might provide a "pro-drug" concept, since their oxidation generates quinones. The

studied catechols, viz. caffeic acid and dopamine and α-methyldopa are relatively non-toxic and thus might be applicable in some cells. Caffeic acid is widely distributed in the plant kingdom, while the catecholamines are present in high concentration in the nervous system. Catecholamines are alo known for their role in melanogenesis. Especially those cells with high concentrations of the GST pi-class and with optimal conditions for quinone formation might be sensitive to irreversible inactivation, since the GST of the pi-class is by far the most sensitive to the inactivation by the quinones derived from both groups of compounds. However, unlike the catecholamines, caffeic acid was also an effective reversible inhibitor of the major GST isoenzymes studied. Both the glutathione conjugate of either caffeic acid and the catecholamines display reversibly inhibitory capacity of all GST isoenzymes studied. This product-inhibition of the enzyme, seems to be a common property of almost any glutathione conjugate studied sofar (Van Bladeren and Van Ommen, 1991).

The pharmacokinetics of these compounds will strongly influence the in vivo inhibition of GST. In this context it was shown that a single oral dose of caffeic acid, gave no major irreversible inhibition of GST in intestinal mucosa, kidney, or liver. The use of radioactive labelled compounds, as well the estimation of the glutathione conjugation with model substrates (to determine the total inhibition by glutathione depletion, reversible and irreversible inhibition) is indicated.

11.2 Ethacrynic acid

Ethacrynic acid is a potent diuretic drug, introduced as therapeutic agent in the middle sixties (Williamson 1977). This compound has recently been studied in a phase I clinical trial, for its application in the treatment of drug resistance of the alkylator phenotype (O'Dwyer *et al.* 1991). Relatively high doses of ethacrynic acid could be administered without toxic effects occurring.

Ethacrynic acid may react spontaneously or GST catalyzed with glutathione. For most isoenzymes the catalytic activity of GST towards ethacrynic acid is not high. This may be compensated by the high concentration of several isoenzymes (Mannervik 1985, Van Ommen *et al.* 1990). From ethacrynic acid, in principle two diastereomeric

glutathione conjugates may be formed, due to the formation of a new optically active center during the conjugation reaction. However, NMR studies indicated that both diastereomers are formed in about equal amounts (unpublished results), indicating that the enzymatic reaction does not proceed with an appreciable stereoselectivity.

In vivo, in the rat during the first 60 minutes after intravenous injection, [^{14}C]ethacrynic acid is rapidly excreted, with 80-90% of the label appearing in bile and the remainder in urine (Klaassen and Fitzgerald 1974, Wallin *et al.* 1978). In bile, the major metabolite is the glutathione conjugate, comprising about 60% of the labelled material (Klaassen and Fitzgerald 1974, Wallin *et al.* 1978). Induction of hepatic GST by phenobarbital increased biliary excretion of the glutathione conjugate (Wallin *et al.* 1978). This supports the view that the enzymatic catalysis is predominant.

Ethacrynic acid has been shown to inhibit rat GST reversibly (Ahokas *et al.* 1985). In this thesis the concentrations of ethacrynic acid resulting in 50% inhibition of the enzymatic activity (I_{50}-values) towards CDNB were determined. Strong inhibition of both human and rat GST isoenzymes of the alpha-, mu-, and pi-class was obtained with ethacrynic acid. The mu-class was most sensitive to the reversible inhibition by ethacrynic acid, as has also been shown by Hansson *et al.* (1991).

The dibromo dihydro derivative of ethacrynic acid showed a similar potential for reversible inhibition as ethacrynic acid itself. This suggests that the α,β-unsaturated ketone moiety is not very critical for the inhibitory action. Presumably, the important structural element in the molecule is the 2,3-dichloro-phenoxyacetic acid moiety. Two compounds with this moiety, tienilic acid and indacrynic acid, have been shown to be strong inhibitors as well (Ahokas *et al.* 1985). On the other hand, the closely related 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and some structural analogs did not efficiently inhibit GST (Vessey and Boyer 1988). Thus, newly developed derivatives of ethacrynic acid should contain this critical element.

The glutathione conjugate of ethacrynic acid reversibly inhibited GST isoenzymes of alpha- and mu-class even more efficiently than the parent compound, especially the human isoenzymes. In human liver cytosol, the glutathione conjugate was also more potent (approximately one order of

magnitude) than ethacrynic acid (Takamatsu and Inaba 1992). The inhibitory effects of the cysteine conjugate and the mercapturate produced by further metabolism of the initial glutathione conjugate of ethacrynic acid, were approximately two orders of magnitude less than the parent ethacrynic acid in human liver cytosol (Takamatsu and Inaba 1992). This suggest that the glutathione moiety is critical for the inhibitory action of the glutathione conjugate. Since ethacrynic acid is very efficiently conjugated with glutathione, this compound will be responsible for the major part of the (reversible) inhibition in vivo.

Already in 1978, it was shown that a small but consistent portion (approximately 1%) of an administered dose of [^{14}C]ethacrynic acid covalently binds to both rat liver and kidney cytosol (Wallin *et al.* 1978). Upon further purification, it was shown that ethacrynic acid specifically binds to rat mu-class GST isoenzyme 3-4 (Yamada and Kaplowitz 1980).

We performed studies to assess the relevance of this covalent binding to the enzymatic activity, which revealed no significant inactivation of GST 3-4 by covalent binding of ethacrynic acid, or for any of the other isoenzymes studied. On the other hand, using slightly more drastic conditions, the catalytic activity of the GST of the pi-class isoenzymes were strongly inactivated by covalent binding. It was shown that the reaction of ethacrynic acid takes place with cysteine 47 of GST P1-1. Thus ethacrynic acid reacts with a cysteine residue, which has been recognized as a highly reactive thiol in the proximity of the catalytic site of GST 7-7 and human GST P1-1 (Tamai *et al.* 1990, Ricci *et al.* 1991). The structural integrity of this cysteine seems to be crucial for maintaining an active-enzyme conformation, despite its none-involvement in either substrate binding or catalysis (Reinemer *et al.* 1992).

The dibromo dihydro derivative of ethacrynic acid was synthesized in an effort to enhance the covalent binding characteristics. Dibromo dihydro ethacrynic acid inactivated pi-class GST more efficiently than the parent compound, while reversible inhibitory capacity of the compound was similar to ethacrynic acid (see above). Thus, this compound combines the properties of a strong lipophilic irreversible and reversible inhibitor, making it a potential tool to inhibit GST in biological systems. This was confirmed in rat hepatoma cells, in which the DNPSG

excretion was more efficiently inhibited by dibromo dihydro derivative than ethacrynic acid itself.

Normally, in *irreversible* inhibition the inhibitor is covalently linked to the enzyme so tightly that its dissociation from the enzyme is very slow. However, the Michael addition of glutathione with the α,β-unsaturated aldehydes and ketones is a well-known reversible reaction (Witz 1989). Via this so-called retro Michael cleavage, the inactivation of the pi-class GST may be reversed by release of ethacrynic acid, when the concentration of free ethacrynic acid decreases (e.g. as a result of transport or conjugation with glutathione). We showed that full restoration of the catalytic activity of GST P1-1 which was inactivated by ethacrynic acid (>90%), could actually occur by prolonged incubation in the presence of glutathione. The velocity of the retro Michael cleavage at room temperature was relatively low. However the rate of this reaction will be increased by the higher temperature in vivo. This regeneration of the unmodified native GST P1-1 enzyme by release of a covalently bound inhibitor may have several implications. Although under normal physiological conditions the inhibition of GST P1-1 by covalent binding of ethacrynic acid would be reversed by glutathione, leaving reversible inhibition by the glutathione conjugate and ethacrynic acid itself as the main mechanism of inhibition, when glutathione levels are low, the covalent inhibition might be predominant resulting in a completely different time course of inhibition.

In conclusion, ethacrynic acid is a particularly interesting inhibitor of GST, since it inhibits all GST *reversibly* and GST of pi-class *irreversibly*. A major part of the inhibition due to covalent binding of ethacrynic acid to GST P1-1 may be reversible in vivo. As also concluded for the quinones, studies using model substrates to assess glutathione conjugation in vivo as well as the use of [^{14}C]ethacrynic acid are clearly indicated.

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Samenvatting

Glutathion S-transferases (GST) vervullen een belangrijke rol in de biotransformatie van een groot aantal reaktieve verbindingen. De GST van rat en mens komen voor als een groep van isoenzymen, die ingedeeld kan worden in 4 klassen: alpha, mu, pi en theta.

De belangrijkste functie van de GST isoenzymen is de katalyse van de conjugatie van electrofiele, hydrofobe verbindingen met het tripeptide glutathion. Deze reaktie kan in het algemeen beschouwd worden als een detoxificatie-stap.

De inhibitie van GST wordt om een aantal redenen bestudeerd. Zo worden remmers van GST gebruikt om het katalytisch mechanisme en de opbouw van het aktieve centrum te bestuderen. Isoenzym selektieve remmers worden eveneens gebruikt om de indeling van GST in verschillende klassen te onderbouwen. Daarnaast kan remming van GST mogelijk ook relevantie hebben voor bepaalde vormen van geneesmiddel-resistentie. Met name de resistentie tegen alkylerende cytostatica (zoals chlorambucil, melphalan e.d.) wordt in verband gebracht met GST. Zo wordt een verhoogde GST expressie waargenomen in tumorcellen resistent tegen deze alkylerende cytostatica. Deze cytostatica blijken geschikte substraten van GST te zijn, waardoor GST deze cytostatica kan inaktiveren.

De studies beschreven in dit proefschrift zijn gericht op de inhibitie van GST van rat en mens. Verschillende benaderingen kunnen gekozen worden voor de ontwikkeling van remmers. Zo bestuderen verschillende groepen onderzoekers de glutathion analoga. In dit proefschrift is de aandacht gericht op de zogenaamde "tweede substraten", de xenobiotica, en dan met name stoffen die GST remmen middels een covalente interaktie. Twee groepen van verbindingen werden bestudeerd: de gehalogeneerde chinonen en gerelateerde catecholen, en etacrynezuur (EA) en derivaten.

In hoofdstuk 1 wordt een overzicht gegeven van de eigenschappen, funkties, expressie en katalytische aspekten

van GST isoenzymen, alsmede de rol van GST in geneesmiddel resistentie. Verder worden enkele theoretische aspekten van reversibele en irreversibele remming, toegespitst op GST, beschreven.

De verbinding tetrachloor-1,4-benzochinon (TCBQ) is in onze werkgroep gebruikt als modelstof om de irreversibele remming van GST te bestuderen. TCBQ bindt aan alle bestudeerde rat GST isoenzymen, waardoor GST geïnaktiveerd wordt. Het glutathionconjugaat van TCBQ (GSTCBQ) blijkt GST nog sneller te inaktiveren dan TCBQ. De affiniteit van het glutathiongedeelte voor het aktieve centrum verhoogt de selektiviteit van de remmer aanzienlijk.

In hoofdstuk 2 wordt beschreven dat TCBQ en GSTCBQ ook *humane* GST isoenzymen kunnen remmen. Vrijwel volledige irreversibele remming van GST van alpha-, mu-, en pi-klassen werd bereikt met tienmaal overmaat chinon. Deze inhibitie ging gepaard met de inbouw van één molecuul TCBQ per GST subunit. Het isoeńzym GST A2-2 bevat als enige geen cysteines, en bleek ook het enige bestudeerde isoenzym te zijn dat niet geremd werd en geen TCBQ inbouwde. Chinonen reageren over het algemeen snel met cysteines. Het lijkt dus aannemelijk dat in de buurt van het aktieve centrum een cysteineresidu aanwezig is.

Bij de humane GST werd alleen voor het alpha-klasse isoenzym A1-1 een aanzienlijk snellere inaktivatie door GSTCBQ waargenomen.

Hoofdstuk 3 handelt over de irreversibele remming van rat GST isoenzymen door chinonen gevormd uit het catechol caffeic acid, alsmede de reversibele remming door caffeic acid zelf. Deze verbinding kan beschouwd worden als een "pro-drug", daar de oxidatie van caffeic acid het chinon genereert. Caffeic acid is relatief niet-giftig en komt voor in vrijwel alle planten in relatief hoge concentraties. Caffeic acid bleek een redelijk goede *reversibele* remmer van GST te zijn. Het glutathionconjugaat van caffeic acid (2-GSCA) was een nog sterkere reversibele remmer. Aan de andere kant, resulteerde alleen de oxidatie van caffeic acid in sterke *irreversibele* remmer van GST, met name van de pi-klasse.

In Hoofdstuk 3 wordt ook het effekt van een eenmalige orale dosis van caffeic acid op de *irreversibele* remming van de GST aktiviteit in lever, nier en darmmucosa beschreven. Alléén in de lever werd een marginale irreversibele remming waargenomen. Geadviseerd werd om in toekomstige studies vooral aandacht te schenken aan

reversibele remming door caffeic acid en zijn glutathionconjugaat.

Hoofdstuk 4 behandelt de remming van humane GST door de catecholamines dopamine en methyldopa. Ook oxidatie van catecholamines leidt tot de vorming van reaktieve chinonen. Catecholamines spelen een rol als neurotransmitters in het zenuwstelsel, maar zijn ook bekend vanwege hun rol in de pigmentvorming in de huid. Methyldopa wordt ook toegepast als anti-hypertensium. De catecholamines zelf bleken GST niet reversibel te remmen, terwijl hun glutathion conjugaten wel over reversibele remmingseigenschappen bleken te beschikken. De chinonen gevormd uit de catecholamines remde het GST van de pi-klasse (P1-1) sterk *irreversibel*. Ook het glutathionconjugaat van dopamine (5-GSDA) bleek dit isoenzym (in mindere mate) irreversibel te remmen. Aanwijzingen werden verkregen dat de remming van GST P1-1 door dopamine deels het gevolg was van disulfide vorming in het enzym, terwijl de remming door 5-GSDA volledig het gevolg was van de vorming van disulfides.

Hoofdstuk 5 behandelt de effekten van niet-giftige concentraties TCBQ en GSTCBQ (waarvan de glycine carboxylgroep veresterd was om de absorptie te verbeteren) op de uitscheiding in het medium van het intracellulair gevormde glutathionconjugaat van 1-chloro-2,4-dinitrobenzeen (DNPSG) bij rat hepatoma cellen. In deze cellen werd geen significante reductie van deze uitscheiding gevonden en geen effekt op de GST aktiviteit in het supernatant. Dus TCBQ en zijn afgeleide glutathionconjugaat zijn niet geschikt om GST te remmen in celsystemen.

Hoofdstuk 6 vat kort alle studies met de chinonen samen.

De studies beschreven in hoofdstuk 7, 8 en 9 handelen over de remming van GST door etacrynezuur (EA). Sinds de zestiger jaren wordt EA als diureticum toegepast. In 1985, werd aangetoond dat EA over goede remmingskarakteristieken van rat GST beschikt. Bovendien kan EA in relatief hoge dosis aan mensen worden toegediend.

In de literatuur bestond enige discussie over de aard van de remming: zowel reversibele remming als covalente binding aan rat GST 3-4 was beschreven. In hoofdstuk 7 werd de aard van de remming onderzocht. Er werd geen significante remming van GST door EA opgespoord onder condities waarbij TCBQ de GST isoenzymen irreversibel remde. Vastgesteld werd dat EA vooral een sterke *reversibele* remmer van rat en humane GST is. Het

glutathionconjugaat van EA (GSEA) bleek de alpha- en mu-klasse GST zelfs nog sterker reversibel te remmen. GSEA blijkt een van de meest potente reversibele remmers van GST te zijn.

Door gebruik te maken van meer drastische omstandigheden (hogere temperatuur, langere incubatietijd) werd in hoofdstuk 8 vastgesteld dat selektieve *irreversibele* remming van GST van de pi-klasse door EA toch kan optreden. Ca. 0.8 nmol EA per nmol humaan of rat GST van de pi-klasse kon worden ingebouwd, hetgeen gepaard ging met 65-95% remming.

Hoofdstuk 8 handelt ook over de remmingseigenschappen van een gesynthetiseerd dibromo dihydro EA derivaat. Dit derivaat bleek de GST pi-klasse zeer efficiënt irreversibel te remmen, terwijl bovendien ook de mu-klasse geremd werd.

De chemische reactie betrokken in de conjugatie van EA en aanverwante α,β-onverzadigde ketonen, een Michael additie, is in principe reversibel. Daarom werd de reversibiliteit van de conjugatie reactie van EA met glutathion en GST P1-1 bestudeerd (hoofdstuk 9). Door lange tijd te incuberen (tot 150 uur), werd aangetoond dat EA gebonden aan zowel glutathion als GST P1-1, volledig kan overstappen, op een overmaat van respectievelijk N-acetyl-L-cysteine of glutathion. Afhankelijk van de concentratie van de stoffen, kunnen transfer reacties in beide richtingen optreden.

Middels Edman degradatie van het peptide waaraan EA gebonden was, werd het cysteine residu waarmee EA reageert, geïdentificeerd (Cysteine 47).

De effekten van EA (en zijn dibromo dihydro derivaat) op de uitscheiding van DNPSG in rat hepatoma cellen, werd beschreven in hoofdstuk 5. EA en dibromo dihydro derivaat bleken deze uitscheiding effectief te remmen, met een maximum van 30% en 50% reductie voor respectievelijk EA en zijn derivaat. Aanwijzingen werden verkregen dat deze remming middels reversibele GST remming tot stand komt.

Hoofdstuk 10 vat kort alle experimenten met EA samen. In hoofdstuk 11 worden de resultaten met de chinonen en EA bediscussieerd en vergeleken met andere studies. Geconcludeerd wordt dat de gehalogeneerde chinonen vanwege hun goede remmingseigenschappen van humane GST gebruikt kunnen worden als basisstof voor de ontwikkelingen van een in vivo werkzame suicide-inhibitor. Bij de chinonen zullen toekomstige studies o.a. gericht zijn op het vaststellen van het aminozuur van GST waarmee GSTCBQ reageert, om

zodoende een op chinonen gebaseerde rationele drug-design te vereenvoudigen. De toepasbaarheid van irreversibele remming van GST van de pi-klasse in biologische systemen door de catecholen, kan onderzocht worden in cellen rijk aan dit GST en/of met optimale condities voor chinonvorming (zoals b.v. in melanoma cellen). Op grond van de bepaalde remmingsconstantes kan verwacht worden dat caffeic acid en zijn glutathionconjugaat ook geschikt zijn om GST reversibel te remmen in vivo. Voor EA kan geconcludeerd worden dat het een belangwekkende remmer van GST is, omdat het reversibel alle GST remt en irreversibel GST van de pi-klasse. De reversibele remming kan in vivo nog vergroot worden door de vorming van het glutathionconjugaat van EA. Een belangrijk gedeelte van de inhibitie als gevolg van covalente binding van EA aan GST P1-1, kan in vivo reversibel zijn. Het gebruik van radioaktief gelabelde stoffen van de catecholen, chinonen en/of EA wordt aanbevolen om de remming in vivo te onderzoeken.

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Curriculum vitae

Jan-Peter Ploemen werd op 1 november 1963 te Beek (L) geboren. De middelbare school periode werd doorlopen op de Scholengemeenschap "groenewald" te Stein, waarop in 1982 het diploma Atheneum B werd behaald. In dat zelfde jaar werd begonnen met de studie Humane Voeding aan de Landbouwuniversiteit Wageningen. Als specialisatie-richting binnen de studie werd de oriëntatie Toxicologie gekozen. Tijdens de doctoraalfase werden de hoofdvakken Humane Voeding (Prof.Dr. M.B. Katan), Toxicologie (Prof.Dr. J.H. Koeman), en Biochemie (Prof.Dr. F. Müller) afgelegd. Een stage Toxicologie (Dr. J.W.G.M. Wilmer) werd afgelegd op de afdeling Biologische Toxicologie van het TNO Instituut voor Toxicologie (TNO-ITV) te Zeist. Het doctoraalexamen werd in juni 1988 afgelegd. Aansluitend werd de militaire dienst vervuld.

Op 1 september 1989 trad hij in dienst als onderzoeker in opleiding op een project gefinancieerd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, gebied Medische Wetenschappen), bij de afdeling Biologische Toxicologie van TNO-ITV, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd onder leiding van Prof.Dr. P.J. van Bladeren. Vanaf september 1993 is hij aldaar werkzaam als onderzoeker op een Stichting Technisch Wetenschappen gefinancieerd project onder leiding van Prof.Dr. P.J. van Bladeren en Prof.Dr. N.P.E. Vermeulen.

Nawoord

En eindelijk kun je dan het laatste gedeelte schrijven. Vrijwel de laatste produktieve handeling leidt tot de waarschijnlijk meest gelezen pagina's van dit proefschrift! Op de achtergrond hoor ik de muziek van MTV, en in het bijzonder één regel valt me op: "...was it worth it?..." (Petshop Boys). Ja, het was het waard!

Aan dit proefschrift heeft een aantal mensen een cruciale bijdrage geleverd.

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Bill Johnson, working together was not only very productive, but also big fun. Dear Bill, moreover the barbaric Dutch changed his view on politics much more than he admitted to the best race-biking republican!

Sonja Jespersen (afdeling Structuuropheldering) much progress was made by the great job you did in the analysis of the peptides.

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Abbreviations

CDNB:	1-chloro-2,4-dinitrobenzene
DNPSG:	S-(2,4-dinitrophenyl)glutathione
2-GSCA:	2-S-glutathionylcaffeic acid
5-GSDA:	5-S-glutathionyldopamine
GS(ethyl)TCBQ:	2-S-[(γ-L-glutamyl-L-cysteinyl(ethyl glycinate)]-3,4,5-trichloro-1,4-benzoquinone
GSH:	glutathione
5-GSMDOPA:	5-S-glutathionyl-α-methyldopa
GST:	glutathione S-transferases
GSTCBQ:	2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone
TCBQ:	tetrachloro-1,4-benzoquinone
TCHQ:	tetrachloro-1,4-hydroquinone
TFA:	trifluoroacetic acid