GLUTATHIONE S-TRANSFERASE ISOENZYMES IN RELATION TO THEIR ROLE IN DETOXIFICATION OF XENOBIOTICS

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Proefschrift
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STELLINGEN

1 Chinonen vormen een aantrekkelijke klasse van verbindingen als uitgangspunt voor de ontwikkeling van een selectieve en irreversibele in vivo remmer van de glutathion S-transferases.
- Dit proefschrift

2 Het genetisch polymorfisme van humane glutathion S-transferases behorende tot de klasse mu is van complexere aard dan in het verleden is aangenomen.
- Strange et al. An. Hum. Genet. 48 (1984) 11-20
- Carmichael et al. Carcinogenesis 9 (1988) 1617-1621
- Dit proefschrift

3 Voor een juiste interpretatie van de fysiologische en toxicologische consequenties van het mu-phenotype is de ontwikkeling van technieken die specifiek de individuele mu-klasse glutathion S-transferase isoenzymen kunnen onderscheiden van groot belang.

4 Glutathion S-transferase isoenzym 7-7 is een betere tumormarker voor de lever van de rat dan γ-glutamyltranspeptidase.
- Tatematsu et al. Carcinogenesis 9 (1988) 215-220

5 Het toepassen van diagnostische tests gebaseerd op glutathion S-transferase π als tumormarker is alleen zinvol in weefsels waarin dit isoenzym onder normale omstandigheden in zeer lage concentraties voorkomt.

6 De poging van Kilpikari en Savolainen om de glutathion S-transferase activiteit in erythrocyten te correleren aan blootstelling aan toxische stoffen bij de verwerking van rubber, gaat voorbij aan de verschillen in glutathion S-transferase isoenzympatronen tussen erythrocyten en de belangrijkste metaboliserende organen.
- Kilpikari, I. & Savolainen, H. Int. Arch. Occup. Environ. Health 53 (1984) 299-302

7 De conclusie van Singh et al. dat de basische humane glutathion S-transferases heterodimeren zijn van subunits met verschillende molecuulmassa's is onjuist.
- Singh et al. Biochem. J. 232 (1985) 781-790

8 Classificatie van isoenzymen van verschillende species op basis van aminozuursequentie homologie vormt de enige hanteerbare wijze van indeling.

9 Bij het bepalen van de lekkage van lactaatdehydrogenase als vitaliteitsparameter voor een celcultuur welke is blootgesteld aan een toxische verbinding, wordt in het algemeen te weinig rekening gehouden met directe of indirecte effecten van de toxische stof op de activiteit van dit enzym.

10 Het enthousiasme waarmee de hypothese wordt gepropageerd dat de activatie van het enzym protein kinase C een rol zou spelen in de werking van 12-O-tetradecanoylphorbol-13-acetaat (TPA) als tumorpromoter is niet terecht, daar experimenten die deze hypothese zouden kunnen voorzien van een mechanistische basis ontbreken.
- Castagna, M. Biol. Cell 59 (1987) 3-14
- Aylsworth, C. et al. Cell Biol. Toxicol. 5 (1989) 27-37

11 In tegenstelling tot hetgeen Poland en Glover beweren, is de toxiciteit van polygechloreerde bifenylen en dioxines vermoedelijk niet rechtstreeks gerelateerd aan hun vermogen tot binding van de Ah-receptor.
- Poland, A. & Glover, E. Mol. Pharmacol. 17 (1980) 86-94
- McKinney, J.D. et al. J. Med. Chem. 28 (1985) 375-381
- Brouwer, A. et al. Toxicol. Appl. Pharmacol. 78 (1985) 180-189

12 Een belegger in aandelen heeft verschillende opties.

Stellingen behorende bij het proefschrift "Glutathione S-transferase isoenzymes in relation to their role in detoxification of xenobiotics." Ria M.E. Vos. Wageningen, 24 mei 1989.

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CHAPTER 1

INTRODUCTION

1.1
GLUTATHIONE S-TRANSFERASES: FUNCTIONS AND CLASSIFICATION OF ISOENZYMES

General

In the course of evolution, organisms have developed several enzyme systems to deal with the toxic challenges exerted by an increasing number of xenobiotics. These enzyme systems may be divided into phase I and phase II enzyme systems, involved in transformation (i.e. oxidation, reduction, hydrolysis) and conjugation reactions respectively. Their concerted action generally increases the hydrophilic character of the compound involved, and hence facilitates its excretion from the body. Biotransformation may lead to detoxification as well as to activation of a compound.

The studies described in this thesis are concerned with the most important phase II enzyme system in the detoxification of electrophilic alkylating agents, the glutathione S-transferases (GST) (EC 2.5.1.18). Epoxide hydrolase is the second enzyme involved in the detoxification of electrophylic species, but in contrast to the glutathione S-transferases, which demonstrate a broad substrate selectivity, its action is confined to epoxides as substrates. The existence of the glutathione S-transferases was first recognized in 1961[1]. Since then, these enzymes have been the subject of numerous investigations and it has become clear that their catalytic activity serves a major role in the biotransformation of many reactive compounds.

Most studies so far, have focussed on the glutathione S-transferases from the rat. Data on transferases from other species, in particular those of human origin, are, however, accumulating. Several isoenzymes have been characterized thoroughly in recent years, and their number is still growing. The considerable interest in this group of isoenzymes is reflected in the organisation of an international workshop dedicated to the relationship between glutathione S-transferases and carcinogenesis (Dublin, 1986).

Functions

The main function of the glutathione S-transferases is the catalysis of the conjugation of electrophilic, hydrophobic compounds with the tri-

peptide glutathione (GSH). As a family of isoenzymes, this enzyme system is capable of handling a large variety of electrophilic compounds[2,3]. Several classes of potential substrates are listed in table 1. In general, the catalysis involves a nucleophilic attack of GSH on an electrophilic carbon resulting in the substitution of, for example, a halogen atom. In some cases, e.g. α, β-unsaturated compounds and several quinones, GSH may react via a Michael-addition. Substitution at elements other than carbon occurs in the reaction of GSH with organic nitrates and organic thiocyanates[4].

Table 1. Some classes of substrates for glutathione S-transferases[a].

compound class	example	leaving group
alkyl halides	iodomethane	I^-
aralkyl halides	p-nitrobenzyl chloride	Cl^-
allyl halides	3-chloro-propene	Cl^-
nitroalkanes	1-nitrobutane	NO_2^-
halogenonitrobenzenes	1,2-dichloro-4-nitro-benzene	Cl^-
	2,3,5,6-tetrachloro-nitrobenzene	NO_2^-
epoxides	benzo(a)pyrene-4,5-oxide	-
organophosphorus triesters	OSS-trimethyl phosphoro-dithioate	O-dealkylated organophosphate
phenoltetrabromophthaleins	sulfobromophthalein	Br^-
quinones	2-methyl-1,4-naphtho-quinone	-
α,β-unsaturated compounds	ethacrynic acid	-
organic hydroperoxides	cumene hydroperoxide	RO^-
alkyl nitrates	glyceryl trinitrate	NO_2^-
organic thiocyanates	octyl thiocyanate	CN^-
organic isothiocyanates	benzyl isothiocyanate	-

[a] Adapted from Chasseaud[2,3].

The glutathione conjugates thus formed may be excreted into the bile. Alternatively, they may be transported to the kidney, where they are transformed through the subsequent actions of the enzymes γ-glutamyl-transpeptidase, cysteinyl glycine dipeptidase and N-acetyltransferase to their corresponding mercapturic acids, which are excreted into the urine[5].

Although conjugation with GSH can generally be regarded as a detoxification pathway, several compounds may be activated through this reaction. As was indicated in a recent review[6], glutathione conjugates possessing toxic properties may be divided into three groups: i) the conjugate may be more reactive than the parent compound. A well-known example of this kind is 1,2-dibromoethane. The conjugation of this compound with GSH leads to the formation of a reactive thiiranium ion[7]; ii) the conjugate may be metabolized to toxic intermediates, e.g. the conjugate of hexachlorobutadiene, which is activated in the kidney by the action of β-lyase, resulting in the formation of a reactive thiol-compound[8]; iii) the conjugation may be reversible: the parent compound is released, when conditions are favourable to do so. Examples are the conjugates formed between GSH and isothiocyanates[9,10].

Besides the formation of toxic glutathione conjugates, it should also be mentioned, that activation may result from the release of a toxic metabolite. OSS-trimethyl phosphorodithioate, for instance, reacts with GSH to give the toxic compound SS-dimethyl phosphorodithioate in addition to the conjugate S-methylglutathione[11].

Apart from the catalysis of conjugation with GSH, the glutathione S-transferases are involved in several other important functions. Some of the GST isoenzymes possess Δ^5-3-ketosteroid-isomerase activity, i.e. catalyze the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids indicating a role for the enzymes in steroid metabolism[12,13]. The transferases are also involved in prostaglandin metabolism, which is shown, for instance, by their catalysis of the conversion of prostaglandin H_2 to prostaglandins E_2 and D_2[14]. The fact that glutathione S-transferases are capable of catalyzing the reaction of GSH with organic hydroperoxides (non-Se-peroxidase activity) and with several epoxides, suggests a protective function for this group of enzymes against the potential hazardous effects of oxidative metabolism[15,16].

A number of non-substrate ligands, including several steroids, bilirubin and bile acids, are bound non-covalently by the glutathione S-transferases[17,18,19]. In this respect, glutathione S-transferases may serve a role as intracellular binding and transport proteins, analogous to the transport function of albumin in blood. The covalent binding of electrophilic compounds by glutathione S-transferases has also been reported[19,20]. The abundance of the proteins in liver is presumably of relevance in this type of binding. This property of the glutathione S-transferases may therefore be relatively non-specific, extending to other cellular proteins.

Classification of isoenzymes

The glutathione S-transferase isoenzymes each consist of two subunits. Most isoenzymes are present in the cytosol, although a microsomal form has also been identified[21]. The cytosolic transferases from rat, man, and mouse have been divided into 3 classes, alpha, mu and pi, based on similar structural and catalytic properties of their subunits[22]. Individual isoenzymes belonging to these classes are given in table 2. In the rat, 8 subunits have been characterized, which are denoted by arabic numerals, according to the nomenclature described by Jakoby et al.[23]. These subunits give rise to 8 homodimeric and 4 heterodimeric forms, named by their respective subunit compositions[24,25].

The human glutathione S-transferases, have so far been designated by Greek letters. A number of basic enzymes have been described ($\alpha,\beta,\gamma,\delta,\varepsilon$) in addition to a near-neutral form (μ) and several acidic proteins (π,ρ,λ)[24,26,27,28,29,30]. It was recently found by Stockman et al.[31] that the basic isoenzymes α-ε are formed by binary combinations of 2 immunologically and catalytically distinct subunits, B_1 and B_2. The acidic transferase from erythrocytes, isoenzyme ρ, is probably identical to GST π from placenta[32]. Acidic transferases from other human tissues may also correspond to transferase π[24].

The nomenclature used for mouse GST isoenzymes has been described by Warholm et al.[33]. Similar to the nomenclature for the rat, it is based on subunit composition. A letter is added to indicate the strain from which the isoenzyme was isolated[33,34,35]. Although Cl-1, Dl-1 and Nl-1 are presumably the same protein, the existence of possible strain

Table 2. Classification of cytosolic glutathione S-transferases from rat, man and mouse.[a]

species	class		
	alpha	mu	pi
rat[b]	1-1	3-3	7-7
	1-2	3-4	
	2-2	4-4	
	8-8	3-6	
		4-6	
		6-6	
man[c]	α, β, γ (B2B2)	μ	π
	δ (B1B2)	ψ	
	ε (B1B1)		
	skin "9.9"		
mouse[d]	N4-4	N1-1	N3-3
		C1-1	
		C2-2	
		D1-1	

[a] Based on data from ref. 24.

[b] Rat isoenzyme 5-5 has not yet been classified.

[c] Other acidic enzymes found in human tissues are very closely related and possibly identical to isoenzyme π and are therefore considered pi-class enzymes[32].

[d] N: NMRI mouse strain; N4-4, N1-1 and N3-3 were formerly refered to as MI, MIII and MII respectively[33]. C: CD1 mouse strain; C1-1 and C2-2 were previously named GT8.7 and GT9.3 respectively[34]. D1-1 was called F3 and originates from DBA/2J mice[35].

variants has not been excluded[24].

The structural basis for the classification has been derived from the determination of immunological cross reactivities, NH_2-terminal amino acid sequence analysis and sequencing of cDNA clones. The structural homology of transferases belonging to the same class but obtained from different species, is generally greater than the structural homology between different classes of transferases from the same species.

cDNA sequences for the two alpha-class subunits from human GST's were found to be approx. 80 % identical base-for-base to the rat cDNA's for subunits 1 and 2[36,37]. Similarly, the amino acid sequence homology is 68 % and 79 % for subunits 1 and 2 and subunits 3 and 4 respectively, whereas only approx. 25 % protein sequence identity is shared by subunits 1/2 as compared with subunits 3/4[38,39,40].

Some enzymatic properties of the rat isoenzymes are listed in table 3. The isoenzymes demonstrate different but overlapping substrate selectivities, which is also the case for mouse and human isoenzymes. Some substrates may be used for the classification of transferases. Thus class alpha isoenzymes generally exhibit high peroxidase activity, whereas class pi isoenzymes are highly active towards ethacrynic acid, although in the rat, alpha class isoenzyme 8-8 has a higher specific activity with this compound than pi class isoenzyme 7-7. Trans-stilbene oxide has been found a useful compound for the classification of class mu transferases[24].

It should be noted that rat GST isoenzyme 5-5 has not yet been classified. This isoenzyme has not been studied thoroughly, presumably because it is not bound to the S-hexylglutathione affinity column generally used for purification of the glutathione S-transferases. Furthermore, the enzyme is rather labile[41].

The structural properties of isoenzyme 5-5 differ from those of class alpha and class mu isoenzymes, since no cross-reactivity was noticed with antibodies raised against rat transferases 1-2 and 3-4[42]. The isoenzyme is further characterized by an extremely low activity towards the standard substrate 1-chloro-2,4-dinitrobenzene (CDNB) (< 0.15 units/mg), and a high activity with the epoxide 1,2-epoxy-3-(p-nitrophenoxy)propane, in addition to its being the most active transferase known, possessing peroxidase activity with organic hydroperoxides[43,44].

Table 3. Specific activities (μmol/min/mg protein) of rat glutathione transferases.[a]

	class	alpha			mu			pi
substrate	enzyme	1-1	2-2	8-8	3-3	4-4	6-6	7-7
1-chloro-2,4-dinitrobenzene		50	17	10	58	17	190	24
1,2-dichloro-4-nitrobenzene		<0.04	<0.04	0.12	5.3	0.18	2.85	0.048
bromosulfophthalein		<0.01	<0.01	-	0.94	0.04	-	0.01
ethacrynic acid		0.08	1.24	7.0	0.08	0.62	0.057	3.84
trans-4-phenyl-3-buten-2-one		<0.004	<0.004	0.10	0.05	1.18	0.019	0.22
4-hydroxynonenal		2.6	0.67	170	2.7	6.9	-	-
leukotriene A_4		0.002	0.0005	-	0.002	0.077	-	-
1,2-epoxy-3-(p-nitrophenoxy)propane		<0.1	<0.1	-	0.53	1.37	-	-
trans-stilbene oxide		0.001	0.003	0.033	0.10	2.0	0.13	0.005
benzo(a)pyrene-7,8-diol-9,10-oxide		-	0.006	0.18	0.012	0.68	-	5.5
cumene hydroperoxide		3.1	7.9	1.10	0.35	0.72	0.19	0.048
H_2O_2		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Δ^5-androstene-3,17-dione		4.2	0.36	-	0.02	0.002	-	-
p-nitrophenyl acetate		0.79	0.20	-	1.01	0.28	0.19	-

[a]Adapted from ref. 24.

1.2
TOXICOLOGICAL IMPLICATIONS OF GLUTATHIONE S-TRANSFERASE ISOENZYME PATTERNS

General

The presence of a large number of isoenzymes with a differential, overlapping, substrate selectivity, affords the organism the possibility to detoxify a wide range of reactive xenobiotics, by catalyzing their conjugation with GSH, or by "trapping" them through non-covalent or covalent binding. The extent to which detoxification and/or activation will occur in a certain tissue, and thereby its sensitivity towards hazardous effects of electrophilic compounds, clearly depends on the number and amount of specific isoenzymes present, i.e. the actual isoenzyme pattern. Besides substrate selectivity, important factors to be considered in this respect are tissue distribution, developmental patterns with age, hormonal influences, sensitivity to inhibitors and inducibility of individual isoenzymes.

Tissue distribution

The tissue distribution of glutathione S-transferases has received considerable attention over the past few years. In the rat, each subunit seems to be present in almost every organ, however, the level of expression differs significantly from tissue to tissue (table 4). The highest amount of total GST protein is present in the liver (3-5 % of total soluble protein). In this tissue, GST activity is mainly accounted for by isoenzymes containing subunits 1, 2, 3 and 4. Trace amounts of subunit 7 and minor amounts of subunits 6 and 8 have been reported[45]. In rat kidney, the expression of subunit 3 is extremely low, whereas differential levels of expression of subunit 4 have been noticed, possibly dependent on the strain of the rat[45,46,47,48,49]. Subunit 7 is present in larger amounts in kidney than in liver.

Subunit 1 seems to be virtually lacking from rat lung, on the other hand considerable amounts of subunits 7 and 8 are present. The concentration of 8-8 seems to be highest in this organ, which is of interest in view of its high activity with 4-hydroxy-non-2-enal, a peroxidative degradation product of polyunsaturated fatty acids[45,50,51].

Table 4. Tissue distribution of rat glutathione S-transferase subunits.[a]

tissue	subunits						
	1	2	3	4	6	7	8
liver (4)[b]	++	++	++	++	±	-	±
kidney (1.2)	++	++	-	±	-	++	±
lung (0.9)	-	++	++	++	±	++	++
intestine (1.6)	++	±	±	++	-	++	+
heart (0.4)	-	-	-	++	+	±	-

[a] Based on data from ref. 45-50, 52-54. Data on isoenzyme 5-5 are limited and have not been included. ++ : major component; + : moderate amounts; ± : minor amounts; - : traces or not detectable.

[b] Approx. percentage of cytosolic protein bound to (S-hexylglutathione and glutathione) affinity matrix[45].

Table 5. Tissue distribution of glutathione S-transferase classes alpha, mu and pi in man.[a]

tissue	class		
	alpha	mu[b]	pi
liver	++	+/-	-
kidney	++	+/-	++
lung	±	+/-	++
intestine	++	?	+
adrenals	++	+/-	-
stomach	±	+/-	++
spleen	±	+/-	++
erythrocytes	-	-	++

[a] Based on data from ref. 28, 29, 60, 62, 63, 65-68.

++ : major component; + : moderate amounts;

± : minor component; - : traces or absent; ? : not known

[b] Mu-class isoenzymes are absent in approx. 40-50 % of the individuals.

In rat small intestine, 7-7 is a major enzyme form, accounting for approx. 40 % of total cytosolic activity. Subunits 1 and 4 are also major components, whereas subunits 2 and 3 are present in minor amounts[52].

Rat heart lacks the majority of basic isoenzymes such as 1-1 and 2-2. The predominant part of GST activity in heart (± 80 %) is accounted for by near-neutral and acidic proteins. Isoenzyme 4-4 is a major form and moderate amounts of subunit 6 are present[45,53,54]. When present, subunit 6 is usually expressed at low levels. However, an exception is rat testis. In this organ approx. 50 % of activity is represented by an acidic isoenzyme (pI 5.9) which has been identified as isoenzyme 6-6[42,55,56].

Little is known on the tissue distribution of isoenzyme 5-5. It seems to be present in liver, kidney, lung and brain[41,46,50,57]. However, no quantitative data have been reported.

Besides large variability in tissue distribution, GST isoenzymes may also demonstrate differential localization within a certain tissue and even within cells. Thus, in liver, highest activity and immunohistochemically stainable protein with antibodies raised against isoenzymes 1-2, 3-4 and 5-5, have been reported in the centrilobular region[58,59]. Anti 3-4 and anti 5-5 were found to produce intense staining within bile duct epithelium, whereas 1-2 is not apparent within the bile duct. Isoenzyme 5-5 is present in parenchymal cell nuclei. Anti 1-2 and anti 3-4 on the other hand caused perinuclear staining[59].

The considerable polymorphism demonstrated by alpha-class and mu-class glutathione S-transferases has complicated studies on the tissue distribution in humans (table 5). With the exception of liver and adrenal gland, pi-class isoenzymes have been found in considerable amounts in virtually every tissue investigated. They are major components in human lung, stomach, spleen, placenta, erythrocytes, lymphocytes, platelets and kidney[28,30,42,60,61,62,63,64].

Mu-class isoenzymes are not expressed in 40-50 % of the livers examined[65]. When present however, expression has also been noticed in a number of other tissues, e.g. brain, lung, heart, spleen, kidney, stomach and lymphocytes[60,64,66].

Alpha-class isoenzymes are major components in liver, adrenal gland,

kidney and small intestine[26,63,67,68].

Patterns of alpha-class isoenzymes differ with the individual analyzed. In addition, the occurrence of this polymorphism is not a constant individual characteristic, but may vary from tissue to tissue[60].

The reason for the extensive differences noticed for GST isoenzyme patterns in different tissues is not clear, but may partly reflect adaptations to specific functions of these tissues. This is possibly also the case for the developmental patterns observed in several tissues. Most studies of this kind have been performed on human tissues.

Developmental patterns

In foetal kidneys, brain and intestine a pi-class isoenzyme has been observed as a representative of the glutathione S-transferases[69]. Since the adult pattern in these tissues is somewhat more complex, several changes must occur with age. Thus, in kidney, the pi-class isoenzyme is constantly expressed at a high level until approx. 40 weeks post-natal age, after which a relative decline is noticed, in favour of the development of alpha-class transferases[68].

Foetal liver and adrenal gland contain a major alpha-class isoenzyme in addition to the pi-class transferase[68,70]. For foetal liver, the relative contribution of the pi-class enzyme to total GST activity declines rapidly during gestation, so that at birth, this isoenzyme is only expressed weakly. Little change occurs in the relative proportions in adrenal gland[68]. Interestingly, only one type of alpha-class isoenzyme has been found in foetal liver, whereas several isoenzymes are expressed in adults[71].

The near-neutral glutathione S-transferases are generally not detected in foetal tissues until approx. 30 weeks of gestation[72]. The levels of these isoenzymes are variable in adrenal gland, kidney and spleen, and no developmental patterns have been observed for these tissues[68].

It should be stressed that the descriptions of isoenzyme patterns above, are based on relative proportions of total GST activity. This activity is also subject to developmental changes. Although more than 90 % of GST activity is accounted for by a pi-class enzyme in both foetal and adult lung, the specific activity in this tissue declines

during gestation from approx. 2 units/mg protein at 10 weeks gestation to approx. 0.5 units/mg at birth; this value does not demonstrate further changes until at least 2 years post-natal age[73]. Similarly, the specific activity in liver changes from approx. 0.8 units/mg protein during foetal life to approx. 1.3 units/mg in adults[27,74,75,76].

Hormonal influences

Some evidence exists that GST isoenzyme patterns may be subject to hormonal influences. Sex differences have been noticed both in the rat and in the mouse, for which hormonal differences may be responsible[77,78]. Both male rat and male mouse demonstrate higher specific activities in the liver than their female counterparts. Whereas male rat liver contains higher levels of subunits 3 and 4, female rat liver shows a preferential expression of subunits 1 and 2[77]. In male mouse, high levels of a pi-class subunit have been found in the liver, which only constitutes a minor form in females[78].

Beckett et al.[79] have demonstrated that surgical thyroidectomy in the rat results in a 30 % increase in isoenzyme 1-1. Prolonged oral administration of thyroxine or triiodothyronine causes a decline in GST activity in rat liver, which is at least partly due to a decrease in the levels of expression of isoenzymes 1-1, 2-2 and 3-3[79]. Drastic changes in isoenzyme pattern have also been noticed in the liver of female mice, following intraperitoneal injection of triiodothyronine[80].

Induction

The factors involved in the sensitivity towards alkylating agents discussed so far, are more or less determined on a genetic basis. A given isoenzyme pattern may, however, also be subject to modulation by external factors. Thus, the activity of a set of isoenzymes may be modified by the presence of specific inhibitors or the level of expression of each of these isoenzymes may be changed as a result of exposure to an inducing agent.

Early reports on the induction of glutathione S-transferases focussed on changes in total GST activity, using a number of different substrates thought to be representative for the activities of individual isoenzymes, which were, at that time, called S-alkyltransferase, S-aryl-

transferase, S-aralkyltransferase and S-epoxide transferase[81]. The recognition of the existence of a number of isoenzymes with overlapping substrate specificities gave rise to studies giving attention to the induction of the subunits constituting these isoenzymes.

The induction of individual isoenzymes in rat liver has been studied extensively for phenobarbital and 3-methylcholanthrene[38,40,82,83,84,85,86,87]. Both compounds cause an elevation of the relative amounts of subunits 1 and 3, which is reflected in a relative increase of the isoenzymes containing those subunits[38,82,83]. The increase in protein results from an increase in translationally active mRNAs, which, in turn, reflects at least partly a stimulation of the transcription of the corresponding genes[38,40,85,87]. Although the extent of transcriptional activation seems sufficient to account for the increases in mRNA and protein, an increase in translational efficiency of mRNA and/or a decrease of turnover rates of mRNA and protein have not been excluded.

It is not known by which mechanism the transcription is activated, whether e.g. specific receptors are involved or whether the genes possess a common regulatory region important in the sensitivity towards xenobiotics. Interesting in this context, however, is the observation that induction by simultaneous administration of a number of xenobiotics is more or less additive and greater than the increase caused by either of the substances alone[84,88]. This suggests the possibility of the existence of more than one mechanism of transcriptional activation.

Besides phenobarbital and 3-methylcholanthrene, some other compounds, e.g. trans-stilbene oxide and hexachlorobenzene have also been found to cause a relative increase in the levels of hepatic isoenzymes containing subunits 1 and 3[84,89,90].

Induction of glutathione S-transferase activity in extrahepatic tissues has been reported for a number of antioxidants (small intestine, lung)[88,91], 3-methylcholanthrene (lung, small intestine, kidney)[88,92], and phenobarbital (small intestine, testis)[92,93]. Little data are, however, available on effects on subunit composition in extrahepatic tissues. In rat testis, subunit 4 is selectively induced by phenobarbital[93]. Trakshel et al.[94] found that cis-platinum treatment causes a significant alteration in subunit composition of the isoenzyme profile in rat kidney. Subunits 3,4 and 8 showed a relative increase compared

to subunits 1 and 2. Little change was noticed for subunits 6 and 7. However, it is not clear from their report, whether these changes in the isoenzyme pattern were accompanied by an increase in total GST activity.

Inhibition

An extensive list of inhibitors of GST activity was recently published by Mannervik and Danielson[24]. A shortened version of this list, containing some additional classes of compounds, is given in table 6[95-121].

Most of the studies on inhibition have been performed on a mixture of purified isoenzymes or on cytosol. However, data on inhibition of individual isoenzymes are accumulating. Some of the known data have been adapted to give table 7.

The inhibitors triphenyltin chloride, bromosulfophthalein, cibacron blue and hematin are generally used in the classification of isoenzymes as belonging to class alpha, class mu or class pi[22]. However, as is shown in table 7, a considerable overlap exists between individual classes with respect to these inhibitors, making it essential to use additional criteria.

Differences are also found in the susceptibilities of corresponding classes from rat and human tissues. Although 7-7 is most sensitive to S-(p-bromobenzyl)-glutathione in the rat, isoenzyme μ shows the highest inhibition in humans.

Interesting compounds in the inhibition of the glutathione S-transferases are the chlorophenoxy acetic acid herbicides 2,4-dichlorophenoxy acetic acid (2,4-D) and 2,4,5-trichloro-phenoxy acetic acid (2,4,5-T). These compounds are most inhibitory towards class mu isoenzymes, both in rats and in humans[101-103].

Rat isoenzyme 2-2 is unusual in that the activity of this transferase is activated instead of inhibited (240 % and 120 % by 2,4,5-T and 2,4-D respectively, at a 1 mM concentration)[102]. Neither of these compounds interacts with GSH or CDNB in a competitive way, suggesting that the inhibitory activity is exerted at a position other than the active site[101]. This is also indicated by the results of double inhibition experiments with the bile acid taurochenodeoxycholate, a known non-

Table 6. Classes of inhibitors of glutathione S-transferases.[a]

compound class	examples	references
glutathione derivatives	S-hexylglutathione	95-97
glutathione analogues	γ-Lglu-Lser-gly	98
sulfhydryl reagents	N-phenylmaleimide	99,100
	2,2'-dithiopyridine	
chlorophenoxy alkyl acid herbicides	2,4-dichlorophenoxy acetic acid	101-103
	2,4,5-trichlorophenoxy acetic acid	
diuretic drugs	ethacrynic acid	105
bile acids	cholate	106
steroid hormone derivatives	17β-estradiol disulfate	107
quinones	3,4,5,6-tetrachloro-1,2-benzoquinone	108,109
plant phenols	quercetin	110
porphyrins and related compounds	hematin	18,116
	bilirubin	
chalcones	4'-phenylchalcone	111
halogenated anaesthetics	halothane	112
	isoflurane	
anti-inflammatory drugs	indomethacin	113-116
	sulfosalazine	
hypolipidemic drugs	ciprofibrate	117,118
	clofibrate	
dyes	bromosulfophthalein	116
	cibacron blue	
metal compounds	tributyltin acetate	116
isothiocyanates	benzyl isothiocyanate	119
other drugs	misonidazole	120
	propylthiouracil	121

[a] Adapted from ref. 24.

Table 7. Sensitivities of isoenzymes towards several inhibitors.[a]

inhibitor	rat isoenzymes
S-(p-bromobenzyl) glutathione	7-7 > 2-2 > 4-4 > 3-3 > 1-1
2,4-dichlorophenoxy acetic acid	3-3 > 4-4 > 1-1 > 2-2[b]
2,4,5-trichlorophenoxy acetic acid	3-3 > 4-4 > 1-1 > 2-2[b]
hematin	1-1 > 3-3 = 4-4 > 2-2 > 7-7
halothane	3-3 = 4-4 > 1-1 > 1-2[b]
indomethacin	4-4 > 1-1 > 2-2 = 3-3
ciprofibrate	1-1 > 4-4 > 3-3 > 2-2
bromosulfophthalein	4-4 > 1-1 > 3-3 > 7-7 > 2-2
cibacron blue	4-4 > 3-3 > 7-7 > 1-1 > 2-2
triphenyltin chloride	3-3 > 4-4 > 1-1 > 7-7 > 2-2
triethyltin bromide	3-3 > 2-2 > 4-4 > 7-7 > 1-1
benzyl isothiocyanate	3-3 > 2-2 > 4-4 > 1-1
	human isoenzymes
S-(p-bromobenzyl) glutathione	μ > π = α - ε
2,4-dichlorophenoxy acetic acid	μ > π > α - ε
2,4,5-trichlorophenoxy acetic acid	μ > π > α - ε
hematin[c]	μ > B1B1 > π > B2B2
bromosulfophthalein	μ > B1B1 > π > B2B2
cibacron blue	μ > π > B1B1 > B2B2
triphenyltin chloride	B1B1 > μ > B2B2 > π
triethyltin bromide[d]	B2B2 > B1B1 > μ > π

[a] Adapted from ref. 24, 101-103, 112, 116, 117, 119.

[b] Activation instead of inhibition.

[c] Data from ref. 24 indicate that α-ε is more sensitive than μ.

[d] Data from ref. 24 indicate that α-ε is least sensitive.

substrate ligand[102,106]. Since the binding of bile acid under non-inhibitory conditions reduced the extent of activation of 2-2 by 2,4,5-T, this effect is probably also not due to interactions in the active site[102]. Interestingly, the effect of 2,4,5-T on isoenzyme 2-2 was found to vary with the substrate used[104]. The activity of 2-2 with ethacrynic acid demonstrated less activation than the activity with CDNB. Activities towards cumene hydroperoxide, p-nitrophenyl acetate and p-nitrobenzyl chloride were actually inhibited. Vessey and Boyer[104] have suggested that the activation may be the result of allosteric modification of the enzyme and that substrates with variant active site orientations are differentially affected by the conformational changes. In contrast to the rat isoenzymes, none of the human transferases have been shown to be activated by 2,4,5-T[103]. Differential inhibitory and activating effects have recently also been reported for the anaesthetic halothane[112].

Whether the inhibition found with purified isoenzymes has any in vivo relevance, is a question yet to be answered. In vivo inhibition of GST activity with CDNB has been reported in rats, following treatment with the hypolipidemic drugs clofibrate or ciprofibrate[117,118]. Remaining activities were 79 % and 43 % for these compounds respectively. Oral dosage of clofibrate (20 mg/100 g, 4 days) was also found to reduce GST activity in small intestine[118].

A single i.p. injection of misonidazole, a radiosensitizing drug, resulted in significant inhibition of GST activity in the liver of male mice, 1 to 6 hours following administration. After 12 hours cytosolic activities had returned to control levels[120].

In contrast, a number of the in vitro inhibitors listed in table 6, are known to cause a significant induction of hepatic GST activity in vivo, e.g. propylthiouracil, 2,4,5-T and benzyl isothiocyanate[80,90,122]. Since propylthiouracil is known to cause hypothyroidism and its effect on hepatic GST activity could be largely reversed by the administration of triiodothyronine, its effect may be at least partly of an indirect nature.

1.3
CLASS ALPHA ISOENZYMES

Class alpha generally comprises isoenzymes with a high isoelectric point. In the rat, isoenzymes containing subunits 1, 2 or 8 are members of this class, whereas in man, class alpha is represented by isoenzymes α-ε and a recently characterized skin transferase "9.9"[123]. Most of the alpha class enzymes, not including subunit 2 and skin transferase "9.9", have N-blocked termini, which has prevented direct chemical analysis of their N-terminal amino acid sequences[24]. The majority of sequences of alpha class proteins have therefore been determined by use of corresponding cDNA-clones.

Although it was previously assumed that the human enzymes α-ε are charge isomers, arising through the process of deamidation[26], it is now firmly established that at least two immunologically and catalytically distinct subunits exist, which have been designated B1 and B2[31,124]. The three possible dimers, B1B1, B1B2 and B2B2 correspond to ε, δ and γ respectively. Whether the presence of α and β results from the existence of a third subunit, or represents post-translational modification of isoenzyme γ, is not known at present.

Considerable polymorphism has been noticed for the human liver alpha class enzymes. The ratio of B2/B1 subunits, as determined by radioimmunoassay, was found to range from 0.16 to 1.43[125]. However, 6 out of 8 livers showed a ratio smaller than or approx. equal to 0.5. The majority of livers thus seem to contain relatively high amounts of B1B1 and B1B2, but minor to moderate levels of B2B2. Expression of isoenzymes α and β was only noticed in the livers with a high B2/B1 ratio[125].

Characteristic properties of the alpha class glutathione S-transferases include the catalysis of the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids and the non-selenium-dependent glutathione peroxidase activity towards organic hydroperoxides.

As steroid isomerases, the glutathione S-transferases are active towards C19 and C21 Δ^5-3-ketosteroids[12]. The compound Δ^5-androstene-3,17-dione is generally used for the determination of steroid isomerase activity. In the rat, the highest activity with this substrate is

associated with isoenzymes 1-1 and 1-2. Isoenzyme 2-2 is at least ten times less efficient and mu-class isoenzymes 3-3 and 4-4 hardly demonstrate any activity at all[13,24].

Although all human alpha class transferases possess catalytic activity with Δ^5-androstene-3,17-dione, isoenzymes ε and δ are most active, which is indicative of a role for the B1-subunit[13]. In contrast, none of the mouse transferases (N4-4, N1-1, N3-3) exhibits significant catalysis of Δ^5-3-ketosteroid isomerization[24].

The glutathione peroxidase reaction, catalyzed by glutathione S-transferases, is thought to involve two steps: in the first step, GSH reacts with the organic peroxide, to form the corresponding alcohol and the sulfenic acid of glutathione, GSOH. This sulfenic acid reacts with another molecule of GSH in the second step, producing oxidized glutathione and water. Only the first step is enzyme-catalyzed[15].

Next to isoenzyme 5-5, which is the most active non-selenium-peroxidase known, alpha class glutathione S-transferases, notably those containing subunit 2 in the rat and the human B2-containing isoenzymes, demonstrate the highest activity with the standard substrate cumene hydroperoxide[24,41,124]. However, all rat isoenzymes seem to possess the capacity for catalyzing the conversion of organic peroxides. For instance, isoenzyme 7-7, which is not very active with cumene hydroperoxide as a substrate, is quite capable of catalyzing the reaction of GSH with the hydroperoxides derived from linoleate and arachidonate. Isoenzyme 1-1 shows the highest activity towards these compounds[43].

Both rat transferases 1-2 and 2-2, but not isoenzymes 3-3 and 3-4, have been found capable of detoxifying lipid peroxides formed in microsomes by treatment with ADP-Fe^{3+} and NADPH. The presence of active phospholipase A2 was required to establish the release of free-fatty acyl hydroperoxides from the membrane phospholipids[16]. These results indicate that in the rat, alpha class isoenzymes and possibly isoenzyme 5-5 may play a role in the repair of oxidative damage to membrane phospholipids[16,43].

It was recently suggested that the peroxidase activity of glutathione S-transferases may be involved in the detoxification and repair of radical damage to DNA[44]. Although the thymine-derived peroxide 5-peroxy-methyluracil was found to be a good substrate for isoenzymes

2-2, 6-6 and 7-7, their activity towards peroxidized DNA was extremely low, class mu enzymes 3-3 and 4-4 being the best catalysts. Significant activity was, however, only noticed for a newly isolated glutathione S-transferase, found to be closely associated with chromatin. This isoenzyme demonstrated enzymatic and physical properties similar to 5-5, but was not identical to this cytosolic form, and therefore tentatively received the designation 5*-5*[44].

The capacity for non-covalent binding of a variety of hydrophobic compounds, including heme and its metabolites, steroids, bile acids, thyroid hormones and a number of drugs and carcinogens, was first recognized for the glutathione S-transferases 1-1 and 1-2[19]. These isoenzymes were at that time thought to constitute one protein, called "ligandin". In recent years, it has become clear that this binding capacity extends to other glutathione S-transferases. However, because most studies have involved alpha-class "ligandin" and also because isoenzyme 2-2 seems to differ from the other GST isoenzymes, the binding properties of the glutathione S-transferases are discussed in this section.

Most studies on the binding of non-substrate ligands have used the compound bilirubin. Binding of bilirubin to glutathione S-transferases gives rise to a characteristic circular dichroism spectrum, with positive extrema at 405 and 515 nm and a negative extremum at 455 nm (figure 1a)[126,127,128,129]. The band at 405 nm increases in magnitude up to a 1:1 molar ratio of bilirubin to dimeric protein (figure 1b). At this ratio the band at 455 nm has reached approx. 80 % of its maximal ellipticity and no further increases occur after addition of 2 moles bilirubin per mole of protein. These bands are indicative of binding to a high affinity site on the protein. The band at 515 nm requires addition of higher bilirubin concentrations and represents binding to a secondary low affinity site, which is presumably associated with the active site, since its appearance can be abolished by the addition of GSH[126-128]. The alpha-class isoenzymes 1-1, 1-2 and the mu-class isoenzymes 3-3, 3-4 and 4-4 seem to possess one high-affinity binding site per dimer, although one study mentions the presence of two sites per dimer for the mu-class transferases[18,19,128-130]. One high-affinity binding site per dimer would

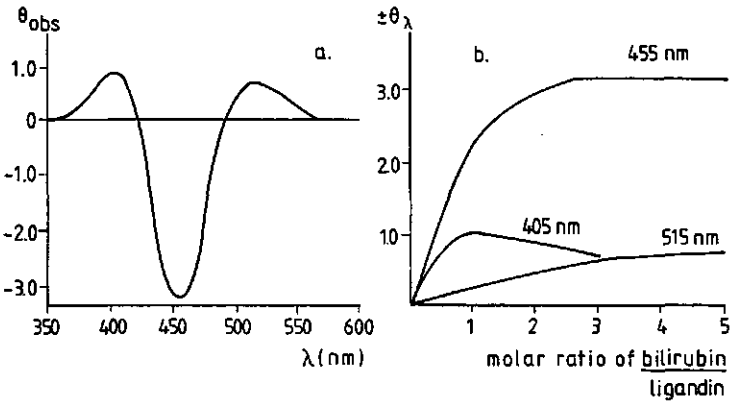

Figure 1. Circular dichroism spectrum of the "ligandin"-bilirubin complex (figure 1a). Figure 1b shows titration of ligandin with bilirubin as reflected by the ellipticity bands at 515, 455 and 405 nm.
Adapted from ref. 126.

suggest its formation to be due to an interaction between subunits, rather than its location on a specific subunit.

Isoenzyme 2-2 differs from the other transferases in that it lacks a high-affinity binding site. In spite of this fact, subunit 2 does seem to be involved in the formation of the high-affinity site on isoenzyme 1-2[131]. A [^{14}C]-labeled enol ester derivative of bilirubin (bilirubin Woodward's reagent K), which binds to the high-affinity site in a similar fashion to bilirubin, was shown to label subunits 1 and 2 from 1-2 equally[131]. These data seem to substantiate the suggestion concerning the location of the binding site.

Binding of non-substrate ligands generally causes a loss of activity. A higher inhibition is noticed for mu-class isoenzymes than for alpha-class enzymes as a result of the binding of steroids. Consistent with this inhibition, mu-class transferases also exhibit a higher affinity for these compounds[17,107,129].

Similar to the rat, human alpha- and mu-class isoenzymes also contain a binding site different from the catalytic site[132,133].

1.4
CLASS MU ISOENZYMES

Class mu comprises the homo- and heterodimeric isoenzymes containing subunits 3,4 and 6 in the rat and the human isoenzymes μ and ψ. The most interesting aspect of this class of transferases is the genetic polymorphism noticed for the human enzymes. This phenomenon was first recognized in human liver, where isoenzyme μ was found to be expressed in only approx. 60 % of the samples analysed[27,65].

Isoenzyme ψ was found to be a member of the mu-class family on the basis of amino acid composition in addition to comparison of the N-terminal amino acid sequences of a number of different transferases[134]. Its first 23 N-terminal amino acid residues are identical to the sequence of isoenzyme μ, whereas only approx. 30 % sequence identity is shared with isoenzyme π from human placenta. Analysis of the isoenzyme composition of 6 liver samples demonstrated the presence of significant amounts of isoenzyme ψ in 3 specimen, representing more than 50 % of total GST activity in one sample.

The presence of ψ is not always associated with expression of isoenzyme μ. Singh et al.[134] found significant amounts of both isoenzymes in only 1 out of 6 liver samples. Hussey et al.[125] found a positive reaction with antibodies against isoenzyme μ for 5 out of 8 liver specimen, by Western blot analysis. Isoelectric focussing of these samples demonstrated the presence of μ in 3 samples and the presence of ψ in one. The near-neutral enzyme in the fifth positive sample was not identified.

The genetic basis for the heterogeneity of the mu-class isoenzymes was provided by Board[135]. He proposed that the near-neutral enzymes are the products of a single gene locus GST 1. Four phenotypes may be formed by the combinations of three alleles: GST 1 * 0, GST 1 * 1 and GST 1 * 2. The GST 1 0 phenotype (GST 1 * 0/GST 1 * 0) is characterized by an absence of near-neutral enzymes, whereas individuals with the GST 1 1 (GST 1 * 1/GST 1 * 1; GST 1 * 1/GST 1 * 0) or the GST 1 2 (GST 1 * 2/GST 1 * 2; GST 1 * 2/GST 1 * 0) phenotype each express one near-neutral transferase. The GST 1 2-1 phenotype is heterozygous for the GST 1 * 1 and GST 1 * 2 alleles and possesses 3 isoenzymes, resulting

from the homo- and heterodimeric combinations of the products of the 2 alleles. It has recently become clear that the near-neutral enzymes from the phenotypes GST 1 1 and GST 1 2 correspond to transferases ψ and μ, respectively[136].

No information is available on the heterodimeric protein of the GST 1 2-1 phenotype, apart from its existence, which has been demonstrated by means of starch-gel electrophoresis and chromatofocusing[60,72]. This lack of information is presumably partly due to the low frequency of occurrence of the GST 1 2-1 phenotype (table 8).

The recent finding that the genetic differences in hepatic expression of mu-class isoenzymes are reflected in mononuclear lymphocytes, offers several perspectives for relatively easy phenotyping of individuals. Seidegard et al.[137] demonstrated that the cytosolic GST activity in mononuclear leukocytes towards the compound trans-stilbene oxide falls into three groups with low, high and very high activity (figure 2). The pattern of activity towards the standard GST substrate 1-chloro-2,4-dinitrobenzene did not follow this kind of distribution. On the other hand, activities towards styrene-7,8-oxide were found to correlate with high and low activities towards trans-stilbene oxide, although the existence of differential groups was not noticed[137].

Table 8.: The observed and expected numbers of GST 1 phenotypes in liver samples from a North European population sample of 49 individuals[a].

Phenotype	GST 1 1		GST 1 2		GST 1 2-1	GST 1 0	Total
Genotype	1-1	1-0	2-2	2-0	2-1	0-0	
Observed	9		17		3	20	49
Expected	0.83	8.15	2.59	14.43	2.93	20.07	49

[a] Data from Strange et al.[60].
The expected distribution was calculated assuming Hardy-Weinberg equilibrium and gene frequencies GST 1 1 = 0.13, 2 = 0.23 and 0 = 0.64.

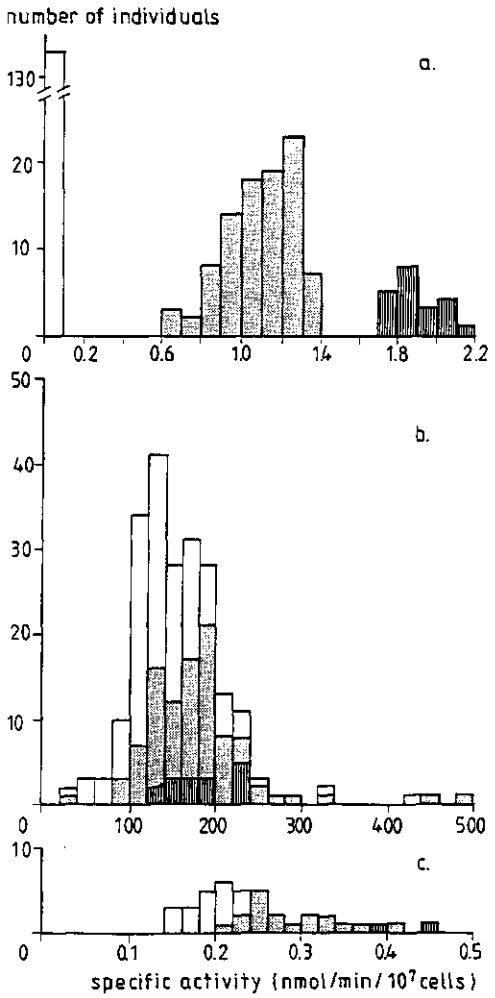

Figure 2. The distribution of glutathione transferase activity in mononuclear lymphocytes towards trans-stilbene oxide (a), 1-chloro-2,4-dinitrobenzene (b), and styrene-7,8-oxide (c). Individuals measured in figures b and c were also assayed for their glutathione transferase activity with trans-stilbene oxide. Open and striped bars in these figures correspond to the activity ranges with trans-stilbene oxide in figure a.
Adapted from ref. 137

The glutathione S-transferase responsible for the activity with trans-stilbene oxide has been shown to be identical with isoenzyme μ[64]. This isoenzyme has a 2500-fold higher activity with this compound than the alpha-class enzymes and transferase π. The activity towards trans-stilbene oxide in lymphocytes was found to match the activity in liver from the same individual indicating that screening the lymphocytes of individuals for the presence of μ is a suitable way for phenotyping them. In this respect, it should be noted that it is not clear at present to what extent ψ is involved in the findings described above. The fact that Seidegard et al.[137] report a similar percentage for null-phenotypes as found by other investigators, suggests that both μ and ψ may be expressed in lymphocytes when expressed in liver. The isoenzymes possess similar activities towards trans-stilbene oxide (Bogaards, Van Ommen, Van Bladeren, unpublished results) and it is tempting to speculate that the group with "very high activity" constitutes the heterozygotes for the GST 1 * 1 and GST 1 * 2 alleles, expressing both transferases. It can be calculated from figure 2 that this group presents approx. 8.5 % of the total number of individuals tested, which is similar to the frequency reported by Strange et al.[60] (6.1 %, table 8).

The genetic polymorphism exhibited by mu-class transferases may be a factor in determining an individual's susceptibility to the toxic effects of various xenobiotics. The high activity of isoenzyme μ towards epoxide metabolites from polycyclic aromatic hydrocarbons (which are generally thought to possess considerable mutagenic and carcinogenic potential) may be of particular significance[132].

Determination of trans-stilbene oxide activity in lymphocytes from 144 individuals, including 66 lung cancer patients and 78 controls (matched for smoking habits), demonstrated a lower percentage of mu-class positive lung cancer patients (34.8 %) than mu-class positive control smokers (59.0 %). Based on these results, Seidegard et al.[138] suggested that isoenzyme μ could serve as a genetic marker in the susceptibility for lung cancer in smokers.

Recent findings, concerning the existence of additional mu-class proteins, indicate that the genetic polymorphism demonstrated by these enzymes may be somewhat more complicated than previously assumed. The

glutathione S-transferases GST 4 and GST 5, described by Suzuki et al.[66] and present in human skeletal muscle and brain respectively, are closely related to μ and ψ[66,136]. Carmichael et al.[139] found a cross reactive protein on Western blot with antibodies against μ, for all human lung samples tested. Interestingly, the subunit molecular mass of this protein was slightly lower than the subunit molecular mass of the hepatic standard.

Since no kinetic data are available and information on tissue distribution is extremely limited, the impact of these isoenzymes on individual susceptibility towards xenobiotics is not clear.

1.5
CLASS PI ISOENZYMES

The class pi glutathione S-transferases comprise the rat isoenzyme 7-7 in addition to a number of anionic isoenzymes in man ($\pi, \rho, \lambda, \omega$) and the mouse isoenzyme N3-3.

The human isoenzymes ρ, λ and ω correspond closely to the placental isoenzyme π[24]. The erythrocyte transferase ρ is probably identical to GST π, whereas the amino acid sequences of lung isoenzyme λ and GST π, as deduced from their respective cDNA-clones, were found to differ at only 2 positions[32,140]. Since human GST π has been studied most, this denotation will be used as representative for human class pi isoenzymes.

Several reports have appeared in recent years on the possible use of isoenzyme 7-7 and GST π as tumormarkers[141,142,143,144,145,146,147]. Isoenzyme 7-7 is present at low levels in normal liver, but its expression is markedly increased (10- to 30-fold) in hyperplastic (preneoplastic) nodules and hepatocellular carcinoma. This increase in protein results directly from an increase in the amount of mRNA, indicating that regulation of the expression occurs at a transcriptional level[141,147,148].

The presence of 7-7 in hyperplastic nodules is not associated with rapid growth ("a return to the foetal state"), since foetal liver does not contain higher amounts of 7-7 than adult liver and mRNA or protein are not increased in regenerating liver following 70 % partial hepatectomy[141,148].

Similarly, the increase in 7-7 does not simply result from the administration of carcinogens. Isoenzyme 7-7 is not inducible by short-term exposure to 3-methylcholanthrene, phenobarbital, polychlorinated biphenyls and 3'-methyl-4-dimethyl-aminoazobenzene, although a slight (approx. 2-fold) increase has been noticed for butylated hydroxyanisole in periportal areas[141]. Furthermore, the increase in 7-7 is not apparent prior to the appearance of preneoplastic foci and hyperplastic nodules. It seems, therefore, that the elevated expression of isoenzyme 7-7 is a secondary effect and a characteristic feature of the (pre) neoplastic state of liver cells.

In spite of the fact that the increase in subunit 7 is most marked, analysis of the GST isoenzyme pattern of hyperplastic nodules has demonstrated, that alpha-class and mu-class isoenzymes still account for the major part of the GST protein (figure 3). Both alpha- and mu-class subunits show an approx. 2-fold increase, which is associated with elevated levels of corresponding mRNAs[40,149].

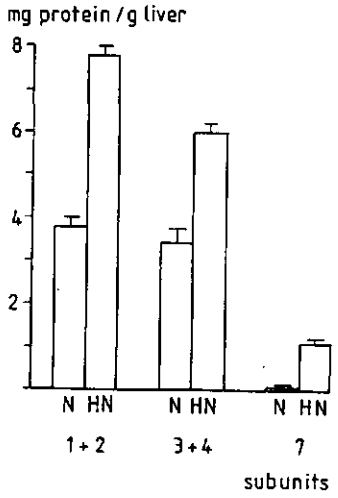

Figure 3. Levels of isoenzymes containing subunits 1+2, subunits 3+4 and subunit 7, in non-tumour liver (N) and hyperplastic nodules (HN). Adapted from ref. 149.

Similar to the rat, GST π contents are significantly higher in human hepatic tumours than in surrounding non-tumour tissue or normal liver. This phenomenon has been noticed for both primary tumours and metastatic hepatic tumours, originating from stomach or colon[76].
There has been a tendency to view GST π as a more general tumour marker, in a variety of human tissues, including colon, kidney, uterine cervix and lung[62,63,145,146,150]. However, this does not seem to be justified in all cases.

Immunohistochemical examination of uterine cervix demonstrated increased staining with anti-GST π in severe dysplasia, carcinoma in situ and invasive carcinoma. Normal squamous epithelial cells were found to be almost negative for GST π-binding, whereas various staining intensities were noticed for moderately dysplastic specimen[145]. Total GST activity and GST π-content showed a parallel increase (approx. 3 to 14-fold) in 4 squamous cell carcinoma as compared with normal cervical epithelial tissue.

Shea et al.[146] analysed the isoenzyme composition of a number of tumours, from different origins (e.g. colon, kidney, breast, lung and stomach). Although total GST activity was found to show a large variation, ranging from 40 to 1010 mUnits/mg protein, isoelectric focusing of 17 tumour samples demonstrated that GST π was a major transferase in each sample. In only 3 out of 17 tumours, approx. 10-20 % of GST activity was recovered in the alkaline region, indicative of the presence of class alpha enzymes. Unfortunately, no comparison was made with corresponding non-tumour tissue, making it impossible to evaluate whether actual changes in activity and/or isoenzyme patterns had occurred. This seems essential, especially in tissues where GST π is already a major contributing enzyme.

In human lung, for instance, more than 90 % of activity is accounted for by GST π in both tumour and non-tumour tissue. Since only 23 out of 38 individuals exhibited a higher specific activity in tumour tissue, which was claimed to be associated with an increase in GST π, GST π does not seem to be a suitable marker for lung tumour[62]. Indeed, Carmichael et al.[139] found that the tumour/non-tumour ratio in specific activity in lung was not correlated with the tumour/non-tumour ratio of GST π, but instead with the ratio of the amount of a neutral enzyme, cross-reactive with anti-μ.

Similarly, GST π may not be a suitable marker for renal tumours. In non-tumour tissue alpha-class enzymes have been shown to demonstrate a high level of expression in addition to GST π, whereas renal tumours contain only GST π as a major isoenzyme[63]. Since total GST protein demonstrates a decrease from approx. 2 % of cytosolic protein in non-tumour tissue to approx. 0.5 % in tumour samples, tumour development in human kidney may be more characterized by a decrease in alpha-class

enzymes than by an actual increase in GST π.

The recognition of the pi-class transferase as a tumour marker in some tissues offers a number of perspectives. For instance, GST π may be used for diagnostic purposes in clinical practice. The expression of 7-7 in rat liver could be useful in the screening of compounds with hepatocarcinogenic properties. A test system for this kind of screening was recently described by Ito et al.[151].

In cancer therapy, acquired drug resistance of tumour cells against chemotherapeutic agents poses a major problem. Several alterations in cell function may be responsible for the development of cellular resistance, including decreased drug uptake, increased DNA repair, decreased drug metabolism and/or increased drug detoxification. The glutathione S-transferases may be involved in acquired drug resistance, in view of their general role in detoxification of xenobiotics in addition to the fact that their concentration is usually higher in resistant than in non-resistant cells[140,152,153,154].

Emphasis has generally been placed on the class pi-GST isoenzyme, since it accounts for the major part of GST activity in most types of tumours and is present at high levels in both resistant and non-resistant cells. It should be noted, however, that a role in the development of cellular resistance is by no means restricted to this class of isoenzymes[154].

An elevated level of anionic GST π has been reported for the multidrug-resistant MCF-7 human breast cancer cell line, the cis-platinum-resistant human SCC-25 squamous carcinoma cell line and the adriamycin-resistant P388-leukemia cell line[140,152,153]. In contrast, the increase in GST activity in nitrogen-mustard resistant Walker 256 rat breast cells was attributed to an over-expression of GST 2-2[154].

It would seem reasonable to assume that an increase in total GST activity will lead to an increased detoxification of a number of alkylating agents and thereby an increased resistance against certain classes of chemotherapeutic agents. The classes of agents to which resistance will occur, will depend on the type of isoenzyme(s) "induced".

1.6
OBJECTIVE OF THE INVESTIGATION

The studies described in this thesis were aimed at the relationship: glutathione S-transferase isoenzyme pattern versus sensitivity towards alkylating agents. In this context, several important features of the glutathione S-transferases, as discussed in the preceding sections, were studied.

Chapter 2 deals with the aspect of substrate selectivity. The activity of individual isoenzymes towards the 9,10-mono-ozonide of methyl linoleate, a postulated intermediate in the toxicity of ozone, was determined, as well as the reaction pathway involved.

The isoenzyme pattern may be subject to changes in response to external challenges by xenobiotics, which may influence an individual's sensitivity to the toxic effects of both these compounds and other xenobiotics. The aspect of induction was studied using four inducing agents with different chemical structures. The results of this study are described in chapter 3.

In chapter 4 inhibitory effects of a number of quinones were investigated, both towards purified glutathione S-transferase isoenzymes and in a cellular system, which resembles the in vivo situation more closely.

Monoclonal antibodies are useful tools for specific recognition of individual isoenzymes. In the first part of chapter 5, the synthesis of monoclonal antibodies against rat isoenzymes 2-2 and 3-3 is described. Their cross-reactivity with other rat GST isoenzymes and with human transferases was studied.

The isoenzyme pattern of the human glutathione S-transferases is subject to considerable genetic polymorphism. Most interesting is the absence of mu-class isoenzymes in 40-50 % of the population. In the second part of chapter 5 the occurrence of this genetic deficiency was determined in human mononuclear lymphocytes by use of a monoclonal antibody. The implications for the handling of xenobiotics in the work-environment are discussed.

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CHAPTER 2

METHYL LINOLEATE OZONIDE AS A SUBSTRATE FOR RAT GLUTATHIONE S-TRANSFERASES: REACTION PATHWAY AND ISOENZYME SELECTIVITY

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ABSTRACT

The 9,10-mono-ozonide of methyl linoleate was shown to be a substrate for rat hepatic cytosolic, rat lung cytosolic and rat hepatic microsomal glutathione S-transferases. The activities of lung cytosol and liver microsomes with methyl linoleate ozonide were found to be high relative to the activity demonstrated by liver cytosol, as compared with their respective activities towards 1-chloro-2,4-dinitrobenzene. Only a slight catalytic activity towards the ozonide was noticed for rat lung microsomes. Isoenzyme 2-2 exhibited the highest specific activity (208 nmol/min/mg) when isoenzymes 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 7-7 were compared. This isoenzyme accounts for approx. 25 % of cytosolic glutathione S-transferase protein in rat lung, while in rat liver it represents approx. 9 %. This may partly explain the high activity towards the ozonide noticed for rat lung cytosol.

No stable conjugates were formed as products of the reaction of methyl linoleate ozonide with glutathione; although two glutathione-conjugates were noticed on TLC, they were only formed as intermediate compounds.

Coupling of an aldehyde dehydrogenase assay or a glutathione reductase assay to the glutathione S-transferase-catalyzed conjugation, demonstrated that oxidized glutathione and aldehydes are formed as the major products in the reaction.

To further confirm the formation of aldehydes, the products of the glutathione S-transferase-catalyzed reaction were incubated with 2,4-dinitrophenylhydrazine, which resulted in hydrazone formation.

In conclusion, the activity of the glutathione S-transferases towards the ozonide of methyl linoleate is similar to their peroxidase activity with lipid hydroperoxides as substrates.

INTRODUCTION

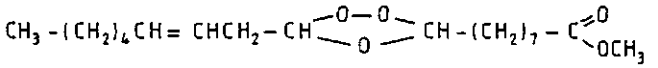
Ozone is one of the most toxic components of photochemical air pollution. Several studies indicate that its toxic action is mediated by the formation of fatty acid ozonides, resulting from the oxidation of unsaturated fatty acid components of membrane phospholipids[1-3].

Exposure of thin films or suspensions of unsaturated fatty acids to ozone leads to the formation of such ozonides as major products[4-6]. Furthermore, fatty acid ozonides display a toxicity very similar to the toxicity of ozone itself. Menzel et al.[1] demonstrated that both ozone and fatty acid ozonides cause Heinz body inclusions, hemolysis and formation of methemoglobin in human and mouse erythrocytes, following in vitro exposure. Similarly, Cortesi and Privett[2] found that intravenous injection of the model compound methyl linoleate ozonide (MLO, fig. 1) results in effects on rat lung resembling those induced by ozone intoxication.

In addition to the similarities in toxic effects, antioxidant protection against ozone and MLO shows comparable characteristics[3].

One of the antioxidants involved in the protection against ozone toxicity is the cellular thiol glutathione (GSH). The toxicity of methyl linoleate ozonide towards rat alveolar macrophages is reduced considerably by preincubation of the ozonide with GSH, while preincubation with GSH and a mixture of rat hepatic glutathione S-transferases (GST) completely abolishes toxicity. MLO was subsequently shown to be a substrate for rat GST[3].

The present study focusses on the GST isoenzyme selectivity towards MLO and the elucidation of the reaction pathway.



MLO

Figure 1. The 9,10-ozonide of methyl linoleate (MLO).

MATERIALS AND METHODS

Materials. 1-chloro-2,4-dinitrobenzene (CDNB) and GSH were purchased from Aldrich Chemical Co. (Milwaukee, USA) and Janssen Chimica (Beerse, Belgium) respectively. Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid) was obtained from Fluka AG (Buchs, Switzerland). 2,4-dinitrophenylhydrazine was from Merck (Darmstadt, FRG). Aldehyde dehydrogenase (ALDH, EC 1.2.1.5), glutathione reductase (GR, EC 1.6.4.2), glutathione peroxidase (GPx, EC 1.11.1.9) and methyl linoleate were obtained from Sigma Chemical Co. (St. Louis, MO). The 9,10-mono-ozonide of methyl linoleate was synthesized as described by Rietjens et al.[3], by leading a nitrogen/ozone gas stream through a solution of methyl linoleate (0 °C). The resulting product was purified on a Silicagel 60 (70-230 mesh) column eluted with 5 % (v/v) ether in petroleum ether.

Purification of glutathione S-transferase isoenzymes. Rat hepatic cytosolic and rat lung cytosolic glutathione S-transferase isoenzymes were purified on a S-hexylglutathione Sepharose 6B affinity column and separated by means of FPLC-chromatofocusing, as described previously[7]. Isoenzyme 7-7 was only present in rat lung cytosol, where it co-eluted with isoenzyme 3-4 and was further purified by cation exchange chromatography on a Mono S column. The isoenzyme was eluted with a 0-250 mM NaCl gradient in 15 mM acetate buffer pH 5.2 containing 0.2 mM dithiothreitol, and was pure as judged by SDS-PAGE analysis. Relative amounts of protein per GST isoenzyme were calculated from FPLC isoenzyme fractions, using the total activity of each isoenzyme fraction with CDNB, divided by their individual specific activities. Microsomes used for the determination of microsomal GST activity were washed twice in 0.15 M Tris-HCl pH 8.0 to remove contaminating cytoplasm.

Enzyme assays. GST activity towards CDNB was measured at 25 °C, according to Habig et al.[8]. GST activity with MLO was determined using Ellman's reagent, as described by Rietjens et al.[3]. In short, the assay mixtures, containing 0.1 M potassium phosphate buffer pH 6.5, 1 mM EDTA, 1 mM GSH, 390 µM MLO and differing amounts of glutathione S-transferases in a total incubation volume of 1.5 ml, were incubated at 37 °C, in a shaking water bath. Every 30 seconds, up to 5 minutes, a 100 µl sample was drawn from the incubation mixture and added to 10 µl 33 % (w/v) trichloro-

acetic acid. Precipitated protein was removed by centrifugation. 50 µl of the clear supernatant was added to 0.95 ml of Ellman's reagent (0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid in 0.1 M potassium phosphate buffer pH 6.5) and absorbance was read at 412 nm after 5 minutes. The amount of unreacted GSH in the incubation mixture was calculated using an appropriate calibration curve. Although the concentration of MLO used was rather high, Lineweaver-Burk plots have previously shown that a continuous increase in GSH-conversion is obtained at MLO concentrations ranging from 230 to 1300 µM, thus demonstrating that at 390 µM, all MLO is available as a substrate[3].

GST activity with MLO was also measured spectrofotometrically by coupling of an aldehyde dehydrogenase assay or a glutathione reductase assay to the reaction of MLO with GSH.

For the aldehyde dehydrogenase assay, final concentrations used were: 0.1 M potassium phosphate buffer pH 6.5, 1 mM EDTA, 1 mM GSH, 390 µM MLO, 1 mM NAD^+, 0.5 units/ml aldehyde dehydrogenase (with acetaldehyde as a substrate at pH 8, 25 °C, in the presence of potassium ions and sulfhydryl compounds) and differing amounts of GST. The activity of aldehyde dehydrogenase was also determined with n-octanal, n-nonanal and n-decanal (final concentration 0.1 mM) at pH 6.5, 37 °C. Specific activities amounted to 1.50, 1.58 and 0.45 µmol/min/mg respectively, demonstrating that all compounds could serve as substrates under the circumstances used and that the assay was suitable for the determination of the formation of long-chain aldehydes from the ozonide.

Final concentrations amounted to 0.1 M potassium phosphate buffer pH 6.5, 1 mM EDTA, 1 mM GSH, 390 µM MLO, 0.2 mM NADPH, 1 unit/ml glutathione reductase (at pH 7, 25 °C) and differing amounts of GST for the glutathione reductase assay.

Both reactions were followed spectrofotometrically at 340 nm, 37 °C. Glutathione peroxidase activity towards hydrogen peroxide (pH 7.0, 25 °C) and MLO (pH 6.5, 37 °C) was measured according to the method of Lawrence and Burk[9].

Preparation and identification of hydrazones. Hydrazones were prepared by the method of Ariga[10]. In short: 1.5 ml of the assay mixture described under enzyme assays for the determination of GSH conversion, was incubated at 37 °C in a shaking water bath. After 15 minutes 150 µl of 33 % (w/v)

trichloroacetic acid was added. The precipitated protein was removed by centrifugation and 1.1 ml of the clear supernatant was neutralized with 0.2 ml 1.07 N NaOH. 0.5 ml of the neutralized sample was incubated with 0.25 ml of 2.5 mM 2,4-dinitrophenylhydrazine in 1.2 N HCl, at 30 °C for 30 minutes. 1 ml of 100 % ethanol and 0.75 ml of 1.07 N NaOH were added and the resulting sample was scanned spectrofotometrically from 470 to 370 nm. The assay mixture without GST was used as a blank.

Thin-Layer Chromatography (TLC). MLO was incubated with GSH in 50 % (v/v) dioxane, pH 8, for 3 hours. The incubation mixture was subjected to TLC, using a solvent system of n-propanol/acetic acid/aqua dest (16:3:5 (v/v)). The plates were sprayed with 0.5 % (w/w) ninhydrin in acetone.

RESULTS

Isoenzyme selectivity. Table 1 shows that both rat hepatic cytosolic and rat hepatic microsomal GST were capable of catalyzing the conjugation of MLO with GSH. The reaction was also catalyzed by cytosolic GST from rat lung, which demonstrated a rather high activity compared with liver cytosol, taking their respective activities towards CDNB into account. Only a slight catalytic activity towards MLO was found for rat lung microsomes.

The specific activities of individual isoenzymes 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 7-7 were determined (table 2). Although most isoenzymes exhibited some activity towards the ozonide, the highest activity was noticed for isoenzyme 2-2. No detectable activity was found for isoenzyme 7-7.

In addition to the catalysis demonstrated by glutathione S-transferases, MLO was also shown to be a substrate for glutathione peroxidase. The specific activity of the enzyme amounted to approx. 70 nmol/min/mg at pH 6.5, 37 °C, as compared with a specific activity of 200 µmol/min/mg towards hydrogen peroxide (pH 7.0, 25 °C). The activity of glutathione peroxidase noticed towards MLO was not due to the presence of contaminating glutathione S-transferases, since the enzyme did not demonstrate conjugative activity towards CDNB.

Table 1. GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) and methyl linoleate ozonide (MLO) in rat liver and rat lung.[a]

	specific activity with	
	CDNB (nmol/min/mg)	MLO (nmol/min/mg)
liver cytosol	2100 ± 70	5.5 ± 1.1
lung cytosol	140 ± 3	2.0 ± 0.4
liver microsomes[b]	99 ± 8	2.4 ± 1.5
lung microsomes[b]	17 ± 0	≤ 0.9

[a] Assays were performed using concentrations of 1 mM GSH and 1 mM CDNB or 0.39 mM MLO. The reaction rates were determined spectrofotometrically at 340 nm according to Habig et al.[8] for CDNB and by taking a 100 µl sample every 30 sec. for 5 min. and determining the amount of unreacted GSH with Ellman's reagent for MLO[3]. Results are given as means ± S.D. of duplicate determinations.

[b] Microsomes washed twice in 0.15 M Tris-HCl pH 8.0.

Table 2. Specific activity of individual GST isoenzymes with 1-chloro-2,4-dinitrobenzene (CDNB) and methyl linoleate ozonide (MLO).[a]

isoenzyme	specific activity with	
	CDNB (µmol/min/mg)	MLO (nmol/min/mg)
1-1	48 ± 1	83 ± 24
1-2	28 ± 1	29 ± 3
2-2	14 ± 0	208 ± 8
3-3	35 ± 2	36 ± 18
3-4	19 ± 1	47 ± 3
4-4	6 ± 0	17 ± 6
7-7	18 ± 1	≤ 10

[a] Isoenzymes were purified as described previously[7]. For assay conditions see table 1. Results represent means ± S.D. of duplicate or triplicate determinations.

The glutathione peroxidase activities with hydrogen peroxide were 0.06 µmol/min/mg and 0.35 µmol/min/mg for rat lung cytosol and rat liver cytosol respectively.

Product identification. Following an incubation of MLO with GSH, two GS-conjugates were observed on TLC after spraying with ninhydrin, in addition to large amounts of GSH and oxidized glutathione (GSSG). The R_f's of the conjugates amounted to 0.64 ± 0.03 and 0.81 ± 0.04 respectively. Using ^3H-labeled GSH in an effort to quantify these derivatives however, the label recovered from these conjugates was not above background levels, GSH and GSSG accounting for 89-91 % of the radioactivity. Similarly, subjecting the incubation mixture to HPLC did not result in the isolation of any conjugates. 96 % of the radioactivity was recovered in the polar fraction, which only contained GSH and GSSG (results not shown).

The activity of GST towards MLO was measured using Ellman's reagent, to determine the amount of GSH converted (fig. 2).

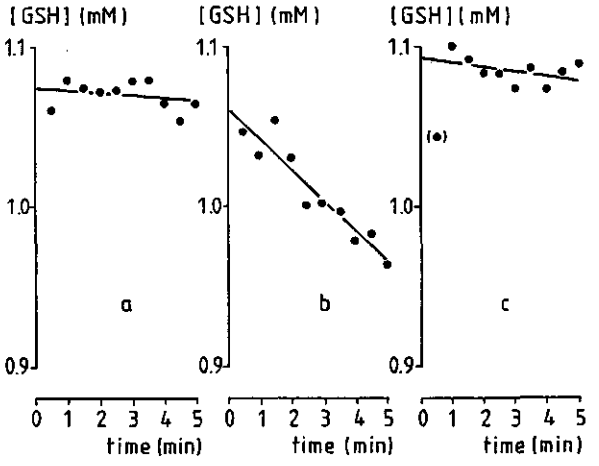


Figure 2. Reaction of MLO with GSH

A. Chemical reaction

B. Reaction catalyzed by a mixture of GST isoenzymes purified on S-hexylglutathione Sepharose 6B

C. Reaction catalyzed by GST in the presence of glutathione reductase and NADPH

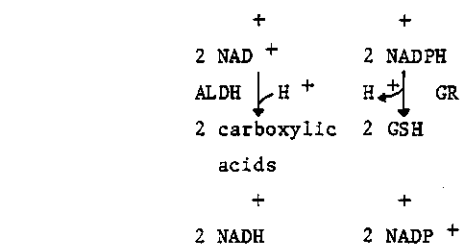
When glutathione reductase and NADPH were added to the GST-catalyzed reaction of MLO with GSH, no GSH-conversion was found (fig. 2c), indicating the formation of oxidized glutathione as a major product. Coupling of an aldehyde dehydrogenase assay or a glutathione reductase assay to the GST-catalyzed reaction showed an approx. stoichiometric relationship between GSH-conversion, formation of aldehydes (NADH-formation) and formation of GSSG (NADPH-conversion) (table 3).

Table 3. Stoichiometry of conversion of reduced glutathione (GSH), formation of aldehydes, and formation of oxidized glutathione (GSSG), in the glutathione S-transferase-catalyzed reaction of methyl linoleate ozonide with GSH.[a]

	specific activity nmol/min/mg GST
GSH-conversion	6.6 ± 1.3 nmol GSH/min/mg GST
Aldehyde-formation	10.0 ± 0.3 nmol NADH/min/mg GST
GSSG-formation	8.2 ± 1.4 nmol NADPH/min/mg GST

[a] GSH-conversion was measured by use of Ellman's reagent. Aldehyde-formation and formation of GSSG were determined by coupling of an aldehyde dehydrogenase assay or a glutathione reductase assay to the GST-catalyzed reaction of MLO with GSH.

Schematically the reactions measured were:



Results represent means ± S.D. of three or four determinations.

The products of the reaction of MLO with GSH were incubated with 2,4-dinitrophenylhydrazine and scanned from 470 to 370 nm. A spectrum characteristic for 2,4-dinitrophenylhydrazones was found (λ_{max} = 435 nm), confirming the presence of one or more carbonyl compounds (results not shown).

DISCUSSION

Fatty acid ozonides formed upon ozone-induced lipid oxidation may be detoxified by glutathione S-transferases. GST from both rat liver and rat lung cytosol are capable of catalyzing the reaction of MLO with GSH. In addition, MLO is a substrate for microsomal GST. Although, with the exception of isoenzyme 7-7, all isoenzymes used in the present study demonstrate catalytic activity towards MLO to some extent, isoenzyme 2-2 exhibits the highest specific activity.

The ultimate products formed in the reaction of MLO with GSH are GSSG and aldehydes. The two conjugates noticed on TLC therefore only serve a role as intermediate compounds.

The high activity demonstrated by isoenzyme 2-2 and by hepatic microsomal GST suggests that the GST activity towards MLO may be similar to the GST activity with lipid hydroperoxides, since both these GST isoenzymes exhibit glutathione peroxidase activity[11,12]. Thus, the most likely reaction pathway is an attack of the nucleophilic thiol moiety of GSH on one of the two oxygen atoms of the peroxide part of the ozonide ring structure (fig. 3). Two possible conjugates may be formed, which are unstable and prone to a second attack by GSH, leading to the formation of GSSG and aldehydes. A similar mechanism has been proposed for the GST-catalyzed reaction of organic hydroperoxides with GSH[11].

Rat liver cytosol contains approx. 3.5 $\pm$ 1.8 µg isoenzyme 2-2/mg protein, accounting for approx. 9 % of total GST protein. In rat lung, approx. 25 % of the GST protein is represented by isoenzyme 2-2 (1.4 $\pm$ 0.3 µg/mg cytosolic protein). The relatively high amount of isoenzyme 2-2 in rat lung may partly explain the high activity of lung cytosol towards MLO as compared with liver cytosol.

In addition however, it should be noted that the purification procedure

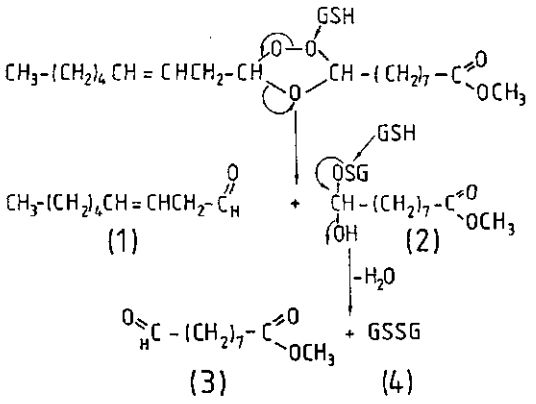


Figure 3. A possible mechanism of the reaction of MLO with GSH, attack taking place on one of the oxygen atoms. Products are aldehydes (1,3) and oxidized glutathione (4), with the glutathione-conjugate (2) occurring as an intermediate. A similar mechanism has been proposed for the reaction of organic hydroperoxides with GSH[11].

used in the present study does not result in the isolation of all cytosolic GST isoenzymes.

Approx. 5 % and 15 % of the total CDNB activity applied, is not retained by the S-hexylglutathione Sepharose 6B column for rat liver cytosol and rat lung cytosol respectively. This activity can be accounted for by the presence of GST isoenzyme 5-5[13-15]. Isoenzyme 5-5 demonstrates an even higher catalytic activity towards organic peroxides than isoenzyme 2-2[15,16] and may therefore also be partly responsible for the relatively high MLO conjugative activity in rat lung cytosol.

Although glutathione S-transferases are capable of catalyzing the detoxification of MLO in vitro, the in vivo implications of this phenomenon remain to be established. The presence of active phospholipases may be essential, to establish a release of fatty acid ozonides from oxidized membrane phospholipids into the cytoplasm prior to catalysis by GST, as was found in the case of lipid hydroperoxides[17].

In addition, ozone exposure could well lead to induction of GST in the

lung and consequently to a possibly increased resistance towards ozone intoxication, depending on the changes in the isoenzyme pattern. The induction of several glutathione pathway enzyme activities has been described following in vivo exposure to ozone[18,19].

Although glutathione peroxidase was capable of catalyzing the reaction of MLO with GSH in vitro in the present study, its involvement in vivo may be limited. The contribution of glutathione peroxidase to the cytosolic conversion of MLO may be calculated from the cytosolic glutathione peroxidase activities with hydrogen peroxide and the specific activities of purified glutathione peroxidase towards hydrogen peroxide and MLO. This contribution was found to amount to 0.02 nmol GSH/min/mg and 0.12 nmol GSH/min/mg for rat lung and rat liver cytosol respectively, which is less than 2.5 % of the total conversion noticed. In addition, following ozone exposure in vivo (1.5 mg/m^3, 4 days), the sensitivities of alveolar macrophages and type II lung cells towards ozone were unchanged in vitro, in spite of a marked increase in their glutathione peroxidase activities[18].

In summary, the GST-catalyzed reaction of MLO with GSH has been studied on a molecular basis. The involvement of GST in the resistance against ozone-induced lipid oxidation in vivo remains to be established. However, the high activity with MLO noticed for rat lung cytosol indicates that the lung may be relatively well protected against ozone intoxication.

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CHAPTER 3

DIFFERENTIAL INDUCTION OF RAT HEPATIC GLUTATHIONE S-TRANSFERASE ISOENZYMES BY HEXACHLOROBENZENE AND BENZYL ISOTHIOCYANATE: COMPARISON WITH INDUCTION BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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ABSTRACT

Male Wistar rats were treated with hexachlorobenzene, benzyl isothiocyanate, phenobarbital or 3-methylcholanthrene. Hepatic cytosolic glutathione S-transferase (GST) activity was determined with the substrates 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and trans-4-phenyl-3-buten-2-one. Cytosolic glutathione peroxidase activity was measured with cumene hydroperoxide.

GST activity towards 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene and ethacrynic acid was enhanced by all compounds, hexachlorobenzene and 3-methylcholanthrene causing the largest and the smallest increase respectively. Trans-4-phenyl-3-buten-2-one-conjugating activity exhibited only small changes, while peroxidase activity with cumene hydroperoxide was not changed by any of the inducing agents.

GST isoenzymes were purified on S-hexylglutathione Sepharose 6B and separated by means of FPLC-chromatofocusing, to evaluate effects on the GST isoenzyme pattern.

Hexachlorobenzene and phenobarbital both caused an increase in the relative amounts of subunits 1 and 3 when compared with subunits 2 and 4 respectively. For 3-methylcholanthrene only induction of subunit 1 was observed, possibly due to the relatively low induction levels of total GST activity.

In benzyl isothiocyanate-treated animals, an induction of subunit 3 was found as well as an increase in the relative amount of subunit 2. Thus, benzyl isothiocyanate behaves differently from hexachlorobenzene, phenobarbital and 3-methylcholanthrene as an inducing agent of rat hepatic glutathione S-transferases.

INTRODUCTION

The glutathione S-transferases (EC 2.5.1.18) are a family of isoenzymes catalyzing the conjugation of a wide variety of hydrophobic electrophilic compounds with glutathione (GSH). At least ten different isoenzymes occur in rat liver cytosol of which six have been well documented[1-4]. They are dimeric proteins formed by binary combinations of four subunits (1 to 4) and exhibit a broad but overlapping substrate specificity.

Three classes of cytosolic glutathione S-transferases (alpha, mu and pi), common to several mammalian species, have recently been identified[5]. Each class comprises isoenzymes with similar structural and enzymatic properties. Rat liver isoenzymes containing subunits 1 and/or 2 belong to class alpha, while glutathione S-transferases formed by combinations of subunits 3 and/or 4 are members of class mu[5].

Glutathione S-transferase (GST) activity in rat liver can be induced by several classes of compounds[6-9]. Not only total GST activity may be enhanced but GST isoenzyme patterns may be changed as well[10-13]. In view of the differences in substrate specificity between the GST isoenzymes, such changes may have a considerable influence on the toxic effects of various xenobiotics, both with regard to detoxification[14] as well as activation[15].

Phenobarbital and 3-methylcholanthrene have been well described as inducers of rat hepatic glutathione S-transferases[6,7,11-13,16-19]. A number of these studies were concerned with total GST activity towards several second substrates[7,19]. From studies using immunoprecipitation and from studies comparing hepatic concentrations of translationally active GST mRNAs, it is clear that both compounds cause elevated levels of subunits 1 and 3, resulting in an increase of isoenzymes containing those subunits[12,13,16-18]. In rat testis, however, subunit 4 is induced by phenobarbital[20].

The present study describes a comparison of the effects of hexachlorobenzene and benzyl isothiocyanate on rat hepatic glutathione S-transferase isoenzymes with the effects of phenobarbital and 3-methylcholanthrene. Isoenzymes were separated by means of FPLC-chromatofocusing, as described by Ålin et al.[2], a technique which allows evaluation of

relative changes for six isoenzymes in a single run.

Hexachlorobenzene is a waste product in the manufacture of various chlorinated compounds. Prolonged exposure leads to the development of porphyria cutanea tarda, resulting from a defect in the heme biosynthesis pathway[21]. In vivo studies of its biotransformation have demonstrated the occurrence of oxygen- and sulphur-containing metabolites, indicating that both cytochrome P-450 and glutathione S-transferases are involved in its metabolism[22-24]. Elevated levels of GST activity following hexachlorobenzene-treatment have been found in rat liver[25,26] and in the liver of the Japanese quail, a species very susceptible to the development of porphyria[27].

Benzyl isothiocyanate is a naturally occurring, anti-carcinogenic agent, which is also metabolized by glutathione S-transferases[28]. Benzyl isothiocyanate-treatment causes a substantial induction of GST activity in several organs of the mouse[9]. No studies on effects of benzyl isothiocyanate on rat glutathione S-transferases are as yet available.

MATERIALS AND METHODS

Materials. Hexachlorobenzene (BDH Chemicals Ltd., Poole, U.K.), phenobarbital (Interpharm B.V., Rotterdam, The Netherlands), 1-chloro-2,4-dinitrobenzene (Aldrich Chemical Co., Milwaukee, U.S.A.), ethacrynic acid (Sigma Chemical Co., St. Louis, MO), GSH (Merck, Darmstadt, FRG), Servalyt 4-9T and Servalyt 9-11T (Serva Feinbiochemica, Heidelberg, FRG) were purchased from the companies indicated. Benzyl isothiocyanate and 3-methylcholanthrene were obtained from Fluka AG (Buchs, Switzerland). Trans-4-phenyl-3-buten-2-one and 1,2-dichloro-4-nitrobenzene were from Janssen Chimica (Beerse, Belgium). All other chemicals used were standard commercial products of analytical purity.

Epoxy-activated Sepharose 6B and Bio-Gel P-6 DG were from Pharmacia Fine Chemicals (Uppsala, Sweden) and from Bio-Rad Laboratories (Richmond, California) respectively.

S-hexylglutathione was synthesized according to Vince et al.[29] and coupled to epoxy-activated Sepharose 6B as described by Mannervik and

Guthenberg[30].

Treatment of animals. Male Wistar rats (260-310 g) received one of the following treatments: 1 g hexachlorobenzene/kg food, prepared from a 1 % (w/v) solution in olive oil, ad libitum, for 14 days; 5 g benzyl isothiocyanate/kg food, prepared from a 5 % (w/v) solution in olive oil, ad libitum, for 14 days; 0.1 % (w/v) phenobarbital in the drinking-water, ad libitum, for 7 days; 3 intraperitoneal injections of 3-methylcholanthrene (30 mg/kg, in olive oil) on 3 consecutive days. Control rats received similar treatments, i.e. olive oil in food, plain drinking-water or 3 intraperitoneal injections of olive oil.

Purification of glutathione S-transferase isoenzymes. Glutathione S-transferase isoenzymes were purified essentially as described by Ålin et al.[2]. Livers were perfused with 25 mM Tris-HCl pH 7.4, containing 0.25 M sucrose, and homogenized in the same buffer. The homogenates were centrifuged at 16,000 g for 20 min to remove cell debris. The cytosol fraction was obtained by centrifugation of the resulting supernatant at 90,000 g for 90 min.

The cytosol fraction from one liver was filtered over glass-wool and loaded onto an affinity column of S-hexyl GSH Sepharose 6B (2.5 x 8 cm), equilibrated with 100 mM Tris-HCl pH 7.8/1 mM EDTA/0.2 mM dithiothreitol (DTT) (buffer A). The column was washed with approx. 400 ml of buffer A, containing 200 mM NaCl, until no more protein could be detected in the effluent. The bound glutathione S-transferases were eluted with a 200 ml gradient of 0-2.5 mM S-hexyl GSH in the salt-fortified buffer. The GST-containing fractions (approx. 90 ml) were pooled, concentrated by ultrafiltration (Amicon Diaflo YM-10 filter) to approx. 6 ml and desalted on a Bio-Gel P-6 DG column (2.5 x 11 cm), equilibrated with 5 mM Tris-HCl pH 8.0/1 mM EDTA/0.2 mM DTT. The resulting GST-pool was again concentrated by ultrafiltration to approx. 6 ml. GST isoenzymes were subsequently separated by chromatofocusing on a Mono P™ HR 5/20 column (Pharmacia FPLC system), equilibrated with 25 mM triethylamine-HCl pH 10. One ml fractions of the GST concentrate, containing approx. 5 mg of protein, were adjusted to pH 9 with 25 mM triethylamine immediately before chromatofocusing. The eluent consisted of a 1:200 dilution of a mixture of 1 ml Servalyt 4-9T and 1 ml Servalyt 9-11T, adjusted to pH 7 with HCl.

A prerun of 2 ml of the eluent was carried out before injection of the above 1 ml sample onto the column. A flow rate of 1 ml/min was used throughout the run, and the effluent was monitored continuously at 280 nm. Individual isoenzymes were collected in separate fractions and samples from each fraction were analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[31], using 17.5 % (w/v) polyacrylamide gels. Finally, ampholytes were removed on a Bio-Gel P-6 DG column (1.5 x 16 cm), equilibrated with 25 mM potassium phosphate buffer pH 7.4/1 mM EDTA/0.2 mM DTT. All steps up to chromatofocusing were performed at 4 °C. Chromatofocusing and subsequent removal of ampholytes were carried out at room temperature.

Enzyme assays. Glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and trans-4-phenyl-3-buten-2-one was measured spectrophotometrically at 25 °C, according to Habig et al.[32].

Peroxidase activity was measured with cumene hydroperoxide as described by Lawrence and Burk[33].

Microsomal GST activity was measured with 1-chloro-2,4-dinitrobenzene as the second substrate, using microsomes washed twice in 0.15 M Tris-HCl pH 8.0 to remove contaminating cytoplasm. The extent of removal was assessed by measuring lactate dehydrogenase activity according to Mitchell et al.[34]. Less than 3 % of the lactate dehydrogenase activity in unwashed microsomes (20-40 $\mu molmin^{-1}$) remained after 2 washes.

Protein. Protein concentrations were determined by the method of Lowry et al.[35], with bovine serum albumin as a standard. For the isoenzyme fractions, a modified procedure, described by Peterson[36], was followed.

RESULTS

Effects on body weights and liver weights. Initial body weights, final body weights and liver weights were recorded during the experiments. Hexachlorobenzene-treated animals exhibited lower weight gain (+ 13 $\pm$ 13 g) than their controls (+ 49 $\pm$ 11 g), while benzyl isothiocyanate-treatment resulted in a loss of body weight (- 35 $\pm$ 6 g).

All compounds, with the exception of benzyl isothiocyanate, caused an increase in liver weight expressed as a percentage of body weight. No change was observed for benzyl isothiocyanate; actual liver weights of benzyl isothiocyanate-treated rats were lower than those of their respective controls, due to the overall loss of body weight.

Effects on glutathione S-transferase activities. Cytosolic hepatic GST activities were determined using several second substrates (table 1). Hexachlorobenzene increased GST activity with 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene to 3 to 4 times control levels, while in benzyl isothiocyanate-, phenobarbital-, and 3-methylcholanthrene-treated animals an approx. 2-fold stimulation was found. GST activity towards ethacrynic acid was enhanced by all compounds, hexachlorobenzene and 3-methylcholanthrene again causing the largest and the smallest increase respectively.

Changes in the formation of the conjugate of trans-4-phenyl-3-buten-2-one were relatively small. Peroxidase activity towards cumene hydroperoxide was unchanged in all groups.

Microsomal GST activity with 1-chloro-2,4-dinitrobenzene was determined in microsomes washed twice in 0.15 M Tris-HCl pH 8.0 and was not enhanced by any of the inducing agents (results not shown).

Effects on glutathione S-transferase isoenzyme patterns. Glutathione S-transferases were purified by use of affinity chromatography. Approx. 5 % of the activity loaded onto the affinity column was not retained. This percentage was similar for control and for treated samples. The total amount of GST protein recovered from the affinity column followed by the Bio-Gel P-6 DG desalting column was increased from 1.7 $\pm$ 0.3 mg/g liver in control rats to 3.9 $\pm$ 0.3 mg/g liver in hexachlorobenzene- and 3.0 $\pm$ 0.5 mg/g liver in benzyl isothiocyanate-treated animals. Following phenobarbital- and 3-methylcholanthrene-treatment, GST protein amounted to 2.8 $\pm$ 0.2 mg/g liver and 2.1 $\pm$ 0.0 mg/g liver respectively.

Isoenzymes were separated by means of FPLC-chromatofocusing, and identified by their position in the elution profile, their subunit molecular weights and their specific activities towards 1-chloro-2,4-dinitrobenzene. SDS-PAGE of FPLC isoenzyme fractions showed that isoenzyme 2-2 was always contaminated with subunit 1, while isoenzyme 3-3

Table 1. Effects of hexachlorobenzene (HCB), benzyl isothiocyanate (BITC), phenobarbital (PB) and 3-methylcholanthrene (3MC) on rat hepatic cytosolic glutathione S-transferase activities toward several substrates

treatment	n	enzyme activity with				
		CDNB* ($nmolmin^{-1}mg^{-1}$)	DCNB* ($nmolmin^{-1}mg^{-1}$)	EA* ($nmolmin^{-1}mg^{-1}$)	TPBO* ($nmolmin^{-1}mg^{-1}$)	cumene hydroperoxide ($nmolmin^{-1}mg^{-1}$)
none (HCB/BITC control)	4	1590 ± 50	54 ± 4	34 ± 2	28 ± 3	530 ± 50
HCB	4	5570 ± 170 (350)≠	157 ± 12 (291)≠	160 ± 14 (471)≠	37 ± 3 (132)	490 ± 80 (92)
BITC	4	3160 ± 400 (199)≠	118 ± 11 (219)≠	60 ± 4 (176)≠	38 ± 4 (136)+	540 ± 60 (102)
none (PB control)	2	2220 ± 100	69 ± 1	38 ± 4	33 ± 7	570 ± 40
PB	2	4860 ± 390 (219)≠	149 ± 9 (216)≠	68 ± 4 (179)‡	39 ± 1 (118)	640 ± 20 (112)
none (3MC control)	2	1780 ± 10	56 ± 2	32 ± 3	21 ± 8	540 ± 40
3MC	4	2980 ± 180 (167)‡	79 ± 3 (141)≠	44 ± 2 (138)‡	28 ± 1 (133)	500 ± 50 (93)

Male Wistar rats (260 - 310 g) were treated with 0.1 % (w/w) hexachlorobenzene in food for 14 days, 0.5 % (w/w) benzyl isothiocyanate in food for 14 days, 0.1 % (w/v) phenobarbital in drinking-water for 7 days, or 3 i.p. injections of 3-methylcholanthrene (30 mg/kg/day) on 3 successive days. Controls received olive oil in food, plain drinking-water, or i.p. injections of olive oil. GST activities towards 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, ethacrynic acid and trans-4-phenyl-3-buten-2-one were measured at 25 °C according to Habig et al.[32]. Activity with cumene hydroperoxide was measured at 25 °C by the method of Lawrence and Burk[33]. Values are expressed as means ± S.E.M. Percentage of control is given in parentheses.

* CDNB: 1-chloro-2,4-dinitrobenzene; DCNB: 1,2-dichloro-4-nitrobenzene; EA: ethacrynic acid; TPBO: trans-4-phenyl-3-buten-2-one. + $P < 0.05$ (one-sided Student's t-test); ‡ $P < 0.025$ (one-sided Student's t-test); ≠ $P < 0.01$ (one-sided Student's t-test); ≠ $P < 0.005$ (one-sided Student's t-test).

contained a small amount of subunit 2 only in some separations.

FPLC isoenzyme patterns are shown in figure 1. Since no differences could be detected between the different controls, only the hexachlorobenzene/benzyl isothiocyanate control pattern is given (figure 1A). From figures 1B and 1D it is clear that both hexachlorobenzene and phenobarbital caused an increase in the relative concentrations of isoenzymes 1-1 and 3-3 as well as a decrease in the relative amounts of isoenzymes 2-2 and 4-4, the effect of hexachlorobenzene being somewhat stronger than the effect of phenobarbital.

The induction of subunits 1 and 3 by hexachlorobenzene and phenobarbital was also demonstrated by the relative amounts of protein contained in subunits 1 to 4 (figure 2). Both treatments resulted in an increase of the relative amounts of protein represented by subunits 1 and 3 when compared with subunits 2 and 4 respectively.

Interestingly, the majority of protein was accounted for by subunits 1 and 2 in treated animals, while in controls the reverse situation was observed.

Benzyl isothiocyanate did not induce subunit 1, but comparing figures 1A and 1C isoenzyme 2-2 seems to be enhanced. The induction of subunit 2 was confirmed by an increase in the relative amount of protein represented by this subunit (figure 2).

Although the effect of benzyl isothiocyanate on isoenzyme 3-3 was less pronounced than in the case of hexachlorobenzene and of phenobarbital, isoenzymes 3-3 and 3-4 were increased when compared with isoenzyme 4-4, suggesting that subunit 3 was in fact induced. The latter also seemed to hold for 3-methylcholanthrene (figure 1E). However, in this case the relative amount of protein contained in subunit 3 was not increased, indicating that no induction of subunit 3 had occurred. 3-Methylcholanthrene-treatment only resulted in an induction of isoenzyme 1-1 (figure 1E).

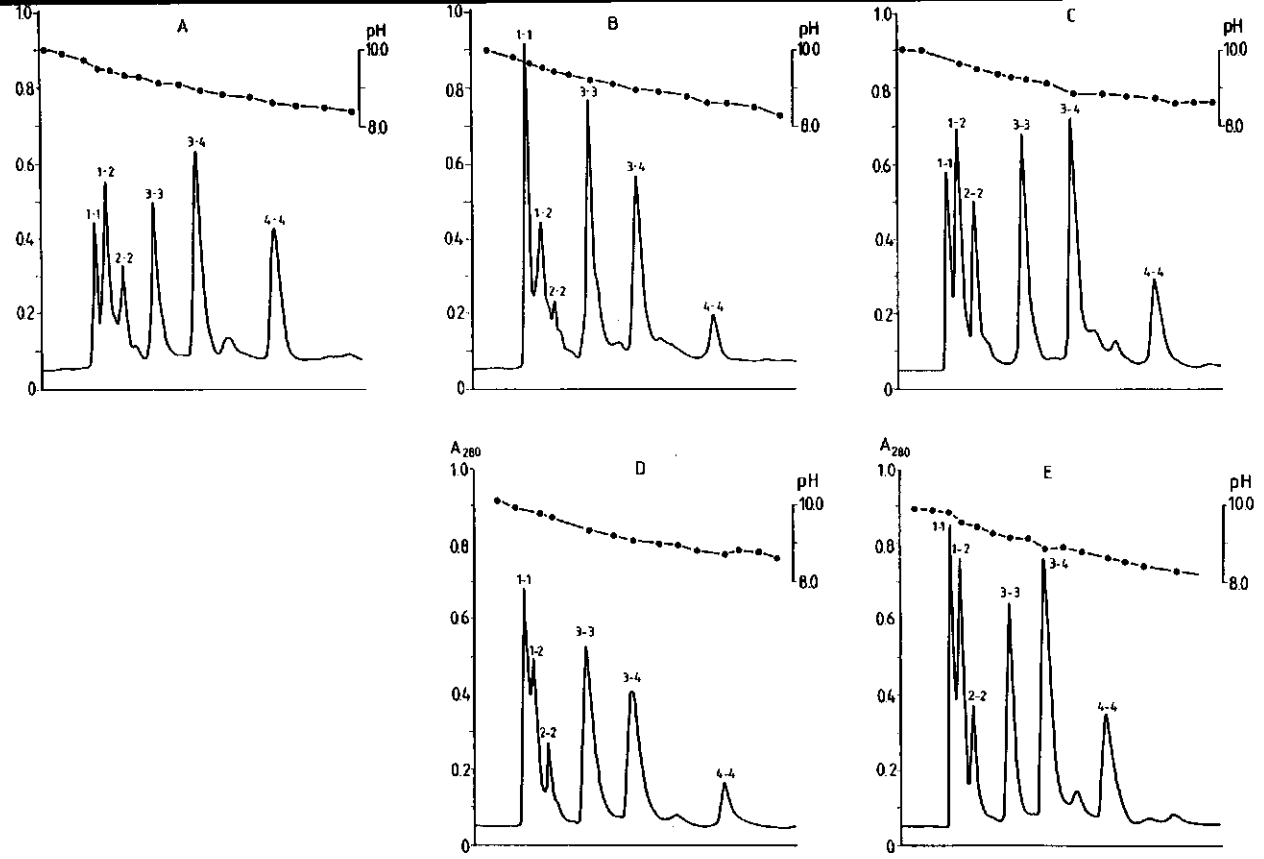

Figure 1. Glutathione S-transferase isoenzyme patterns in rat liver from control (A), hexachlorobenzene-(B), benzyl isothiocyanate-(C), phenobarbital- (D), and 3-methylcholanthrenetreated (E) rats.

For treatment of animals see Materials and Methods. GST were purified on S-hexylglutathione Sepharose 6B and isoenzymes were separated by means of chromatofocusing on a Mono P™ HR 5/20 column. The eluent for chromatofocusing consisted of a 1:200 dilution of a mixture of 1 ml Servalyt 4-9T and 1 ml Servalyt 9-11T, adjusted to pH 7 with HCl. Individual isoenzymes were collected in separate fractions, and identified by their position in the elution profile, their subunit molecular weights and their specific activities towards 1-chloro-2,4dinitrobenzene.

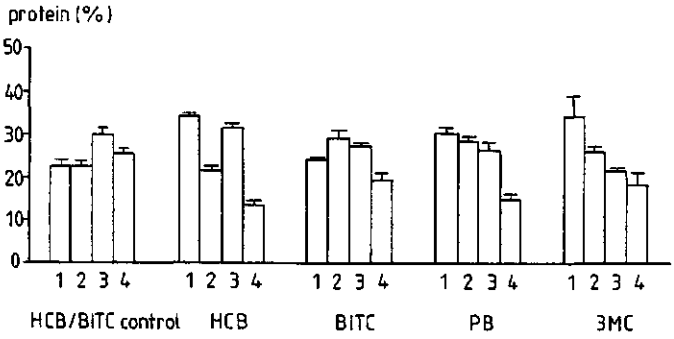

Figure 2. Effects of hexachlorobenzene, benzyl isothiocyanate, phenobarbital and 3-methylcholanthrene on the relative amounts of protein represented by subunits 1 to 4.
Relative amounts of protein were calculated from FPLC isoenzyme fractions, as follows:

$$\text{Protein}_{\text{isoenzyme}} = \frac{\text{Activity}^{*}_{\text{isoenzyme}}}{\text{Mean specific activity}^{+}_{\text{isoenzyme}}}$$

$$\%\ \text{Protein}_{\text{subunit}} = \frac{\text{Protein}_{\text{homodimer}} + \tfrac{1}{2}\,\text{Protein}_{\text{heterodimer}}}{\text{Protein}_{\text{isoenzymes 1-1 to 4-4}}} \times 100$$

Values represent means $\pm$ S.E.M.

*total 1-chloro-2,4-dinitrobenzene-conjugating activity in FPLC isoenzyme fractions.
+specific activities with 1-chloro-2,4-dinitrobenzene were determined in isoenzyme fractions from which ampholytes had been removed on Bio-Gel P-6 DG.
Mean specific activities used were:
isoenzyme 1-1:60 $\mu molmin^{-1}mg^{-1}$; isoenzyme 1-2: 39 $\mu molmin^{-1}mg^{-1}$; isoenzyme 2-2: 30 $\mu molmin^{-1}mg^{-1}$; isoenzyme 3-3: 46 $\mu molmin^{-1}mg^{-1}$; isoenzyme 3-4: 39 $\mu molmin^{-1}mg^{-1}$; isoenzyme 4-4: 19 $\mu molmin^{-1}mg^{-1}$.

DISCUSSION

The present study demonstrates that treatment of male Wistar rats with hexachlorobenzene, benzyl isothiocyanate, phenobarbital or 3-methylcholanthrene not only results in elevated levels of hepatic cytosolic glutathione S-transferases, but causes changes in the GST isoenzyme pattern as well. The technique of FPLC-chromatofocusing has proved a unique tool in the evaluation of relative changes in the GST isoenzyme pattern, both on an isoenzyme and on a subunit level.

Although the four compounds investigated have rather different chemical structures, three of these, hexachlorobenzene, phenobarbital and 3-methylcholanthrene demonstrate similar effects, i.e. a preferential induction of subunits 1 and 3. Benzyl isothiocyanate differs from the other inducing agents in that this compound does not enhance the relative amount of subunit 1, but causes an increase in subunit 2 instead. However, all inducers demonstrate one common effect: alpha-class subunits are induced to a greater extent than mu-class subunits, since subunits 1 and 2 represent 53 to 60 % of the GST protein in treated animals, whereas only 38 to 45 % of the protein can be accounted for by these subunits in controls.

The mechanism of induction of rat hepatic cytosolic glutathione S-transferases is still unknown. Alpha-class subunits 1 and 2 demonstrate approx. 68 % amino acid sequence homology, but they are coded for by separate mRNAs, which are regulated independently[37-39]. Similarly, mu-class subunits 3 and 4 are closely related but distinctly different polypeptides, for which separate mRNAs exist[18,40]. Little sequence homology is, however, shared by alpha- and mu- class subunits, which, therefore, seem to represent two distinct gene families[41].

By use of hybridization experiments with specific cDNA clones, Ding et al.[18] and Pickett et al.[17,41] demonstrated that phenobarbital and 3-methylcholanthrene cause a 5- to 10-fold increase in the level of subunit 1-mRNA and a 5- to 6-fold elevation of mRNAs coding for subunits 3 and 4, in rat liver, 16 to 24 hours following administration. For subunit 2-mRNA, only a 2-fold induction was found[17,18,41]. Activation of the transcription of glutathione S-transferase genes by phenobarbital and 3-methylcholanthrene appeared to account at least

partly for the observed increase in mRNA-levels[41,42].

No information is at present available on the mechanism of transcriptional activation of glutathione S-transferase genes by xenobiotics. There is no evidence for the involvement of specific receptors in rat liver to which xenobiotics or their metabolites could bind, in analogy to the AH-receptor-mediated induction of P450c by 3-methylcholanthrene[41,42].

Our results clearly confirm on a protein level that the synthesis of individual subunits is regulated independently. The absolute amount of protein per subunit per gram liver can be estimated from the relative amounts of protein represented by individual subunits and the total amount of GST protein per gram liver. Following treatment with phenobarbital or 3-methylcholanthrene, no increase in subunit 4 is then found, while the increase in subunit 2 as compared with subunits 1, 3 and 4 is larger than expected on the basis of the increases in mRNA levels described above. Differences in translational efficiency and/or turnover rates between mRNAs coding for individual subunits may exist in vivo, indicating the importance of evaluating the induction of glutathione S-transferase isoenzymes on a protein level.

The differential behaviour of benzyl isothiocyanate as compared with the other compounds used, indicates that, although the exact mechanism of transcriptional activation of GST genes remains to be elucidated, some relationship may exist between chemical structure of the inducing agent and subunit induction. However, irrespective of the chemical structure of the administered xenobiotic, alpha-class subunits seem to be enhanced preferentially.

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CHAPTER 4

IRREVERSIBLE INHIBITION OF RAT GLUTATHIONE S-TRANSFERASE ISOENZYMES BY QUINONES

4.1

IRREVERSIBLE INHIBITION OF RAT HEPATIC GLUTATHIONE S-TRANSFERASE ISOENZYMES BY A SERIES OF STRUCTURALLY RELATED QUINONES

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ABSTRACT

The effect of several structurally related 1,4-benzoquinones and 1,4-naphthoquinones on the activity of rat hepatic glutathione S-transferases was studied. For the 1,4-benzoquinones, the extent of inhibition increased with an increasing number of halogen substituents. Neither the type of halogen nor the position of chlorine-atoms was of major importance. Similarly, 2,3-dichloro-1,4-naphthoquinone demonstrated a considerably higher inhibitory activity than 5-hydroxy-1,4-naphthoquinone. 2-Methyl derivatives of 1,4-naphthoquinone did not inhibit glutathione S-transferase activity at all. The irreversible nature of the inhibition was shown both by the time-course of the inhibition as well as by the fact that removal of the inhibitor by ultrafiltration did not restore the enzymatic activity. Incubation of quinones and enzyme in the presence of the competitive inhibitor S-hexylglutathione, slowed the inhibition considerably, indicating an involvement of the active site. Isoenzyme 3-3 was found to be most sensitive towards the whole series of inhibitors, whereas the activity of isoenzyme 2-2 was least affected in all cases.

The inhibition by quinones is probably mainly due to covalent modification of a specific cysteine residue in or near the active site. The differential sensitivities of individual isoenzymes indicate that this residue is more accessible and/or easier modified in isoenzyme 3-3 than in any of the other isoenzymes tested. The findings suggest that quinones form a class of compounds from which a selective *in vivo*-inhibitor of the glutathione S-transferases might be developed.

INTRODUCTION

The cytosolic glutathione S-transferases (EC 2.5.1.18)(GST) from several mammalian species can be divided into three classes (alpha, mu and pi), based on similar structural characteristics as well as enzymatic properties with standard substrates and inhibitors[1]. Although several classes of inhibitors of these enzymes are known[2-8], none of them have been shown to work well in vivo.

A selective, in vivo inhibitor would be an important tool, for elucidation of metabolic pathways of drugs and other xenobiotics but especially for use as a potentiating agent for alkylating anticancer drugs. Several classes of glutathione S-transferase isoenzymes have been found to be overexpressed in tumour cells that have become resistant to cytotoxic drugs[9-11]. A selective inhibitor of the GST might well result in overcoming this resistance.

A number of quinones have been found to be capable of inhibiting GST activity, both naturally occurring as well as synthetic ones[12-15]. Recently, 2,3,5,6-tetrachloro-benzoquinone and its mono-substituted glutathione conjugate were shown to be very strong inhibitors of rat glutathione S-transferases[15]. A five-fold molar excess of the quinone as compared with the monomeric enzyme concentration results in 70-80 % inhibition of GST activity after 5 min. of incubation at 25 °C[15]. The inhibition is due to arylation of the enzyme, presumably in or near the active site, i.e. the glutathione-binding site, since at 0 °C, the glutathione-conjugate demonstrates a substantial faster inhibition than the corresponding β-mercaptoethanol conjugate, indicating a targeting effect of the glutathione moiety. In addition, the presence of the competitive inhibitor S-hexylglutathione slows down the inhibition considerably[15].

However, in spite of its obvious selectivity the reactivity of the quinone is such that it may cause considerable toxicity in vivo. In a first attempt, therefore, to find a quinone with a suitable selectivity/reactivity ratio, the inhibition of GST activity by several structurally related 1,4-benzoquinones (BQ) and 1,4-naphthoquinones (NQ) was studied, to determine which structural characteristics are important determinants for the inhibitory activity towards glutathione S-transferases.

The nature of the inhibition was investigated using three quinones with different chemical structures as model compounds, to establish whether the inhibition characteristics found for tetrachloro-BQ apply to quinones more generally; 2-tert-butyl-BQ is a metabolite of the food antioxidant 2(3)-tert-butyl-4-hydroxyanisole[16], which has been shown to possess both carcinogenic as well as anti-carcinogenic properties[17-19]; 5-hydroxy-NQ (juglone) is a naturally occurring quinone, found in the shells of unripe walnuts; 2,3-dichloro-NQ (dichlone) is a synthetic compound, used as a fungicide. The individual sensitivities of several rat hepatic isoenzymes were compared.

MATERIALS AND METHODS

Materials. 1,4-Benzoquinone (Janssen Chimica, Beerse, Belgium), tetrabromo-BQ, tetrachloro-BQ (Merck, Darmstadt, FRG), 2,6-dichloro-BQ and 2,5-dichloro-BQ (Eastman Kodak Co., Rochester, USA) were purchased from the companies indicated. Tetramethyl-BQ, tetrafluor-BQ, 2,3-dichloro-NQ, 5-hydroxy-NQ, 2-methyl-NQ, 2-methyl-5-hydroxy-NQ, 2-methyl-3-phytyl-NQ (vit. K_1), 2-tert-butyl-1,4-hydroquinone and 2-chloro-1,4-hydroquinone were from Aldrich Chemical Co. (Milwaukee, WI). 2-Tert-butyl-BQ and 2-chloro-BQ were prepared from their respective hydroquinones by oxidation with $KBrO_3/H_2SO_4$ in aqueous 1,4-dioxane, according to Grinev and Terent'ev[20,21]. Melting points were in agreement with literature values. 2-chloro-1,4-hydroquinone was recrystallized from chloroform twice before use.

Purification of glutathione S-transferase isoenzymes. Glutathione S-transferase isoenzymes were purified on S-hexylglutathione Sepharose 6B and separated by FPLC-chromatofocusing (Mono P^{TM} HR 5/20 column) as described elsewhere[22]. Purity of the isoenzymes was evaluated by means of SDS-PAGE analysis.

Enzyme assays. Glutathione S-transferase activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25 °C, pH 6.5, according to Habig et al[23]. Incubations with quinones were performed using a monomeric enzyme concentration of 1 μM.

Enzyme and quinones were incubated for 15 min. at 25 °C, in a 25 mM potassium phosphate-buffer pH 7.4, supplemented with 1mM EDTA. A 10 to 25 µl-sample was drawn from the incubation mixture for determination of GST activity according to Habig et al.[23], using 1 mM glutathione (GSH) and 1 mM CDNB in a final volume of 1 ml. Concentrations of quinones in this final incubation mixture were generally lower than 1.25 µM, practically ruling out competitive inhibition by quinone-glutathione conjugates, formed during activity measurements.

RESULTS

The inhibition of rat GST activity by several structurally related 1,4-benzoquinones and 1,4-naphthoquinones is shown in figures 1A and 1B. Fully halogenated benzoquinones were most inhibitory, demonstrating 65 to 80 % inhibition at a 2 µM concentration after 15 min. of incubation at pH 7.4, 25 °C. 2,5-Dichloro-BQ and 2,6-dichloro-BQ were somewhat less inhibitory, reaching approximately 70 % inhibition at 15 µM. 2-Chloro-BQ and 2-tert-butyl-BQ inhibited GST activity only slightly more than 1,4-benzoquinone itself, while 2,3,5,6-tetramethyl-BQ did not show any inhibitory activity.

2,3-Dichloro-NQ was the strongest inhibiting 1,4-naphthoquinone, followed by 5-hydroxy-NQ. 2-Methyl-derivatives did not inhibit GST activity at any of the concentrations used.

The extent of the inhibition was strongly dependent on incubation time, as was demonstrated for 2-tert-butyl-BQ, 5-hydroxy-NQ and 2,3-dichloro-NQ (fig. 2), suggesting the inhibition was of an irreversible nature. When the concentration of unreacted inhibitor was reduced approximately 10.000 times by means of ultrafiltration (Amicon Diaflo YM-10 filter) in 25 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA and 5 % (v/v) ethanol, the activity of the glutathione S-transferases was not increased (shown in fig. 3 for 5-hydroxy-NQ), which also indicated that the inhibition was irreversible.

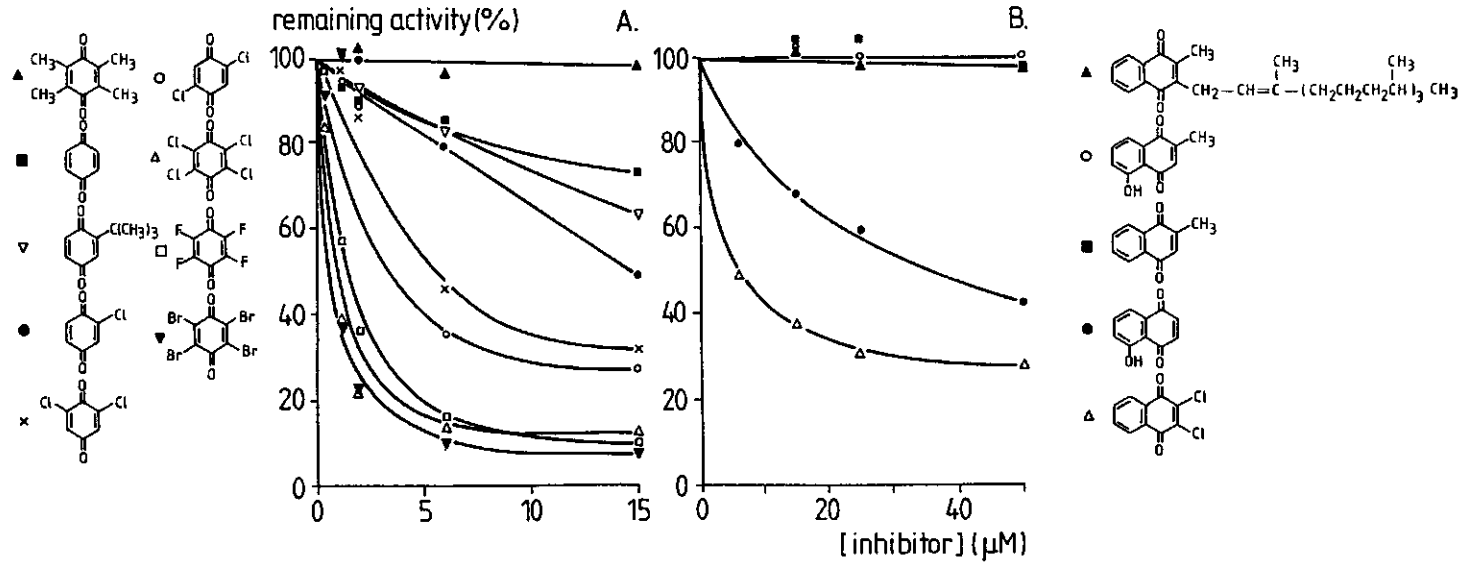
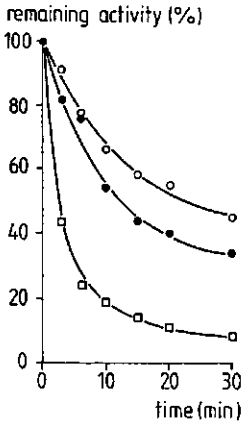


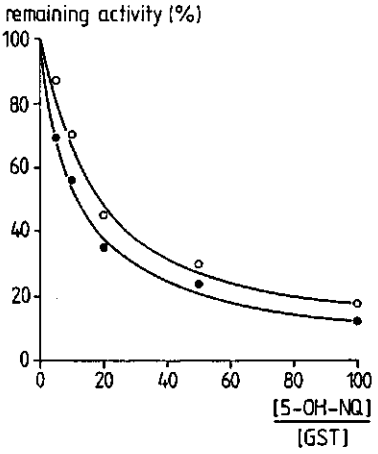
Figure 1. Inhibition of rat glutathione S-transferase activity by structurally related 1,4-benzoquinones (A) and 1,4-naphthoquinones (B). A mixture of glutathione S-transferase isoenzymes (monomeric concentration 1 μM) and inhibitor were incubated for 15 min. at 25 °C in 25 mM potassium phosphate buffer pH 7.4/1mM EDTA. A 25 μl-sample was drawn from the incubation mixture for determination of the glutathione S-transferase activity according to Habig et al.[23].
Results are presented as means of duplicate determinations of one out of two experiments. Standard deviations were generally less than 5 %.

Figure 2.



Time-course of the inhibition of rat glutathione S-transferase activity by 2-tert-butyl-1,4-benzoquinone (200 µM) (●), 5-hydroxy-1,4-naphtho-quinone (20 µM) (o) and 2,3-dichloro-1,4-naphthoquinone (50 µM)(□).
Incubations were performed as described in the legend of figure 1. Results are presented as means of duplicate or triplicate determinations of one experiment representative of at least three. Standard deviations were generally less than 4 %.

Figure 3.



Effect of reduction of the concentration of unreacted 5-hydroxy-1,4-naphthoquinone by means of ultrafiltration on the inhibition of rat glutathione S-transferase activity.
Enzyme (monomeric concentration 9.6 µM) and quinone were incubated at pH 7.4, 25 °C. After 30 min of incubation a small sample was drawn for determination of glutathione S-transferase activity. In the remainder of the incubation mixture the concentration of unreacted quinone was reduced approximately 10.000 times by means of ultrafiltration (Amicon Diaflo YM-10 filter), after which the glutathione S-transferase activity was measured again. Blanks without quinone were treated similarly. ●: without ultrafiltration; o: with ultrafiltration.
Results are presented as means of two separate experiments. Standard deviations were generally less than 5 %.

For 5-hydroxy-NQ and 2,3-dichloro-NQ the time-course of the inhibition was studied in the presence or absence of a four-fold concentration of S-hexylglutathione, as compared with the concentrations of the quinones. As demonstrated in figure 4, S-hexylglutathione slowed down the inhibition considerably.

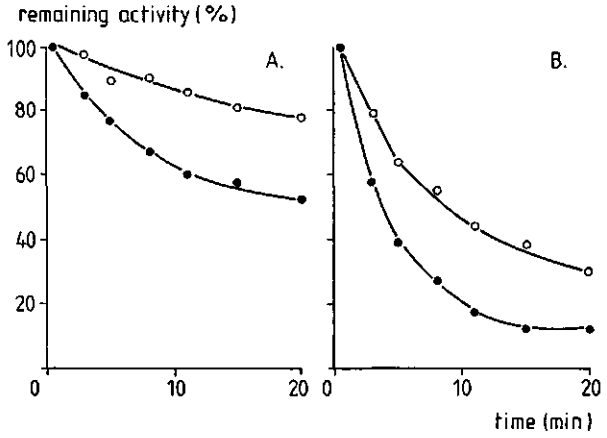


Figure 4. Effect of S-hexylglutathione on the time-course of inhibition of rat glutathione S-transferase activity by 5-hydroxy-1,4-naphthoquinone (20 µM)(A) and 2,3-dichloro-1,4-naphthoquinone (10 µM)(B). •: incubation without S-hexylglutathione; o: incubation in the presence of a fourfold concentration of S-hexylglutathione as compared with the concentration of quinone. Incubations were performed as described in the legend of figure 1. Results are presented as means of triplicate determinations of one experiment. Standard deviations were generally less than 8 %.

The influence of 2-tert-butyl-BQ, 5-hydroxy-NQ and 2,3-dichloro-NQ on the activity of individual isoenzymes, is shown in fig. 5. Isoenzyme 3-3 was found to be most sensitive towards all three inhibitors. Isoenzymes 2-2 and 1-1 demonstrated similar inhibition characteristics for 5-hydroxy-NQ and 2,3-dichloro-NQ, and were least inhibited. The activity of isoenzyme 2-2 was also least affected by 2-tert-butyl-BQ. Isoenzyme 4-4 demonstrated intermediate sensitivity.

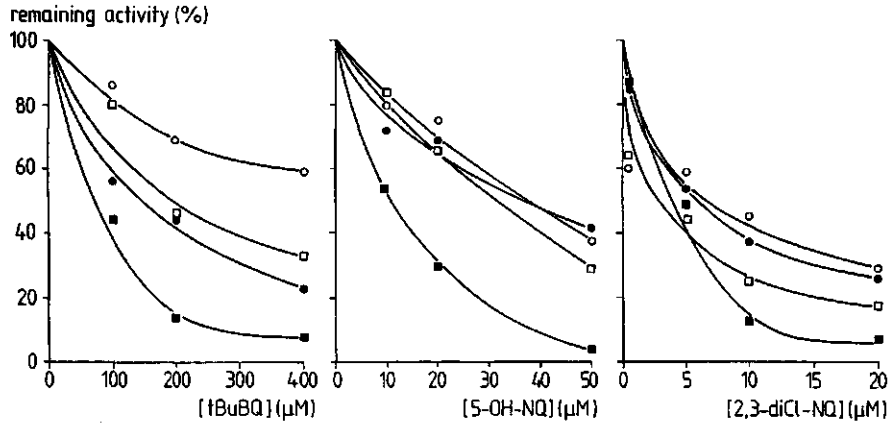


Figure 5. Sensitivity of glutathione S-transferase isoenzymes 1-1 (•), 2-2 (o), 3-3 (■) and 4-4 (□) towards 2 tert-butyl-1,4-benzoquinone, 5-hydroxy-1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone. Incubations were performed as described in the legend of figure 1. Results are presented as means of duplicate determinations of one experiment. Standard deviations were generally less than 5 %.

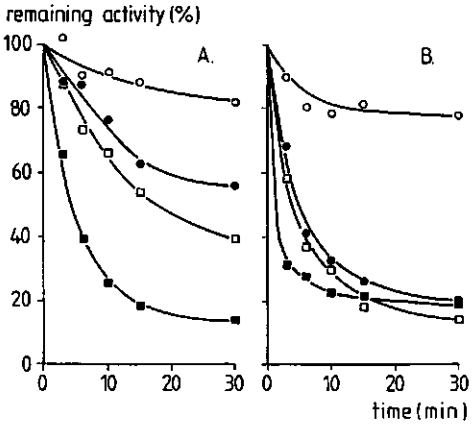


Figure 6. Time-course of inhibition of glutathione S-transferase isoenzymes 1-1 (•), 2-2 (o), 3-3 (■) and 4-4 (□) by 5-hydroxy-1,4-naphthoquinone (15 μM) (A) and 2,3-dichloro-1,4-naphthoquinone (15 μM) (B). Incubations were performed as described in the legend of figure 1. Results are presented as means of duplicate determinations of one out of two or three experiments. Standard deviations were generally less than 6 %.

A time-course experiment, using 15 μM concentrations of 5-hydroxy-NQ and 2,3-dichloro-NQ, also demonstrated that isoenzymes 3-3 and 2-2 are most and least sensitive towards the inhibitors, respectively (fig. 6). Although after 30 min. of incubation, remaining activities were similar for isoenzymes 3-3, 4-4 and 1-1 with respect to the inhibitor 2,3-dichloro-NQ, fastest inhibition was noticed for isoenzyme 3-3 (fig. 6B).

DISCUSSION

For 1,4-benzoquinones the extent of inhibition of the GST increases with an increasing number of halogen substituents. The type of halogen does not seem to be of major importance, at least for fully substituted benzoquinones. Similarly, the position of the chlorine-atoms does not influence the inhibition noticed for the dichloro-BQ isomers. In agreement with these results, 2,3-dichloro-NQ is a much more effective inhibitor than 5-hydroxy-NQ, which does not contain 2,3-substituents, while methyl-substitution at the 2-position abolishes the inhibitory action completely.

The presence of electron-withdrawing substituents on the quinone ring thus results in an increase of the inhibitory activity as compared with the parent quinone, whereas the presence of electron-donating substituents results in a decrease of the extent of inhibition. Partly analogous experiments have been performed by Motoyama et al.[12] and Dierickx[13]. Although the irreversible nature of the inhibition was not specifically studied, it is also apparent from their data that the presence of electron-donating substituents, such as hydroxy-, methyl- or methoxy-groups reduces the loss of activity when compared with the unsubstituted quinones. Motoyama et al.[12] reported a much higher inhibition for 2-methyl-derivatives of 1,4-naphthoquinones than found in the present study. Besides a different source of enzyme (housefly), this discrepancy may be explained by the use of a high pH (pH 9) for incubations and by the use of 1,2-dichloro-4-nitrobenzene as a substrate, which is not a substrate for all GST isoenzymes.

The loss of activity caused by quinones is of an irreversible nature,

as is demonstrated by the time-course of the inhibition as well as by the lack of influence of removal of unreacted inhibitor. The slower inhibition found when enzyme and quinone were incubated in the presence of the competitive inhibitor S-hexylglutathione, was previously also observed for the mono-glutathione conjugate of tetrachloro-BQ, at 0 °C[15]. Raising the temperature of the incubation mixture to 25 °C for 10 min resulted in the same extent of inhibition for this quinone, with or without S-hexylglutathione present[15]. Thus, the effect of S-hexylglutathione is not due to a reduction of the effective concentration of the quinones by a direct reaction with this compound, but clearly indicates an involvement of the active site. It can be concluded, therefore, that the inhibition characteristics of tetrachloro-BQ are shared by other quinones.

Quinones may be involved in two types of irreversible interactions, i.e. oxidation and/or alkylation. The involvement of cysteine residues in the inhibition of GST activity has been demonstrated for tetrachloro-BQ. Incubation of isoenzyme 4-4 with tetrachloro-BQ completely prevented the subsequent alkylation of the enzyme by the sulfhydryl reagent iodoacetamide. In addition the number of [^{14}C]-labeled tetrachloro-BQ molecules bound per monomer, exactly equalled the number of cysteine residues present[24]. A role for cysteines is not entirely unexpected in view of the high reactivity of quinones in general towards protein sulfhydryls and small sulfhydryl-containing compounds such as GSH[25,26].

Both under aerobic and anaerobic conditions the reaction of NQ or 2-methyl-NQ with GSH leads to the formation of glutathione-conjugates and oxidized glutathione (GSSG)[27,28]. Under aerobic conditions the formation of GSSG is not only due to a direct redox reaction between the quinone and GSH, but also results from the production of super oxide anion radicals and hydrogen peroxide[28]. Wefers and Sies have demonstrated that super oxide anion radicals formed by xanthine/xanthine oxidase or by hydroquinone auto-oxidation are capable of inducing the formation of small amounts of glutathione sulfonate in addition to large amounts of GSSG (6-15 % of the GSSG formed)[29]. For bovine serum albumin and papain, it was found that incubations with 2-methyl-NQ resulted in covalent modification of cysteine residues. The decrease in protein

sulfhydryl groups was accompanied by a concomitant increase in absorption at 430 nm, indicative of a thioether linkage[30].

Several results from the present study in addition to studies presented by other investigators indicate that the inhibition of GST activity by quinones is mediated by modification of one specific cysteine residue. This has already been firmly established for tetrachloro-BQ[24]. Although isoenzyme 4-4, containing 3 cysteines per monomer, was capable of binding 3 tetrachloro-BQ molecules per monomer, an almost complete inhibition was found after modification of the first cysteine residue, located in or near the active site[15,24]. Similarly, Carne et al.[31], who used several reagents for cysteine modification in ligandin (isoenzymes 1-1 and 1-2), found that the enzymatic activity is most affected by the loss of one particular residue (although in this case it was the third residue modified). The 40-65 % inhibition and 65-80 % inhibition observed in the present study for completely halogenated benzoquinones at concentrations only 1.2-fold and 2-fold higher than the monomeric enzyme concentrations, also suggests that modification of all cysteines is not necessary to obtain maximum inhibition. It seems unlikely therefore that the inhibition by quinones results from the formation of a cystine disulfide linkage. Although the involvement of a cysteine-sulfonate can as yet not be excluded, the similar inhibition characteristics of the quinones tested in the present study as compared with tetrachloro-BQ as well as the type of interaction reported for 2-methyl-NQ with bovine serum albumin and papain strongly suggest that the inhibition is mainly due to covalent modification.

The differential sensitivities of individual GST isoenzymes thus indicate that the cysteine residue involved is more accessible and/or easier modified in isoenzyme 3-3 than in any of the other isoenzymes used.

In view of their general role in the detoxification of xenobiotics and because resistant tumour cells exhibit higher levels of certain classes of isoenzymes than non-resistant cells, the glutathione S-transferases have been implicated in the development of cellular resistance of tumour cells against the alkylating class of chemotherapeutic agents[9-11]. The findings presented in the present study indicate that the quinones form a class of compounds possibly suitable for the development of

selective *in vivo* inhibitors. The similar inhibition characteristics make it possible to select one or more quinones with the right properties concerning reactivity towards glutathione S-transferases and *in vivo* toxicity. Whether or not quinones are capable of inhibiting glutathione S-transferase activity in a cellular system is presently under investigation.

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4.2

IRREVERSIBLE INHIBITION OF GLUTATHIONE S-TRANSFERASE ISOENZYMES IN RAT H35-HEPATOMA CELLS BY CHLORINATED QUINONES

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Submitted

ABSTRACT

Rat H35-hepatoma cells were exposed to 2,3,5,6-tetrachloro-1,4-benzoquinone ($\leq$ 10 µM), 2,3-dichloro-1,4-naphthoquinone ($\leq$ 20 µM) or 5-hydroxy-1,4-naphthoquinone ($\leq$ 20 µM) for one hour. Cell viability was over 90 % after this exposure period, as judged by trypan blue exclusion and leakage of lactate dehydrogenase activity. Although all three quinones are strong, irreversible inhibitors of purified glutathione S-transferases, only the first two, 2,3,5,6-tetrachloro-1,4-benzoquinone and 2,3-dichloro-1,4-naphthoquinone were capable of inhibiting glutathione S-transferase activity in H_{35}-cells, causing a 50 % and an 80 % loss of activity respectively, at a 10 µM concentration. Removal of unreacted inhibitor or inhibiting glutathione conjugates from the cell homogenate by means of ultrafiltration did not increase glutathione S-transferase activity, demonstrating that the inhibition was irreversible. All three quinones tested caused a considerable depletion of intracellular levels of reduced glutathione. From the results it was concluded that starting from compounds like 2,3,5,6-tetrachloro-1,4-benzoquinone and 2,3-dichloro-1,4-naphtoquinone an in vivo inhibitor of the glutathione S-transferase might be developed which could be used to decrease the resistance certain tumour cells display against chemotherapeutic agents.

* Abbreviations used:

TCBQ	: 2,3,5,6-tetrachloro-1,4-benzoquinone;
2,3-diCl-NQ	: 2,3-dichloro-1,4-naphthoquinone;
5-OH-NQ	: 5-hydroxy-1,4-naphthoquinone;
EBSS	: Earl's Balanced Salt Solution (1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.12 M NaCl, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5.6 mM D-glucose, 20 mM HEPES);
PBS	: phosphate buffered saline (0,14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3);
GSH	: reduced glutathione;
GSSG	: oxidized glutathione;
CDNB	: 1-chloro-2,4-dinitrobenzene;
TFA	: trifluoroacetic acid.

** Unpublished results

INTRODUCTION

The glutathione S-transferases (EC 2.5.1.18) are a family of isoenzymes which catalyze the conjugation of a broad range of electrophilic xenobiotics with glutathione[1]. The isoenzymes are divided into three classes, alpha, mu and pi, on the basis of structural characteristics as well as kinetic behaviour towards standard substrates and inhibitors[2].

The glutathione S-transferases have been implicated in acquired drug resistance of tumour cells against alkylating cytostatic agents, for two reasons: i) one of their main functions is detoxification of alkylating agents, and ii) resistant tumour cells display higher levels of some classes of glutathione S-transferase isoenzymes than non-resistant tumour cells[3-6].

The use of selective inhibitors of glutathione S-transferases would thus presumably be an effective way of overcoming this resistance.

Several inhibitors have been described, but most of these act in a more or less competitive manner, implying that their action is temporary, and strongly dependent on the concentration of the inhibitor, thereby limiting their usefulness in vivo.

Recently, 2,3,5,6-tetrachloro-1,4-benzoquinone (TCBQ)* as well as the mono-glutathione conjugate derived from it, were demonstrated to be very efficient, covalently binding inhibitors of the glutathione S-transferases. A 5-fold molar excess as compared with the monomeric enzyme, results in 80-90 % inhibition of the enzyme within 5 min of incubation at 25 °C[7].

The glutathione-conjugate is especially interesting, because it still possesses a quinone structure, and therefore retains its reactivity towards sulfhydryl groups. Although both TCBQ and 2-S-glutathionyl-3,5,6-trichloro-1,4-benzoquinone are equally effective inhibitors at 25 °C, the glutathione conjugate demonstrates faster inhibition at 0 °C than the parent compound (or the analogous mercaptoethanol derivative) demonstrating a targeting effect of the glutathione part of the molecule[7]. However, evidence exists for the involvement of the active site in the action of both inhibitors, since incubation (at 0 °C) in the presence of the competitive inhibitor S-hexylglutathione also slows

the inhibition by TCBQ considerably. Glutathione conjugates are not thought to be effectively taken up by cells[8]. TCBQ therefore seems a promising starting compound for the development of a selective and irreversible in vivo inhibitor of the glutathione S-transferases.

In addition to TCBQ, several other 1,4-benzoquinones (e.g. 2-tert. butyl-1,4-benzoquinone) as well as some 1,4-naphthoquinones (e.g. 5-hydroxy-1,4-naphthoquinone) have been found to be capable of inhibiting the activity of purified glutathione S-transferases in an irreversible manner at concentrations in the micromolar range ($\leq$ 50 µM)[9].

The aim of the present study was to investigate whether quinones are capable of inhibiting glutathione S-transferase activity in a cellular system. In addition to TCBQ, 2,3-dichloro-1,4-naphthoquinone (2,3-diCl-NQ)* and 5-hydroxy-1,4-naphthoquinone (5-OH-NQ)* were chosen as model compounds, being the strongest inhibitory 1,4-naphthoquinones tested so far[9].

Similar to TCBQ, 2,3-diCl-NQ is a strong alkylating agent and retains its quinone structure following conjugation with e.g. glutathione. Reaction of 5-OH-NQ with glutathione on the other hand, results in the formation of a hydroquinone conjugate which is no longer reactive. Both 2,3-diCl-NQ and 5-OH-NQ have been found to be capable of inducing oxidative stress by generation of reactive oxygen species[10-12].

METHODS

Rat H_{35}-hepatoma cells were cultured in Ham's F10 medium (Flow, Irvine, Scotland), supplemented with $NaHCO_3$ (1.2 g/l), 10 % newborn calf serum (Gibco, Glasgow, Scotland), 50 IU/ml penicillin and 50 µg/ml streptomycin, at 37 °C in a humid atmosphere containing 5 % CO_2 in air. For each experiment approx. 0.8 * 10^6 cells were plated onto a petri dish (growth area 56 cm^2) and cultured for 2 days until a semi-confluent monolayer was obtained. Cells were exposed to TCBQ (Merck, Darmstadt, FRG), 2,3-diCl-NQ (Aldrich Chem. Co., Milwaukee, WI) or 5-OH-NQ (Aldrich Chem. Co, Milwaukee, WI) (added from a stock solution in 100 % ethanol (TCBQ, 5-OH-NQ) or acetone (2,3-diCl-NQ)) in Earl's Balanced Salt Solution (EBSS)* for 1 hour, using a total volume of 10 ml. With

the exception of the 10 µM TCBQ incubation, the final concentration of ethanol or acetone was 0.5 % and was also included in control incubations. After incubations cells were rinsed twice with 2.5 ml of phosphate buffered saline (PBS)* to remove any remaining quinone.

Viability was evaluated by determination of the percentage of trypan blue excluding cells after trypsinization of the exposed cells, and by measuring leakage of lactate dehydrogenase activity into the surrounding medium according to Mitchell[13]. The condition of the cells following exposure was also evaluated by microscopic investigation.

For enzyme assays, cells were harvested by scraping with a rubber policeman in 250 µl PBS and disrupted by sonication on ice for 5 minutes. The cell homogenates were centrifuged and the supernatant was used for determination of glutathione S-transferase activity and concentrations of reduced glutathione (GSH)*. Glutathione S-transferase activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB)* as a substrate, at 25 °C, according to Habig et al.[14]. GSH concentrations were determined fluorimetrically by the method of Hissin and Hilf[15]. Protein was measured according to Lowry[16] or Peterson[17]. Remaining glutathione S-transferase activity was calculated as a percentage of the specific activity in control cells.

Whether the inhibition was of an irreversible nature was determined by removing the majority of low molecular weight compounds (unreacted inhibitor plus glutathione conjugates) by means of ultrafiltration (Amicon Diaflo YM10-filter), for homogenates of cells exposed to 10 µM TCBQ or 10 µM 2,3-diCl-NQ. Theoretically, the maximum concentration, i.e. under assumption that the whole dose was taken up by the cells and recovered in the homogenate, was reduced to approx. 2 µM. Since not all the quinone was present in the cell homogenate, as was indicated by the highly coloured pellet after centrifugation of disrupted cells, the real concentration remaining was presumably much lower. Total activity and total protein were also determined to see if any increase in the remaining activities was due to an increase in total activity or a decrease in total protein.

Glutathione S-transferases were purified from H_{35}-cells on S-hexyl-glutathione Sepharose 6B, as described elsewhere[18]. The subunit composition was determined by injecting the isoenzyme mixture onto a Vydac

Protein & Peptide C18 reversed phase column (5 µm (Alltech, Hesperia, USA)), equilibrated with 35 % acetonitrile + 0.1 % trifluoroacetic acid (TFA)*/65 % water + 0.1 % TFA. A linear gradient was used over 30 min from 35 % to 50 % acetonitrile + 0.1 % TFA, followed by a 10 min gradient from 50 % to 100 % acetonitrile + 0.1 % TFA. A flow rate of 1 ml/min was used. Protein was monitored at 220 nm[19]. Subunits were identified by comparison of retention times with subunits from purified isoenzymes from rat liver and rat lung (subunit 7)[20].

RESULTS

Glutathione S-transferase subunit composition in rat H_{35}-hepatoma cells. The subunit composition of the glutathione S-transferase fraction of H_{35}-cells was determined by use of HPLC reversed phase chromatography. Subunits were identified by comparison of retention times with those of purified isoenzymes. As shown in figure 1A, rat H_{35}-hepatoma cells mainly contain subunits 4 and 7, as well as minor amounts of subunits 2 and 3. No subunit 1 was noticed. The subunit composition implies the presence of small amounts of isoenzymes 2-2, 3-3 and 3-4 in addition to isoenzymes 4-4 and 7-7. Figure 1B is given as a reference pattern.

Viability of cells exposed to TCBQ, 2,3-diCl-NQ or 5-OH-NQ. Cells were exposed to 1-10 µM TCBQ, 5-20 µM 2,3-diCl-NQ or 5-20 µM 5-OH-NQ. Viability of exposed cells was evaluated by determination of the percentage of trypan blue excluding cells in trypsinized samples and by measuring leakage of lactate dehydrogenase activity into the surrounding medium. Both assays demonstrated a cell viability of over 90 % for all quinones tested at the concentrations indicated. In the trypan blue exclusion assay, the number of cells per ml cell suspension was similar for control and treated samples and was not influenced by the trypsinization procedure.

Microscopic investigation demonstrated that the cells were, however, affected by the exposure. At 5 µM concentrations or higher, cells were no longer stretched, but showed a rounded appearance.

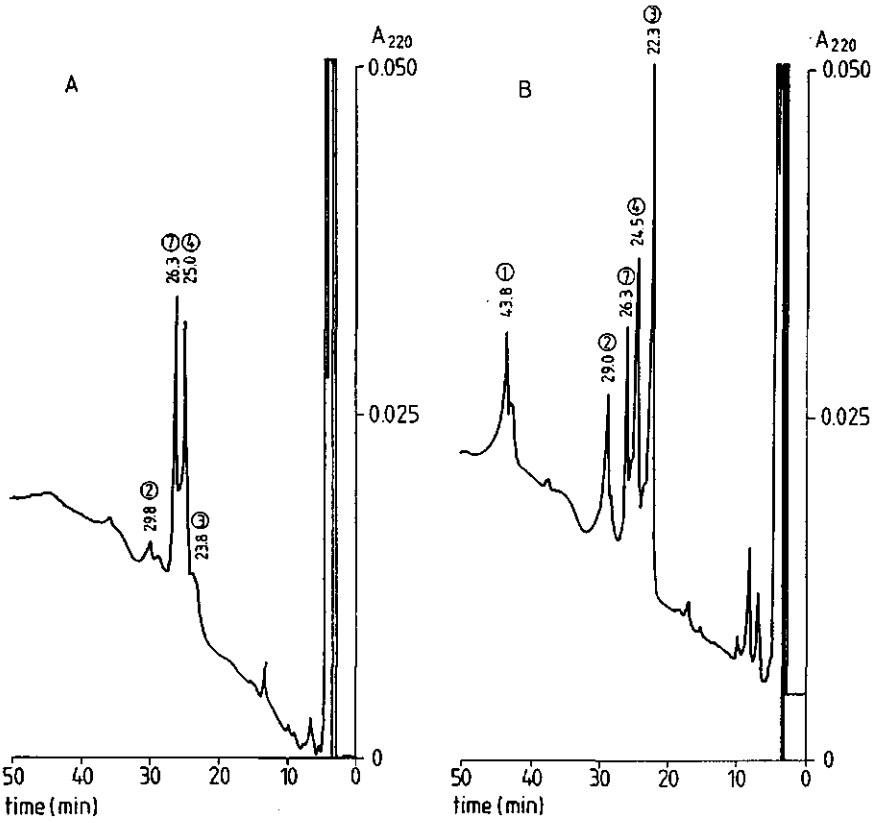

Figure 1. Subunit composition of glutathione S-transferases in rat H_{35}-hepatoma cells (A) and in a 2-day old culture of rat hepatocytes (B).

Purification of glutathione S-transferases and the separation of the subunits are described in the Methods section. Subunits were identified by comparison of retention times with subunits from purified isoenzymes from rat liver and rat lung.

Inhibition of glutathione S-transferase activity by TCBQ, 2,3-diCl-NQ and 5-OH-NQ. As shown in figure 2, both TCBQ and 2,3-diCl-NQ caused a considerable inhibition of intracellular glutathione S-transferase activity after a one hour exposure to concentrations in the micromolar range. 2,3-diCl-NQ was a stronger inhibitor than TCBQ, causing approx.

80 % inhibition at a concentration of 10 µM. The maximum inhibition caused by TCBQ was approx. 50 %. However, higher concentrations than 10 µM could not be tested, due to the limited solubility of TCBQ. 5-OH-NQ was used at similar concentration levels as TCBQ and 2,3-diCl-NQ, but no inhibition was noticed for this compound.

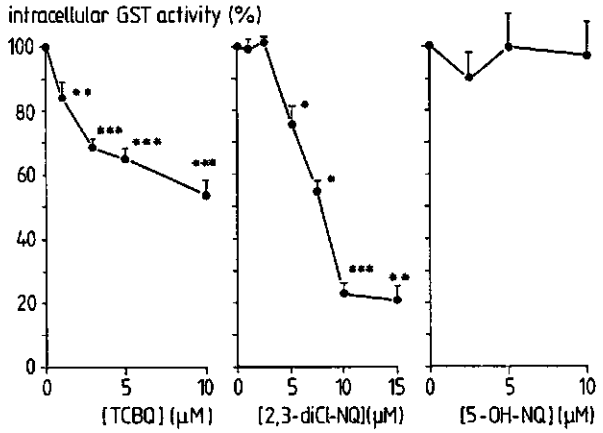

Figure 2. Effect of TCBQ, 2,3-diCl-NQ and 5-OH-NQ on intracellular glutathione S-transferase (GST) activity in rat H_{35}-hepatoma cells. Cells were exposed for 1 hour to the concentrations indicated. Glutathione S-transferase activity was measured according to Habig et al[14]. Protein was measured by the method of Lowry[16]. Remaining activities were determined as a percentage of the specific activities in control cells. Results are given as means ± SEM from at least 3 experiments, except for 1, 2.5 and 7.5 µM 2,3-diCl-NQ which are means ± SEM from 2 experiments. Specific activity in control cells amounted to 0.79 ± 0.04 µmol/min/mg protein.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (paired Student's t-test).

The inhibition demonstrated by TCBQ and 2,3-diCl-NQ was of an irreversible nature (table 1). For 2,3-diCl-NQ, no increase was noticed in the extent of inhibition after removal of "unreacted" inhibitor by ultrafiltration. The remaining activity for cells exposed to TCBQ was some-

what higher after ultrafiltration than before. However, the difference was not statistically significant and was mainly due to effects on protein. No increase was found in the total number of enzyme activity units recovered (results not shown).

Table 1. Influence of reduction of the concentration of "unreacted" inhibitor by ultrafiltration on the extent of inhibition by TCBQ and 2,3-diCl-NQ[a].

	n	remaining activity before ultrafiltration (%)	remaining activity after ultrafiltration (%)
10 μM TCBQ	3	49 ± 7	60 ± 10
10 μM 2,3-diCl-NQ	2	36 ± 1	37 ± 6

a. Remaining activities were determined as a percentage of specific activities (μmol/min/mg) in control cells and are expressed as means ± SEM.

Effects of TCBQ, 2,3-diCl-NQ and 5-OH-NQ on intracellular GSH concentrations. GSH levels in cells exposed to TCBQ, 2,3-diCl-NQ and 5-OH-NQ are shown in figure 3. All quinones caused a depletion of intracellular GSH, TCBQ demonstrating the strongest effect. The relative decrease in GSH levels was somewhat larger than the decrease in glutathione S-transferase activity for both TCBQ and 2,3-diCl-NQ, at a given concentration. The decrease in GSH levels was not due to leakage into the surrounding medium.

DISCUSSION

Three quinones were compared with respect to their ability to inhibit glutathione S-transferase activity in a cellular system. Whereas all three compounds are strong, irreversible inhibitors of purified gluta-

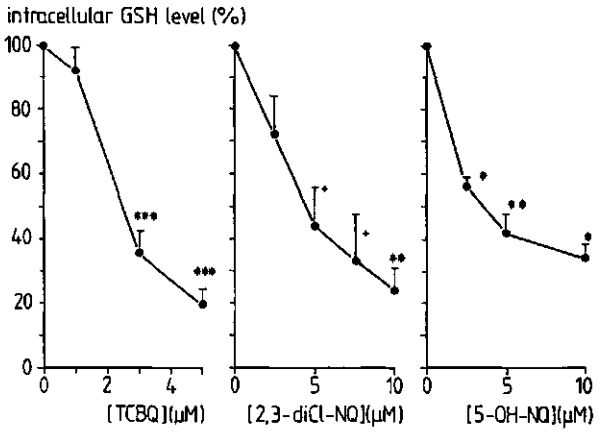

Figure 3. Effect of TCBQ, 2,3-diCl-NQ and 5-OH-NQ on intracellular glutathione (GSH) levels in rat H_{35}-hepatoma cells. Cells were exposed for 1 hour to the concentrations indicated. GST was determined by the method of Hissin and Hilf[15]. Protein was measured according to Lowry[16]. Results are given as means ± SEM from at least 3 experiments, except for 2.5, 5 and 7.5 µM 2,3-diCl-NQ and 2.5 and 10 µM 5-OH-NQ, which are means ± SEM from 2 experiments. In control cells the intracellular GSH concentration amounted to 38.1 ± 2.3 µg/mg protein. + $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (paired Student's t-test).

thione S-transferases[7,9], only two of them, TCBQ and 2,3-diCl-NQ, inhibit glutathione S-transferase activity in rat H_{35}-hepatoma cells. 2,3-diCl-NQ, causing approx. 80 % inhibition at a 10 µM concentration, is clearly a more efficient inhibitor under these conditions than TCBQ. The inhibition caused by both TCBQ and 2,3-diCl-NQ in the cells is due to covalent interactions, as is indicated by the lack of influence of reduction of the concentration of "unreacted" inhibitor by means of ultrafiltration. The sensitivity of all purified isoenzymes tested[9], as well as the high loss of activity obtained with 2,3-diCl-NQ, suggests that in H_{35}-hepatoma cells all isoenzymes are inhibited to a certain extent.

The depletion of GSH caused by quinones has received considerable attention over the past few years[21-23]. Three mechanisms may be involved: i) formation of GSSG, resulting from the generation of reactive oxygen species (redox cycling) and/or direct oxidation by the quinone; ii) formation of (hydro)quinone-SG-conjugates; iii) formation of protein mixed disulfides. The type of quinone seems to determine which mechanism is mainly responsible for the loss of GSH. For menadione, a well-known redox cycling compound, approx. 75 % of the decrease in cellular GSH levels in isolated hepatocytes is accounted for by formation of GSSG, which is rapidly excreted into the medium[22]. For some benzoquinones, however, e.g. 2-methyl-BQ and 2-bromo-BQ, which do not cause oxidative stress, the depletion of GSH is mainly due to conjugate formation[23].

TCBQ, 5-OH-NQ and 2,3-diCl-NQ all cause a considerable loss of intracellular GSH. One of the explanations for the lack of inhibition of glutathione S-transferase activity by 5-OH-NQ, could be a higher tendency for redox cycling for this compound. An attractive alternative explanation could be that the inhibition is (at least partly) mediated by quinone-SG conjugates, since the glutathione moiety can target the inhibitor to the active site[7]. In contrast to TCBQ and 2,3-diCl-NQ, 5-OH-NQ is conjugated via a Michael-addition, resulting in a hydroquinone conjugate, which no longer possesses alkylating properties.

Whether the inhibition is of a selective nature still needs to be established. The involvement of the glutathione S-transferase active site has been firmly established for the inhibition of purified enzymes[7,9]. In addition, several quinones have been described as substrates for glutathione S-transferases, also indicating a possible preferential reaction in or near the active site. However, 2,3-diCl-NQ also seems to be capable of inhibiting the activity of purified glutathione reductase, by preventing reoxidation of the active site by substrate[24]. In 2,3-diCl-NQ fed rats, activities of glutathione reductase and glutathione peroxidase are depressed[24]. Menadione has been found to inhibit glutathione reductase activity in isolated hepatocytes, following a 20 min exposure at a concentration of 25 to 200 μM[21]. Since H_{35}-hepatoma cells do not contain detectable amounts of glutathione peroxidase and glutathione reductase**, it could not be studied whether

inhibition of these enzymes occurs at the exposure levels used in the present study. The aspect of selectivity is presently under investigation.

In summary, two strong alkylating inhibitors of purified glutathione S-transferases, TCBQ and 2,3-diCl-NQ, have been found capable of inhibiting glutathione S-transferases in a cellular system. The inhibition is of an irreversible nature, making these compounds attractive starting points for the development of an in vivo inhibitor to decrease the resistance of certain tumour cells against chemotherapeutic agents.

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CHAPTER 5

DEVELOPMENT AND APPLICATION OF MONOCLONAL ANTIBODIES AGAINST RAT AND HUMAN GLUTATHIONE S-TRANSFERASES

5.1
MONOCLONAL ANTIBODIES AGAINST RAT GLUTATHIONE S-TRANSFERASE ISOENZYMES 2-2 AND 3-3

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ABSTRACT

Monoclonal antibodies were prepared against rat glutathione S-transferase isoenzymes 2-2 and 3-3. Three clones were capable of specifically differentiating their respective antigens from other rat isoenzymes as well as human isoenzymes, in ELISA and on Western blot. One hybridoma produced antibodies specific for isoenzyme 2-2, and 2 hybridomas were specific for isoenzyme 3-3.

Balb/c mice did not respond to immunization with glutathione S-transferase isoenzymes 1-1 and 4-4. However, an immune response was obtained in some other strains of mice, with differential H-2 haplotypes, notably CBA/BrARij mice and CBA/CaHRij-T6 mice for isoenzyme 1-1 and CBA/BrARij mice for isoenzyme 4-4, which offers perspectives for obtaining additional specific monoclonal antibodies against these glutathione S-transferases.

+ Abbreviations: GST: glutathione S-transferases; i.p.: intraperitoneal; PBS: phosphate buffered saline; ELISA: Enzyme-linked immunosorbent assay; DMEM: Dulbecco's Modification of Eagle's medium; DMSO: dimethylsulfoxide; SDS: sodium dodecyl sulfate; PAGE: polyacrylamide gel electrophoresis.

INTRODUCTION

The glutathione S-transferases (GST)+ (EC 2.5.1.18) are a group of dimeric proteins, important in the biotransformation of xenobiotics[1]. Several homo- and heterodimeric isoenzymes exist, which catalyze the conjugation of a wide range of hydrophobic, electrophilic compounds with glutathione, and are capable of binding a variety of non-substrate ligands noncovalently[2,3]. Although the activity of GST isoenzymes towards a certain compound will generally result in reduced toxicity, several examples of activation through conjugation with glutathione are known[4].

The toxicity of a compound which may serve as a substrate or a non-substrate ligand is clearly dependent on isoenzyme profiles. Specific antibodies raised against individual isoenzymes (i.e. subunits) may be useful in analysing several aspects of relevance in this context, including, for instance, immunohistochemical analysis of tissue distribution and qualitative/quantitative analysis of changes caused by inducing agents. In addition, antibodies may be of use in the purification of isoenzymes, or the determination of isoenzyme selectivities in cytosolic preparations.

GST isoenzymes from rat, man and mouse have been divided into three classes, alpha, mu and pi, on the basis of enzymatic properties and structural homologies[5]. Transferases belonging to the same class share approx. 70-80 % protein sequence identity, whereas isoenzymes from different classes are more distantly related (approx. 25 % sequence identity)[6]. In view of the considerable structural homology, the use of monoclonal antibodies is prefered over the use of polyclonal antibodies, which are generally less specific.

The present paper reports the production of monoclonal antibodies against rat isoenzymes 2-2 and 3-3.

MATERIALS AND METHODS

Purification of isoenzymes. GST isoenzymes were purified from rat liver by S-hexylglutathione affinity chromatography and separated by FPLC-chromatofocusing, as described previously[7]. Isoenzyme 7-7 (rat kidney) and human glutathione S-transferases (liver) were purified, using essentially the same procedure.

Immunization of mice. Balb/c mice (H-2^d haplotype), CBA/BrARij mice (H-2^q haplotype), CBA/CaHRij-T6 mice (H-2^k haplotype) or C57Bl/LiARij mice (H-2^b haplotype) received an i.p. injection of 50-100 µg of the antigen in PBS, mixed with an equal volume of Freund's complete adjuvant. After 2-3 weeks, an i.p. injection of 50-100 µg of the antigen was administered in Freund's incomplete adjuvant (1:1 ratio). Serum antibody titers were determined by means of ELISA[8], 7-14 days following the second injection. In the case of a cell fusion experiment, a booster of 100 µg of the antigen was given i.p., 3 days prior to cell fusion. Besides the protocol described above, different immunization procedures, i.e. injection in denatured form following SDS-PAGE or SDS-PAGE and transfer to nitrocellulose, were also used for immunization of Balb/c mice with GST isoenzymes 1-1 and 4-4.

Cell fusion and preparation of ascites fluid. Cell fusions were performed with SP 2/0-Ag 14 mouse myeloma cells, essentially as described by Galfré and Milstein[9], using 40 % polyethyleneglycol 4000 GA/5 % DMSO (Merck, Darmstadt, FRG) as the fusion agent. After the fusion procedure, cells were taken up in a hybridoma selective medium, consisting of DMEM (Gibco, Glasgow, Scotland), supplemented with 10 % foetal calf serum, 5 % horse serum, 2 mM glutamine, 1 mM pyruvate, 0.1 mM hypoxanthine, 50 IU/ml penicillin, 50 µg/ml streptomycin, 1 µg/ml amphotericin B and 6 µM azaserine. Macrophages were removed by incubation at 37 °C for 90 min in a 150 cm^2 cell culture bottle. Subsequently, the cells were plated in 96 well culture plates and cultured in the hybridoma selective medium for 2 weeks. After this period, the medium was replaced by medium without azaserine.

The culture supernatants of the hybrid cells were screened for the presence of specific antibodies by use of ELISA, spot-blot and SDS-PAGE immunoblotting[8,10]. Antibody-producing cells were cloned by limiting

dilution and the resulting clones were tested for the secretion of antibodies as mentioned. A number of positive clones were chosen for preservation.

For the preparation of ascites fluid, female Balb/c mice, pretreated with pristane (2,6,10,14-tetramethylpentadecane (Aldrich Chem. Co., Milwaukee, WI)), were given an i.p. injection with 5.10^6 hybrid cells. After the development of an ascitic tumour, samples of peritoneal fluid were collected, freed from cells by centrifugation and stored at -20 °C until use.

Heavy chain analyses. The isotype of the obtained hybridoma clones was determined by means of ELISA, using purified rabbit immunoglobulins specific for IgA, IgG1, IgG2a, IgG2b and IgM mouse immunoglobulins[8].

RESULTS

Rat hepatic isoenzymes 1-1, 2-2, 3-3 and 4-4 or a mixture of affinity-purified GST were used as antigens. Serum of Balb/c mice injected with the GST mixture was reactive towards isoenzymes 1-1, 2-2 and 3-3. Balb/c mice injected with isoenzymes 2-2 or 3-3 gave high serum antibody titers against their respective antigens. Fusion experiments were carried out with splenocytes from Balb/c mice immunized with the GST-mixture or with isoenzyme 3-3, resulting in one anti-2-2 clone (6G3) and two anti-3-3 clones (13H11/14E8), respectively. These hybridomas are listed in table 1, along with their antibody isotype.

Table 1. Monoclonal antibodies against rat glutathione S-transferase isoenzymes 2-2 and 3-3.

hybridoma	reactive with isoenzyme	isotype
6G3	2-2	IgG2b
13H11	3-3	IgG1
14E8	3-3	IgG1

Reactivities of the isolated clones towards rat hepatic isoenzymes 1-1, 2-2, 3-3 and 4-4 were determined by means of ELISA and Western blot analysis. Western blot experiments were also used to investigate cross-reactivity with rat isoenzyme 7-7, and human class alpha- and class mu isoenzymes. Results are given in figures 1 and 2. Since clones 13H11 and 14E8 showed similar specificities, only results for 13H11 are presented.

All clones were capable of differentiating their respective antigen from other rat isoenzymes, both in ELISA and on Western blot. From figure 1, it is clear that clones 6G3 and 13H11 specifically recognized isoenzymes 2-2 and 3-3 respectively. Only at high antibody-concentrations a slight cross reaction was noticed for 6G3 with isoenzyme 3-3, whereas 13H11 demonstrated some binding to isoenzyme 4-4.

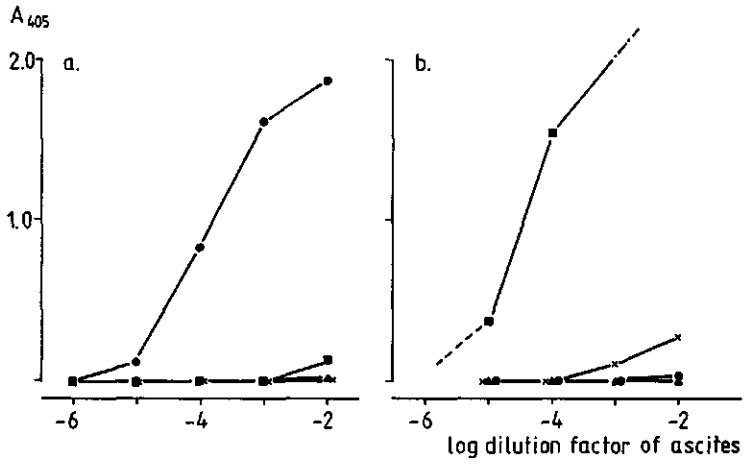

Figure 1. Specificities of anti-2-2-clone 6G3 (A) and anti-3-3-clone 13H11 (B), as determined by ELISA.
One experiment typical of at least 4. ELISA's were performed according to Goding[8]. Wells were coated with 100 ng of antigen.
▲ : isoenzyme 1-1; ● : isoenzyme 2-2; ■ : isoenzyme 3-3; x : isoenzyme 4-4.
---- in figure 1B indicates: not measured
-.-. in figure 1B indicates: $A_{405} > 2.0$

Similar specificity results were obtained with immunoblotting experiments (figure 2). At high antibody concentrations 6G3 and 13H11 showed a slight cross-reactivity with isoenzymes 3-3 and 4-4 respectively (not shown). None of the monoclonals demonstrated affinity for rat isoenzyme 7-7 and human class alpha- or class mu isoenzymes on Western blot (figure 2, results for 7-7 not shown for 6G3).

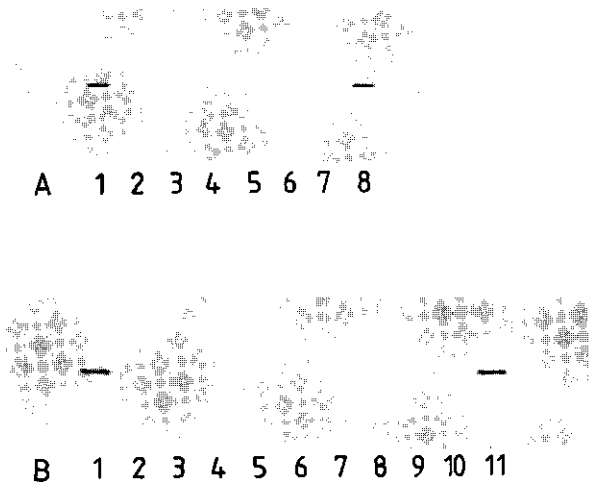

Figure 2. Western blot of glutathione S-transferases probed with anti-2-2 clone 6G3 (A) and anti-3-3 clone 13H11 (B).
Panel A: 10^2-fold dilution of purified IgG fraction of 6G3. Lanes 1 and 8: 100 ng 2-2; lanes 2 and 7: - ; lane 3: 100 ng 1-1; lane 4: 100 ng 3-3; lane 5: 100 ng 4-4; lane 6: 4 µg affinity-purified mixture of human isoenzymes.
Panel B: 10^3-fold dilution of ascites of 13H11. Lanes 1 and 11: 100 ng 3-3; lanes 2 and 10: - ; lane 3: 100 ng 1-1; lane 4: 100 ng 2-2; lane 5: 100 ng 4-4; lane 6: 100 ng 7-7; lane 7: 100 ng human mu class transferase µ; lane 8: 100 ng human alpha class transferase pI 8.5; lane 9: 100 ng human alpha class transferase pI 8.0.

Isoenzymes 1-1 and 4-4 failed to elicit an immune response in Balb/c mice, by any of the immunization protocols used (figure 3). To study if an immune response could be obtained in strains of mice of differential H-2 haplotypes, CBA/BrARij mice (H-2^q), CBA/CaHRij-T6 mice (H-2^k) and C57Bl/LiARij mice (H-2^b) were immunized with GST isoenzymes 1-1 and 4-4. Both CBA/BrARij mice (H-2^q) and CBA/CaHRij-T6 mice (H-2^k) gave high serum antibody titers after injection with isoenzyme 1-1. CBA/BrARij mice were also responsive towards isoenzyme 4-4. Similar to Balb/c mice, C57Bl/LiARij mice (H-2^b) did not respond to injection with either of the 2 antigens (figure 3).

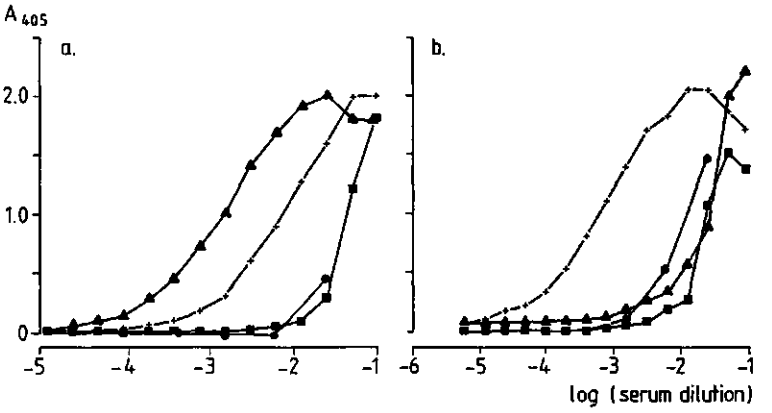

Figure 3. Immune response of isoenzymes 1-1 (A) and 4-4 (B) in four strains of mice with differential H-2 haplotypes. ELISA's were performed according to Goding[8]. Wells were coated with 100 ng of antigen.

● : Balb/c mice (H-2^d)

+ : CBA/BrARij mice (H-2^q)

▲ : CBA/CaHRij-T6 mice (H-2^k)

■ : C57Bl/LiARij mice (H-2^b).

DISCUSSION

In the present study, monoclonal antibodies have been obtained, which can be considered specific for GST isoenzymes 2-2 and 3-3. The results clearly demonstrate the potential of the antibodies for specific differentiation of their respective antigens from other rat GST isoenzymes as well as human isoenzymes, belonging to the same or a different GST class.

Despite the high degree of structural homology between isoenzymes 3-3 and 4-4, which are both class mu transferases[6], only a slight cross-reactivity of 13H11/14E8 with 4-4 was noticed at high antibody concentrations. The slight affinity found at high antibody concentrations of antibodies raised against isoenzyme 2-2 for isoenzyme 3-3 has not been reported previously. Isoenzyme 2-2 is a class alpha transferase. The degree of protein sequence identity with 3-3 is relatively low, and this identity does not seem to be confined to a specific region of the protein, but is scattered throughout the sequence[6]. However, it has been shown previously, that monoclonal antibodies may in some cases even exhibit cross-reactivity with proteins structurally unrelated to the respective antigens, the extent of cross-reactivity being dependent on antibody concentration[11]. It appears, therefore, that the specificity of a monoclonal antibody is not an intrinsic property of the antibody, but is related to affinity instead[11-14].

The lack of immune response in Balb/c mice for isoenzyme 1-1 has also been reported by Wang et al.[15]. These authors used AKR-mice to obtain monoclonal antibodies against this transferase. In addition to the host's regulatory mechanisms, the immune response is dependent on structural differences between the antigen administered and the proteins of the host[16]. A high degree of structural homology with rat isoenzymes has been reported for GST from NMRI-mice[17]. Structural identity may therefore at least partly explain the lack of immune response noticed.

However, the high serum antibody titers observed in some strains of mice with a H-2 haplotype differing from that of Balb/c mice, notably CBA/BrARij mice for isoenzymes 1-1 and 4-4, and CBA/CaHRij-T6 mice also for isoenzyme 1-1, offer perspectives for the production of specific monoclonal antibodies against these GST isoenzymes in the near future.

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5.2
GENETIC DEFICIENCY OF HUMAN CLASS MU ISOENZYMES IN RELATION TO MERCAPTURIC ACID EXCRETION

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ABSTRACT

Mononuclear lymphocytes were isolated from the blood of 12 individuals, who had been exposed to the vapor of the soil fumigant 1,3-dichloropropene (DCP). Western blot experiments were performed on the crude lymphocyte homogenates, using a monoclonal antibody against hepatic isoenzyme μ, to determine the presence or absence of mu-class isoenzymes μ and/or ψ. Nine of the individuals were found to be positive for μ and/or ψ, the remaining 3 individuals being negative. In addition, all individuals showed a positive staining on immunoblot of a protein of somewhat lower molecular mass than the hepatic standard. This protein was bound by the S-hexylglutathione affinity column, and presumably constitutes a new mu-class isoenzyme, which is not subject to genetic polymorphism.

Urinary excretion levels and elimination half-lives of the mercapturic acids of the cis-(Z-) and trans-(E-)isomers of DCP were compared with the data on the presence or absence of mu-class isoenzymes μ and/or ψ. No significant differences were observed between mu-class positive and mu-class negative individuals.

The importance of the establishment of this type of correlation, as well as correlations with the occurrence of certain diseases, is discussed.

INTRODUCTION

The glutathione S-transferases (GST)(EC 2.5.1.18) from several mammalian species can be divided into three classes, alpha, mu and pi, on the basis of similarities in structural and enzymatic properties[1]. The main function of these proteins involves the catalysis of the conjugation of electrophilic xenobiotics with the tripeptide glutathione (GSH)[2]. Although the reaction with GSH generally results in reduced toxicity, several examples of activation are known[3].

The existence of a large number of GST isoenzymes with differential though overlapping substrate selectivities enables the enzyme system to handle a wide range of reactive compounds, but at the same time implies, that the extent to which detoxification and/or activation occurs in a certain tissue, is dependent on the actual profile of isoenzymes present.

Of substantial interest in this context is the genetic polymorphism of the human class mu transferases μ and ψ[4,5]. Mu class GST isoenzymes are not expressed in 40-50 % of human individuals. This genetic polymorphism may have a considerable impact on the susceptibility for toxic compounds, particularly in view of the high activity of isoenzyme μ with reactive epoxide metabolites from polycyclic aromatic hydrocarbons[6].

Seidegard et al.[7] have recently shown that three groups of individuals may be discriminated on the basis of the cytosolic GST activity in mononuclear lymphocytes with the model substrate trans-stilbene oxide, i.e. a group with low, high and very high activity, respectively. The GST isoenzyme responsible for the high activity with this compound was identified as isoenzyme μ[8]. In addition, the range of activity in lymphocytes was found to correspond with the range of activity in the liver of the same individual[8]. The fact that the mu-phenotype is reflected in the GST isoenzyme profile of mononuclear lymphocytes, offers perspectives for relatively easy (non-invasive) phenotyping of individuals.

In the present study, a monoclonal antibody, raised against hepatic GST isoenzyme μ, was used in the screening for the presence of mu-class isoenzymes in crude lymphocyte homogenates of 12 individuals. These

individuals had worked with the soil fumigant 1,3-dichloropropene (DCP), used for preplant soil treatment in bulb fields to control parasitic nematodes, and had been exposed to the vapor of this agent in the field. The cis-(Z-) and trans-(E-) isomers of DCP have been shown to be directly mutagenic in the Ames-test, the Z-isomer being approx. twice as active[9]. Conjugation with GSH is a major route of metabolism for these compounds, both in the rat and in man[10,11]. Urinary excretion levels and elimination half-lives of the mercapturic acids of the two isomers were compared with the data on the presence or absence of mu-class isoenzymes to establish the existence of a possible correlation.

MATERIALS AND METHODS

Materials. Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All chemicals used for Western blotting, including affinity-purified goat-anti mouse IgG (H + L) alkaline phosphatase conjugate, were purchased from Bio-Rad Laboratories (Richmond, CA). Dulbecco's phosphate buffered saline (PBS) was from Oxoid Ltd. (Basingstoke, England).

Isolation of human mononuclear lymphocytes. Mononuclear lymphocytes were isolated by Percoll-density gradient centrifugation. Approx. 5 ml of blood was layered on top of 10 ml of a Percoll solution with a starting density of 1.077 g/ml in 0.15 M NaCl. After centrifugation at 400 g for 20 min, the buffy layer, containing the mononuclear lymphocytes, was isolated, diluted with PBS and centrifuged at 400 g for 20 min. The resulting pellet was resuspended in 250 µl PBS. Cells were homogenized by three times freeze-thawing, followed by centrifugation to remove cell debris. The resulting supernatant was used for Western blot experiments. Protein concentrations were determined according to Lowry[12].

Monoclonal antibody preparation. A monoclonal antibody against GST isoenzyme µ was obtained by fusion of mouse myeloma cells with spleen cells from female Balb/c mice, immunized with GSH-agarose purified human hepatic glutathione S-transferases, using a similar procedure as described previously for uridine 5'-diphosphate-glucuronosyltransfe-

rase[13]. Hybridoma culture medium was used for Western blot experiments.

Immunoblotting. Western blotting was performed according to Towbin et al.[14]. Briefly, 250 µg of lymphocyte homogenate protein or 1.25 µg of S-hexylGSH-affinity purified human hepatic GST isoenzymes[15], containing both isoenzymes µ and ψ [16], was loaded onto a 17.5 % polyacrylamide gel. After electrophoresis, protein was transfered to nitrocellulose electrophoretically, at 200 mA, for 3 hours. Free binding sites were blocked with 1.5 % (w/v) gelatin in 10 mM Tris-HCl pH 7.4, containing 150 mM NaCl and 0.05 % (w/v) Tween-20 (TBST). The nitrocellulose was successively incubated with primary antibody (1:5 dilution in TBST, 1 hour) and the goat-anti mouse alkaline phosphatase conjugate (1:1000 dilution in TBST, 1 hour). Between steps, the nitrocellulose was washed thoroughly with TBST. Protein was visualized with 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium chloride.

Determination of mercapturic acids of Z- and E-1,3-dichloropropene. Urine samples were collected before, during and for 24 hours after finishing soil fumigation with DCP, and were stored at -20 °C until analysis. The mercapturic acids of Z- and E-DCP were determined as described elsewhere[17]. In short, urine samples were acidified with HCl to pH 1-2 and extracted twice with ethyl acetate. After evaporation of ethyl acetate, the residues were subjected to methylation with diazomethane. The resulting products were analysed by gas chromatography with sulfur-selective detection[17].

Assessment of respiratory exposure to Z- and E-DCP. Time weighed 8 hour air concentrations of Z- and E-DCP were measured to assess respiratory exposure. Personal air samplers, connected to the applicator's collar, were used, which pumped air through a tube containing charcoal. Z- and E-DCP were desorbed from the charcoal and were analysed by gas chromatography[18].

RESULTS

The monoclonal antibody raised against human hepatic isoenzyme µ, was not cross-reactive with human class alpha or class pi GST isoenzymes, but recognized both isoenzymes µ and ψ (Peters, unpublished results).

In figure 1 a Western blot of 6 lymphocyte homogenates, probed with the anti- μ monoclonal antibody, is presented, showing 5 mu-class positive samples and 1 mu-class negative sample, in addition to a mixture of affinity-purified hepatic glutathione S-transferases containing both isoenzymes μ and ψ. Out of 12 samples tested, 9 were found to be positive and 3 samples were negative for mu-class isoenzymes μ and/or ψ. In addition, all samples contained a protein which reacted with the monoclonal antibody and demonstrated a somewhat lower molecular mass than μ and/or ψ. This protein was bound by the S-hexylGSH Sepharose 6B affinity column (results not shown).

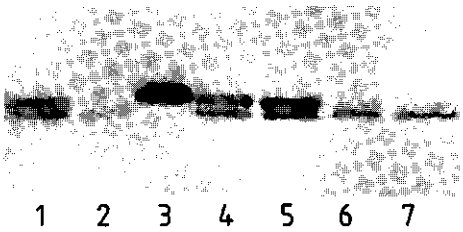

Figure 1. A Western blot of crude lymphocyte homogenates, probed with a monoclonal antibody against hepatic isoenzyme μ.
Lanes 1,2,4-7: 250 μg of protein from crude lymphocyte homogenates of 6 individuals.
Lane 3: 1.25 μg of a mixture of S-hexylGSH Sepharose 6B-affinity purified human hepatic GST isoenzymes.

The urinary excretion levels and half lives of elimination of Z- and E-DCP mercapturic acids are given in table 1 for mu-class positive and mu-class negative individuals. Both the levels of excretion of the Z- and E-DCP mercapturic acids and the half lives of elimination of the 2 isomers showed a large inter-individual variation, and no significant differences were noticed between mu-class positive and mu-class negative individuals (unpaired Student's t-test).

Table 1. Urinary excretion levels and half-lives of elimination of Z- and E-DCP mercapturic acids of mu-class positive and mu-class negative individuals.[a]

mu-class isoenzymes and/or	urinary excretion level ($\frac{\text{mg mercapturic acid (0–36 h)} \cdot m^3}{\text{mg DCP (8 h)}}$)	
	Z-DCP-mercapturic acid	E-DCP-mercapturic acid
+	6.54 ± 3.21 (7)	2.00 ± 2.15 (7)
-	5.26 ± 2.19 (3)	1.42 ± 0.71 (3)
	half-live of elimination $T_{\frac{1}{2}}$ (h)	
	Z-DCP-mercapturic acid	E-DCP-mercapturic acid
+	9.45 ± 3.08 (7)	9.07 ± 3.25 (6)
-	11.53 ± 3.59 (3)	7.16 ± 2.01 (3)

a. Urinary excretion levels represent total excretion of Z- and E-DCP mercapturic acids in 0-36 h urine, corrected for the time weighed 8 hour exposure to Z- and E-DCP. Values are expressed as means ± S.D. for the number of individuals indicated in parentheses.

DISCUSSION

At present, the exact physiological and toxicological consequences of the genetic polymorphism of mu-class GST isoenzymes are poorly understood. In the past, attention has mainly been focussed on the deficiency of isoenzyme μ. It has only recently become clear that an additional mu-class isoenzyme exists, which is also subject to genetic polymorphism, i.e. isoenzyme ψ[19]. The genetic basis for the heterogeneity has been provided by Board[20]. He suggested that the near-neutral transferases are the products of a single gene locus. Four phenotypes may be formed by combinations of three alleles: GST 1 * 0, GST 1 * 1

and GST 1 * 2. Individuals of the GST 1 0 phenotype do not possess mu-class isoenzymes, whereas isoenzymes ψ and μ are expressed by the phenotypes GST 1 1 and GST 1 2 respectively. The GST 1 2-1 phenotype expresses both the homodimeric forms μ and ψ in addition to a heterodimeric near-neutral transferase. The frequency of occurrence of this phenotype is, however, rather low[4,20,21].

Studies on the existence of possible correlations between the mu phenotype and the levels or profiles of excretion of mercapturic acids or the occurrence of certain diseases could be useful in: i. increasing our understanding of the possible consequences of the mu phenotype; in ii. explaining interindividual differences in urinary excretion of mercapturic acids; and in iii. establishing safety regulations concerning the handling of toxic chemicals.

The importance of this type of investigation is stressed, for instance, by the finding that the GST isoenzyme(s) active with trans-stilbene oxide may serve as a genetic marker in the susceptibility to lung cancer in smokers. A larger percentage of individuals exhibited a low activity towards this compound in a group of lung cancer patients than in a group of controls, matched for smoking habits[22].

The present study describes the first attempt of correlating genetic deficiency of mu-class isoenzymes with mercapturic acid excretion. Several explanations may be given for the lack of significant differences between mu-class positive and mu-class negative individuals concerning the levels and elimination half lives of Z- and E-DCP mercapturic acids.

The most obvious possibilities include a high rate of spontaneous conjugation under physiological conditions, or a relatively low affinity of mu-class transferases for these compounds as compared with the other classes of GST isoenzymes. Despite the fact however, that the urinary excretion of mercapturic acids of Z- and E-DCP does not seem to depend on the presence or absence of mu-class transferases, this type of study may provide useful information for other xenobiotics.

It should be noted, that no method is at present available, capable of discriminating μ from ψ in mononuclear lymphocytes. Both isoenzymes are approx. equally active towards trans-stilbene oxide[16]. Similarly, the anti- μ antibody used in the present study also recognizes transferase

ψ (Peters, unpublished results). In order to obtain a maximum degree of information on the implications of the mu-phenotype, the development of quick and sensitive methods for the specific recognition of either μ or ψ, is of utmost importance.

The extra protein band noticed on Western blot, with a somewhat lower molecular mass than the hepatic class mu transferases, presumably constitutes an additional mu-class isoenzyme, which is not subject to genetic polymorphism. This is indicated by its cross-reactivity with the monoclonal antibody against class mu isoenzymes, in combination with the fact that it is bound by the S-hexylglutathione affinity column.

Carmichael et al.[23] have reported the presence of a protein in human lung, cross-reactive with a polyclonal antiserum against isoenzyme μ, with a slightly lower molecular mass than the hepatic isoenzyme μ, and present in all samples tested. These authors found that the ratio of GST activity in tumour tissue as compared with normal lung tissue was related to the amount of this isoenzyme present, and suggested that this protein might correspond to the Y_n subunit (i.e. subunit 6) in the rat.

Summarizing, the use of a monoclonal antibody has been described for quick and sensitive screening of human lymphocytes for the presence or absence of mu-class isoenzymes μ and/or ψ. An additional mu-class isoenzyme has been found, which is not subject to genetic polymorphism. A first attempt to correlate the presence or absence of mu-class isoenzymes μ and/or ψ with urinary mercapturic acid excretion in individuals exposed to 1,3-dichloropropene, has not revealed significant differences between mu-class positive and mu-class negative individuals. However, the establishment of this type of correlation or correlations with the occurrence of certain diseases may provide substantially important information in future studies. The development of methods capable of discriminating μ from ψ (e.g. specific monoclonal antibodies) is of considerable interest in this context.

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SUMMARY AND CONCLUDING REMARKS

The glutathione S-transferases (GST) are a family of isoenzymes serving a major part in the biotransformation of many reactive compounds. The isoenzymes from rat, man and mouse are divided into three classes, alpha, mu and pi, on the basis of similar structural and enzymatic properties.

The main function of the glutathione S-transferases is the catalysis of the conjugation of electrophilic, hydrophobic compounds with the tripeptide glutathione (GSH). In addition, some of the isoenzymes are capable of binding a number of non-substrate ligands non-covalently. The reaction with GSH generally results in the formation of less toxic compounds, however, several cases of activation are also known.

Since the individual isoenzymes demonstrate differential though overlapping substrate selectivities, the actual isoenzyme pattern determines the extent to which detoxification and/or activation occurs. Consequently, an individual's susceptibility towards electrophilic compounds is not only dependent on substrate specificity, but also on factors determining the isoenzyme profile of the glutathione S-transferases, including genetic factors as well as external factors causing changes in the levels or activities of individual isoenzymes.

The studies in this thesis were aimed at the relationship: glutathione S-transferase isoenzyme patterns versus sensitivity towards alkylating agents, and focussed on several aspects of relevance in this context, i.e. substrate selectivity, induction, inhibition and the genetic deficiency of human class mu isoenzymes.

In chapter 1 a review is presented on properties of the glutathione S-transferases. Functions of the enzyme system and the classification of isoenzymes are discussed in section 1.1. Section 1.2 deals with aspects of GST isoenzymes influencing an individual's susceptibility towards alkylating agents, i.e. tissue distribution, developmental patterns with age, hormonal influences, induction and inhibition. Sections 1.3, 1.4 and 1.5 describe the current knowledge on specific properties of class alpha, class mu and class pi isoenzymes respectively.

Chapter 2 describes a study on the 9,10-mono-ozonide of methyl linoleate (MLO), a postulated intermediate in the toxicity of ozone, as a substrate for rat glutathione S-transferases. The reaction of MLO with GSH was found to result in the formation of oxidized glutathione and aldehydes as major products, two glutathione-conjugates being formed as unstable intermediates only. The reaction was catalyzed by rat liver cytosol, rat lung cytosol and rat liver microsomes. Relatively high activities were noticed for rat lung cytosol and rat liver microsomes when compared with rat liver cytosol, taking the respective activities towards 1-chloro-2,4-dinitrobenzene (CDNB) into account. Comparison of the specific activities of isoenzymes 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 7-7 showed that isoenzyme 2-2 was most active with MLO, whereas 7-7 did not demonstrate detectable activity. The relatively high activity in rat lung cytosol may be partly explained by the fact that isoenzyme 2-2 constitutes a larger percentage of GST protein in rat lung than in rat liver.
Although the ozonide was also found to be a substrate for glutathione peroxidase, the activity of this enzyme did not contribute significantly to the cytosolic conversion of MLO.
From the results it was concluded that the activity of the glutathione S-transferases towards MLO is similar to their activity with lipid hydroperoxides.

In chapter 3, effects of four inducing agents with different chemical structures on rat hepatic isoenzyme patterns were studied. Hexachlorobenzene, benzyl isothiocyanate, phenobarbital and 3-methylcholanthrene all caused an increase in the cytosolic GST activity towards CDNB, 1,2-dichloro-4-nitrobenzene and ethacrynic acid. Changes in the activity towards trans-4-phenyl-3-buten-2-one were relatively small and the activities with cumene hydroperoxide were essentially unchanged. The largest and smallest effects were noticed for hexachlorobenzene and 3-methylcholanthrene, respectively. Microsomal GST activity was not induced by any of the compounds.
Isoenzyme patterns obtained by FPLC-chromatofocusing showed that hexachlorobenzene and phenobarbital both cause an increase in the relative amounts of subunits 1 and 3 as compared with subunits 2 and 4, respec-

tively. For 3-methylcholanthrene only an induction of subunit 1 was observed.
Benzyl isothiocyanate differed from the other agents in that this compound did not enhance the relative amount of subunit 1, but caused an increase in subunit 2 instead. Subunit 3 was also induced by benzyl isothiocyanate.
Alpha class subunits seemed to be enhanced preferentially: subunits 1 and 2 represented 53 to 60 % of the GST protein in treated animals, but only 38 to 45 % in controls.
The exact mechanism of induction of glutathione S-transferases is unknown, but the differential behaviour of benzyl isothiocyanate suggests that there may be a relationship between chemical structure of the inducing agent and GST subunit induction.

Chapter 4 deals with the inhibitory effects of quinones on rat glutathione S-transferases. In section 4.1, inhibition by a series of structurally related 1,4-benzoquinones (BQ) and 1,4-naphthoquinones (NQ) was studied towards a mixture of affinity-purified glutathione S-transferases. The nature of the inhibition was investigated for three quinones with different chemical structures, including 2-tert-butyl-BQ (a metabolite of the food additive 2(3)-tert-butyl-4-hydroxy-anisole), 5-hydroxy-NQ (a naturally occurring quinone) and 2,3-dichloro-NQ (a synthetic compound used as a fungicide). The sensitivities of individual rat hepatic isoenzymes towards these quinones were compared.
The inhibitory activity of BQ and NQ was found to increase with an increasing number of electron-withdrawing substituents on the quinone ring, whereas the presence of electron-donating substituents resulted in a decrease in the extent of inhibition as compared with the parent quinone.
The inhibition was of an irreversible nature and most likely due to covalent modification of a specific cysteine residue located in or near the active site. The three quinones tested were most inhibitory towards isoenzyme 3-3, isoenzyme 2-2 being least sensitive.
Three strongly inhibitory quinones, tetrachloro-BQ, 5-hydroxy-NQ and 2,3-dichloro-NQ, were tested for their inhibitory capacity towards GST activity in a cellular system, using rat H_{35}-hepatoma cells (section

4.2). The GST fraction of these cells mainly contained subunits 4 and 7, with subunits 2 and 3 present as minor constituents. Tetrachloro-BQ and 2,3-dichloro-NQ both inhibited GST activity in rat H_{35}-hepatoma cells in an irreversible manner, 2,3-dichloro-NQ being most efficient. All isoenzymes present were presumably inhibited to some extent. All quinones caused a considerable depletion of cellular GSH-levels.
The lack of inhibition noticed for 5-hydroxy-NQ may be explained by two mechanisms. First, this compound could possess a higher tendency for redox cycling. Alternatively, the inhibition may be at least partly mediated by glutathione-conjugates. In contrast to the chlorinated quinones, conjugation of 5-hydroxy-NQ with GSH results in the formation of a hydroquinone-conjugate, which no longer possesses alkylating properties.
Tetrachloro-BQ and 2,3-dichloro-NQ seem attractive starting points for the development of an in vivo inhibitor.

In section 5.1 the production of monoclonal antibodies against rat GST isoenzymes 2-2 and 3-3 is described. One hybridoma against isoenzyme 2-2 and 2 hybridomas against isoenzyme 3-3 were capable of specifically differentiating their respective antigens from other rat isoenzymes as well as human isoenzymes in ELISA and on Western blot. Isoenzymes 1-1 and 4-4 did not elicit an immune response in Balb/c mice. However, high serum antibody titers were obtained for these isoenzymes in some other strains of mice, of different H-2 haplotype, notably CBA/BrARij mice and CBA/CaHRij-T6 mice for isoenzyme 1-1, and CBA/BrARij mice also for isoenzyme 4-4 after two injections with the antigen.
In section 5.2 mononuclear lymphocytes from the blood of 12 human individuals were screened for the presence or absence of mu-class GST isoenzymes μ and/or ψ, using a monoclonal antibody against human hepatic isoenzyme μ. These individuals had worked with a commercial preparation of the soil fumigant 1,3-dichloropropene (DCP), and had been exposed to the vapor of this agent in the field. Nine samples were found to be positive and 3 were negative for the presence of these mu-class isoenzymes. In all samples a protein was noticed, staining positively with the anti-μ antibody, with a somewhat lower molecular mass

than the hepatic standard. This protein presumably constitutes an additional mu-class isoenzyme, since it was bound by the S-hexylglutathione affinity column.
The data on the presence or absence of GST isoenzymes μ and/or ψ were compared with excretion levels and excretion profiles of the mercapturic acids from cis-(Z-) and trans-(E-)DCP. No significant differences were observed between mu-class positive and mu-class-negative individuals, with respect to the levels and the half-lives of elimination of Z- and E-DCP mercapturic acids. The importance of establishing correlations between the mu-phenotype and mercapturic acid excretion and/or the occurrence of certain diseases is discussed in section 5.2.

Most studies described in this thesis have focussed on glutathione S-transferases from the rat. The recognition of the existence of three classes of isoenzymes common to several mammalian species, including man, and with a high degree of structural homology within the same class, could in principle simplify the extrapolation of data from rat to man. Main obstacles to overcome include the relative lack of knowledge on the substrate selectivity of human isoenzymes, the fact that isoenzymes α- ε have generally been studied as a group and not individually, and the genetic polymorphism of human class mu enzymes for which no equivalent is known in the rat.
Studies on the substrate selectivities of individual human isoenzymes would therefore be quite useful. In addition, there is a need for studies on the mechanisms of transcriptional activation of genes coding for transferase subunits in the rat. In the context of induction it is interesting to note that in human liver, alpha class subunit B1 is generally present at much higher levels than subunit B2[1]. B1B1 has a higher pI, displays a higher activity with Δ^5-androstenedione and a lower activity with cumene hydroperoxide, than isoenzyme B2B2[2]. B1 and B2 may therefore be similar in function to subunits 1 and 2 respectively. It seems possible that the polymorphism noticed for alpha class subunits might partly reflect exposure to xenobiotics, with a preferential increase in subunit B1, similar to the increase of subunit 1 in the rat.

The development of resistance of tumour cells against chemotherapeutic agents presents a major problem in the treatment of various types of cancer. An increase in the levels of GST isoenzymes has been implicated as one of the possible causes. A selective *in vivo* inhibitor could possibly overcome this problem and would also be useful in the studies on *in vivo* metabolism of xenobiotics. The data presented in chapter 4 suggest that quinones may form a suitable starting point. Future studies will have to focus on the mechanism of inhibition, the aspect of selectivity and the structural requirements for the quinone to be capable of causing inhibition in a physiological environment.

Evidence is increasing that the genetic deficiency of human class mu isoenzymes is more complicated than previously assumed. Besides the enzymes μ and ψ, which are both subject to genetic polymorphism, an additional mu class isoenzyme exists with a slightly lower subunit molecular mass, present in all human individuals. The physiological and toxicological implications of this isoenzyme are unknown and certainly warrant further investigation.

The determination of correlations of the genetic deficiency of isoenzymes μ and ψ with certain diseases or with excretion profiles of mercapturic acids will be useful in increasing our understanding of the toxicological consequences of this polymorphism, in explaining inter-individual differences in mercapturic acid excretion and in establishing safety regulations for the handling of xenobiotics in the work-environment. In this respect, the development of techniques capable of discriminating μ from ψ is of considerable interest.

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SAMENVATTING EN SLOTOPMERKINGEN

De glutathion S-transferases (GST) vormen een groep van isoenzymen, die een belangrijke rol spelen in de biotransformatie van een groot aantal reactieve verbindingen. De isoenzymen van de rat, de mens en de muis zijn ingedeeld in drie klassen, alpha, mu en pi op grond van overeenkomsten in structuur en enzymatische eigenschappen.
De katalyse van de conjugatie van electrofiele, hydrofobe verbindingen met het tripeptide glutathion (GSH) is de voornaamste functie van de glutathion S-transferases. Daarnaast zijn verschillende isoenzymen in staat tot niet-covalente binding van een aantal stoffen die niet als substraat fungeren. De reactie met GSH kan in het algemeen beschouwd worden als een detoxificatie-stap. Er zijn echter ook voorbeelden bekend waarin de reactie met GSH leidt tot de vorming van toxische glutathion-conjugaten.
De mate waarin detoxificatie en/of activatie plaatsvindt, wordt bepaald door het isoenzympatroon, daar de individuele isoenzymen een verschillende, doch overlappende substraatselectiviteit vertonen. De gevoeligheid van een individu is dientengevolge niet alleen afhankelijk van substraatspecificiteit, maar ook van factoren die het isoenzymprofiel van de glutathion S-transferases bepalen. Deze factoren omvatten zowel genetische factoren alsook externe factoren, die een modulerende invloed hebben op de gehaltes en/of de activiteiten van individuele isoenzymen.
De studies in dit proefschrift waren gericht op de relatie glutathion S-transferase isoenzympatronen versus gevoeligheid voor alkylerende verbindingen. Verschillende aspecten van de GST isoenzymen zijn in dit verband bestudeerd, te weten substraatselectiviteit, inductie, remming en genetische deficiëntie van humane isoenzymen behorende tot de klasse mu.

In hoofdstuk 1 wordt een overzicht gegeven van de eigenschappen van de glutathion S-transferases. De functies van het enzymsysteem en de classificatie van isoenzymen worden besproken in paragraaf 1.1. Paragraaf 1.2 behandelt aspecten van de GST isoenzymen die van belang zijn voor de individuele gevoeligheid t.a.v. alkylerende verbindingen: weefselverdeling, leeftijdsafhankelijkheid van de isoenzympatronen, hormonale invloeden, inductie en remming. De paragrafen 1.3, 1.4 en 1.5 beschrijven de huidige kennis over de specifieke eigenschappen van respectievelijk de klasse alpha, klasse mu en klasse pi isoenzymen.

De toxiciteit van ozon verloopt vermoedelijk via de vorming van ozonides uit onverzadigde vetzuren in membraan fosfolipiden. In hoofdstuk 2 wordt een onderzoek beschreven naar het 9,10-mono-ozonide van methyl linoleaat (MLO), als substraat voor de glutathion S-transferases van de rat.
De reactie van MLO met GSH resulteerde in de vorming van geoxideerd glutathion en aldehydes als voornaamste produkten.
Twee instabiele glutathion-conjugaten werden gevormd als intermediairen. De reactie werd gekatalyseerd door levercytosol, longcytosol en levermicrosomen. De activiteiten t.o.v. MLO waren relatief hoog voor longcytosol en levermicrosomen in vergelijking tot levercytosol; grotere verschillen werden waargenomen voor activiteiten met het standaardsubstraat 1-chloor-2,4-dinitrobenzeen (CDNB).
Door vergelijking van de specifieke activiteiten van de isoenzymen 1-1, 1-2, 2-2, 3-3, 3-4, 4-4 and 7-7 bleek dat isoenzym 2-2 de hoogste activiteit bezit, terwijl 7-7 de reactie van MLO met GSH nauwelijks katalyseert. De relatief hoge omzetting door longcytosol kan mogelijk verklaard worden door het feit dat isoenzym 2-2 in de long van de rat een hoger percentage van het GST eiwit vormt dan in de lever.
Het ozonide bleek tevens een substraat te zijn voor glutathion peroxidase. De activiteit van dit enzym vormde echter een verwaarloosbare bijdrage aan de cytosolaire omzetting van MLO.
Uit de resultaten werd geconcludeerd dat de activiteit van de glutathion S-transferases t.o.v. MLO vergelijkbaar is met hun activiteit ten opzichte van lipide peroxides.

In hoofdstuk 3 worden de effecten vergeleken van vier inductoren met verschillende chemische structuren op het GST isoenzympatroon in de lever van de rat. Hexachloorbenzeen, benzyl isothiocyanaat, fenobarbital en 3-methylcholanthreen veroorzaakten allen een toename in de cytosolaire GST activiteit t.o.v. de substraten CDNB, 1,2-dichloor-4-nitrobenzeen en ethacryne zuur. Veranderingen in de activiteiten met trans-4-fenyl-3-buten-2-on waren slechts klein en de activiteiten t.o.v. cumeen hydroperoxide waren onveranderd. Het grootste effect werd waargenomen voor hexachloorbenzeen, terwijl 3-methylcholanthreen het

kleinste effect vertoonde. Het microsomale glutathion S-transferase werd door geen van de verbindingen geinduceerd.
Uit de GST isoenzympatronen, verkregen d.m.v. FPLC-chromatofocusing, bleek dat hexachloorbenzeen en fenobarbital beiden een relatieve toename veroorzaken van subunits 1 en 3 t.o.v. respectievelijk subunits 2 en 4. 3-Methylcholanthreen induceerde alleen subunit 1. Benzyl isothiocyanaat verschilde van de andere inductoren. Deze verbinding veroorzaakte naast een toename in subunit 3, tevens een verhoging van subunit 2 i.p.v. subunit 1.
De belangrijkste toename leek op te treden voor klasse alpha subunits: in behandelde dieren bestond 53-60 % van het GST eiwit uit subunits 1 en 2, terwijl dat percentage in controles slechts 38-45 % bedroeg.
Het exacte mechanisme van inductie van de glutathion S-transferases is onbekend. Het afwijkende effect van benzyl isothiocyanaat suggereert echter een mogelijk verband tussen de chemische structuur van de inductor en de inductie van GST subunits.

Hoofdstuk 4 behandelt de remming van GST activiteit door chinonen. In paragraaf 4.1 werden de remmende effecten van een serie structureel verwante 1,4-benzochinonen (BQ) en 1,4-naftochinonen (NQ) bestudeerd, t.a.v. een mengsel van glutathion S-transferases, gezuiverd d.m.v. affiniteits-chromatografie. De aard van de remming werd onderzocht voor drie chinonen met een verschillende chemische structuur, te weten 2-tertbutyl-BQ (een metaboliet van het voedseladditief 2(3)-tert-butyl-4-hydroxy-anisol), 5-hydroxy-NQ (een natuurlijk voorkomend chinon) en 2,3-dichloor-NQ (een synthetische verbinding toegepast als fungicide). De gevoeligheden van een aantal isoenzymen werden vergeleken t.a.v. deze drie chinonen.
De remmende invloed van BQ en NQ steeg met een toenemend aantal electronenzuigende substituenten aan de chinonring. De aanwezigheid van electronenstuwende groepen veroorzaakte een daling in de remming, t.o.v. het ongesubstitueerde chinon.

De remming bleek irreversibel van aard en was waarschijnlijk het gevolg van covalente modificatie van een specifieke cysteine, gelokaliseerd in of bij het actieve centrum van het enzym. Isoenzym 3-3 werd het sterkst geremd door de drie geteste chinonen, terwijl isoenzym 2-2 het minst gevoelig was.
De invloed van drie sterk remmende chinonen, tetrachloor-BQ, 5-hydroxy-NQ en 2,3-dichloor-NQ, op de GST activiteit in rat H_{35}-hepatoma cellen werd bestudeerd in paragraaf 4.2. De GST fractie van deze cellen bevatte voornamelijk de subunits 4 en 7, alsook kleine hoeveelheden subunits 2 en 3.
Tetrachloor-BQ en 2,3-dichloor-NQ veroorzaakten beiden een irreversibele remming van de GST activiteit in H_{35}-cellen, waarbij 2,3-dichloor-NQ het sterkste effect vertoonde. Alle aanwezige isoenzymen werden waarschijnlijk in zekere mate geremd. De drie chinonen veroorzaakten een aanzienlijke daling in de cellulaire GSH gehaltes.
Het feit dat 5-hydroxy-NQ niet in staat bleek tot het remmen van GST activiteit in een cellulair systeem, zou in de eerste plaats verklaard kunnen worden door een hogere neiging tot redox cycling van dit chinon. Een mogelijk alternatieve verklaring is dat in ieder geval een deel van de remming verloopt via de vorming van glutathion-conjugaten. Conjugatie van 5-hydroxy-NQ met GSH resulteert in de vorming van een hydrochinon-conjugaat, dat geen alkylerende eigenschappen meer bezit, terwijl de twee gechloreerde chinonen bij conjugatie hun geoxideerde toestand en daarmee hun alkylerende activiteit behouden.
De remming door tetrachloor-BQ en 2,3-dichloor-NQ lijkt een mogelijk uitgangspunt voor de ontwikkeling van een in vivo remmer van de GST activiteit.

In paragraaf 5.1 wordt de isolatie van monoclonale antilichamen tegen de GST isoenzymen 2-2 en 3-3 beschreven. Eén hybridoma tegen isoenzym 2-2 en 2 hybridoma's tegen isoenzym 3-3 werden verkregen, die in staat waren hun respectievelijke antigenen specifiek te onderscheiden van andere isoenzymen van de rat, alsook van humane isoenzymen, zowel in de ELISA als op Western blot. De isoenzymen 1-1 en 4-4 veroorzaakten geen immuunrespons in Balb/c muizen. Hoge serum antibody titers werden voor deze isoenzymen echter waargenomen in enkele muizestammen met een ander

H-2 haplotype, te weten CBA/BrARij muizen en CBA/CaHRij-T6 muizen voor isoenzym 1-1, en CBA/BrARij muizen tevens voor isoenzym 4-4 na 2 injecties met het antigen.
In paragraaf 5.2 werden mononucleaire lymfocyten van 12 individuen gescreend op de aan- of afwezigheid van de mu-klasse isoenzymen μ en/of ψ, m.b.v. een monoclonaal antilichaam tegen het humane lever isoenzym μ. Deze individuen waren tijdens hun werk blootgesteld aan de damp van het grondontsmettingsmiddel 1,3-dichloorpropeen (DCP). De isoenzymen μ en/of ψ waren aanwezig in 9 van de 12 lymfocyt-monsters. De overige monsters waren negatief. In alle monsters werd echter een eiwit waargenomen met een iets lagere molecuulmassa dan de leverstandaard, dat kruisreageerde met het monoclonale antilichaam tegen μ. Dit eiwit is vermoedelijk eveneens een mu-klasse GST isoenzym, aangezien het werd gebonden door de S-hexylglutathion affiniteitskolom.
De gegevens omtrent de aan- of afwezigheid van de GST isoenzymen μ en/of ψ , werden vergeleken met de excretie-niveaus en excretie-patronen van de mercaptuurzuren van cis-(Z-) en trans-(E-)DCP. Er werden geen significante verschillen waargenomen tussen mu-klasse negatieve en mu-klasse positieve individuen m.b.t. de niveaus en eliminatie halfwaarde tijden van Z- en E-DCP mercaptuurzuren. Het belang van het vaststellen van correlaties tussen het mu-phenotype en mercaptuurzuur-excretie of de incidentie van bepaalde ziekten wordt in paragraaf 5.2 besproken.

De meeste studies in dit proefschrift zijn gericht op de glutathion S-transferases van de rat. De indeling van de isoenzymen van de rat, de mens en de muis in drie klassen, alpha, mu en pi, waarbij de isoenzymen binnen een klasse een hoge mate van structurele homologie vertonen, zou in principe de extrapolatie van gegevens van de rat naar de mens kunnen vereenvoudigen. Belangrijke problemen die zich hierbij voordoen omvatten het relatieve gebrek aan kennis over de substraatselectiviteit van de humane isoenzymen, het feit dat de isoenzymen α - ε tot dusver voornamelijk als groep en niet individueel zijn bestudeerd, en het genetisch polymorfisme van de humane klasse mu isoenzymen, waarvoor geen analoge situatie bekend is in de rat. Studies naar de substraatspecificiteit van de individuele humane isoenzymen zijn derhalve wenselijk.

Onderzoek naar het mechanisme van activatie van de transcriptie van de coderende genen voor de transferase subunits in de rat zijn eveneens van belang. In het kader van inductie is het interessant dat de alpha klasse subunit B1 i.h.a. in hogere concentratie aanwezig is in humane lever dan de subunit B2[1]. B1B1 bezit een hoger isoelectrisch punt, vertoont een hogere activiteit met Δ^5-androsteendion en een lagere activiteit met cumeen hydroperoxide dan isoenzym B2B2[2], hetgeen suggereert dat B1 en B2 mogelijk vergelijkbaar zijn in functie met respectievelijk subunits 1 en 2. Het is niet onmogelijk dat het polymorfisme t.a.v. de klasse alpha subunits gedeeltelijk een gevolg is van blootstelling aan xenobiotica, waarbij de B1 subunit bij voorkeur toeneemt, analoog aan subunit 1 in de rat.

De ontwikkeling van tumorresistentie tegen chemotherapeutica is een groot probleem in de behandeling van verschillende vormen van kanker. Een toename in de gehaltes van GST isoenzymen wordt beschouwd als één van de mogelijke oorzaken. Een selectieve in vivo remmer zou van belang kunnen zijn bij de aanpak van het probleem van tumorresistentie, en zou tevens nuttig kunnen zijn bij studies naar in vivo metabolisme van xenobiotica. De resultaten in hoofdstuk 4 geven aan dat de remming door chinonen een mogelijk uitgangspunt vormt voor de ontwikkeling van een in vivo remmer. In de nabije toekomst zal aandacht besteed moeten worden aan het mechanisme van de remming in een cellulair systeem, aan het aspect van selectiviteit alsmede aan de structurele eisen die gesteld worden aan het chinon om remming te kunnen veroorzaken onder fysiologische condities.

De genetische deficientie problematiek rond de humane klasse mu isoenzymen lijkt gecompliceerder dan voorheen is aangenomen. Naast de isoenzymen μ en ψ, die beiden onderhevig zijn aan genetisch polymorfisme, is een derde mu-klasse isoenzym waargenomen, met een iets lager subunit molecuulgewicht, dat in alle individuen aanwezig is. De fysiologische en toxicologische consequenties van de aanwezigheid van dit enzym zijn onbekend, maar vormen wellicht een interessant onderwerp voor toekomstig onderzoek.

Het vaststellen van het al of niet bestaan van correlaties tussen de genetische deficientie van de isoenzymen μ en ψ en excretiepatronen van mercaptuurzuren of het optreden van bepaalde ziekten, zou een bijdrage

kunnen leveren aan een beter begrip van de toxicologische consequenties van dit genetisch polymorfisme. Tevens zouden deze correlaties een aanknopingspunt kunnen vormen voor het verklaren van verschillen in excretiepatronen alsook voor het vaststellen van veiligheidseisen bij het omgaan met xenobiotica. De ontwikkeling van gevoelige technieken die μ van ψ kunnen onderscheiden is in dit opzicht van belang.

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CURRICULUM VITAE

Ria M.E. Vos werd geboren op 15 maart 1961 te Rhenen. Na het behalen van het diploma Gymnasium β aan het Titus Brandsma Lyceum te Oss, werd in 1979 begonnen met de studie Humane Voeding aan de Landbouwuniversiteit te Wageningen. De doctoraalfase omvatte de hoofdvakken Humane Voeding (prof.dr. J.G.A.J. Hautvast) en Toxicologie (prof.dr. J.H. Koeman) en het bijvak Biochemie (prof.dr. F. Muller). De studie werd in januari 1986 afgerond met lof. Op 1 februari 1986 werd het in dit proefschrift beschreven onderzoek gestart, bij de vakgroep Toxicologie van de Landbouwuniversiteit.

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