Interactions of α,β-unsaturated carbonyl compounds with the glutathione-related biotransformation system

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Stellingen

1. De remming van glutathion-S-transferase activiteit in cellen door α,β-onverzadigde carbonylverbindingen komt tot stand via covalente binding en competitieve inhibitie (*dit proefschrift*).

2. De rol van GST bij biotransformatie op cellulair niveau dient bestudeerd te worden in samenhang met glutathionnivo's en de multidrug resistance associated protein (MRP) (*dit proefschrift*).

3. De betrokkenheid van GSTP1-1 in het metabolisme van de endogene verbinding prostaglandine A_2, duidt op een mogelijke rol van dit isoenzym in de regulatie van celproliferatie (*dit proefschrift*).

4. Bij beoordeling van detoxificatie en/of bioactivering van verbindingen door glutathionconjugatie dient rekening gehouden te worden met stereoselectiviteit van GSTs (*dit proefschrift*).

5. De inductie van apoptose door ceramiden is tot nu toe onderbelicht als mechanisme van de chemopreventieve werking van NSAIDs (*T.A. Chan et al. (1998) PNAS USA **95**, 681-686*).

6. Fysiologische bindingsstudies naar de interactie van humane voedselcarcinogenen en de eiwitcomponenten in de voeding zijn, gezien de overheersende invloed van vet in het dieet, fysiologisch niet relevant (*E.H. Vis*).

7. Het Amerikaanse beleid met betrekking tot hormoonverstorende stoffen loopt vooruit op de wetenschap (*Dr. T.G. Osimitz, 1998*)

8. Het feit dat GST van de pi-klasse onafhankelijk van de catalytische activiteit een reductie van de effectiviteit van bevruchting veroorzaakt, bevestigt de vruchtbaarheid van het onderzoek naar inhibitie van GST activiteit (*B. Gopalakrishnan et al. (1998), Biochem. J. 329, 231-241*).

9. Het feit dat de Viagra-pil veel eerder op de markt is dan de anticonceptiepil voor mannen, duidt erop dat de pharmaceutische wereld voornamelijk door mannen geleid wordt.

10. Voor fundamenteel onderzoek naar metabolisme en kinetiek is de toepassing meestal heel duidelijk.

11. Van een president mag worden verwacht dat hij zijn buitenechtelijke relaties beter geheimhoudt.

12. Het lezen van een 'Tom Poes' is qua moeite omgekeerd evenredig met het eten van een tompoes (*A. van Iersel*).

Stellingen behorende bij het proefschrift 'Interactions of α,β-unsaturated carbonyl compounds with the glutathione-related biotransformation system', door Marlou van Iersel, te verdedigen op vrijdag 13 November, 1998 te Wageningen.

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Marlou L. P. S. van Iersel

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'In de wetenschap gelijken wij op kinderen, die aan den oever der kennis
hier en daar een steentje oprapen, terwijl de wijde oceaan
van het onbekende zich voor onze oogen uitstrekt'

John Newton (1725-1807)

Contents

Chapter 1

General introduction

Context of the current thesis

The research described in this thesis focuses on interactions of α,β-unsaturated carbonyl compounds with various parts of the glutathione-related biotransformation system. Especially the parts of this system which may play a role in multidrug resistance are investigated. These comprise three main topics: glutathione (GSH) levels, glutathione S-transferase (GST) activity and the multidrug resistance associated protein pump (MRP).

The tripeptide glutathione is the major intracellular nonprotein thiol present in virtually all cells. It protects cells against noxious compounds, including carcinogens (Meister, 1983b). Variations in GSH content of tumour cells indicate that it may protect cells from alkylating anti-

cancer drugs (Arrick and Nathan, 1984).

Glutathione S-transferases (GSTs) are an important class of enzymes that play a role in the detoxification of a wide variety of electrophilic compounds by catalysing glutathione conjugation of these compounds (recently reviewed in Hayes and Pulford, 1995; Armstrong, 1997). The GST π-class is especially interesting as this isoenzyme is over expressed in certain tumours (Sato, 1989) and therefore may be a main factor in drug resistance against alkylating anticancer drugs (Waxman, 1990).

The third key factor is the MRP pump, which has first been described in 1992 by Cole and co-workers in a multidrug resistant human lung cancer cell line (Cole *et al.*, 1992) and which has been shown to transport numerous, mainly conjugated compounds of both endogenous and exogenous origin out of the cell (Lautier *et al.*, 1996). The overexpression of this protein in several human cancers (Nooter and Stoter, 1996) and in various multidrug resistant cancer cell lines (Lautier *et al.*, 1996) suggests a role in multidrug resistance.

Modulation of each of these glutathione-related biotransformation processes i.e. depletion of GSH, inhibition of GST activity and/or inhibition of MRP activity would provide a possible approach to overcome drug resistance and thus has been the subject of intense research in the last few years.

One of the inhibitors most widely studied is the α,β-unsaturated ketone, ethacrynic acid (EA). EA is conjugated to glutathione (GSH) through a process referred to as Michael addition (Ahokas *et al.*, 1985), both chemically as well as catalysed by GSTs (Habig *et al.*, 1974). Interestingly, this Michael addition can be reversible (Ploemen *et al.*, 1994b). EA has been shown to inhibit GST activity both competitively and by covalent binding (Ploemen *et al.*, 1993a,b) and the glutathione conjugate of ethacrynic acid is a good substrate for MRP (Zaman *et al.*, 1996). Treatment of tumour cell lines with ethacrynic acid sensitized these cells to the cytotoxic effects of alkylating agents (Tew *et al.*, 1988; Hansson *et al.*, 1991). These observations led to the hypothesis, that other compounds with an α,β-unsaturated carbonyl moiety (i.e. aldehydes and ketones) might possess similar properties.

α,β-Unsaturated carbonyl compounds are a class of compounds to which man is ubiquitously exposed. Some originate from external sources such as automobile exhaust (acrolein, crotonaldehyde) and food (cinnamaldehyde, curcumin). However, a number of endogenous bioactive compounds also belong to this group (prostaglandin A_2, lipid peroxidation product 4-hydroxy-2-nonenal). They are known to be conjugated to GSH both chemically (Esterbauer *et al.*, 1975) and catalysed by GSTs (Boyland and Chasseaud, 1967). These properties make them an interesting group to investigate in relation to the GSH-related biotransformation pathway.

This is the basis for the research presented in this thesis. The next paragraphs emphasize subjects which are relevant within the context of the present thesis.

Glutathione-related metabolism and cancer

The three main aspects of the glutathione-related biotransformation system in relation to cancer are, as mentioned above, glutathione levels, GST activity and MRP activity.

Glutathione

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH) is present in basically every cell in concentrations varying from 0.5 mM up to 10 mM in hepatocytes. It plays a role in the protection of cells against reactive oxygen species (ROS) and electrophilic compounds by preventing these compounds from attacking and damaging cellular proteins and DNA. The ROS are reduced with concomitant formation of oxidized glutathione (GSSG) and the electrophilic compounds are conjugated to GSH forming thioether compounds, which are usually less toxic and more hydrophilic. Furthermore, GSH participates in thiol-disulfide exchange reactions with low molecular weight disulfides and with protein disulfide bonds. The latter are involved in the breakdown of proteins and regulation of certain enzymes (Meister, 1983b). As the association of GSH with cancer is highly related to GSTs, the role of GSH in detoxification, but also activation of (potential) carcinogens will be described in the next paragraph.

Glutathione S-transferases

Glutathione S-transferases (EC 2.5.1.18) are Phase II biotransformation enzymes catalysing the conjugation of electrophilic compounds to glutathione (Jakoby, 1978; Mannervik, 1985; Hayes and Pulford, 1995; Armstrong, 1997). In most cases this is a detoxification reaction. GSTs and high cellular levels of glutathione are among the most important protection mechanisms of mammalian cells against toxic and carcinogenic compounds (Chasseaud, 1979; Coles and Ketterer, 1990; Hayes and Pulford, 1995; Talalay *et al.*, 1995). Substrates for GSTs range from naturally occurring agents such as certain α,β-unsaturated carbonyl compounds, chemicals, several anticancer drugs, to endogenous compounds such as cyclopentenone prostaglandins (Cagen *et al.*, 1975) and products of lipid peroxidation (4-hydroxyalk-2-enals) (Ålin *et al.*, 1985).

However, in a number of cases glutathione conjugation results in the formation of more reactive compounds (activation). Three possible mechanisms have been described for this phenomenon (Van Bladeren, 1988; Commandeur *et al.*, 1995). Firstly, the conjugate is more toxic than the parent compound. This is the case for the glutathione conjugate of 1,2-dibromoethane that is transformed into a reactive episulfonium ion. Secondly, the generated conjugates are further metabolized to reactive species, either by subsequent oxidation and redox cycling or by the action of γ-glutamyl transpeptidase and β-lyase. This has recently been reviewed by Monks and Lau (1997) and is observed for instance with the GSH conjugate of

hydroquinones. The third mechanism is based on the phenomenon that glutathione conjugation can be reversible for some substrates and the electrophilic compound can be released under different circumstances, indicating that the conjugate is a storage form. Examples of this mechanism are isothiocyanates and α,β-unsaturated carbonyl compounds.

An interesting aspect in the balance between detoxification and activation by GSTs is the existence of genetic polymorphisms in the human GST mu, theta and pi gene. People having a homozygous deletion of the GST M1 locus, which occurs in 52% of the English population, are associated with a higher risk for cancer of the bladder, colon, skin, and lung (Hayes and Pulford, 1995). Polymorphism of the hGSTT1-1 gene is documented (Pemble *et al.*, 1994), but the consequences of this polymorphism for cancer susceptibility are not yet clear (Hayes and Pulford, 1995). The recent discovery of polymorphism of hGSTP1- 1 (Harries *et al.*, 1997) has triggered new research on the implications for cancer susceptibility. An effect of this polymorphism has already been shown for the glutathione conjugation of the carcinogen 7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (*anti*-BPDE) (Hu *et al.*, 1997).

In addition to the detoxification and activation of potential carcinogenic compounds by glutathione conjugation, GSTs are associated with cancer in two other ways as well. Firstly, GSTs, especially the pi-class GST is upregulated in numerous cancer tissues. GSTP1-1 is highly expressed in tumours of, among other organs, breast, kidney, melanoma, colon, uterus (as reviewed in Tsuchida and Sato, 1992). Harries *et al.* (1997) described two polymorphic GSTP1-1 forms differing in amino acid 105 (isoleucine in the wild type (GSTP1a) and valine in the mutant enzyme (GSTP1b)). The latter allele produces a less active enzyme and is strongly associated with bladder, testicular and prostate cancer (Harries *et al.*, 1997). Ali-Osman *et al.* (1997) found three different enzymes, the third one (hGSTP1c) with an additional modification in codon 113 resulting in a change from alanine to valine, besides the isoleucine to valine change on codon 104. The GSTP1c genotype had a 4-fold higher frequency in malignant glioma compared to normal tissue (Ali-Osman *et al.*, 1997). *In vitro* screening of human tumour cell lines for glutathione associated enzymes revealed that in all but two cell lines GSTP1-1 was the predominant isoenzyme (Tew *et al.*, 1996). In addition, pi-class GST in rats, GST7-7, has thus become one of the most useful markers of early hepatocarcinogenesis, because this isoenzyme is normally not expressed in rat liver (Sato, 1989). In healthy human liver GSTP1-1 only appears in the biliary epithelium (Hayes *et al.*, 1989), but it is increased in hepatic tumours, especially cholangiocarcinomas and metastatic tumour tissue in livers (Sato, 1989).

Another aspect of the differences in GSTP1-1 amount in various tissues is the presence of GSTP1-1 in human fetal, but not adult liver (Kashiwada *et al.*, 1991). This indicates a role in cell differentiation/proliferation. Taking in mind that pi-class GST is upregulated in initiated cells and that tumour cells undergo dedifferentiation, this would further support this theory. Interestingly, the reverse also seems true, inhibition of GSTs resulted in decreased cell proliferation in the K562 cell line (Sato *et al.*, 1990). Moreover, several groups reported

changing GST activity during cell cycle (Senjo and Ishibashi, 1988; Jungnelius *et al.* 1994), although this could not be shown for murine leukemic cells (Ramachandra *et al.*, 1995). Although a precise role of GSTP1-1 in cell proliferation is certainly not clear at the moment, McCaughan *et al.* (1994) have reported that GSTP1-1 down regulates apoptosis and has a permissive role in cell cycle activity. GSTP1-1 might possibly have evolved to protect proliferating cells.

Secondly, GSTs are reported to play a role in chemoprevention, as they are induced as an adaptive response to chemical stress. High levels of expression of GSTs are associated with increased tolerance of cells to noxious compounds and can thus be advantageous for mammals. However, one should consider that GSTs are part of a complex detoxification system, so a direct association is difficult to demonstrate. The regulation of GST expression has been the subject of numerous studies and is still an intensively studied field of research (for review: Talalay *et al*, 1988; 1995; Hayes and Pulford, 1995). GSTs are induced by various chemicals and drugs and this induction is often isoenzyme specific. Four categories of inducers with different mechanisms are generally identified, involving separate responsive elements (as reviewed in Hayes and Pulford, 1995; Ciaccio and Tew, 1996). The first mechanism is regulation of GST expression by polycyclic aromatic hydrocarbons (PAH) and involves the XRE (xenobiotic-responsive element); secondly, induction by phenolic antioxidants, Michael acceptors and ROS involves the antioxidant-responsive element (ARE), electrophile-responsive element (EpRE), AP-1 binding sites, and Nuclear Factor (NF)-κB binding sites; the third group of inducers include barbiturates. The corresponding regulatory element, Barbie box, has been identified in GST genes. The fourth mechanism involves a glucocorticoid-responsive element (GRE) for the induction by glucocorticoids (Hayes and Pulford, 1995). Clearly, induction of detoxification enzymes by xenobiotics is highly complex.

Multidrug resistance-associated protein (MRP)

MRP was first detected by Cole *et al.* (1992) in a multidrug resistant human lung cancer cell line. It is a 190 kDa membrane bound glycoprotein and its overexpression is a general phenomenon in non-P-glycoprotein (Pgp) mediated drug resistance. MRP transports a wide variety of mainly conjugated substrates among which are the endogenous leukotriene C_4 (Leier *et al.*, 1994) and glutathione, glucuronate and sulfate conjugates from both endogenous as well as xenobiotic compounds (Müller *et al.*, 1994; Jedlitschky *et al.*, 1996; Evers *et al.*, 1997). Transporters with these substrate characteristics are known as GS-X pumps (Ishikawa, 1992), multispecific organic anion transporters (MOAT) (Jansen and Oude Elferink, 1993), or leukotriene C_4 (LTC_4) transporter (Keppler, 1992) and they are all member of the ATP-binding cassette superfamily of transmembrane transporters (Lautier *et al.*, 1996). Low MRP levels are found in most healthy human tissues and overexpression is observed in several human cancers, among which are leukemia, non-small cell lung cancer, and cancers of the breast and ovary

(Nooter and Stoter, 1996). For the transport of anti-cancer drugs, glutathione seems to be required (Loe *et al.*, 1996; Versantvoort *et al.*, 1995), and it has been suggested that a co-transport mechanism might also be involved or that GSH causes a change in MRP structure (Lautier *et al.*, 1996). Since GSH plays a role both in this pump mechanism and in GST activity and especially pi class GST is able to sequester electrophilic compounds and/or glutathione conjugates, it is tempting to speculate on a possible cooperation between MRP and GSTP1-1. However, efforts to demonstrate such a link have been unsuccessful so far (Ploemen and Cnubben, unpublished results). Synergy in the action of both proteins (glutathione conjugation and glutathione conjugate efflux) has recently been indicated for the protection against toxicity of 4-nitroquinoline 1-oxide in MCF7 cells (Morrow *et al.*, 1998).

Glutathione-related metabolism and multidrug resistance

General

The upregulation of the GSH-related biotransformation system in tumour tissue implicates a major problem for the treatment of cancer with chemotherapeutic agents, as these drugs are better detoxified and hence less effective. This phenomenon is referred to as drug resistance. This drug resistance can either be an intrinsic property of tumours or develops during multiple courses of chemotherapy. Numerous studies have already been carried out to find mechanisms which contribute to drug resistance and to discover ways in which this problem might be overcome (for review Nooter and Stoter, 1996; Laing and Tew, 1997). Processes involved in multidrug resistance include alterations in drug uptake, metabolism, and excretion as well as modulation of the target sites, repair mechanisms and cell survival pathways (Bates *et al.*, 1994).

Glutathione

The relationship between GSH levels and multidrug resistance has already been described in 1984 by Arrick and Nathan (1984). Depending on the class of drugs, GSH metabolism can influence the therapeutic efficacy of anticancer drugs, by detoxification, toxification, and drug delivery (Arrick and Nathan, 1984). Cell lines, that are resistant to various cytostatics, among which are cisplatin, phenylalanine mustard and melphalan, were found to contain higher GSH levels compared to sensitive cells. Tumours, that were resistant *in vivo* (in patients) generally had higher GSH content than did tumours obtained before any treatment (reviewed in Meister, 1994; O'Dwyer *et al.*, 1995).

Glutathione S-transferases

GST isoenzymes are reported to be involved in the detoxification of numerous anti-cancer

drugs viz. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Smith *et al.*, 1989), cyclophosphamide (Dirven *et al.*, 1994), melphalan (Dulik *et al.*, 1986), chlorambucil (Ciaccio *et al.*, 1991) and thiotepa (Dirven *et al.*, 1995). Furthermore, GSTs might be involved indirectly by scavenging reactive oxygen species, which arise after metabolism of certain cytostatics (Tew *et al.*, 1996) or sequester the parent drugs or their glutathione conjugates. *In vitro* studies have shown that GSTs are overexpressed in a variety of human and rodent cell lines, that were selected for resistance to various cytostatic drugs (as reviewed in Hayes and Pulford, 1995). In most cases class pi GST was overexpressed in these resistant cell lines, but levels of alpha and mu class GSTs are sometimes increased as well. In human tumour cell lines a correlation was found between GSTP1-1, but not GSH levels, and drug resistance against alkylating agents (Tew *et al.*, 1996). But it should be emphasized that often other multidrug resistance related enzymes and proteins are overexpressed as well in these cell lines as for example the multidrug resistance associated protein (MRP) pump or γ-glutamylcysteine synthetase (γ-GCS) (Ciaccio and Tew, 1996). Further evidence that GSTs play a role in drug resistance is obtained by the use of GST inhibitors. Inhibition of GSTs in cell lines results in an increase of the cytotoxicity of several chemotherapeutic agents as will be described in the next paragraph (Tew *et al.*, 1988; Hansson *et al.*, 1991; Morgan *et al.*, 1996).

Transport pumps

Another mechanism which plays a role in drug resistance and which is part of metabolism of anti-cancer drugs, is the efflux of the compounds, free or conjugated with glutathione, mediated by transporters which belong to the ATP-binding cassette superfamily. At this moment P-glycoprotein (Pgp) and the above mentioned multidrug resistance associated protein (MRP) pump are thought to be the two most important pumps regarding multidrug resistance. Pgp is a 170 kDa drug efflux pump, with a broad substrate specificity, which is detected in virtually all tumour types, but also in many normal tissues. This trans membrane protein is able to extrude drugs directly from the plasma membrane and is responsible for the 'classical' multidrug resistance (MDR) (Nooter and Stoter, 1996; Twentyman, 1997).

Reversal of glutathione-related multidrug resistance

Glutathione

Modulation of glutathione levels can play a multiple role in reversal of multidrug resistance. As mentioned in previous paragraphs, it is not only a general intracellular scavenger of reactive compounds (Meister, 1983b), but also a cofactor for both GST activity and the MRP efflux pump. Already in the 1950s it was shown that GSH depletion resulted in sensitization of resistant cells to ionizing radiation (Alper, 1956). Numerous studies have since been conducted

to investigate the effect of GSH depletion on reversal of drug resistance. The synthesis of L-buthionine sulfoximine (BSO) (Griffith, 1982), a specific inhibitor of γ-glutamylcysteine synthetase (γ-GCS), opened possibilities to deplete GSH levels without severe toxicity. Depletion of glutathione by BSO treatment increased the cytotoxicity of various electrophilic anti-cancer drugs in for instance human breast cancer cells (Chen and Waxman, 1994).

Glutathione S-transferases

As GSTP1-1 is the major GST isoenzyme in drug resistance and is considered to play a role in cell proliferation (McCaughan *et al.*, 1994; Sato *et al.*, 1990), inhibition of this isoenzyme might be an important tool in the struggle against cancer and has been the subject of research in recent years. Inhibition of enzymes may be divided in two types: reversible and irreversible inhibition. An extensive list of reversible GST inhibitors is described in reviews from Mannervik and Danielson (1988) and Van Bladeren and Van Ommen (1991). They include glutathione analogs, nonsubstrate ligands, isothiocyanates, plant phenols as caffeic acid and quercitine, metal compounds and are thus widely spread. Irreversible inhibitors modify enzymes covalently, which leads in most cases to irreversible loss of activity. A well known example of this class of inhibitors are quinones (Van Bladeren and Van Ommen, 1991). hGSTP1-1 is known to be quite susceptible to covalent modification on the cysteine residues of the enzyme, the two most reactive ones located on position 47 and 101 (Ricci *et al.*, 1991). Especially Cys-47, which is located quite near the active site, has been observed to be a reactive and accessible moiety for electrophilic compounds (LoBello *et al.*, 1990). Although cysteine residues are not essential for catalytic activity of the enzyme (Kong *et al.*, 1991), modification of Cys-47 by site-directed mutagenesis resulted in lower activity (LoBello *et al.*, 1990) and GST activity can be regulated by its oxidation status and by SH/SS exchange reactions (Ricci *et al.*, 1991; Nishihira *et al.*, 1991). Furthermore covalent modification results in a loss of activity. This has been shown for, among other compounds, H_2O_2 (Tamai *et al.*, 1990; Shen *et al.*, 1991), the commonly used GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Caccuri *et al.*, 1992), and ethacrynic acid (Ploemen *et al.*, 1994b).

Multidrug resistance associated protein (MRP)

Reversal of MRP-associated resistance has appeared to be a difficult field of research; several compounds have been reported to (partially) reverse MRP-related resistance, but often this is dependent on the drug and the type of cells used and little or no structural similarity could be detected thus far (Lautier *et al.*, 1996; Twentyman, 1997). MRP activity can also be influenced by GSH levels, which has been shown in multidrug resistant human lung tumour cell lines overexpressing MRP (Versantvoort *et al.*, 1995). BSO treatment of resistant MCF7 cells overexpressing MRP resulted in sensitization of these cells (Schneider *et al.*, 1995), but the effect of BSO treatment again seems to be dependent on the drug and cell type used

(Lautier *et al.*, 1996). Another approach to reverse MRP-mediated drug resistance is the use of antisense oligonucleotides or synthetic catalytic RNAs to reduce MRP expression by targeting the mRNA coding for MRP (Lautier *et al.*, 1996).

Clinical relevance

Drug resistant cell lines were used to study the influence of GST inhibitors and GSH depletors on the cytotoxicity of anti-cancer drugs. Incubation of cells with ethacrynic acid resulted in increased cytotoxicity of chloroambucil (Tew *et al.*, 1988), melphalan (Hansson *et al.*, 1991), mitomycin C (Singh *et al.*, 1992) and doxorubicin (Awasthi *et al.*, 1996). Several clinical trials have been performed with the aim to modulate glutathione levels and/or GST activity in tumour tissue to enhance the efficiency of anti-cancer drugs (O'Dwyer *et al.*, 1995). For example, Phase I clinical trials were performed with L-buthionine SR-sulfoximine (BSO) in combination with melphalan. GSH levels were decreased to below 20% of control and the combination resulted in severe myelosuppression (O'Dwyer *et al.*, 1996). A Phase I study with ethacrynic acid in combination with thiotepa, showed a decreased GST activity in peripheral mononuclear cells with considerable interpatient variability (O'Dwyer *et al.*, 1991). In a case study with a patient with chronic lymphocytic leukaemia with extremely high GST activity in lymphocytes, it was shown that ethacrynic acid reversed chloroambucil resistance (Petrini *et al.*, 1993). The development of isoenzyme specific inhibitors is still a current research subject (Adang *et al.*, 1990; Flatgaard *et al.*, 1993; Ouwerkerk-Mahadevan *et al.*, 1995; Ploemen *et al.*, 1996; Morgan *et al.*, 1996). Another approach to overcome drug resistance related to high tumour GST levels is the generation of compounds that are iso-enzyme specifically activated to a cytotoxic compound (Lyttle *et al.*, 1994).

α,β-Unsaturated carbonyl compounds

General introduction

α,β-Unsaturated carbonyl derivatives are a class of compounds to which man is ubiquitously exposed. They occur naturally in the environment, are formed after combustion of organic materials and are identified in food and used as food flavouring substances. For instance, the highly reactive acrolein and crotonaldehyde are found in tobacco smoke and automobile exhaust. Cinnamaldehyde is the main element of cinnamon and curcumin is the main component of turmeric from which the spice currie is obtained. Additionally, these reactive compounds are formed endogenously during lipid peroxidation (4-hydroxy-2-nonenal; HNE), DNA-degradation (base propenals) and metabolism of certain drugs (acrolein formation after biotransformation of cyclophosphamide) (Witz, 1989; Feron *et al.*, 1991; Eder *et al*, 1993; Berhane *et al.*, 1994; Stoner and Mukhtar, 1995). For these widely spread compounds a variety

of biological effects has been described: acrolein and crotonaldehyde form DNA adducts (Wilson *et al.*, 1991), but for instance curcumin has anticarcinogenic potential (Stoner and Mukhtar, 1995).

α,β-Unsaturated carbonyl compounds and glutathione-related metabolism

These electrophilic carbonyls are thiol reactive compounds and have been shown to be conjugated to glutathione catalysed by glutathione S-transferases (Boyland and Chasseaud, 1967; Van Bladeren, 1988; Berhane and Mannervik, 1990; Berhane *et al.*, 1994). The chemical reaction of these compounds with thiols is an important pathway as well, and this has been described by Esterbauer *et al.* (1975). However, because this class of compounds are Michael-acceptors, retro-Michael cleavage can occur under certain circumstances. This has been reported or suggested previously for glutathione conjugates of this group of xenobiotics (Vroomen *et al.*, 1988; Witz, 1989; Baillie and Slatter, 1991; Ploemen *et al.*, 1994b; Ramu *et al.*, 1995).

Furthermore, α,β-unsaturated carbonyl compounds have been observed to inhibit GSTs (Berhane and Mannervik, 1990; Chien *et al.*, 1994; Linderman *et al.*, 1994).

Upon glutathione conjugation of α,β-unsaturated carbonyl compounds, two possible diastereoisomers can be formed. Catalysis by GSTs often leads to the preferential formation of one of the two diastereoisomers. For instance, the glutathione conjugation of 4-phenyl-3-buten-2-one (PBO) is stereoselectively catalysed by rat GSTM2-2, but not rat GSTM1-1 (Chen and Armstrong, 1995). Lately, isoenzyme specific stereoselectivity has been shown for the catalysis of the glutathione conjugation of the endogenous compounds prostaglandins A_2 and J_2 (Bogaards *et al.*, 1997). Stereoselective formation of one of two diastereoisomers can have physiological consequences, especially when glutathione conjugates are the actual active compounds. When looking at glutathione conjugation as a bioactivation reaction instead of detoxification, the formed diastereoisomer can be the toxic compound or the substrate for further processing enzymes, leading to the formation of a toxic agent. In this latter case it is possible that only one diastereoisomer is a substrate for the enzymes, and the other one is not; this of course having impact on the actual toxicity of certain compounds.

Besides the fact that α,β-unsaturated carbonyl compounds are substrates for GSTs, Talalay and co-workers pointed out already in 1988 that induction of chemoprotective enzymes (among which are GSTs) by numerous seemingly unrelated anticarcinogens can be attributed to the presence of a Michael reaction acceptor (Talalay *et al.*, 1988). Regulation of these enzymes has been and still is a major subject of research in recent years. Michael acceptors, among other compounds, are believed to act via the antioxidant-responsive element (ARE), which can be activated by heterodimers of various transcription factors, including Maf and Nrf proteins and probably Fos and Jun (Hayes and Pulford, 1995; Itoh *et al.*, 1997). Most likely, Michael acceptors and some other GST inducers are recognized by a redox and/or electrophile-sensitive protein, perhaps containing one or more cysteine residues, for which the oxidation status is a

determinant in the binding activity of the transcription factors (Hayes and Pulford, 1995; Talalay *et al.*, 1995; Primiano *et al.*, 1997).

Summarizing, α,β-unsaturated carbonyl compounds are in many ways related to GST activity, being substrate, inhibitor and inducer of these enzymes. In the research presented in this thesis various α,β-unsaturated carbonyl compounds are used, that are relevant for human exposure, ethacrynic acid being a model compound. Their structures, sources and properties are presented in Table 1.1.

Ethacrynic acid

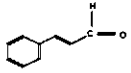
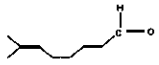
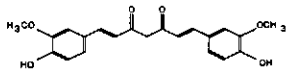
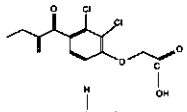
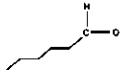
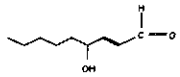
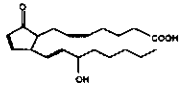
The diuretic drug ethacrynic acid ([2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy)]acetic acid), an α,β-unsaturated ketone and a Michael acceptor, is conjugated to glutathione both chemically as well as catalysed by GSTs (Habig *et al.*, 1974). Besides, it is a potent inhibitor of rat and human hepatic glutathione S-transferases (Ahokas *et al.*, 1985; Takamatsu and Inaba, 1992). Both the parent compound ethacrynic acid (EA) and the ethacrynic acid glutathione conjugate (EASG) are potent reversible inhibitors of GSTs with I_{50}-values in the range of <0.1-11μM (Ploemen *et al.*, 1990; Awasthi *et al.*, 1993) and EA inhibits GST of the pi-class by covalent binding (Ploemen *et al.*, 1993a). These GST inhibiting properties were used to enhance cytotoxicity of alkylating drugs in various drug resistant cell lines, as described above. Furthermore, ethacrynic acid was used in a Phase I clinical trial with thiotepa in 1991 and it was found that pi-class GST activity was decreased (O'Dwyer *et al.*, 1991).

It has only recently been shown that the ethacrynic acid glutathione conjugate is a very good substrate for MRP (Zaman *et al.*, 1996). So the effect of ethacrynic acid on the reversal of multidrug resistance is most likely the result of several modes of action i.e. depletion of glutathione, inhibition of GSTs and competitive inhibition of transport by MRP.

Human colon carcinoma cells, that were made resistant to EA showed increased levels of GSTπ transcript, increased glutathione levels (Kuzmich *et al.*, 1992) and the half lifes of GSTπ, γ-GCS, and dihydrodiol dehydrogenase (DDH) transcripts were also increased (Shen *et al.*, 1995). These changes together with an elevated expression of MRP resulted in a 5 times enhanced capacity to metabolize and efflux EA in these resistant cells compared to wild-type cells (Ciaccio *et al.*, 1996). Apparently, EA is capable of inducing transcription of various genes encoding for thiol-related enzymes (Ciaccio and Tew, 1996), which is not surprising as mentioned above.

Another facet of glutathione-related metabolism of EA is that it has been implicated that the cysteine conjugate of EA is the active metabolite in the diuretic effect of EA (Koechel and Cafruny, 1973). O'Dwyer *et al.* (1995) too suggested that metabolites of EA may be the active diuretic agents. The fact that two diastereoisomers can be formed upon glutathione conjugation and that GSTs might be stereoselective for the formation of one of the diastereoisomers is thus an interesting issue.

Table 1.1 α,β-Unsaturated carbonyl compounds used in the research described in the present thesis.

compound	structure	source	properties	Ref.
acrolein		cigarette smoke, automobile exhaust, CP metabolite, red wine	DNA adduct formation, mutagenic hepatotoxic, irritant	Feron *et al.*, 1991 Eder *et al.*, 1993 Esterbauer *et al.*, 1991
cinnamaldehyde		cinnamon, cassia, food flavouring	antimutagenic, mutagenic (?)	Feron *et al.*, 1991 Ohta, 1993
citral		citrus fruits, lemon grass, eucalyptus, flavouring agent	carcinostatic, teratogenic, irritant	Diliberto *et al.*, 1990
crotonaldehyde		cigarette smoke, automobile exhaust, red wine	DNA adduct formation, mutagenic genotoxic, hepatotoxic, carcinogenic	Feron *et al.*, 1991 Eder *et al.*, 1993
curcumin		curry, turmeric	antiviral, anticarcinogenic anti inflammatory, antioxidant	Commandeur and Vermeulen, 1996
ethacrynic acid		diuretic drug		
trans-2-hexenal		leaf aldehyde, banana, lipid peroxidation product	DNA adduct formation	Eder *et al.*, 1993
4-hydroxy-2-nonenal		lipid peroxidation product	genotoxic	Esterbauer *et al.*,1991
prostaglandin A_2		endogenous regulatory compound	growth inhibitor, antitumor	Jaffe and Santoro,1977 Hohn *et al.*, 1979

Objective and approach of the present study

The apparently multifaceted relationship between α,β-unsaturated carbonyl compounds and glutathione S-transferases, inspired us to further investigate this association. The significance of especially GST of the π-class in cancer and in the phenomenon of multidrug resistance, further emphasised the possible relevance of the compounds.

To increase understanding of the mechanisms involved in GST inhibition and glutathione conjugation, interactions of α,β-unsaturated carbonyl compounds with purified isoenzymes were investigated.

The mechanistic features, including the retro-Michael reaction, of the covalent interaction of these compounds with human GSTP1-1, were studied, using purified enzyme and three mutant enzymes, missing one or two cysteine residues (*chapter 2*).

The potential relevance of stereoselective formation of glutathione conjugates in relation to toxicity of α,β-unsaturated carbonyl compounds was the basis for *chapter 3*. The formation of diastereoisomers of the glutathione conjugate of model compound ethacrynic acid and the influence of catalysis by human GSTs on this formation is described in this chapter.

As glutathione conjugation was only studied using cytosol or purified enzymes, a method was developed to investigate this pathway in intact cells. In this way it was possible to study glutathione conjugation and GST activity in conjunction with other cellular glutathione-related processes. A human IGR-39 melanoma cell line was used with high levels of especially GSTP1-1. Using this system, the effect of exposure of these cells to various relevant α,β-unsaturated carbonyl compounds (i.e. acrolein, citral, cinnamaldehyde, crotonaldehyde, curcumin, ethacrynic acid, and *trans*-2-hexenal), was studied (*chapter 4*).

Finally, the cellular metabolism and efflux of an important endogenous α,β-unsaturated ketone, prostaglandin A_2, was investigated in the IGR-39 human melanoma cell line. Effects of this compound on the glutathione-related processes in this cell line were studied as well as interactions with purified enzymes (*chapter 5*).

Chapter 2

Interactions of α,ß-unsaturated aldehydes and ketones with human glutathione S-transferase P1-1

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Abstract

In the present study the irreversible inhibition of human glutathione S-transferase P1-1 (GSTP1-1) by α, ß-unsaturated aldehydes and ketones was studied.

When GSTP1-1 was incubated with a 50-fold molar excess of the aldehydes acrolein and 4-hydroxy-2-nonenal and the ketones curcumin and ethacrynic acid at 22°C, all of them inactivated GSTP1-1. The remaining activity after 4 hours of incubation in all cases was lower than 10%. The aldehydes crotonaldehyde, cinnamaldehyde and *trans*-2-hexenal were found to inhibit GSTP1-1 only at a 5000-fold molar excess and even then *e.g.* for cinnamaldehyde a higher remaining activity of 17% was observed.

The same inhibition experiments were conducted with 3 mutants of GSTP1-1: the C47S mutant, the C101S mutant and the double mutant C47S/C101S. Remaining activity for C47S varied between ± 40% for crotonaldehyde, cinnamaldehyde, curcumin and *trans*-2-hexenal and ± 80% for acrolein, ethacrynic acid and HNE. For C101S it varied between 0% and 9% and for the double mutant C47S/C101S, activity after 4 hours of incubation was variable. Again it varied between ± 40% for crotonaldehyde, cinnamaldehyde, curcumin and *trans*-2-hexenal and ± 80% for acrolein, ethacrynic acid and HNE.

Ethacrynic acid is known to react almost exclusively with cysteine 47. When [^{14}C] ethacrynic acid was incubated with the GSTP1-1, modified by the α, ß-unsaturated carbonyl compounds, no [^{14}C] ethacrynic acid was incorporated in the enzyme, indicating that in all cases this cysteine residue was one of the major targets.

Since Michael addition with these reagents is known to be reversible, the results of incubation of the inactivated enzymes with an excess of glutathione were determined. For all compounds, a restoration of the catalytic activity was observed.

The results indicate that α, ß-unsaturated carbonyl derivatives inhibit GSTP1-1 irreversibly mainly by binding to cysteine residues of GSTP1-1, especially Cys-47. This means that some of these compounds (*e.g.* curcumin) might modify GST activity *in vivo* when glutathione concentrations are low by covalent binding to the enzyme.

Introduction

α,ß-Unsaturated aldehydes and ketones are a class of compounds to which man is ubiquitously exposed. They occur naturally in the environment, but are also products of combustion of organic materials and hence compounds like acrolein (ACR) and crotonaldehyde (CRA) are found in tobacco smoke and automobile exhaust. Furthermore they have been identified in food *e.g.* cinnamaldehyde (CA) and curcumin (CUR). The α, ß-unsaturated carbonyl moiety is also found in drugs like *e.g.* the diuretic drug ethacrynic acid (EA).

Moreover, they are formed as products of endogenous lipid peroxidation (*e.g.* 4-hydroxy-2-nonenal (HNE), *trans*-2-hexenal (HEX)) and during metabolism of exogenous compounds such as certain drugs, *e.g.* acrolein formation from cyclophosphamide (Witz, 1989; Feron *et al.*, 1991; Eder *et al.*, 1993; Berhane *et al.*, 1994).

Interactions of these α,ß-unsaturated aldehydes and ketones with cellular macromolecules (Witz, 1989; Cooper *et al.*, 1992) may lead to cytotoxicity and genotoxicity [Feron *et al.*, 1991; Eder *et al.*, 1993; Brambilla *et al.*, 1986; Wilson *et al.*, 1991; Yuan *et al.*, 1992; Dittberner *et al.*, 1995). For example, acrolein (Eder *et al.*, 1993; Wilson *et al.*, 1991), crotonaldehyde and *trans*-2-hexenal (Eder *et al.*, 1993) form DNA-adducts. In contrast cinnamaldehyde has been reported anti-mutagenic in low doses (Neudecker, 1992) and curcumin has been found to have antioxidant and anticarcinogenic properties (Osawa *et al.*, 1995; Stoner and Mukhtar, 1995).

These electrophilic compounds are able to undergo non-enzymatic as well as enzymatically catalysed conjugation with glutathione (GSH) (Berhane *et al.*, 1994; Esterbauer *et al.*, 1975; Van Bladeren, 1988; Chien *et al.*, 1994). This conjugation reaction in general is catalysed by the glutathione-S-transferases (GST). In mammals four cytosolic classes of GSTs are known and one microsomal. The four cytosolic classes are named alpha, mu, pi and theta (Mannervik, 1985; Meyer *et al.*, 1991; Armstrong, 1991). The expression of the different isoenzymes is tissue-specific (Mannervik, 1985) and especially the overexpression of GST of the pi-class in certain tumours is a phenomenon to which considerable attention is directed (Shea *et al.*, 1988; Sato, 1989). This overexpression seems to be related, among other mechanisms, to drug resistance against alkylating anticancer drugs (Waxman, 1990). *In vitro* studies showed that by transfecting the GST π gene into breast cancer cells, the cytotoxicity of some toxins was decreased (Moscow *et al.*, 1989) and by inhibiting GST π in cells, they became more sensitive to some alkylating anticancer drugs (Tew *et al.*, 1988; Hansson *et al.*, 1991) showing its role in drug resistance.

Ethacrynic acid was shown to inhibit GSTP1-1, both by reversible inhibition (Ploemen *et al.*, 1990; Ciaccio *et al.*, 1991; Ploemen *et al.*, 1993b; Awasthi *et al.*, 1993) and inactivation by binding to the cysteine 47 residue (Caccuri *et al.*, 1992; Ploemen *et al.*, 1993a; Ploemen *et al.*, 1994b). Under normal physiological conditions covalent binding of ethacrynic acid to GSTP1-1 will be reversed by glutathione, but when glutathione is depleted the covalent binding might be an important mechanism of inhibition (Ploemen *et al.*, 1994b). It was recently shown in our lab that by incubating human melanoma cells with ethacrynic acid or with curcumin, GST activity was inhibited both reversibly and irreversibly (Van Iersel *et al.*, 1996).

The present study was designed to study a series of α, ß-unsaturated carbonyl compounds in detail, with emphasis on the mechanism of inactivation (using mutant GST). The retro Michael reaction was studied to investigate if a reversible covalent interaction occurred.

Materials and methods

Materials

Acrolein, *trans*-cinnamaldehyde and *trans*-crotonaldehyde and CDNB were purchased from Aldrich Chemie, Bornem, Belgium, curcumin and ethacrynic acid were from Sigma (St Louis, MO, USA), 4-hydroxy-2-nonenal was a generous gift from A. Bast, Free University, Amsterdam and *trans*-2-hexenal was purchased from Acros Chimica, Geel, Belgium. [^{14}C]Ethacrynic acid ([2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy]acetic acid) with a specific activity of 15mCi/mmol was from Amersham, Buckinghamshire, U.K. GSTP1-1 was purified as previously described (Mannervik and Guthenberg, 1981). The three mutants of GSTP1-1, C47S, C101S and C47S/C101S were obtained by site-directed mutagenesis as previously described (Ricci *et al.*, 1995). Acetonitrile was from Biosolve Ltd., Barneveld, The Netherlands and trifluoro acetic acid (TFA) was from J.T. Baker (Deventer, the Netherlands).

To prepare GSTP1-1 modified with [^{14}C] ethacrynic acid, a 75 µl incubation mixture, containing 1.5 nmol GSTP1-1 and 6.67 nmol [^{14}C]ethacrynic acid in 0.2 M potassium phosphate buffer pH 7.4, supplemented with 0.2 mM EDTA, was incubated for two hours at room temperature. Purity (>95%) was determined by RP-HPLC on a Vydac TP5 (200*3mm) column (Ploemen *et al.*, 1994b).

Inhibition studies

Incubations were carried out to determine time-dependent covalent inhibition. 250 µl incubation mixtures contained 0.2 M potassium phosphate buffer pH 7.4, supplemented with 0.2 mM EDTA, 0.5 µM enzyme and 25 µM carbonyl compound were incubated at 22°C. Ethanol was used as solvent (constant at 0.5% v/v). As is shown in Figure 2.1 crotonaldehyde, cinnamaldehyde, citral and *trans*-2-hexenal did not inhibit GSTP1-1 at this concentration. These compounds were thus tested at a concentration of 2.5 mM. The other compounds (*viz.* acrolein, curcumin, ethacrynic acid and HNE) were tested at a concentration of 25 µM.

At various time intervals during 4 hours of incubation GST-activity was measured according to Habig *et al.* (1974) adapted for a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA, USA). A 10 µl sample was added to a well containing 0.2 M potassium phosphate buffer pH 6.5, supplemented with 0.2 mM EDTA, 1 mM GSH (final concentration) and water in a volume of 230 µl. After 2 minutes of incubation at 25 °C, 10 µl CDNB was added to a final concentration of 1 mM and the formation of the CDNB-glutathione conjugate was measured at 340 nm. Ethanol was used as solvent (constant at 4% v/v).

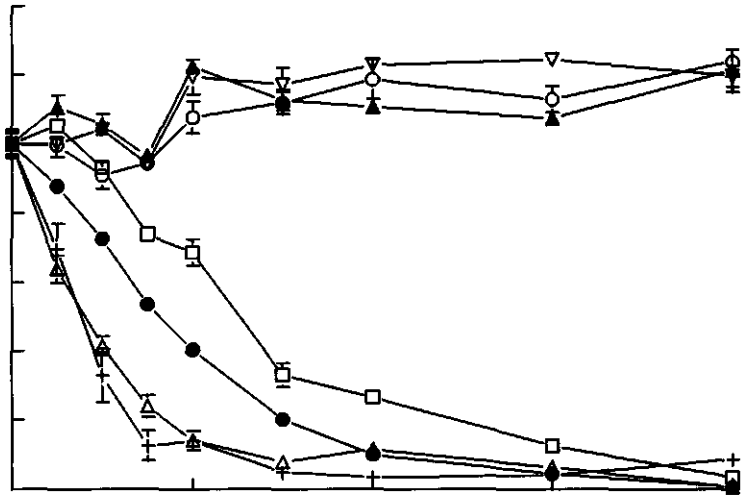

Figure 2.1 Percentage remaining activity of GSTP1-1 during incubation with various α,β-unsaturated carbonyl compounds. GSTP1-1 was incubated for four hours with 25 μM of the various α,β-unsaturated carbonyl compounds. GSTP1-1 activity was determined during 4 hours. Incubations were performed in triplicate. Values are presented as % of control incubations ± SE. Symbols used: +, ethacrynic acid; ●, acrolein; △, curcumin; □, 4-hydroxy-2-nonenal; ▽, crotonaldehyde; ○, cinnamaldehyde; ▲, *trans*-2-hexenal.

Because inactivation of GSTP1-1 by EA was reversed by incubating the modified enzyme with an excess of glutathione (retro Michael cleavage) (Ploemen *et al.*, 1994b), the reversibility of the binding of the α, ß unsaturated carbonyl compounds to GSTP1-1 was studied. Therefore the indicated mixtures were incubated for 4 hours at 22 °C, GST activity was measured (see above) and the mixtures were centrifuged in microcon 10 microconcentrators (Amicon, Beverly, MA, USA). After the mixtures were adjusted to a volume of 225 μl with 0.2 M potassium phosphate buffer pH 7.4, supplemented with 0.2 mM EDTA, GSH was added in a final concentration of 2.5 mM and the mixtures were incubated at 22 °C. The catalytic activity of GSTP1-1 towards CDNB was monitored by transferring 10 μl into wells and measuring DNPSG formation at 340 nm.

Table 2.1 Inactivation rates of GSTP1-1 after 90 minutes exposure to the compounds, expressed as t ½.

Compound	Concentration	t ½ (min)
Acrolein (ACR)	25 μM	38
4-Hydroxy-2-nonenal (HNE)	25 μM	53
Curcumin (CUR)	25 μM	24
Ethacrynic acid (EA)	25 μM	20
Crotonaldehyde (CRA)	2.5 mM	21
Cinnamaldehyde (CA)	2.5 mM	40
trans-2-hexenal (HEX)	2.5 mM	24

GSTP1-1 was incubated with the test compounds for 4 hours. For experimental details see Materials and methods. The inactivation rates were determined and expressed as t ½ in minutes.

Covalent binding

Covalent binding of the aldehydes to GSTP1-1 was analysed by the following experiments: 75 μl reaction mixtures containing 10 μM GSTP1-1 in 0.1 M potassium phosphate buffer pH 7.4, supplemented with 0.1 mM EDTA and 0.5 mM acrolein, curcumin, 4-hydroxy-2-nonenal, or 50 mM crotonaldehyde or 0.25 mM *trans*-2-hexenal or 0.05 mM cinnamaldehyde, were incubated in duplicate for 90 minutes at room temperature, where after the enzyme was incubated with 20 μM [^{14}C] ethacrynic acid for 120 minutes at room temperature. Separation of the GSTP1-1-[^{14}C] ethacrynic acid-conjugate was performed on a Vydac TP5 column (200*3 mm), eluted on a flow rate of 0.6 ml/min, with 0.1% TFA in deionized water (solvent A) and 0.1% TFA in acetonitrile (solvent B), with a linear gradient of 34-50% B in 15 minutes, followed by a linear gradient of 50-60% B in 10 minutes and finally 5 minutes isocratic at 60% B. To identify the enzyme peak UV-detection at 214 nm was used (t=20) and simultaneously radioactivity was measured using an on line radiochemical detector (Packard Flo-One Model Radioactive flow detector with Flo-Scint™ as scintillation cocktail).

Small differences in retention time existed between different runs, which could be attributed to the absence of thermo-equilibration. As a control, samples were spiked with [^{14}C]ethacrynic acid to further demonstrate the identity of the peaks.

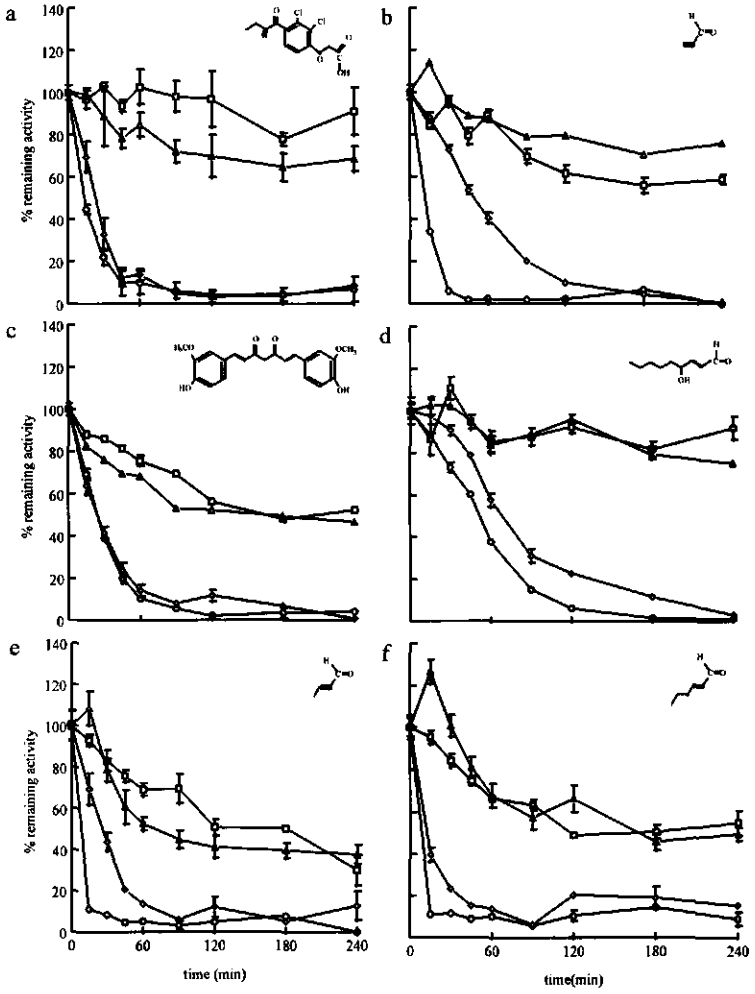

Figure 2.2 Percentage remaining GSTP1-1 activity after incubation with 25 μM ethacrynic acid (a), acrolein (b), curcumin © or 4-hydroxy-2-nonenal (d) and 2.5 mM crotonaldehyde (e), or *trans*-2 hexenal (f). Cinnamaldehyde is not shown. The activity of GSTP1-1(◊), the C47S mutant (△), the C101S mutant (○) or the double mutant C47S/C101S (□) was followed during 4 hours of incubation with the agents. All points are mean values ± SE calculated from three determinations.

Results

Inhibition studies

Time dependent inhibition of GSTP1-1 by the α,ß-unsaturated carbonyl compounds was studied first. It appeared that acrolein, curcumin, ethacrynic acid and 4-hydroxy-2-nonenal inhibited GSTP1-1 in a time dependent way at concentrations as low as 25 μM in the time frame used (Figure 2.1). In contrast, crotonaldehyde, cinnamaldehyde and *trans*-2-hexenal only inhibited GSTP1-1 at concentrations of 2.5 mM. The half-life rates at the concentrations used are shown in table 2.1. The remaining activity after 4 hours of incubation varied between 1% for acrolein and curcumin and 17% for cinnamaldehyde.

When the C47S mutant was incubated with the aldehydes and ketones in the same concentration range, the rate of inactivation was decreased considerably and the activity that remained was between 94% and 45% of the blank (Figure 2.2). However, the C101S mutant did not or only slightly alter the inhibition pattern in comparison with the native enzyme. The remaining activity ranged from 0% to 9%. When cysteine 47 and cysteine 101 were both mutated, the pattern of inhibition resembled the C47S mutant, and the rate of inactivation was decreased markedly.

In order to check if a retro Michael cleavage occurred *i.e.* if the α, ß-unsaturated carbonyl compounds react with a cysteine residue of GSTP1-1, the inactivated GSTP1-1 was incubated

Table 2.2 Restoration of the activity of the inactivated GSTP1-1 by incubation with an excess of glutathione.

compound	% activity at 140 hrs
EA	99.91 ± 0.83
ACR	21.26 ± 9.52
CRA	103.9 ± 9.14
CA	66.47 ± 11.1
HEX	47.45 ± 6.61
CUR	49.15 ± 4.23
HNE	59.47 ± 7.8

Inactivated GSTP1-1 was incubated with 2.5 mM glutathione. For experimental details see Materials and methods. The activity of the inactivated GSTP1-1 after 140 hours of incubation with GSH is expressed as % control incubations (± SE). ACR, acrolein; CRA, crotonaldehyde; CA, cinnamaldehyde; CUR, curcumin; EA, ethacrynic acid; HEX trans-2-hexenal; HNE, 4-hydroxy-2-nonenal.

with 2.5 mM GSH. The catalytic activity of GSTP1-1 towards CDNB was restored (Table 2.2). However the extent of restoration of catalytic activity was quite different for the various compounds.

Covalent binding

The assumption that α,β-unsaturated carbonyl derivatives bind covalently to Cys-47 in the active site of GSTP1-1, was tested by a 'back-titration'-experiment, that is GSTP1-1 was incubated first with the carbonyl compounds, where after [^{14}C]ethacrynic acid was used to determine the amount of unmodified Cys-47.

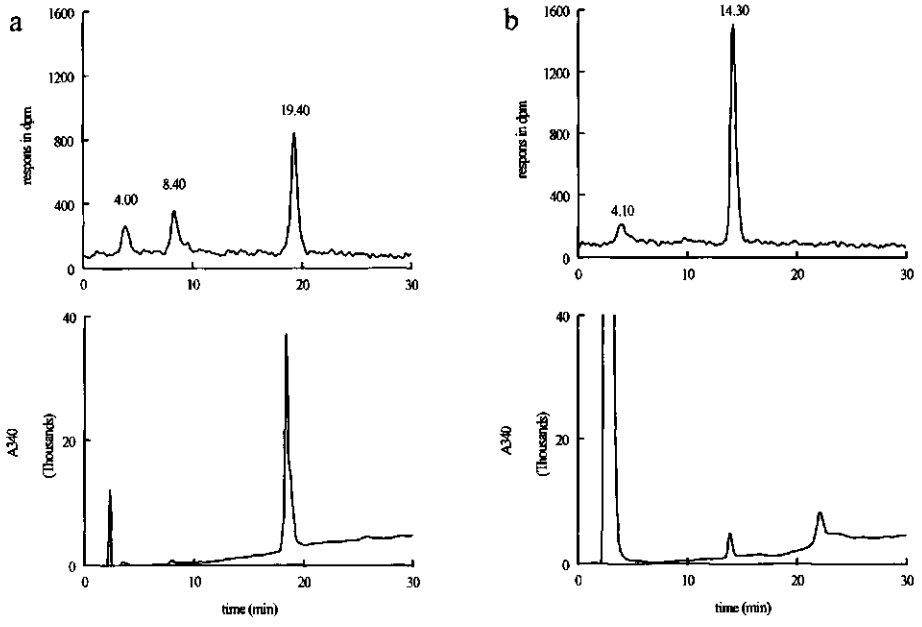

Figure 2.3 Chromatograms of the incubations to determine covalent binding of the test compounds to GSTP1-1. Free [^{14}C]ethacrynic acid eluted at t=14.3 min and [^{14}C]ethacrynic acid modified GSTP1-1 at t=19.4 min. Figure a shows chromatograms of control incubations with virtually most [^{14}C] ethacrynic acid bound to GSTP1-1. Figure b is a typical chromatogram of the incubations with the α,β-unsaturated carbonyl derivatives; virtual no [^{14}C]ethacrynic acid is bound to GSTP1-1. Samples were spiked to further demonstrate the identity of the peaks.

By separating free [^{14}C]ethacrynic acid from enzyme-bound [^{14}C]ethacrynic acid by HPLC, covalent binding of aldehydes to Cys-47 can be determined indirectly. In Figure 2.3a chromatograms from control incubations are depicted, which indicate that the total amount of [^{14}C] ethacrynic acid is bound to GSTP1-1 (t=19.4) as virtually no free [^{14}C] ethacrynic acid (t=14.3) is shown in the chromatograms. Figure 2.3b shows typical chromatographic patterns of the incubations with the carbonyl compounds. It appears that virtually no [^{14}C] ethacrynic acid is bound to GSTP1-1 after incubation of the enzyme with any of the aldehydes and ketones. This indicates that the α, ß unsaturated aldehydes and ketones that are used in these experiments indeed bind to Cys-47 or that at least this amino acid is shielded, which prevents [^{14}C] ethacrynic acid from binding.

Discussion

In the present study we showed that especially acrolein, ethacrynic acid, 4-hydroxy-2-nonenal and curcumin are good inhibitors of GSTP1-1, whereas crotonaldehyde, cinnamaldehyde and *trans*-2-hexenal inhibit GSTP1-1 only at concentrations of 2.5 mM. Unambiguously it was shown that Cys-47 is the principal target for modification by α, ß-unsaturated aldehydes and ketones. Furthermore we demonstrated that the major part of inactivation of GSTP1-1 by these compounds could be reversed by an excess of GSH.

The phenomenon that Cys-47 is the target for inhibition, is well known. It has been demonstrated that Cys-47 is a highly reactive cysteine residue in the proximity of the active site of the GST π class, which can regulate activity by its oxidation status and by SH/SS exchange reactions (Schäffer *et al.*, 1988; Ricci *et al.*, 1991; Nishihira *et al.*, 1991). Covalent modification of Cys-47 results in a loss of GSTP1-1 activity (Caccuri *et al.*, 1992; Ploemen *et al.*, 1993a; Tamai *et al.*, 1990; LoBello *et al.*, 1990) and modification of the cysteine by site directed mutagenesis resulted in a reduced affinity for glutathione (Ricci *et al.*, 1995; LoBello *et al.*, 1995), and thus a lowered activity (Ricci *et al.*, 1995; Kong *et al.*, 1991). As previously described (Ricci *et al.*, 1995) Cys-101 together with Cys-47, may be a factor in stabilizing the G-site structure, thus influencing the activity of GSTP1-1 as well. This study shows again that Cys-47 indeed is the major target for α,β-unsaturated aldehydes and ketones. Furthermore it demonstrates that Cys-101 of GSTP1-1 is only slightly modified by α, ß-unsaturated carbonyl compounds.

However, as expected there might be some lower reactivity sites in the enzyme that can be modified by the compounds if the major target, Cys-47 is absent. Even when both cysteines are mutated some of the compounds still inhibit GSTP1-1 up to 60%. The effect of crotonaldehyde, cinnamaldehyde and *trans*-2-hexenal can be explained by the high concentrations used, but as curcumin is used in a low concentration this effect is quite

remarkable. Apparently curcumin most potently binds to other amino acids as well in low concentrations. Oetari *et al.* (1996) showed that curcumin was stabilized by adding thiols or non thiol proteins indicating that the compound can bind both.

At 25 μM only some of the compounds inactivated GSTP1-1. Esterbauer *et al.* (1975) distinguished three groups of carbonyls, based on reactions with GSH at pH 7.4. The most reactive with stable conjugates, like acrolein, are placed in group 1, the second group contains the 4-hydroxyalkenals with slower reactions but stable conjugates and in the last group carbonyl compounds that react slowly and/or form labile conjugates are situated, including crotonaldehyde and hexenal. The same groups can be identified for the inactivation of GSTP1-1. Indeed, chemical reactivity is the driving force of inactivation of GSTP1-1. For the restoration of activity, the parallel with the classification is less easy to observe. The carbonyls with a ketone moiety (ethacrynic acid and curcumin) seem to belong to the first group. From ethacrynic acid it is known that the glutathione conjugate is quickly formed non-enzymatically, but that the enzyme catalysed reaction is more important *in vivo* (Ploemen *et al.*, 1993b) and that the stability of the conjugate is dependent on pH and thiol concentration (Ploemen *et al.*, 1994b). Curcumin is known to undergo a Michael reaction with glutathione (Mathews and Rao, 1991), but the stability of the conjugate is at present unknown.

Although recently published data show that α, ß-unsaturated carbonyl compounds inhibit other classes of GST too (Chien *et al.*, 1994), the inhibition of GSTP1-1 is the most relevant for *in vivo* situations. In this context it might be interesting to further investigate if these or related compounds can be used in the development of inhibitors for *in vivo* use. Such inhibitors might be useful in relation to drug resistance, one of the major problems in treating cancer (Hayes and Wolf, 1990). It results from a variety of factors, one of which is the abundance of especially GST of the π-class in cancer cells (Shea *et al.*, 1988; Sato, 1989; Waxman, 1990; Hayes and Wolf, 1990). Inhibition of GSTP1-1 might thus be useful in increasing efficacy of alkylating anti-cancer drugs. Recently we showed that in human melanoma cells GST activity was inhibited by incubating them with α,β-unsaturated carbonyl derivatives. Ethacrynic acid and curcumin were shown to inhibit GSTP1-1 reversibly as well as irreversibly after one hour incubation with these cells (Van Iersel *et al.*, 1996).

It was shown that ethacrynic acid inactivated GSTP1-1 but that a retro Michael cleavage occurred by prolonged incubation with an excess of glutathione (Ploemen *et al.*, 1994b). Indeed this study shows that the α, ß-unsaturated carbonyl compounds bound to GSTP1-1 also undergo retro Michael cleavage in the presence of an excess of GSH, although activity is not completely restored. This phenomenon is in agreement with the fact that several of the carbonyl compounds seem to react with other amino acid residues than Cys-47. In addition it has recently been shown that under certain circumstances EA binds GSTP1-1 in a nonproductive mode to the H-site of the enzyme in the absence of GSH *i.e.* showing no covalent modification of the Cys-47 residue. They suggest that the order of addition of the reactants and the reaction conditions

might be important in kinetic analysis (Oakley *et al.*, 1997).

It is tempting to speculate on the usefulness of the observed universal site for modification. The possibility exists that GSTP1-1 acts as a scavenger for reactive intermediates, at least when GSH concentrations are low. In that case it is imaginable that GSTP1-1 is part of a general defense system.

The modification of GSTP1-1 might have another principal function *i.e.* in regulation of cell growth. This latter possibility is suggested because it was shown that inhibitors of GST, *i.e.* ethacrynic acid and bromosulfophthalein had antiproliferative effects in cells (McCaughan *et al.*, 1994; Sato *et al.*, 1990), which seem to be reversible. Furthermore incubation of human glioma cells with the GST inhibitors ascorbyl stearate, ascorbyl palmitate and interferon reduced cell proliferation (Naidu *et al.*, 1993). And curcumin, a reversible and irreversible inhibitor of GSTP1-1 (Van Iersel *et al.*, 1996) is also known to inhibit cell proliferation (Huang et al., 1992). The role of reversible reactions with Cys-47 also warrants further study with regard to this phenomenon.

Acknowledgements

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Chapter 3

GSTP1-1 stereospecifically catalyses glutathione conjugation of ethacrynic acid

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Abstract

Glutathione conjugation of α,β-unsaturated carbonyl derivatives may lead to the formation of two possible diastereoisomers and catalysis by glutathione S-transferases (GST) often results in preferential formation of one of the two diastereoisomers. Here we present the stereochemistry of the GST-catalysed glutathione conjugation of ethacrynic acid (EA), a known diuretic drug and GST inhibitor.

Using ^{1}H NMR the presence of the two diastereoisomers of the ethacrynic acid glutathione conjugate (EASG) as well as of ethacrynic acid could be demonstrated and quantified. Chemically prepared EASG exists as a set of diastereoisomers with a ratio of 48:52. Catalysis of the reaction by GSTP1-1 showed that this isoenzyme stereospecifically forms one of the diastereoisomers. The GSTP1-1 mutant C47S and GSTA1-1 preferentially form the same diastereoisomer of EASG. GSTA2-2 does not show any stereoselectivity.

When human melanoma cells, expressing GSTP1-1, were exposed to ethacrynic acid, diastereoisomer A was the principal conjugate present in the medium after two hours.

Together our data illustrate that chemical and enzyme catalysed equilibria for glutathione conjugation of ethacrynic acid are strongly in favour of product formation. Moreover, GSTP1-1 stereospecifically catalyses the glutathione conjugation of ethacrynic acid and GSTA1-1 is stereoselective for the same diastereoisomer. The stereoselective conjugation plays a role in cellular systems.

Introduction

The diuretic drug ethacrynic acid, an α,β-unsaturated ketone and a Michael acceptor, is known to be conjugated to glutathione (GSH), chemically as well as catalysed by glutathione S-transferases (GST) (Ahokas *et al.*, 1985). Both the parent compound ethacrynic acid and the ethacrynic acid glutathione conjugate (EASG) are potent reversible inhibitors of GSTs with I_{50}-values in the range of <0.1-11μM and ethacrynic acid inhibits GST of the pi-class by covalent binding (Ploemen *et al.*, 1990). Because of these inhibiting properties ethacrynic acid has been studied as an agent to overcome multidrug resistance against alkylating drugs, since GST may play a role in that phenomenon (Tew *et al.*, 1988). Additionally, it has recently been shown that the glutathione conjugate of EA is a very good substrate and an inhibitor for the multidrug resistance associated protein (MRP) or GS-X pump (Zaman *et al.*, 1996). This pump plays a role in drug resistance as well. MRP has first been detected in a multidrug resistant cell line (Cole *et al.*, 1992) and it transports glutathione conjugates of both endogenous and exogenous molecules (Jedlitschky *et al.*, 1994; Müller *et al.*, 1994).

In most cases glutathione conjugation constitutes a detoxification reaction. However, it may

also contribute to bioactivation pathways. Thus, glutathione conjugates can be more reactive than the parent compound or they can be further metabolized to new reactive species. For ethacrynic acid, it has been implicated that metabolites, *viz.* the cysteine conjugate, may be the active diuretic agent (Koechel and Cafruny, 1973; O'Dwyer *et al.*, 1995). For some classes of compounds, glutathione conjugation is reversible and may lead to the release of the reactive electrophile at other sites, implying that the conjugate is only a storage form of the reactive compound (Bruggeman *et al.*, 1986; Van Bladeren, 1988; Monks *et al.*, 1990; Baillie and Slatter, 1991). This last phenomenon is observed when Michael-acceptor substrates are conjugated with GSH and a retro-Michael reaction is favoured under certain circumstances. Ethacrynic acid is a Michael type substrate and a retro-Michael reaction for its glutathione conjugate has been reported previously (Ploemen *et al.*, 1994b).

Glutathione conjugation of α,β-unsaturated carbonyl compounds, such as ethacrynic acid, can lead to the formation of two possible diastereoisomers. Catalysis by glutathione S-transferases often results in preferential formation of one of the two diastereomers (Boehlert and Armstrong, 1984; Dohn *et al.*, 1985; Kubal *et al.*, 1995, Bogaards *et al.*, 1997). For example the GST isoenzyme M2-2 was stereoselective for the formation of one of the diastereomers of 4-phenyl-3-buten-2-one (PBO)(Kubo and Armstrong, 1989).

In view of the multiple effects of ethacrynic acid and especially of its EASG conjugate, taking into account the possible different biological effects of the diastereoisomers, in this study we investigated the stereoselectivity of the glutathione S-transferase catalysed conjugation of ethacrynic acid, using ^{1}H-NMR spectroscopy, both using purified enzymes as well as in human melanoma cells.

Materials and methods

Materials

Ethacrynic acid was obtained from Sigma Chemical Co. (St.Louis, MO, USA). Glutathione was from Boehringer (Mannheim, Germany). Deuterium oxide was purchased from Isotec Inc. (Miamisburg, OH, USA). The racemic glutathione conjugate of ethacrynic acid (EASG) was synthesized according to Ploemen *et al.* (1990). GSTP1-1 and the glutathione S-transferases of the α-class were purified as previously described (Ålin *et al.*, 1985). The C47S mutant was a generous gift from Dr M. LoBello. Since EA is not a substrate for the human mu class isoenzymes, these were not investigated (Ploemen *et al.*, 1993b).

Characterization of EASG diastereoisomers

The two diastereoisomeric glutathione conjugates were characterized by ^{1}H NMR spectroscopy using a Bruker AMX 500 spectrometer. The ^{1}H NMR resonances of both

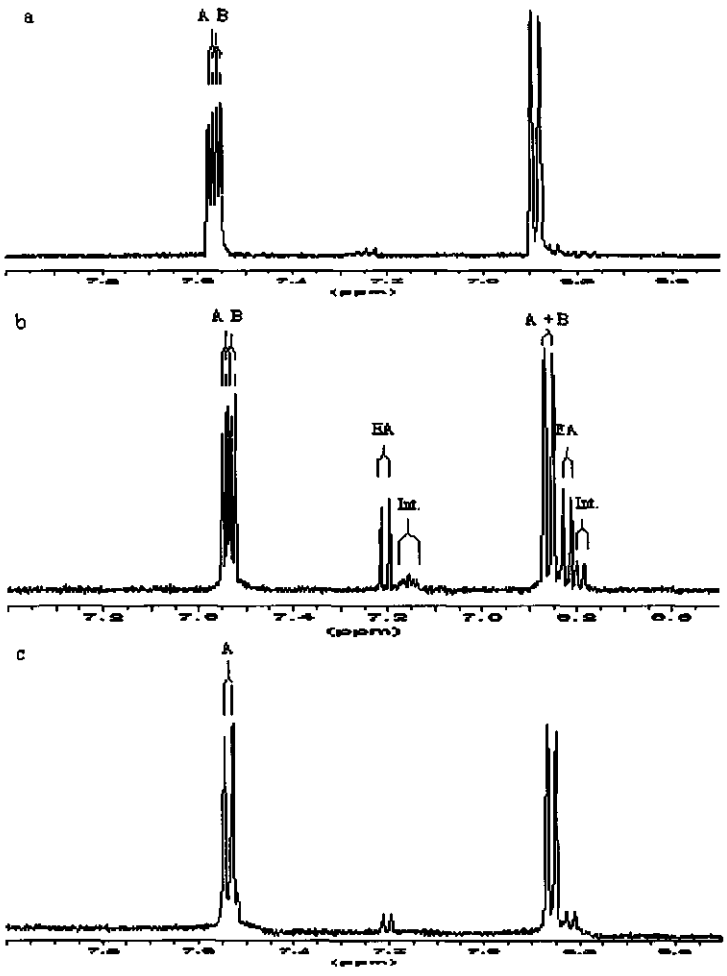

Figure 3.1a Typical part of a ^{1}H NMR spectrum of the ethacrynic acid glutathione conjugate (EASG). EASG was synthesized according to Ploemen *et al.* (1990) and dissolved in D_2O. The distinction between the two diastereoisomers can be seen from the pair of doublet resonances arising from the aromatic moiety around 7.55 ppm; **3.1b** Part of a spectrum of the chemical conjugation reaction of EA and GSH; **3.1c** Part of a spectrum of the conjugation reaction of EA and GSH catalysed by 47.6 μM GSTP1-1. Diastereoisomers A and B are indicated as A and B, respectively; the intermediate conjugate form is marked as Int.

diastereoisomers overlap in the aliphatic region and are as follows (D_2O, p^{2}H 6.1, 25°C); Isomer A/B: 0.80 (tr, 3H, ^3J = 7 Hz, ethacrynic acid CH_3), 1.53 (sex, 1H, ^2J = 14 Hz, ^3J = 7 Hz, ethacrynic acid CH_2), 1.68 (sex, 1H, ^2J = 14 Hz, ^3J = 7 Hz) ethacrynic acid CH_2), 2.04 (q, 2H, ^3J = 7 Hz, γ-Glu β-CH_2), 2.40 (tr, 2H, ^3J = 7 Hz, γ-Glu γ-CH_2), 2.74-2.86 (m, 2H, Cys β-CH_2), 2.74-2.96 (m, 2H, ethacrynic acid CH_2), 3.67 (tr, 1H, ^3J = 7 Hz, γ-Glu α-CH), 3.69 (s, 2H, Gly α-CH_2), 4.43 (q, 1H, ^3J = 6 Hz, ^3J = 7 Hz Cys α-CH), 4.67 (s, 2H, ethacrynic acid CH_2). In the aromatic region the ^{1}H NMR resonances of the two diastereoisomers are clearly separated and as follows; Isomer A: 6.89 (d, 1H, ^3J = 9 Hz, ethacrynic acid, aromatic CH), 7.58 (d, 1H, ^3J = 9 Hz, ethacrynic acid, aromatic CH) ; Isomer B: 6.89 (d, 1H, ^3J = 9 Hz, ethacrynic acid, aromatic CH), 7.56 (d, 1H, ^3J = 9 Hz, ethacrynic acid, aromatic CH) (see also Figure 3.1a). The HDO resonance of D_2O was put at 4.70 ppm.

Nonenzymatic and enzymatic conjugation

Ethacrynic acid and glutathione were mixed in equimolar quantities (5 mM) in 0.2 M potassium phosphate in deuterium oxide p^{2}H 6.1 (or pH 6.5 in H_2O). The loss of ethacrynic acid and the formation of the two diastereoisomeric glutathione conjugates were monitored by collecting a series of ^{1}H NMR spectra at regular time intervals and measuring the changes in integrals of selected resonances.

The enzymatic reactions of glutathione with ethacrynic acid were carried out at 25 °C in 0.2 M potassium phosphate in deuterium oxide p^{2}H 6.1. Equimolar amounts of compounds GSH and EA (5 mM) and various concentrations of GST were mixed and formation of the two diastereoisomers was followed by NMR analysis. GSH was always added prior to ethacrynic acid. Incubations were conducted with 6.5, 22.6 and 47.6 μM GSTP1-1; 11.2 and 29.8 μM C47S mutant of GSTP1-1. For the alpha class GST's, 24 and 120 μM GSTA1-1 and 28.8 and 144 μM GSTA2-2 were used. For the investigation of the rate constant, additional incubations with 2.5 mM EA and 5 mM GSH were performed.

Deconjugation reactions of EASG

Incubations of 5 mM EASG (mixture of diastereoisomers) in 0.2 M potassium phosphate in ^2H_2O p^{2}H 6.1 without enzyme and with 22.6 μM GSTP1-1 were analysed by ^{1}H NMR, monitoring the formation of ethacrynic acid and the ratio of the two diastereoisomers.

Furthermore one of the diastereoisomers was synthesised enzymatically by incubating equimolar quantities of ethacrynic acid and glutathione in ^2H_2O with 47.6 μM GSTP1-1 for 40 minutes at room temperature. The enzyme was precipitated with 18% trichloro acetic acid (TCA), centrifuged and the supernatant was freeze-dried, where after the conjugate was washed several times using subsequent cycles of lyophilization and dissolving in ^2H_2O in order to remove the TCA. The deconjugation of this diastereoisomer was analysed at p^{2}H 6.1 during several hours by ^{1}H NMR spectroscopy in 0.2 M potassium phosphate in ^2H_2O both non-

enzymatically and in the presence of 22.6 μM GSTP1-1.

Cell studies

IGR-39 human melanoma cancer cells, containing a high amount of GSTP1-1 (Van Iersel *et al.*, 1996), were provided by the Dr Daniel den Hoed kliniek (Rotterdam, Netherlands). IGR-39 melanoma cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum, 50 mg/l gentamicin, at 37°C in a humid atmosphere containing 5% CO_2. For the experiments 50 x 10^4 cells/ml were plated onto a 75 cm^2 cell culture flask (Costar, Cambridge, MA) and cultured overnight. Cells were exposed for 2 hours to 50 μM ethacrynic acid in Hanks balanced salt solution (HBSS without phenol red from Gibco), supplemented with $NaHCO_3$ (final concentration of 0.35 gr/l). After two hours HBSS was removed and immediately frozen to -80°C. Cells were trypsinized, resuspended in 10 ml of PBS and immediately frozen to -80°C. Samples were freeze dried, dissolved in 0.5 ml ^2H_2O, p^{2}H 6.1 and analysed by ^{1}H NMR.

Results

^{1}H NMR analysis of the diastereoisomeric ethacrynic acid-glutathione conjugates

The diastereoisomeric ethacrynic acid-glutathione conjugates were characterized by ^{1}H NMR analysis. The most relevant section of a typical NMR spectrum of the EASG diastereoisomeric mixture is shown in Figure 3.1a. Chemically prepared EASG exists as a set of diastereoisomers. Figure 3.2 presents the structures of ethacrynic acid, an intermediate glutathione conjugate and the relevant parts of the two diastereoisomers of EASG. The addition of the sulfur of glutathione to the β-carbon (C10), followed by protonation of the negatively charged Cα centre (C9) thus formed, results in generation of a chiral α-carbon C9. Because the proton most likely originates from the solvent or from a pool of protons that readily exchanges with the solvent (Dohn *et al.*, 1985; Kubal *et al.*, 1995), protonation can occur on either site of the carbon. As we do not know the absolute stereochemistry of A or B, no designation is given in Figure 3.2.

In the ^{1}H NMR spectrum two pairs of doublet resonances arising from the protons in the aromatic moiety of the two diastereoisomers can be readily observed around 7.5 - 7.6 ppm. Due to the small but significant difference in chemical shift of these proton resonances in the two diastereoisomers, these resonances can be used to detect and quantify the two diastereoisomers. From the results presented in Figure 3.1a it can be derived that, upon chemical synthesis of EASG, equal amounts of A and B are formed, since the ratio of the peak areas is 48:52.

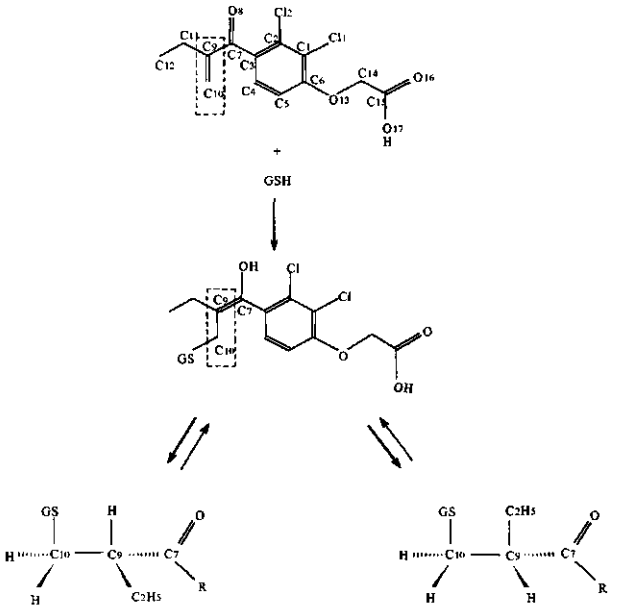

Figure 3.2 Schematic representation of EA (above), the intermediate EASG and the important parts of the two diastereoisomers of EASG. The numbering in the figure is taken from Lamotte *et al.* (1978).

Nonenzymatic conjugation

First, as a control for the enzyme catalysed conjugation, the chemical reaction between ethacrynic acid and glutathione was followed in time by ^{1}H NMR analysis. From spectra obtained at increasing time intervals upon addition of EA to a 5 mM GSH solution the formation of the glutathione conjugates could be followed in time. A spectrum of the incubation at one hour is shown in Figure 3.1b. After one hour, product A and product B were formed in a ratio of 46:54. About 24% of the parent compound ethacrynic acid is still present as well as 9% of an intermediate (peaks are observed around 7.13 and 6.77 ppm). After 60 minutes, the ratio A:B did not change anymore.

In Figure 3.3 the amounts of the various reactants and products of one out of two experiments are shown for the conjugation reaction with 2.5 mM EA and 5 mM GSH, chemically (Figure 3.3a) and catalysed by 22.6 μM GSTP1-1 (Figure 3.3b). From Figure 3.3a it appears that a considerable amount of the intermediate is formed during the conjugation reaction and that it accounts for 36% of the total amount of compound in the reaction mixture after 2 minutes. After 15 minutes still 20% of the compounds is present as this form, decreasing

to 9% after 1 hour. This intermediate is most likely the enol tautomer of the glutathione conjugate, as presented in Figure 3.2. However, the compound was not further identified in the present study.

The rate constant of the chemical reaction was assessed from the formation of the diastereoisomers in time, as shown in Figure 3.3a. The initial velocity was estimated from the slope at t=0 and second order rate constant for the chemical conjugation k was subsequently calculated to be 1.0×10^2 M^{-1} s^{-1}.

Conjugation catalysed by GSTP1-1

Figure 3.1c presents the ^{1}H NMR spectrum of an incubation of glutathione and ethacrynic acid in the presence of 47.6 μM GSTP1-1. The spectrum clearly shows that the GSTP1-1 catalysed reaction between EA and GSH is stereoselective and results in formation of diastereoisomer A. At the highest concentration of GSTP1-1 tested (47.6 μM), 87% of the formed EASG was diastereomer A, leaving 13% product B and about 7% of free ethacrynic acid after 40 minutes. When the enzyme catalysed conjugation reaction was corrected for the contribution of the chemical conjugation reaction it appeared that no diastereoisomer B was formed by the enzyme catalysed conjugation. Thus, GSTP1-1 is 100% stereoselective for formation of diastereoisomer A.

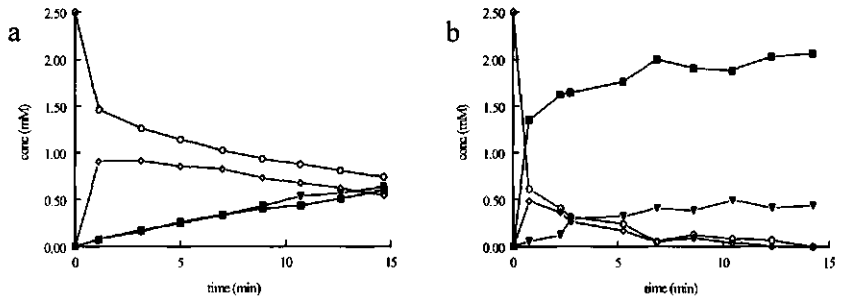

Figure 3.3 The formation of the intermediate enol form of the ethacrynic acid glutathione conjugate, the two diastereoisomeric ethacrynic acid glutathione conjugates and the loss of ethacrynic acid. 2.5 mM ethacrynic acid and 5 mM GSH were mixed without (**3.3a**) or with 22.6 μM GSTP1-1 (**3.3b**) and the loss of ethacrynic acid and the formation of the intermediate and the two EASG diastereoisomers (A and B) was followed in time by ^{1}H NMR analysis. Symbols used: ■, diastereoisomer A; ▼, diastereoisomer B; ◊, intermediate EASG; O, ethacrynic acid.

Table 3.1 Stereoselectivity of GSTP1-1, GSTP1-1 mutant C47S and human alpha class glutathione S-transferases for the conjugation reaction between glutathione and ethacrynic acid, depicted as percentage diastereoisomer of the total amount of conjugate formed, corrected for the contribution of the chemical reaction.

GST class	range enzyme conc (μM)	% diastereoisomer A	% diastereoisomer B
GSTP1-1	20-50	100	0
GSTP1-1/C47S	10-30	100	0
GSTA1-1	20-120	66	34
GSTA2-2	30-150	50	50

Figure 3.3, furthermore, clearly shows that the presence of the intermediate form of the ethacrynic acid glutathione conjugate is much lower in the enzyme catalysed reaction compared with the chemical reaction. In the enzyme catalysed reaction only 18% of the reaction mixture is present as the enol form after 1 minute, rapidly decreasing to 14 % after 2 minutes and to 0% after 12 minutes. When the formation of the intermediate conjugate is corrected for the contribution of the chemical reaction, it appears that the amount of intermediate formed during the enzyme catalysed reaction is essentially the result of the chemical reaction.

The rate for the enzyme catalysed forward reaction was estimated from the formation of the diastereoisomers in time, as shown in Figure 3.3b, using the slope at t=0 i.e. the initial rate of the reaction. For diastereoisomer A the value for v_{A0} thus estimated is 1.3 s^{-1}, the v_{B0} for the formation of diastereoisomer B is approximately 0, so the amount of B formed is almost exclusively the result of the chemical conjugation reaction. The pattern of formation of the two diastereoisomers in this reaction is similar to the incubations with 5 mM EA and 5 mM GSH. The observed initial rates for the formation of the two diastereoisomers are thus equal for 2.5 mM EA as well as 5 mM EA using the same enzyme concentration. Besides, as both the K_m^{GSH} and the K_m^{EA} for GSTP1-1 are much smaller than 1 mM (LoBello *et al.*, 1997), it is likely that the estimated v_{A0} is similar to the V_{max}.

Conjugation catalysed by other GST isoenzymes

Table 3.1 summarizes the stereoselectivity data for GSTP1-1 and other isoenzymes tested in various concentrations, corrected for the contribution of the chemical reaction. At low concentrations of enzyme the reactions were dominated by the cehmical reaction; at higher concentrations of GST the enzyme catalysed reaction became dominant and stereoselectivity could be registered. The GSTP1-1 mutant C47S was used to study the effect of the cysteine 47 residue on the stereoselectivity of the enzyme toward the reaction of EA and GSH. The reaction catalysed by this mutant (C47S) showed equivalent stereochemistry as GSTP1-1. The isoenzymes of the human α-class all catalyse the glutathione conjugation of ethacrynic acid. Only GSTA1-1 showed stereoselectivity, in favour of product A, but the formation of diastereoisomer B was catalysed as well although to a lesser extent. However, for GSTA2-2 no stereoselectivity could be detected.

Deconjugation reactions

First, the chemical deconjugation of the EASG mixture was followed in time by ^{1}H NMR analysis. No significant deconjugation was observed. Even after 16 hours only about 7 % free EA could be detected. Furthermore the ratio between diastereoisomer A and B remained constant during these 16 hours, about 1:1.

In the enzyme catalysed deconjugation reaction of the mixture of diastereoisomers, about 5 % free EA was formed after 16 hours. Although no significant differences in the ratio between diastereoisomer A and B could be detected in time, it seemed that diastereoisomer B predominated diastereoisomer A throughout the time.

In another experiment deconjugation of only diastereoisomer A was investigated chemically as well as catalysed by 22.6 μM GSTP1-1. In the incubation with the purified diastereoisomer A and GSTP1-1, no sign of enzyme-catalysed deconjugation was observed. After about 170 hours, the ratio between diastereoisomer A and B was approximately 1:1 again, with 15% and 8 % free EA for the chemical and enzyme catalysed reaction respectively.

Cell studies

To investigate, whether the stereoselectivity of EA-GSH conjugation observed *in vitro* with purified enzymes would also be relevant for EA-GSH conjugation in whole cells, where pH values are generally above 6.1, IGR-39 human melanoma cells were exposed to ethacrynic acid and the EASG excreted into the medium was analysed for its diastereoisomeric content. Figure 3.4 shows a typical ^{1}H NMR spectrum of the medium after 2 hours of exposure of the cells to EA. The chemical shift of the conjugates in the spectrum is slightly different from the spectrum of the purified EASG. This is the result of the high salt concentration, resulting from the HBSS. However, spiking the samples with purified EASG and EA showed that the proton resonances arose from the ethacrynic acid-glutathione conjugates. From Figure 3.4 it appears that

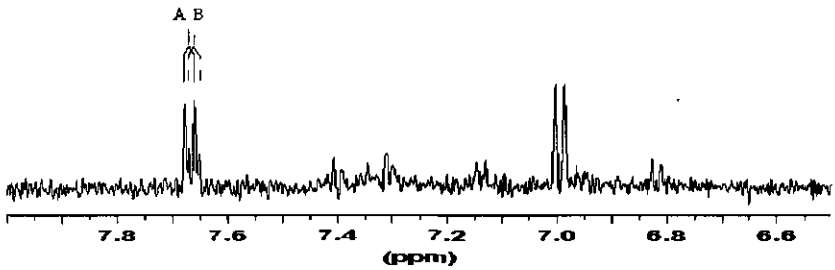

Figure 3.4 Typical part of a ^{1}H NMR spectrum of the medium from cells exposed to ethacrynic acid. Cells were exposed to 50 μM ethacrynic acid for 2 hours and medium was collected, freeze dried, dissolved in ^2H_2O and analysed by ^{1}H NMR.

diastereoisomer A of EASG is the principal conjugate present in the medium of EA exposed IGR-39 cells.

Discussion

In the present paper it was shown for the first time that the conjugation of ethacrynic acid and glutathione catalysed by GSTP1-1 stereospecifically forms one of the diastereoisomers of the glutathione conjugate (EASG). Chemical conjugation results in formation of a mixture of both diastereoisomers with a slight, but not significant preference for diastereoisomer B (ratio A:B = 46:54). From the human GSTs of the alpha class, GSTA1-1 appeared to be stereoselective and to form diastereoisomer A preferentially. For GSTA2-2, the enzyme catalysed EA-GSH conjugation appeared to occur a-selective and resulted in formation of both diastereoisomers. The fact that only GST A1-1 seems to be stereoselective in the conjugation of ethacrynic acid is quite remarkable as GSTA1-1 and GSTA2-2 only differ in 11 amino acids, three of which are in the hydrophobic binding site (as reviewed in Morel *et al.*, 1994). Comparison of both active sites could possibly provide a means to clarify the demonstrated difference in stereoselectivity.

Human GSTM1a-1a has a very low specific activity (Ploemen *et al.*, 1993b), so it was

impossible to test in this study. Furthermore, Ploemen *et al.*(1993b) already showed that rat GST3-3 was not stereoselective towards EA conjugation.

The GSTP1-1 mutant, C47S, was demonstrated to be stereoselective towards EA conjugation in the same way as GSTP1-1, so it can be concluded that the cysteine 47 residue does not influence the stereoselectivity of the enzyme. This result is in line with previous conclusions, that the amino acid does not influence the catalytic mechanism (Ricci *et al.*, 1995).

Although conjugation reactions catalysed by GSTs are widely studied, relatively little attention has been paid to the stereoselectivity of the enzyme catalysed reactions. Since the crystallization of the GST isoenzymes, numerous studies have been performed, characterizing the three-dimensional structures of GST isoenzymes complexed with substrates and/or GSH (conjugates) (Sinning *et al.*, 1993; Garcia-Saez *et al.*, 1994; Dirr *et al.*, 1994; Ploemen *et al.*, 1994a; Cameron *et al.*, 1995; Oakley *et al.*, 1997). For both GSTA1-1 (Cameron *et al.*, 1995) and GSTP1-1 (Oakley *et al.*, 1997) a three dimensional structure of the enzyme in complex with EA and an EASG diastereoisomer has been described and deposited in the Brookhaven PDB databank. However, for both these GST-EASG complexes arbitrarily only one of both EASG diastereoisomers is depicted in complex with the enzyme. The experimental electron density is reported to be not sufficiently well-defined to be conclusive as to whether the EASG bound to the enzyme can be identified as the R or S diastereoisomer. However, it is interesting to notice that in the pdb file of GSTA1-1 the R-form is included whereas the GSTP1-1 pdb file contains the S-form. This is remarkable takin into account the results of the present study showing that both isoenzymes actually form the same diastereoisomer.

For the glutathione conjugation of 7β, 8α-dihydroxy-9α, 10α-oxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene, Hu *et al.* (1997) suggest that the enantioselectivity of GSTP1-1 may be predicted by the structure of the active site. For the glutathione conjugation of ethacrynic acid, such a prediction on the basis of a crystal structure will be more difficult. This becomes clear when looking in detail at the possible mechanisms for glutathione conjugation of ethacrynic acid as a means to find a clue to whether the R or the S isomer is the product to be expected. The diastereoisomeric products are formed by the protonation of C9 of the ethacrynic acid-glutathione intermediate, generating a chiral centre, in principle after the initial attack of the sulfur atom from glutathione. It is very interesting to notice that only one diastereoisomer is actually formed as the proton most likely arises from the solvent or from a pool of protons that readily exchanges with the solvent (Dohn *et al.*, 1985); the enzyme thus determines the direction of this protonation, thereby creating possibilities for stereoselective product formation.

As is shown in Figure 3.3a and b, an intermediate compound is formed during the formation of the actual conjugates, which is most likely the enol tautomer of the glutathione conjugate. The fact that this intermediate is only detected in the chemical conjugation reaction, implicates that in the enzyme catalysed reaction the protonation of the C9 is catalysed and probably conclusive for the formation of diastereoisomer A in the case of GSTP1-1.

The precise mechanism of the GSTP1-1 catalysed reaction is still not known, but it has recently been claimed that the conjugation catalysed by GSTP1-1 follows a rapid equilibrium sequential bi-bi kinetic mechanism with a rate-limiting step, which occurs after binding of the two substrates and before the release of the products (LoBello *et al.*, 1997). The kinetic equilibria expected to occur after substrate binding and before product release can be presented as recently suggested by Chen and Armstrong (1995). They also suggested the existence of a so-called internal equilibrium between the two diastereoisomers while bound to the active site. The observations in the present study together with the fact that EASG is depicted in different ways in GSTA1-1 and GSTP1-1 (Cameron *et al.*, 1995, Oakley *et al.*, 1997) respectively, this might also be true for the GST-catalysed conjugation of EA and GSH.

In additional experiments the possible GST-catalysed deconjugation of EASG was investigated. However, it could not be shown that GSTP1-1 catalyses the deconjugation of EASG under the circumstances used in this study. At p^2H 6.1 no differences can be observed between the chemical and the enzyme catalysed deconjugation neither of the mixture of diastereoisomers nor of diastereoisomer A alone. It has previously been demonstrated that EA but also EASG are potent inhibitors of human GSTP1-1 (Ploemen *et al.*, 1993b; Awasthi *et al.*, 1993). Thus it might be possible that EASG, especially diastereoisomer A, occupies and blocks the active site of GSTP1-1, particularly in circumstances where an excess of conjugate is present.

As a result of chemical deconjugation, eventually equilibrium of equal amounts of diastereoisomer A and B is established for both the chemical as well as the enzyme catalysed incubation mixture. At equilibrium, the rate of EASG to free EA is 94:6. This means that the equilibrium of the reaction is directed far towards the conjugated compound.

All experiments in this study are performed at p^2H 6.1, because although GSTs show optimum activity at pH 7.6, chemical conjugation and deconjugation rates as well as aerobic oxidation of GSH are reduced at this pH and thus maximum effect of the enzyme can be expected (Boyland and Chasseaud, 1967). This leaves a question about the implications of stereochemistry *in vivo*. At physiological pH the GSTP1-1 catalysed rate of EASG-formation is only 1.1-fold higher than the non-enzymatic rate (Awasthi *et al.*, 1993). However, when human melanoma cells were exposed to ethacrynic acid for two hours, diastereoisomer A was the principal conjugate present in the medium. This might be the result of either the stereoselective formation of EASG by GSTP1-1, present in the cells, and/or the stereoselective transport out of the cells by transport pumps, i.e. the multidrug resistance associated protein (MRP). Results from a recent study (Evers *et al.*, 1997) describing non-stereoselective transport of prostaglandin A_2-glutathione conjugates by MRP indicate that very likely no stereoselective transport will occur for the EASG diastereoisomers. Stereoselectivity thus plays a role in cellular systems. Interesting possibilities arise when stereoselectivity actually plays a role *in vivo*, and further research on this topic would be very interesting.

Chapter 4

Inhibition of glutathione S-transferase activity in human melanoma cells by α,ß-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and *trans*-2-hexenal

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Abstract

The glutathione S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene in intact human IGR-39 melanoma cells was determined by the quantification by HPLC-analysis of the excreted glutathione (GSH) conjugate (S-(2,4-dinitrophenyl)glutathione; DNPSG). The major GST subunit expressed in these melanoma cells is the pi-class GST subunit P1.

Using this system, the effect of exposure for one hour to a series of α,β-unsaturated carbonyl compounds at non-toxic concentrations was studied.

Curcumin was the most potent inhibitor (96% inhibition at 25 μM), while 67% and 61% inhibition at 25 μM was observed for ethacrynic acid and trans-2-hexenal, respectively. Moderate inhibition was observed for cinnamaldehyde and crotonaldehyde, while no inhibition was found for citral. The reactive acrolein did not inhibit the DNPSG-excretion at 2.5 μM, the highest non-toxic concentration.

Up to about 50% GSH-depletion was found after treatment with crotonaldehyde, curcumin and ethacrynic acid, however the consequences for GST conjugation are presumably small.

Reversible inhibition of GST was the major mechanism of inhibition of DNPSG-excretion in melanoma cells, except in the cases of curcumin and ethacrynic acid, which compounds also inactivated GSTP1-1 by covalent modification. This was clear from the fact that dependent on the dose between 30% and 80% inhibition was still observed after lysis of the cells, under which conditions reversible inhibition is absent.

Intracellular levels of DNPSG remained relatively high in the case of ethacrynic acid. Possibly ethacrynic acid in some way also inhibits the transport of DNPSG by inhibition of the GSH conjugate export pump (MRP/GS-X pump).

Introduction

Extensive lists of reversibly and irreversibly acting inhibitors of glutathione S-transferases (GST) activity have been published (Mannervik and Danielson, 1988; Ploemen *et al.*, 1996). In general, inhibitors of enzymes can be used to study the mechanism of the catalysis or the architecture of the active site. In the case of the GST, use has also been made of inhibitors to distinguish various isoenzymes (Mannervik, 1985). However, the role of the cytosolic GST in the alkylating drug resistant phenotype, observed in the treatment of tumors (Waxman, 1990; Black and Wolf, 1991) potentially makes inhibition of GST clinically useful.

Especially the GST pi-class, but also the GST alpha-class, have been related to drug resistance (Black and Wolf, 1991). Thus, several groups are in search of isoenzyme-selective inhibitors with improved inhibitory capacity to elucidate the role of GST more definitely (Ouwerkerk-Mahadevan *et al.*, 1995; Morgan *et al.*, 1996). Ethacrynic acid, a diuretic drug,

has been shown to inhibit GST *in vitro* (Ahokas *et al.*, 1985; Ploemen *et al.*, 1990; Ploemen *et al.*, 1993a; Hansson *et al.*, 1991), and *in vivo* (O'Dwyer *et al.*, 1991). Indeed, the use of ethacrynic acid enhanced the action of several alkylating drugs in various drug resistant cells (Tew *et al.*, 1988; Rhodes and Twentyman, 1992; Singh *et al.*, 1992). However, the effectiveness of GST inhibition in conjunction with chemotherapy of resistant cells *in vivo*, is still a matter of debate (Tew, 1994).

Despite the numerous reports on the potentiation of alkylating drugs by GST inhibitors in cells, most GST inhibition data have been obtained using pure enzymes or cytosolic preparations. The extrapolation of results, obtained with these enzyme preparations, to the *in vitro* cell or *in vivo* situation is relatively complicated. The pharmacokinetics and metabolism of the inhibitor determine its intracellular concentration and this concentration may change continuously. Conversely, when studying cytosolic preparations after treatment of cells or in vivo, the extent of reversible inhibition is underestimated, since the non-covalently linked inhibitor is diluted extensively.

This prompted us to develop a system to measure the glutathione (GSH) conjugation in intact cell cultures directly, by the measurement of excretion of the GSH conjugate of CDNB (S-(2,4-dinitrophenyl)glutathione, DNPSG). In such a system, total inhibition (irreversible and reversible) is determined, and moreover the potential contribution of GSH depletion by the inhibitor is included. CDNB was chosen as model substrate since it is the commonly used co-substrate of GST in enzyme assays. The potent inhibitor ethacrynic acid (see above) and several structurally related agents (all containing the α,β-unsaturated carbonyl moiety) to whom potential in vitro GST inhibitory action is attributed (Berhane and Mannervik, 1990; Chien *et al.*, 1994), were studied in this system. Human IGR-39 melanoma cells were used, since they express high levels of the GST isoenzyme of interest (GST of the pi-class, (Ramachandran *et al.*, 1993).

Materials and methods

Materials

Glutathione, NADH, and dithiotreitol were obtained from Boehringer, Mannheim, Germany. Ethacrynic acid, curcumin, S-hexylglutathione, and N-acetyl-L-cysteine were obtained from Sigma Chemical Co., St. Louis, MO, USA. 1-Chloro-2,4-dinitrobenzene (CDNB), *trans*-cinnamaldehyde, *trans*-crotonaldehyde and acrolein were obtained from Aldrich Chemie, Bornem, Belgium. Citral and *trans*-2-hexenal were purchased from Acros Chimica, Geel, Belgium. HPLC-grade trifluoro acetic acid was obtained from Baker (Deventer, The Netherlands). HPLC-grade methanol and acetonitrile were from Labscan Ltd, Dublin, Ireland. Epoxy-activated Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). DNPSG

was synthesized according to Sokolovsky *et al.* (1964) . The N-acetyl-L-cysteine conjugate of CDNB was synthesized analogously to DNPSG.

Drug exposure and cytotoxicity assays

Human melanoma cancer cells (IGR-39) were kindly provided by the Dr. Daniel den Hoed kliniek (Rotterdam, the Netherlands). IGR-39 melanoma cells were cultured in RPMI 1640 medium (Gibco Ltd, Life Technologies, Paisly, UK), supplemented with 10% fetal calf serum, 50 mg/l gentamicin, at 37°C in a humid atmosphere containing 5% CO_2. For each experiment, approximately $20*10^4$/ml cells were plated onto a 6-well tissue cluster (Costar, Cambridge MA, USA) and cultured until a semiconfluent monolayer was obtained (in about 2 days). Cells were exposed to agents in triplicate for one hour at 37°C in Hanks balanced salt solution (HBSS without fenol red and $NaHCO_3$, obtained from Gibco) and $NaHCO_3$ was added to a final concentration of 0.35 gr/l. The final volume was 2 ml. All test compounds were freshly dissolved in ethanol (final concentration in the medium always $\leq$0.5%).

In order to exclude cytotoxic effects during the assay for GSH conjugation, only concentrations of the test compounds giving at least 90% viable cells were used in the experiments. Drug effects on cell proliferation and cytotoxic effects were determined by LDH-leakage assay (Mitchell *et al.*, 1980). Approximately $20*10^4$/ml cells were plated onto a 24-well tissue cluster and cultured as described above. Cells were exposed to agents for 1.5 hours in quadruplicate in HBSS (see above; final volume was 0.5 ml). The 90% cell survival concentrations determined were 2.5, 100, 100, 50, 25, 100, and 75 μM; for acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and *trans*-2-hexenal, respectively. The concentration range of the compound used throughout the experiments was for acrolein: 0.31, 0.63, 1.25 and 2.5 μM; for curcumin: 5, 10, 20 and 25 μM; for cinnamaldehyde, citral and ethacrynic acid: 25, 50, 75 and 100 μM; for crotonaldehyde: 6.3, 12.5, 25 and 50 μM; and for *trans*-2-hexenal: 10, 25, 50 and 75 μM. Control incubations were included with only the solvent.

DNPSG excretion

Cells were cultured for 1 hr with the test compounds (see above). After one hour of exposure, CDNB was added in HBSS in a volume of 100 μl to a final concentration of 10 μM, mixed and at 4 time-points (1, 5, 10 and 20 min) a 0.2 ml sample was taken and mixed with 5 μl 0.04 M N-acetyl-L-cysteine (to remove unreacted CDNB). The mixture was vortexed and immediately stored at -20°C upon analysis. DNPSG was separated from the N-acetyl-L-cysteine conjugate of CDNB by injecting 0.05 ml on a Chromsphere C18 reverse phase column (3.0 * 100 mm; Chrompack, Middelburg, the Netherlands), eluted at a flow rate of 0.8 ml/min, with 0.1% v/v trifluoro acetic acid (solvent A) and 0.1% v/v trifluoro acetic acid in methanol (solvent B) with a linear gradient of 30-70% B in 10 min, followed isocratically at 70% B for

5 min (k'= 3.2, and 4.5, for respectively DNPSG, and the N-acetyl-L-cysteine conjugate of CDNB). Quantification of DNPSG was performed by peak area integration at 340 nm, using concentration/absorbance curves of the DNPSG standard with a Merck Hitachi D2500 Chromato Integrator (HPLC system: Spectra Physics Analytical P2000 pump, AS3000 autosampler and UV1000 UV detector). The new peak visible in the HPLC-chromatogram (peak 2, Figure 4.1), is the conjugate of N-acetyl-L-cysteine and CDNB. DNPSG in the samples (stored at 0-6^{0}C) was stable for at least 2 weeks. For ethacrynic acid, also the formation and excretion of the GSH conjugate of ethacrynic acid (Ploemen *et al.*, 1990) could be followed on HPLC (peak 3, k': 5.3). The total excretion of DNPSG after 20 min incubation with CDNB in the control incubations varied between the various assays from about 10 to 37 nmol/mg protein. The maximal consumption of CDNB was about 15% of the total amount of CDNB present per well.

GSH concentration, intracellular DNPSG level and GST assay in supernatant

The intracellular DNPSG, GST activity in supernatant, and the GSH concentration were determined in the same experiment. The measurements were made at the end of the exposure period (after exposure to the test compounds (1 hr) and the subsequent CDNB exposure (20 min)). Briefly, cells were harvested by scraping with a rubber policeman in ice-cold PBS, followed by centrifugation (5 min 50*g*), whereafter the pellet was carefully rinsed with ice-cold PBS and resuspended in PBS. The cells were disrupted by sonification on ice and supernatant was made by centrifugation at 2800*g* for 5 min. Aliquots were used to determine the GSH concentration immediately (Hissin and Hilf, 1976). Protein concentration was determined by the Bradford assay (using Bio-rad protein assay from Bio-Rad Laboratories HmbH, München, Germany) adapted for 96-wells measurements on a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA, USA) (Bradford, 1976). Moreover, the GST activity was measured in the supernatant towards CDNB according to Habig *et al.* (1974) . The HPLC analysis (see above) was used to quantify DNPSG.

GST subunit composition of IGR-39 melanoma cells

19 dishes (75 cm^2) were cultured with IGR-39 cells until a semiconfluent monolayer was obtained. After trypsinization the cells were centrifuged (5 min 50*g*) in PBS (containing 1 mM dithiotreitol), and disrupted by sonification. The cell homogenates were centrifuged (30 min 15000 *g*, at 6^{0}C) and the supernatants were used to determine the GST subunit composition by S-hexylglutathione Sepharose 6B affinity chromatography followed by wide pore RP-HPLC separation as described (Mannervik and Guthenberg, 1981; Bogaards *et al.*, 1989).

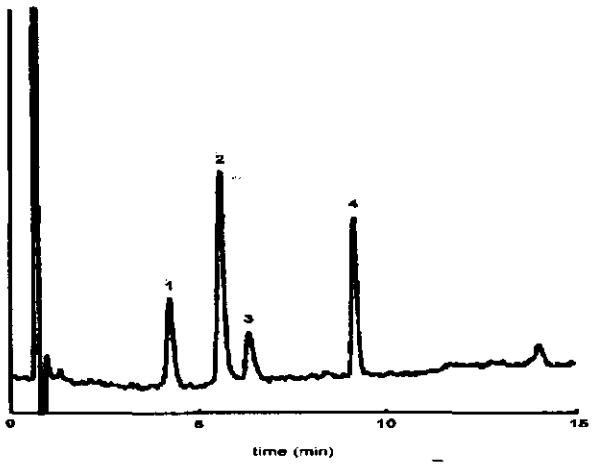


Figure 4.1. Typical HPLC-chromatogram of medium of human IGR-39 melanoma cells exposed to 75 μM ethacrynic acid (1 hour) and CDNB (20 minutes). After 20 minutes exposure to CDNB N-acetyl-L-cysteine was added to scavenge the remaining CDNB (see Materials and methods). Peak 1= DNPSG; Peak 2= N-acetyl-L-cysteine- conjugate of CDNB; Peak 3= EASG; Peak 4= ethacrynic acid. **Y-axis:** full scale 0.02 A at 340 nm.

Statistical Methods

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

Results

GST subunit composition of IGR-39 melanoma

The GST subunit composition was studied to establish whether the GST pi-class was expressed at significant levels, as described for melanoma cells (Ramachandran *et al.*, 1993). Indeed, the principal GST subunit in IGR-39 melanoma cells is P1 (about 70%). An unknown peak (about 30% of the total GST subunits, assuming a similar molar extinction coefficient) is presumably the subunit M1a, judged on historical data (Van Ommen *et al.*, 1990).

Effect on DNPSG excretion

The model substrate CDNB was used to determine the effect of α,β-unsaturated carbonyl derivatives on GSH conjugation in IGR-39 melanoma cells, by following the excretion of its GSH conjugate (DNPSG) in the medium (Figure 4.1). A representative figure of the time-dependent DNPSG excretion and the effect of a test compound (curcumin) is presented in Figure 4.2. The excretion of DNPSG was in general linear over a time span of 20 min (see control, Figure 4.2). Therefore, we used linear regression (y= ax) to describe the time-dependent excretion of DNPSG. The correlation coefficient (r^2) for the regression was in general > 0.95, indicating that linear regression properly described the relation between time and excretion. The effects of different concentrations of α,β-unsaturated carbonyl derivatives are shown in Figure 4.3. Since every compound was tested at 25 μM, with the exception of the highly reactive acrolein, comparison will be made at this concentration (see below). At 25 μM, curcumin was the most potent inhibitor of the DNPSG-excretion, while ethacrynic acid and *trans*-2-hexenal displayed also significant inhibition. Cinnamaldehyde and crotonaldehyde also clearly inhibited the excretion, although the individual points do not reach a significant

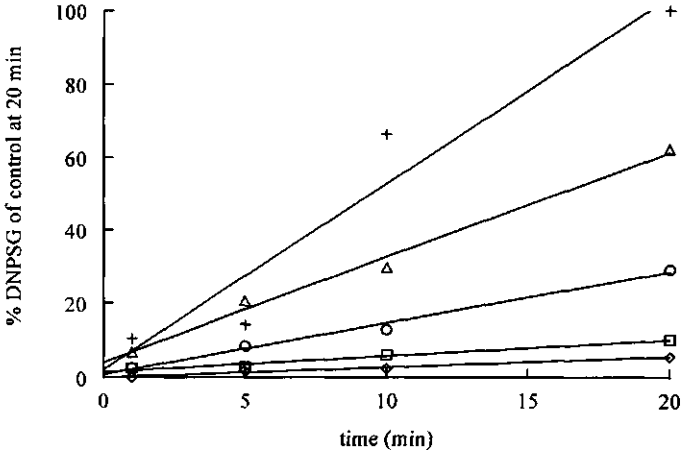


Figure 4.2. The DNPSG excretion of IGR-39 melanoma cells exposed to curcumin. Cells were exposed to curcumin for one hour, and subsequently exposed to 10 μM CDNB for 20 minutes. The GSH conjugation was determined by the excretion of DNPSG into the medium. Incubations were performed in triplicate. Values are presented as % of the DNPSG excretion at 20 min in control incubations. Symbols used: +, control; ∆, 5μM; O, 10 μM; □, 20μM; ◊, 25μM.

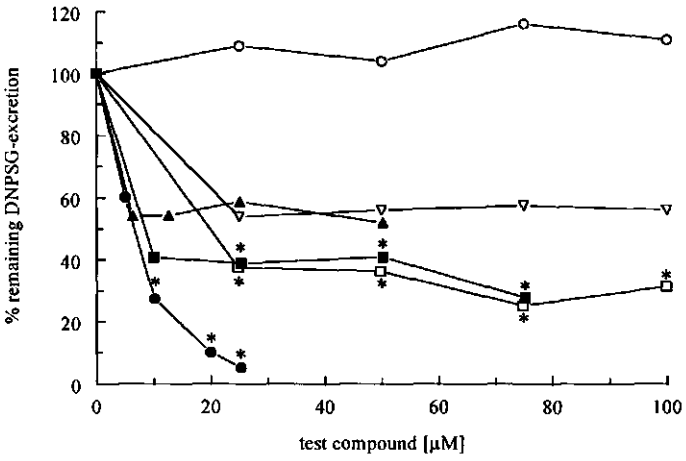


Figure 4.3. The effect of α,β-unsaturated carbonyl derivatives on the DNPSG excretion in human melanoma IGR-39 cells. Cells were preincubated for one hour with the test compounds. Next, the excretion of DNPSG is determined during 20 min exposure to CDNB and expressed as % of control incubations. * significantly different from control incubations ($p < 0.05$). Symbols used: ○, citral; □, ethacrynic acid; ■, trans-2-hexenal; ●, curcumin; △, cinnamaldehyde; ▲, crotonaldehyde.

difference at the level ($\alpha = 0.05$) tested (Figure 4.3). Citral did not show any inhibition of the DNPSG-excretion, while acrolein inhibited the DNPSG-excretion only moderately at the highest non-toxic exposure level, viz. at 2.5 μM 23% (SD= 3.0) inhibition (data not shown).

Only curcumin showed a more or less linear dose-response curve. In general, the major part of the inhibitory effect on the DNPSG-excretion by cinnamaldehyde, ethacrynic acid, *trans*-2-hexenal, crotonaldehyde had already been reached at the lowest exposure level used (Figure 4.3).

Effect on GSH levels

The intracellular GSH levels were quantified at the end of the exposure period (1 hour plus 20 minutes)(Figure 4.4). At 25 μM, crotonaldehyde and curcumin were the strongest GSH depletors (about 50% depletion), while ethacrynic acid also depleted GSH considerably at this concentration. Moderate depleting effects were found for cinnamaldehyde, citral, and to a lesser extent by *trans*-2-hexenal at relative high concentrations (Figure 4.4). The major fraction of the GSH depleting effect is reached with the lowest exposure level for citral, while a small but

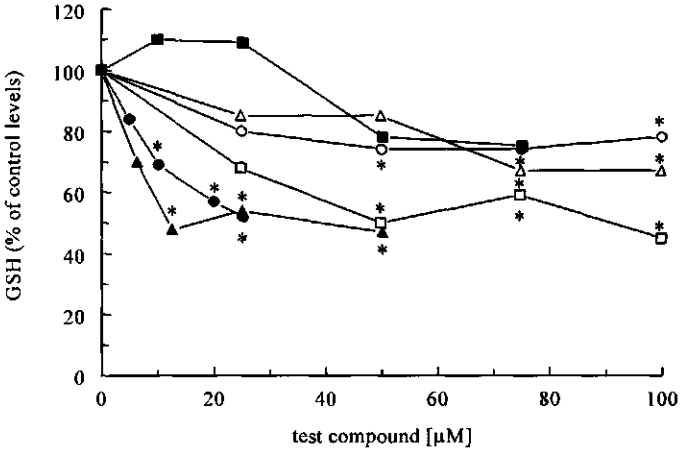


Figure 4.4. The effect of α,β-unsaturated carbonyl derivatives on the GSH levels in human melanoma IGR-39 cells. Cells were preincubated for one hour with the test compounds and subsequently incubated for 20 min with CDNB. The remaining GSH level is determined and expressed as % of control incubations. The coefficient of variation was less than 25% for the individual points. Incubations were performed in triplicate. * significantly different from control incubations ($p < 0.05$). Symbols used: ○, citral; □, ethacrynic acid; ■, trans-2-hexenal; ●, curcumin; △, cinnamaldehyde; ▲, crotonaldehyde.

significant dose-response is observed for cinnamaldehyde, crotonaldehyde, curcumin ethacrynic acid and *trans*-2-hexenal. Acrolein depleted GSH only moderately at 2.5 μM: 14% (SD= 0.8) depletion (data not shown).

Effect on GST activity in supernatant

The effects on the GST activity in the supernatant were determined at the end of the experimental exposure (Figure 4.5). At 25 μM, curcumin was the most potent inhibitor of the GST activity, although at higher concentrations ethacrynic acid is also a potent inhibitor of the GST activity in supernatant (Figure 4.5). Only minor effects were observed for the other test compounds.

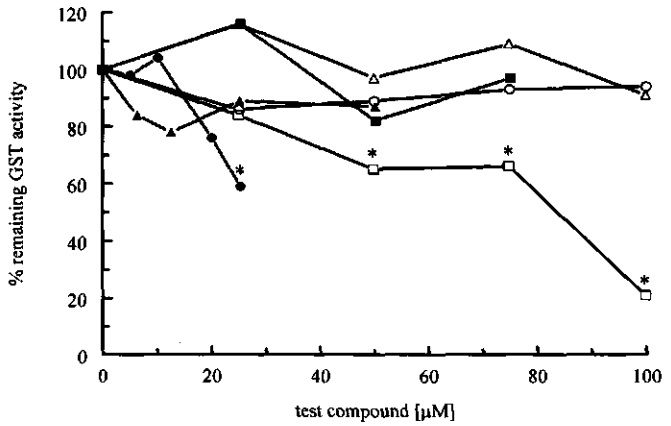


Figure 4.5. The effect of α,β-unsaturated carbonyl derivatives on GST activity in supernatant of human melanoma IGR-39 cells. Cells were preincubated for one hour with the test compounds, and subsequently for 20 min to CDNB. Next, the GST activity towards CDNB was determined (expressed as % of control) in supernatant.

* significantly different from control incubations ($p < 0.05$). The coefficient of variation was less than 25% for the individual points. Incubations were performed in triplicate. Symbols used: ○, citral; □, ethacrynic acid; ■, trans-2-hexenal; ●, curcumin; △, cinnamaldehyde; ▲, crotonaldehyde.

Effect on intracellular DNPSG concentration

The effects of the test compounds on the *intracellular* concentration of DNPSG was determined at the end of the experimental exposure. At 25 μM, a significant decrease of intracellular DNPSG was observed for cinnamaldehyde, *trans*-2-hexenal and crotonaldehyde as expected considering the reduction in DNPSG-excretion (Figure 4.6). However, for ethacrynic acid and to a minor extent also for curcumin at low concentrations, the intracellular levels of DNPSG also decreased, but remained relatively at a high level, as compared with the observed reduction in the DNPSG-excretion (Figure 4.6). Acrolein decreased the intracellular concentration of DNPSG even at the 1.25 μM level of exposure (data not shown).

Discussion

Despite the large number of inhibitors of pure GST isoenzymes known, only few attempts have been made to study GST inhibition in biological systems. The aim of our present study was to develop an assay to determine the GSH conjugation in human melanoma cells by quantification of DNPSG-excretion, and to study the effects of a series of α,β-unsaturated carbonyl derivatives in this assay. Moreover, we studied some determinants involved in the mechanism of inhibition of DNPSG-excretion, like GSH depletion, covalent inactivation of GST, and effects on MRP/GS-X pump.

Although the co-substrate CDNB is a hydrophobic compound that rapidly enters the cell (Oude Elferink *et al.*, 1989), absence of significant GSH depletion was observed even at an exposure of 20 minutes to 40 μM CDNB in hepatocytes (Lindwall and Boyer, 1987). In our assay, the linearity of the DNPSG excretion also suggested that no significant GSH depletion occurred in control incubations, since this would be reflected in a deviation of linearity. Moreover, the fact that excretion of DNPSG is still linear after 20 min exposure to CDNB, indicates that product inhibition of GST (by the GSH conjugates formed) was minimal.

The potential mechanism(s) involved in the inhibition of GST and subsequent DNPSG-0excretion by α,β-unsaturated carbonyl derivatives may be illustrated by the mechanism(s) involved for the most extensively studied inhibitor, *viz.* ethacrynic acid.

Ethacrynic acid and its intracellularly formed GSH conjugate have been identified as potent reversible inhibitors of GST (Morgan *et al.*, 1996; Ploemen *et al.*, 1990). Moreover, the GSH consumption may potentially decrease the rate of spontaneous GSH conjugation and thus may inhibit DNPSG-formation. The K_m-values for GSH were about 50-200 μM for most GST isoenzymes (Mannervik and Danielson, 1988; Mannervik, 1985), while the intracellular GSH concentration is about 1-10 mM (Meister, 1983a). Thus, only massive GSH depletion will inhibit GST activity significantly. The thiols of proteins may react covalently with α,β-unsaturated carbonyl moiety via a Michael addition (Witz, 1989; Esterbauer *et al.*, 1991). Indeed, the human pi-class GST P1-1 has been inactivated by a chemical reaction of a reactive thiol (Cys-47 (Desideri *et al.*, 1991; Tamai *et al.*, 1990)) with ethacrynic acid (Ahokas *et al.*, 1985). Usually, a chemical reaction is considered to be irreversible. The retro-Michael cleavage is a well-known exception on this rule: enzymes with functional SH-groups inactivated with α,β-unsaturated carbonyl derivatives can be reactivated by an excess of GSH or other thiols (Schauenstein *et al.*, 1971). Indeed, reactivation of GST P1-1 which was covalently inactivated by ethacrynic acid, was observed by incubation with an excess of GSH (Ploemen *et al.*, 1994b). However, it should be recognized that the forward reaction (Michael addition) is in general much faster than the reverse reaction (retro-Michael cleavage) (Esterbauer *et al.*, 1991).

In the present study, ethacrynic acid significantly decreased the DNPSG-excretion in melanoma cells, which reflects the balance of the mechanism(s) involved described above. At

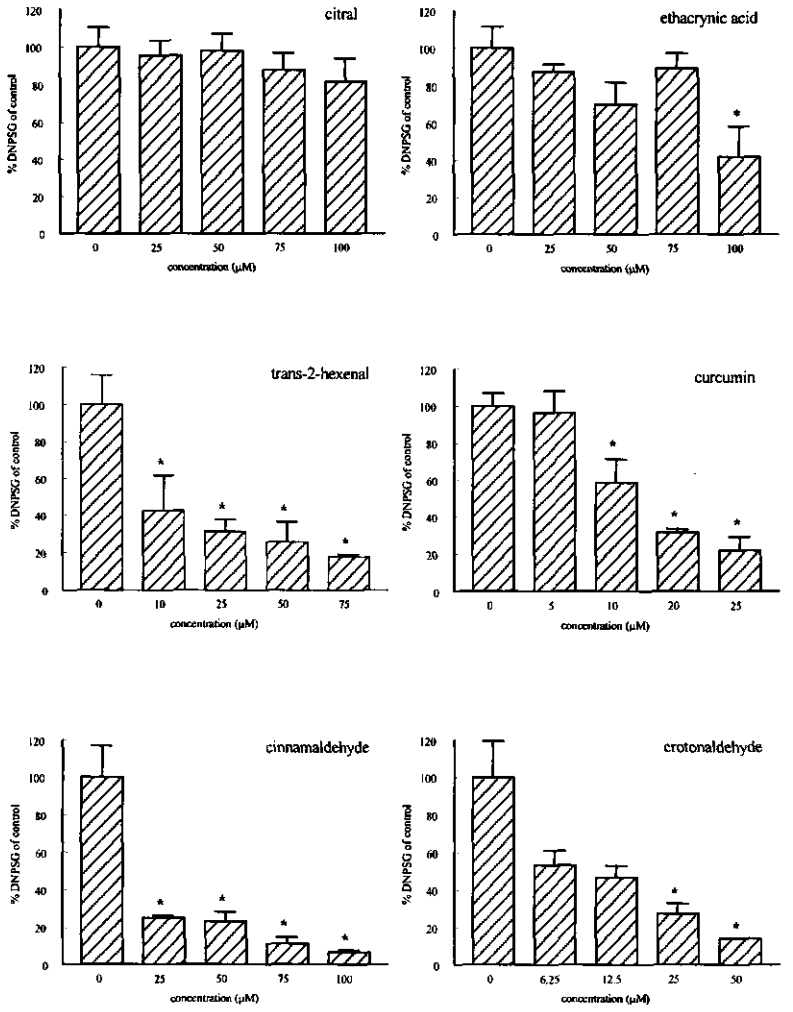
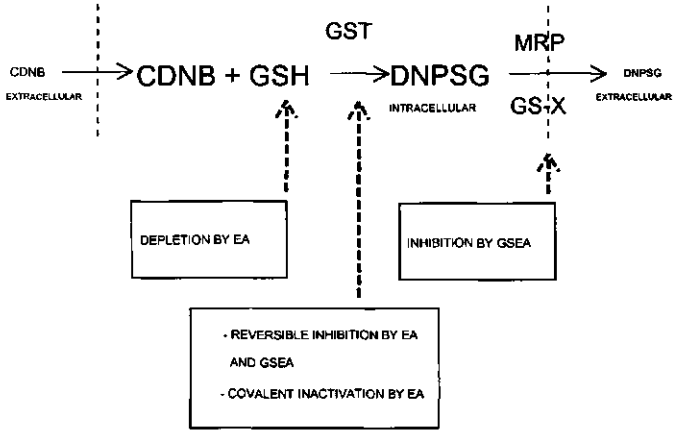


Figure 4.6. The effect of α,β-unsaturated carbonyl derivatives on intracellular DNPSG levels in human melanoma IGR-39 cells. Cells were preincubated for one hour with the test compounds. Next, the intracellular DNPSG level is determined and expressed as % (± SD, n = 3) of control incubations after 20 min exposure to CDNB.

* significantly different from control incubations ($p < 0.05$).

the lowest concentration of ethacrynic acid, the inhibition was already maximal. The absence of a dose-response suggested that a steady-state concentration of ethacrynic acid may have been reached sufficient for strong reversible inhibition (the I_{50} concentration using pure enzymes were about 1-10 μM (Morgan *et al.*, 1996; Ploemen *et al.*, 1990)). However, indications were also found that GSH depletion might be involved in this process: the decrease in DNPSG-excretion correlated with GSH depletion. However, for reasons discussed above, the contribution to the inhibition will probably be small. The GST activity measured in supernatant reflects *only* the inactivation of GSTP1-1 by covalent modification by ethacrynic acid, since the non-covalently incorporated ethacrynic acid is strongly diluted in the preparation of the supernatant and the subsequently used enzymic assay. This type of inactivation of GSTP1-1 was also observed in the melanoma cells, and became relatively more important at high concentrations. This might be explained by the phenomenon that the excess of GSH which normally protects the enzyme from covalent inactivation (see Figure 4.4), has dropped below a protecting level. These events are summarized in Scheme 4.1.

In many different organs and cell types, DNPSG is efficiently excreted by an ATP-dependent GSH conjugate export pump (multidrug resistance-associated protein gene encoding a GS-X pump; MRP/GS-X pump) (Ishikawa, 1992; Müller *et al.*, 1994; Ishikawa *et al.*, 1995). Several GSH conjugates have been shown to competitively inhibit this pump, while the efflux was a



Scheme 4.1. Mechanisms involved in the inhibition of DNPSG excretion by ethacrynic acid (EA). For abbreviations we refer to the list of abbreviations.

Table 4.1. Effect of α,β-unsaturated carbonyl derivatives in human melanoma IGR-39 cells by modulation of the GSH/GST system.

compound	[GSH]	GST activity in cytosol	DNPSG excretion
acrolein	o	o	o
cinnamaldehyde	+	o	+
citral	o	o	o
crotonaldehyde	++	o	+
curcumin	+	+	+++
ethacrynic acid	++	++	++
trans-2-hexenal	+	o	++

Melanoma IGR-39 cells were exposed to non-toxic concentration of the test compounds (for concentration range see Materials and methods) for 1 hr at 37°C. For experimental details see Materials and methods. The effects were summarized according to an arbitrary classification (see below) at the highest concentration tested for the test compound. Inhibition of GST activity in cytosol demonstrates covalent inactivation. Inhibition of DNPSG excretion showes total inhibition.

o = 0-25% inhibition; + = 25-50% inhibition; ++ = 50-80% inhibition and +++ = >80% inhibition (for [GSH] % inhibition = % depletion).

saturable process (Oude Elferink *et al.*, 1989; Lindwall and Boyer, 1987; Kobayashi *et al.*, 1985; Akerboom *et al.*, 1991; Olive and Board, 1994). The intracellularly formed GSH conjugate of ethacrynic acid and to a minor extent the GSH conjugate of curcumin probably inhibited the MRP/GS-X pump in melanoma cells, as indicated by the fact that intracellular levels only drop marginally while the DNPSG-excretion is reduced more efficiently. It is tempting to conclude that MRP/GS-X pump inhibition will eventually lead to more efficient inhibition of GST by the enhanced intracellular concentration of the GSH conjugates of ethacrynic acid (or curcumin) and CDNB (product-inhibition). However, very efficient inhibition of the MRP/GS-X pump by the GSH conjugate of ethacrynic acid , which would lead to an increase of the intracellular level of DNPSG as compared to the control, was not observed.

Several of the other studied α,β-unsaturated carbonyl derivatives inhibited the DNPSG-excretion to relevant levels (summarized in Table 4.1), with the exception of acrolein and citral. The strong electrophile acrolein has been shown to inhibit purified GSTP1-1 very efficiently (Berhane and Mannervik, 1990), however it is not possible to obtain GST inhibition in

melanoma cells at non-toxic concentrations. The mechanism(s) of the inhibition of the DNPSG-excretion were different in some cases: Curcumin was the most potent inhibitor of the DNPSG-excretion in the melanoma cells. The observed covalent type of inhibition and GSH depletion may account for a considerable part of the observed strong inhibition, but direct reversible inhibition will also account for a major part of the inhibition. Recently, curcumin was indeed identified as a potent reversible inhibitor of GST (Oetari *et al.*, 1996). *Trans*-2-hexenal inhibited the DNPSG-excretion in the absence of significant GSH depletion or GST inactivation by a covalent interaction: indicating the significance of reversible inhibition. Reversible inhibition has indeed been reported for structurally related octenal derivatives using purified rat GST isoenzymes (Chien *et al.*, 1994). Interestingly, the major effect of crotonaldehyde was GSH depletion, suggesting that for this compound not the reversible inhibition of GST but the GSH depletion might be a major determinant of the inhibition of the DNPSG-excretion.

α,β-Unsaturated carbonyl compounds are found in many naturally occurring products, but are also formed (endogenously) during metabolism and degradation of natural or man-made compounds (Witz, 1989; Esterbauer *et al.*, 1991). With this assay to measure GST activity in intact melanoma cells, it was shown that several of these compounds produce a significant (acute) inhibition of DNPSG-excretion, at non-toxic concentrations. In particular curcumin seems a promising agent to apply in vivo. In this context, we are presently studying the effects of prolonged exposure to these compounds: Initial inhibition by α,β-unsaturated carbonyl derivatives might lead to subsequent GST induction as was observed in human colon carcinoma cells exposed to ethacrynic acid (Kuzmich *et al.*, 1992). The present assay might prove its value in these studies as well.

Acknowledgements

The melanoma cancer cells (IGR-39) were kindly provided by the Dr. Daniel den Hoed kliniek (Rotterdam, the Netherlands). We would like to thank Bert Spenkelink (Toxicology group, Department of Food Technology and Nutritional Sciences, Wageningen Agricultural University, The Netherlands) for the synthesis of S-(2,4-dinitrophenyl)glutathione and its N-acetyl-L-cysteine derivative.

Chapter 5

Interactions of prostaglandin A_2 with the glutathione mediated biotransformation system

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Abstract

The cyclopentenone prostaglandin A_2 (PGA_2) is known to inhibit cell proliferation and as the α,β-unsaturated ketone moiety is probably necessary for this activity, metabolism of this compound might be important in controlling its ultimate function. The glutathione related metabolism of PGA_2 was therefore investigated both with purified glutathione S-transferase P1-1 (GSTP1-1) and with IGR-39 human melanoma cells.

Firstly, the irreversible inhibition of human GSTP1-1 and its mutant's C47S, C101S and C47S/C101S was studied. PGA_2 inhibits GSTP1-1 mainly by binding to the cysteine 47 moiety of the enzyme. Incubation of the modified enzyme with a molar excess of glutathione (GSH) resulted in a restoration of catalytic activity, indicating that retro-Michael cleavage occurs.

Secondly, studies were conducted to study the prostaglandin A_2-glutathione (PGA_2-SG) conjugate formation in cells. After loading IGR-39 human melanoma cells with [^{3}H] glycine and subsequent exposure to PGA_2, both diastereoisomers of the PGA_2-glutathione conjugate are excreted into the medium, however with a clear excess of the S-form. Previous work indicates that this is the result of the preferential formation of the S-form by GSTP1-1 that is present in the cells and not from a stereoselectivity in transport.

The effect of PGA_2 on intracellular glutathione S-transferase activity was determined by quantification of the excreted glutathione conjugate (S-(2,4-dinitrophenyl)glutathione; DNPSG) after exposure to 1-chloro-2,4-dinitro benzene (CDNB). DNPSG excretion was inhibited after incubation with 10 or 20 μM PGA_2 for 1 or 4 hours, as a result of glutathione depletion, reversible GST inhibition but also covalent modification of intracellular GST. This latter effect was responsible for about 23%-32% of the total inhibition observed. Preincubation with D,L-buthionine-S,R-sulfoximine (BSO) results in an even higher level of irreversible inhibition. Furthermore, PGA_2 also inhibits transport of DNPSG by the multidrug resistance associated protein (MRP) or GS-X pump. This inhibitory effect is reversible and competitive.

In conclusion, PGA_2 modulates all three aspects of the glutathione-mediated biotransformation system i.e. glutathione levels, GSTP1-1 activity and transport of glutathione conjugates. A role for GSTP1-1 as a specific transport protein inside the cell is indicated.

Introduction

Prostaglandin A_2 (PGA_2), a prostaglandin containing an α,β-unsaturated ketone moiety is an inhibitor of cell proliferation of animal and human cell lines (Hohn *et al.*, 1979; Bregman and Meyskens, 1983; Hohn and Marnett, 1985; Bhuyan *et al.*, 1986; Santoro *et al.*, 1986) by arresting the cell cycle in the G_1 phase (Bhuyan *et al.*, 1986; Ohno *et al.*, 1988b). Therefore PGA_2 has been suggested as a potential chemotherapeutic agent (Hohn *et al.*, 1979; Bregman

and Meyskens, 1983; Santoro *et al.*, 1986).

Cyclopentenone prostaglandins are transported into cells by a specific carrier system and subsequently transferred to the nucleus (Narumiya and Fukushima, 1986), where they accumulate (Ohno *et al.*, 1992). Accumulation in the nucleus results in inhibition of DNA synthesis (Hohn *et al.*, 1979), induction of apoptosis (Kim *et al.*, 1993), induction of synthesis of heat shock proteins (Ohno *et al.*, 1988a; Santoro *et al.*, 1989), and regulation of glutathione (GSH) levels by induction of γ-glutamylcysteine synthetase (Ohno and Hirata, 1990). These prostaglandins are further reported to possess antiviral (Santoro *et al.*, 1981) and antitumour (Kato *et al.*, 1986) activity. Although molecular mechanisms are not totally clear, it has recently been shown that cyclopentenone prostaglandins inhibit Nf-κB transcription factor and at the same time activate heat shock transcription factor (Rossi *et al.*, 1997).

The α,ß-unsaturated carbonyl moiety of prostaglandin A_2 seems to be required for the antiproliferative effect (Hohn and Marnett, 1985) and for the reaction with cellular proteins (Narumiya *et al.*, 1987; Ohno and Hirata, 1993; Parker, 1995). Metabolism of PGA_2 thus can be very important in the eventual effect of this compound. It has been shown already in 1975 that prostaglandins of the A class conjugate with glutathione both chemically and catalysed by glutathione S-transferases (Cagen *et al.*, 1975). Recently, Bogaards *et al.* (1997) demonstrated that the various isoenzymes of glutathione S-transferases can stereoselectively catalyse the glutathione conjugation of prostaglandin A_2 and J_2 and that this stereoselective formation of the R- or the S-form of the conjugate is isoenzyme dependent. Furthermore, when L1210 mouse leukemia cells were exposed to PGA_2, it was demonstrated that the prostaglandin A_2-glutathione conjugate (PGA_2-SG) was formed and excreted into the medium (Parker and Ankel, 1992). On the other hand, it appears that glutathione plays an important role in the uptake and metabolism of PGA_2 (Ohno *et al.*, 1992; Ohno and Hirata, 1993), but GSH levels are not critical for the growth inhibitory effect (Ohno *et al.*, 1992).

Another component, which thus is probably a contributing factor in the metabolism of PGA_2, is glutathione S-transferase activity. As previously shown, α, β-unsaturated carbonyl compounds inhibit GSTP1-1 both reversibly and by covalent modification on the cysteine residues (Van Iersel *et al.*, 1996; Van Iersel *et al.*, 1997). This particular isoenzyme is interesting, because it has been associated with drug resistance against alkylating anticancer agents (Waxman, 1990).

Furthermore, Evers *et al.* (1997) lately demonstrated that the glutathione conjugates of prostaglandin A_2 are transported by the multidrug resistance associated protein (MRP) or GS-X pump. No stereoselectivity was observed for the transport of these glutathione conjugates by MRP.

These recent findings prompted us to further investigate the glutathione-related metabolism of prostaglandin A_2 both with purified enzymes as well as in the IGR-39 human melanoma cell line.

Materials and methods

Materials

Glutathione and NADH were obtained from Boehringer, Mannheim, Germany. Prostaglandins A_2 and J_2 were purchased from Sigma, (St, Louis, MO). 1-chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemie, (Bornem, Belgium). HPLC-grade trifluoroacetic acid was obtained from Baker (Deventer, Netherlands). HPLC-grade methanol was from Labscan, (Dublin, Ireland). DNPSG was synthesized analogously to Sokolovsky *et al.*(1964).

[^{3}H] Glycine was purchased from Amersham. GSTP1-1 was purified as previously described (Mannervik and Guthenberg, 1981). The three mutants of GSTP1-1, C47S, C101S and C47S/C101S were a generous gift from Dr. M. LoBello (Dept. of Biology, University of Rome 'Tor Vergata', Rome, Italy).

Inhibition studies with purified GSTP1-1

Incubations were performed to determine time dependent covalent inhibition of glutathione S-transferase P1-1 and its mutants. 250 µl incubation mixtures containing 0.2 M potassium phosphate pH 7.4, supplemented with 0.2 mM EDTA, 0.5 µM enzyme and 10, 25 or 250 μM prostaglandin A_2 were incubated at 25 °C. Methyl acetate was used as a solvent. At various time points during a 4 hours of incubation period GST-activity was measured according to Habig *et al.* (1974) adapted for a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA, USA), as earlier described (Van Iersel *et al.*, 1996). Because inactivation of GSTP1-1 by other α,β-unsaturated carbonyl derivatives was reversed by incubating the modified enzyme with an excess of GSH (retro Michael reaction) (Van Iersel *et al.*, 1997; Ploemen *et al.*, 1994b), the reversibility was of the binding of PGA_2 to GSTP1-1 was investigated as previously described (Van Iersel *et al.*, 1997).

Cellular exposure and cytotoxicity assay

Human melanoma cancer cells (IGR-39) were provided by the Dr Daniel den Hoed kliniek (Rotterdam, Netherlands). IGR-39 cells were cultured in RPMI 1640 medium (Gibco, Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum, 50 mg/l gentamicin, at 37 °C in a humid atmosphere containing 5% CO_2. In order to exclude cytotoxic effects during the assay for GSH conjugation, only concentrations of the prostaglandin A_2 giving at least 90% viable cells were used in the experiments. Cytotoxic effects were determined by LDH-leakage assay (Mitchell *et al.*, 1980). Approximately 50 x 10^4 cells/ml were plated onto a 24-well tissue cluster Costar, and cultured as described above for a maximum period of 24 hours. Cells were exposed for 1.5 or 4 hours in quadruplicate in Hanks balanced salt solution (HBSS without phenol red and $NaHCO_3$, obtained from Gibco), supplemented with $NaHCO_3$ to a final

concentration of 0.35 gr/l. Concentrations used were 2 , 5, 10 and 20 μM prostaglandin A_2, dissolved in methyl acetate (final concentration in the medium always ≤ 0.5%). Control incubations were included containing only the solvent. No cytotoxicity was detected with the concentrations used.

Cellular PGA_2-SG formation

The formation and transport of the prostaglandin A_2 glutathione conjugates (R- and S-form of PGA_2-SG) was studied by loading IGR-39 human melanoma cells, containing primarily GSTP1-1 and a minor amount of probably GSTM1a-1a (Van Iersel *et al.*, 1996), with [^{3}H] glycine, which is subsequently incorporated into intracellular glutathione.

In brief, cells were plated onto 25 cm^2 flasks at a density of 50 x 10^4 cells/ml and incubated at 37 °C for maximal 24 hours in 5 ml medium. The cells were loaded for 3 hours with 45 μCi [^{3}H] glycine (14.8 Ci/mmol) in supplemented medium. After 3 hours the medium was removed and the cells were washed three times with HBSS (w/o phenol red). Hereafter, cells were exposed to 10 or 20 μM PGA_2 in HBSS. At time intervals of 1 hour, 1 ml of HBSS was sampled, immediately frozen on dry ice, and stored at -20 °C until further analysis on the amount of excreted [^{3}H]-labeled glutathione conjugates. After 4 hours, cells were washed three times with HBSS and were harvested in cold PBS. Cells were frozen and stored at -20 °C until further analysis. The effect of glutathione depletion on the formation of the two PGA_2-SG diastereoisomers was studied by incubating the cells overnight with 50 μM D,L-buthionine-S,R-sulfoximine (BSO) and subsequent exposures to [^{3}H] glycine and PGA_2 was conducted in the presence of 50 μM BSO as well.

The PGA_2-SG conjugates were separated and quantitated by HPLC analysis as previously described (Bogaards *et al.*, 1997). In short, 300 μl medium was injected on a Zorbax reversed-phase C_{18} column (250 x 4.6 mm), eluted with a flow rate of 1 ml/min, with 50 mM aqueous ammonium acetate (pH 3.4)/acetonitrile (75:25, v/v) isocratically for 30 minutes, followed by a linear gradient from 25 to 50% acetonitrile in 30 minute. Radioactivity was detected using on-line radiochemical detection (Canberra Packard A500). A 0.5 ml liquid flow cell was used. As scintillation cocktail Flo Scint A (Packard Instrument, Groningen, The Netherlands) was used with a flow of 2 ml/min. The conjugates were identified by comparison of the retention times with that of the synthesized reference compounds.

GST inhibition studies in IGR-39 human melanoma cells

The inhibition of GST-activity towards the substrate CDNB in IGR-39 human melanoma cells was determined as previously described (Van Iersel *et al.*, 1996). Briefly, cells were plated onto 6-well tissue clusters, and exposed for 1 hour to 10 or 20 μM PGA_2 in HBSS (w/o phenol red). Control incubation were included with only the solvent. After 1 or 4 hours of exposure, CDNB was added in HBSS to a final concentration of 10 μM, mixed, and at four time points

(1, 5, 10 and 20 minutes) 0.2 ml aliquots were taken and mixed with 5 µl 0.04M N-acetyl-L-cysteine (NAC). Samples were immediately frozen at -20 °C until further analysis. After 20 minutes cells were harvested in PBS and immediately frozen. The medium samples and the intracellular contents were analysed on HPLC to determine DNPSG formation. Intracellular GSH concentration (Hissin and Hilf, 1976), GST activity (Habig *et al.*, 1974) and protein content (Bradford, 1976) were determined, as previously described (Van Iersel *et al.*, 1996).

The effect of glutathione depletion on the inhibition of GST activity in the IGR-39 human melanoma cells was investigated by incubating cells overnight with 50 µM BSO, after which the exposure to PGA_2 was performed in the presence of 50 µM BSO as well.

Statistical methods

Levels of significance were tested by one-way ANOVA ($p < 0.05$) and comparison between groups were made using Tukey test ($p < 0.05$).

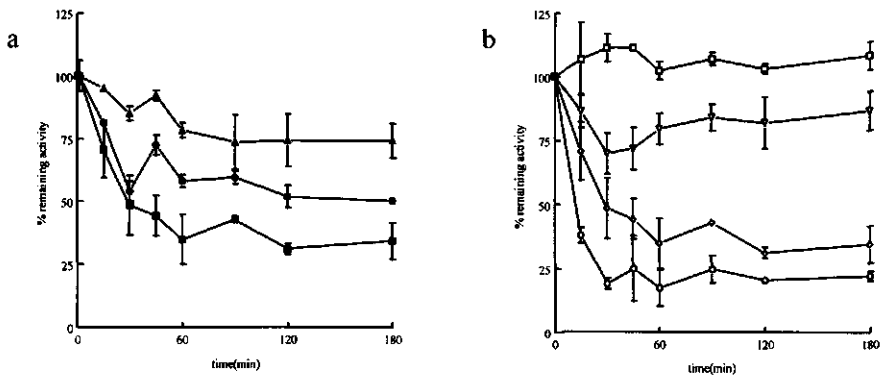

Figure 5.1 a) Inhibition of GSTP1-1 presented as % of control by various concentrations prostaglandin A_2. Symbols used are: ▲, 10 µM; ●, 25 µM; ■, 250 µM. All points are mean values ± S.E. calculated from six determinations. **b) Inhibition of GSTP1-1 and its mutants by 250 µM prostaglandin A_2.** The activity of GSTP1-1(◇), the C47S mutant (▽), the C101S mutant (○) or the double mutant C47S/C101S (□) was followed during 3 hours of incubation with 250 µM PGA_2. All points are mean values ± S.E. calculated from six determinations.

Results

Inhibition studies with purified GSTP1-1

Time-dependent inhibition of GSTP1-1 and the mutants C47S, C101S and C47S/C101S by prostaglandin A_2 was studied. In Figure 5.1a the percentage remaining GST activity is presented after incubating 10, 25 or 250 μM of the prostaglandin A_2 with GSTP1-1. Remaining activity after 3 hours was 74%, 56% and 34% respectively. Incubating these concentrations with the C47S mutant still resulted in a decrease in GST activity, however less evident then with the parent enzyme and only at the highest concentration used (250 μM). Remaining activities were 90%, 85%, and 80%. When the C101S mutant was used in the incubations, the rate of inactivation was somewhat faster, but the remaining activity after 3 hours was similar to incubations with the GSTP1-1. When both cysteine residues were mutated into a serine no inhibition at all could be observed. A typical representation of the inhibition of GST activity of GSTP1-1 and the various mutants after exposure to 250 μM PGA_2 is presented in Figure 5.1b.

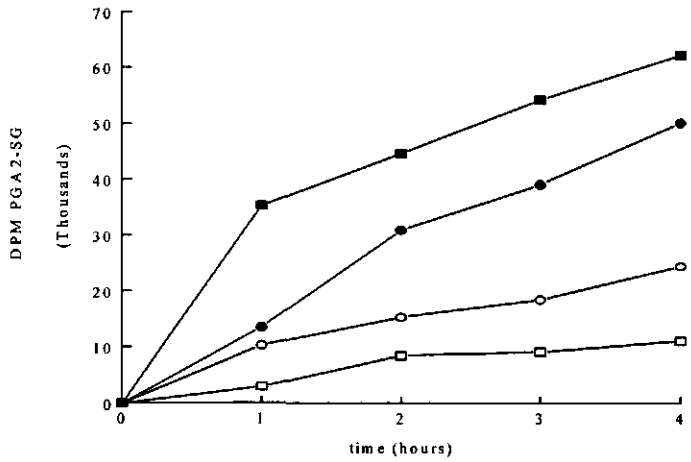

Figure 5.2 Excretion of the S and R form of the prostaglandin A_2-glutathione conjugate, presented as amount of DPM present in the medium during 4 hours. IGR-39 human melanoma cells were loaded with [3H] glycine after which 10 or 20 μM PGA was added and the amount of prostaglandin A_2-glutathione conjugate was measured in the medium. Symbols used are: ■, S-PGA_2-SG after exposure to 10 μM; □, R-PGA_2-SG after exposure to 10 μM; ●, S-PGA_2-SG after exposure to 20 μM; ○, R-PGA_2-SG after exposure to 20 μM.

In order to confirm if PGA_2 reacts with cysteine residues of GSTP1-1, the modified GSTP1-1 was incubated with 2.5 mM GSH to distinguish whether a retro-Michael cleavage occurred. The catalytic activity of GSTP1-1 towards CDNB was restored to almost 100% after 24 hours for both 25 and 250 μM PGA_2 (data not shown).

Cellular PGA_2-SG formation

After IGR-39 human melanoma cells were loaded with [^{3}H] glycine, exposure to 10 or 20 μM prostaglandin A_2 resulted in excretion of both diastereoisomers of the PGA_2-GSH conjugates for at least 4 hours. In Figure 5.2 the amount of excreted PGA_2-SG diastereoisomers into the medium is presented in DPM. Exposure to PGA_2 resulted clearly in a higher amount of the S-form in medium during these 4 hours. The ratio's S:R after 4 hours were 5.7 and 2.0 for the exposure to 10 and 20 μM PGA_2 respectively, but the total amount of excreted PGA_2-SG was similar for the two concentrations. Overnight exposure of the cells to 50 μM BSO before incubation with PGA_2, resulted in no detectable PGA_2-SG conjugate in the medium.

GST inhibition studies in IGR-39 human melanoma cells

The model substrate for GST activity CDNB was used to determine the effect of PGA_2 on the GST activity in IGR-39 human melanoma cells. To determine the effect of prostaglandin A_2 on the cellular glutathione S-transferase activity, cells were exposed to 10 or 20 μM PGA_2 for 1 hour or 4 hours. Two representative figures of the time-dependent DNPSG excretion and the effect of 1 and 4 hour exposure to PGA_2 are presented in Figure 5.3a and 5.3b, respectively. As the excretion of DNPSG was in general linear over a time span of 20 min, linear regression ($y = ax$) was used to describe the time dependent DNPSG excretion. The correlation coefficient (r^2) for the regression was in general greater than 0.95. Exposure for 1 hour to 10 or 20 μM PGA_2 thus resulted in a decrease in DNPSG excretion into the medium. However, the effect at 20 μM was almost equal to the effect at 10 μM. Exposure for 4 hours to the same concentrations resulted in a dose dependent decrease in DNPSG excretion.

As expected, overnight preincubation with 50 μM BSO resulted in no detectable DNPSG excretion into the medium both after 1 and 4 hours exposure to 10 or 20 μM PGA_2.

At the end of the total experimental exposure (1 resp.4 hours plus 20 minutes), intracellular concentration of DNPSG was measured in supernatant. As is shown in Figure 5.4a, intracellular DNPSG after 1 hour exposure to prostaglandin A_2 was significantly higher compared to control values. After 4 hours of exposure to 10 μM or 20 μM PGA_2 intracellular DNPSG was decreased dose dependently to 52% and 20% respectively.

Overnight preincubation of the IGR-39 human melanoma cells with 50 μM BSO resulted in a reduction of intracellular DNPSG to 30% for 1 hour control exposure and to around 20% for 4 hour control exposure and after subsequent exposure to PGA_2 almost no intracellular DNPSG could be detected.

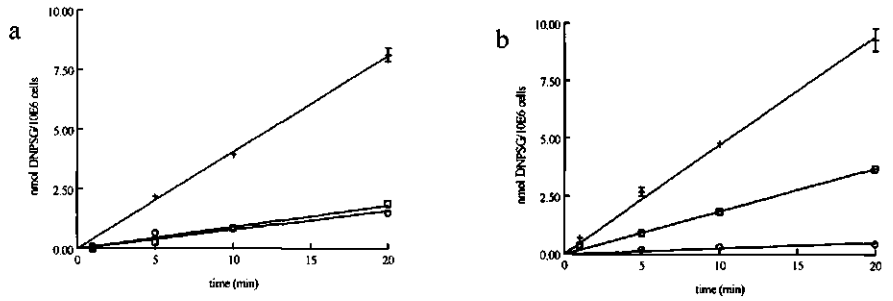

Figure 5.3 The DNPSG excretion of IGR-39 human melanoma cells after exposure to prostaglandin A_2. Cells were exposed to 10 or 20 μM PGA_2 for 1 (Figure 5.3a) and 4 hours (Figure 5.3b) respectively , and subsequently to 10 μM CDNB for 20 minutes. GSH conjugation was determined by the excretion of DNPSG into the medium. Incubations were performed in triplicate. Values are presented as nmol per 10^6 cells. Symbols used are: +, control;□, 10 μM ;○, 20 μM.

In Figure 5.4b and 5.4c the remaining intracellular GSH levels and GST activity after the total exposure period in the supernatant are shown presented as percentage of values obtained from control exposures. After 1 hour exposure to 10 or 20 μM PGA_2 GSH levels are reduced to around 80% as is the GST activity. After 4 hours PGA_2 exposure GSH levels are halved for both concentrations and GST activity decreased to 68% remaining activity after exposure to 20 μM.

When cells are incubated overnight with 50 μM BSO, GSH levels drop to about 50%, but the GST activity is not influenced. Subsequent exposure to 10 or 20 μM PGA_2 for 1 hour does not further deplete GSH levels, but after 4 hours GSH levels are further decreased to 41% of control levels. GST activity is reduced to 74 % and 54% of control values respectively after 1 hour and to 43% and 23% after exposure for 4 hours to 10 or 20 μM PGA_2 respectively.

Discussion

Prostaglandin A_2 is known to inhibit cell proliferation (Hohn *et al.*, 1979; Bregman and Meyskens, 1983; Hohn and Marnett, 1985; Bhuyan *et al.*, 1986; Santoro *et al.*, 1986) and numerous studies have been done on the metabolic fate of this prostaglandin. Recent findings

on formation and transport of the diastereoisomeric glutathione conjugates shed further light on this metabolism (Bogaards *et al.*, 1997; Evers *et al.*, 1997). The present study aimed to get a more complete picture of the glutathione-related metabolism of PGA_2 both by investigating interactions with purified enzymes and in a cellular system using the IGR-39 human melanoma cell line.

To determine whether PGA_2 can inhibit GSTP1-1 by covalent modification on the cysteine residues, PGA_2 was incubated with purified GSTP1-1 and three mutant enzymes, missing one or both cysteine residues. It was demonstrated that PGA_2 inhibits GST activity by binding to the cysteine moieties of the enzyme. After mutation of the cysteine 47 residue of GSTP1-1, the enzyme can still be inhibited by PGA_2, but only at the highest concentration (250 μM) used and to a minor extent compared to native GSTP1-1. When only cysteine 101 was mutated the extent of inactivation reached was similar to native GSTP1-1. When both cysteine 47 and 101 were mutated into a serine no inhibition of GST activity could be detected. Again these findings show that cysteine 47 is the principal target for modification by an α,β-unsaturated ketone, as was previously shown for a number of other α,β-unsaturated carbonyl compounds (Van Iersel *et al.*, 1997; Ploemen *et al.*, 1993a).

The formation of PGA_2-SG conjugates in cells was studied in the IGR-39 human melanoma cell line. In these cells, GSTP1-1 is the major GST expressed, but there is also a minor amount of probably GSTM1a-1a present (Van Iersel *et al.*, 1996). After loading these cells with [^{3}H] glycine and exposure to PGA_2, the PGA_2-SG conjugate excreted into the medium consisted for the most part of the S-diastereoisomer.

As Evers *et al.* (1997) showed that transport of the two PGA_2-SG diastereoisomers by the MRP/GS-X pump is not stereoselective, it can be assumed that the higher amount of the S-form in the medium after exposure of IGR-39 human melanoma cells to PGA_2 is the result of the preferential formation of this form by the glutathione S-transferases present in the cells. GSTP1-1 is stereoselective for the formation of the S-form of PGA_2-SG, whereas GSTM1a-1a showed no stereoselectivity (Bogaards *et al.*, 1997), so the results are in line with these findings. However, although the total amount of excreted PGA_2-SG was similar for both concentrations investigated, the ratio S:R is lower after exposure to 20 μM than after exposure to 10 μM. This might be the result of the increased contribution of the chemical reaction,whichdoes play a role in addition to the enzyme catalysed conjugation.Since GST activity is inhibited covalently to 80-68% after exposure to 20 μM PGA_2 for 1-4 hours it is plausible that the chemical reaction would become more important. On the other hand, degradation of the S-PGA_2-SG might occur extracellularly or intracellularly, or the S-form might be the preferential conjugate for further intracellular metabolism or transport to the nucleus.

Parker and Ankel (1992) ascribed the formation of only one of both PGA_2-SG

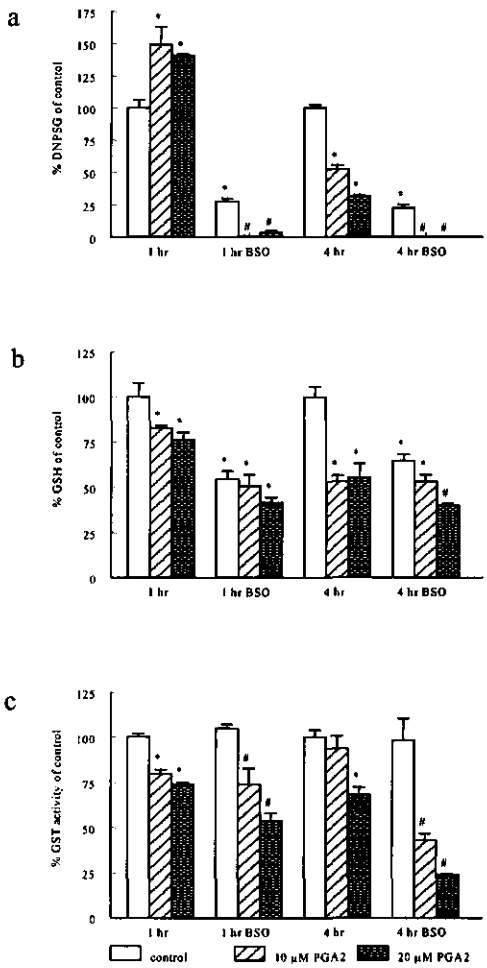

Figure 5.4 The effect of prostaglandin A_2 on intracellular DNPSG (a), GSH levels (b) and GST activity (c) in IGR-39 human melanoma cells. Cells were incubated overnight with 50 μM BSO or in medium and subsequently exposed to 10 or 20 μM PGA2 for 1 or 4 hours (with 50 μM BSO or in medium), after which CDNB was added for 20 minutes. The remaining intracellular DNPSG (a), GSH levels (b) and GST activity (c) are determined and expressed as % of control incubations. Incubations were performed in triplicate. *, significantly different from control incubations ($p < 0.05$); #, significantly different both from control incubations and from control incubations with 50 μM BSO ($p<0.05$).

diastereoisomers in L1210 mouse leukemia cells to enzymatic reduction of the other diastereoisomer. However, in view of the recent findings of Bogaards *et al.* (1997) and the results of the present study, the stereoselective formation by glutathione S-transferases is a more likely explanation. However, it should be emphasized that other metabolic pathways for the prostaglandin A_2 glutathione conjugates might also play a role.

As the total formation of GSH conjugates seems not to increase after exposure to 20 μM compared to exposure with 10 μM, the uptake of PGA_2 might be a limiting factor. The uptake of PGA_2 is mediated by a specific carrier (Narumiya and Fukushima, 1986) and GSH depletion lowers the uptake of PGA_2, although nuclear accumulation is not affected (Ohno *et al.*, 1992). After exposure of IGR-39 human melanoma cells to 20 μM PGA_2, GSH is depleted to 80% after 1 hour to down to 50% after 4 hours, which thus might influence PGA_2 uptake into the cells.

The effect of PGA_2 on GST activity and on transmembrane transport was studied using a method, developed to determine GSH-related metabolism in intact cells by measuring DNPSG excretion (Van Iersel *et al.*, 1996). In the present study, PGA_2 was demonstrated to inhibit DNPSG excretion already after 1 hour exposure to a concentration of 10 μM. However, no clear dose response relationship in excretion could be detected, as was the case for most other α,β-unsaturated carbonyl compounds (van Iersel *et al.*, 1996). Intracellular DNPSG, which was measured in supernatant after the total exposure period, is increased upon 1 hour exposure to 10 or 20 μM PGA_2 and again no dose response relationship could be detected. It appeared that only 10% of the total amount of conjugate is excreted after exposure to PGA_2 compared to 40% in control incubations. This observation and the fact that total DNPSG formation was not decreased after 1 hour incubation with PGA_2, indicates that DNPSG transport out of the cell is inhibited.

After four hours of exposure to PGA_2, the inhibition of DNPSG excretion showed a dose response effect and total DNPSG formation is significantly decreased: a drop in intracellular as well as extracellular DNPSG concentration was observed. Although exposure for four hours to PGA_2 evidently inhibits DNPSG formation, inhibition of transport still seems to play a role as well; after exposure to 20 μM PGA_2 only 12% of the total amount of conjugate formed is excreted compared to 38% and 32% after exposure to control medium and 10 μM PGA_2, respectively.

Recently, it has been demonstrated, that transport of [^{14}C]ethacrynic acid-glutathione conjugate into microsomal vesicles of yeast cells transformed with *MRP1* cDNA, is inhibited by PGA_1 and PGA_2 in the presence of GSH, in a dose dependent way. To investigate whether a glutathione conjugate was involved, both diastereoisomers of PGA_2-SG were synthesized and appeared to be good substrates for MRP and probably two other transporters (Evers *et al.*, 1997). Although it is not clear which transport pumps are present and functional in IGR-39

human melanoma cells, our results indicate that the maximum inhibition of the transport pumps is already reached at the lowest concentration used (10 μM). Assuming that the MRP/GS-X pump plays a role, exposure to this concentration probably results in an intracellular concentration above the K_m value for this pump (appr.1 μM) (Evers *et al.*, 1997). The results after 4 hours exposure indicate that the inhibition of DNPSG transport by PGA_2 is reversible as after 10 μM exposure almost no inhibition is observed anymore.

According to previous findings (Van Iersel *et al.*, 1996), inhibition of DNPSG formation can be the result of the depletion of glutathione, reversible inhibition and/or covalent modification of glutathione S-transferases. Therefore, both GSH concentration and GST activity were measured after the total exposure period in supernatant. GSH was depleted to 80% and 50% of control values after 1 and 4 hours respectively. The loss of GSH notably differs from values obtained after exposure of L1210-cells to similar concentrations of PGA_2 (Parker and Ankel, 1992). GST activity was reduced to 80% (10 μM) and 74% (20 μM) after the 1 hour exposure and to 68% at 20 μM after 4 hours. Although depletion of GSH decreases the spontaneous GSH conjugation, it is not very likely to affect GST activity, as the K_m value of GSH for GSTs is low (Mannervik, 1985; Mannervik and Danielson, 1988) compared to the normal intracellular GSH concentration (Meister, 1983a). The GST activity is inhibited by PGA_2 both by reversible inhibition and covalent modification. Thus PGA_2 covalently modifies GSTP1-1, not only as purified enzyme, but in cells as well. This was previously shown for ethacrynic acid and curcumin (Van Iersel *et al.*, 1996). Further evidence for this phenomenon is obtained when IGR-39 human melanoma cells are exposed to PGA_2 after depletion of GSH to 50% of control values by overnight incubation with BSO. These conditions resulted in a decrease in GST activity to 50% after 1 hour exposure to 20μM and down to 23% after 4 hours. Obviously, GST is covalently modified more effectively when GSH levels are below a certain protecting level.

In L-1210 cells, GSH depletion to about 17% of control values resulted in a reduction of 50% in uptake of PGA_2 (Ohno *et al.*, 1992), causing a drop in cytosolic PGA_2, but not in nuclear amounts of PGA_2 (Ohno and Hirata, 1993). Our results demonstrate, that obviously very small amounts of PGA_2 can already covalently inhibit GST activity especially when GSH is depleted. This indicates that GSTP1-1 is at least one of the cellular proteins which binds PGA_2 as suggested by Ohno and co-workers (1993). This again proves that GSTP1-1 might play a role in scavenging alkylating agents especially when GSH concentrations are low, or conversely might serve as 'storing proteins' for physiologically important compounds such as PGA_2.

The mechanisms which play a role in the inhibition of cell proliferation by cyclopentenone prostaglandins are still not clarified. Binding to cellular proteins seems to be related to inhibition of cell proliferation (Parker, 1995) as is the induction of heat shock proteins (Ohno *et al.*, 1988a; Santoro *et al.*, 1989). Furthermore, c-*myc* expression is inhibited by PGA_2, which

results in an inhibition of cell proliferation (Ishioka *et al.*, 1988). PGA_2 has apoptosis inducing properties and this might also be a way in which the compound influences cell proliferation (Kim *et al.*, 1993).

Clearly PGA_2 is transported by a cytosolic protein to the nucleus where it is most likely bound or transferred to sulfhydryl containing proteins and/or transcription factors, which can regulate cell proliferation and other cellular functions (Ohno and Hirata, 1993; Primiano *et al.*, 1997). This study indicates GSTP1-1 to be a potential candidate for a transport protein.

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Chapter 6

Summary and future perspectives

Introduction

Modulation of glutathione-related biotransformation steps may play a role in important phenomena as anticarcinogenicity and multidrug resistance. Glutathione-related biotransformation comprises three main aspects i.e. glutathione, the glutathione S-transferases and the multidrug resistance associated protein pump. In Figure 6.1 is shown how the levels and relative activities of these three entities interact.

The research presented in this thesis focused on the effects of the ubiquitous class of α,β-unsaturated carbonyl compounds on these glutathione-related processes, especially glutathione S-transferase P1-1, while a secondary aim was to provide insight in the metabolism of these compounds.

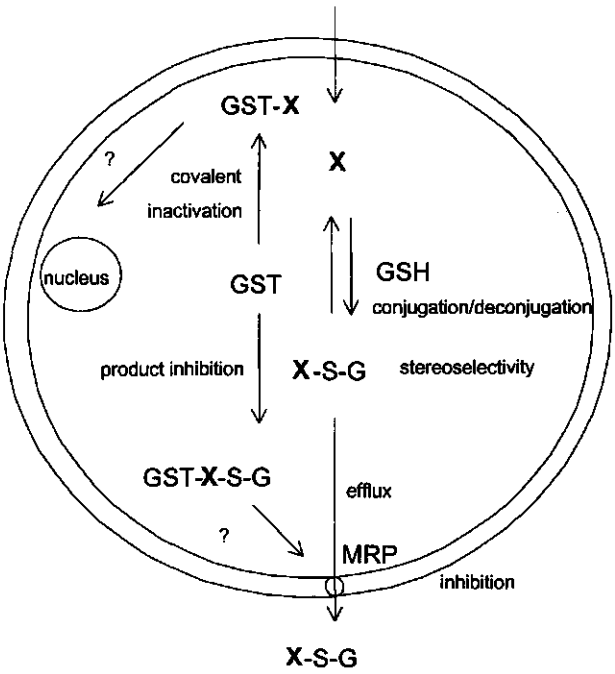

Figure 6.1 Interactions between the three aspects of the glutathione-related biotransformation system.

Firstly, studies were conducted to expand understanding of the mechanisms of both GST inhibition and glutathione conjugation.

Secondly, the effects of a series of exogenous α,β-unsaturated carbonyl compounds on the glutathione-related biotransformation were studied in a cellular system, as all three aspects are integrated in such a system and the relative importance of the various steps can be estimated.

Finally, an important endogenous α,β-unsaturated ketone, prostaglandin A_2, was selected and its metabolism and effects were studied to emphasise the significance of the glutathione-related metabolism for endogenous compounds and obtain insight into the possible role of GST inhibition in regulation of physiological processes.

Summary

To elucidate mechanistic features of the covalent interaction between α,β-unsaturated carbonyl compounds and GSTP1-1 and to study the involvement of the cysteine residues in this interaction, investigations were performed with mutants of GSTP1-1 (*chapter 2*). In these mutants cysteine 47 and/or cysteine 101 were mutated into a serine. The α,β-unsaturated carbonyl compounds, used in this study, inhibited GSTP1-1 activity, but when both cysteine residues were mutated, almost no inhibition of GSTP1-1 could be observed. Mutation of only the cysteine 47 residue already had the same effect. However, especially high concentrations of the α,β-unsaturated compounds still inhibited the double mutant GSTP1-1 to a certain extent, suggesting that lower reactivity sites in the enzyme can be modified as well. From the compounds studied, ethacrynic acid, acrolein, curcumin, and 4-hydroxy-2-nonenal were the most potent covalent inhibitors. As the compounds used are Michael acceptors, reversal of inhibition of activity by an excess of glutathione was investigated. Only for ethacrynic acid and crotonaldehyde, inhibition could be totally reversed. But inhibition by, for instance, acrolein could not be reversed; experiments using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) showed that covalent cross linking between subunits occurred by acrolein (results not shown), as was demonstrated for eugenol previously (Rompelberg *et al.*, 1996). For the other compounds used, partial restoration of GSTP1-1 activity was observed, again indicating that reactions with other amino acids or binding places play a role as well.

The results of this chapter indicate that α,β-unsaturated carbonyl derivatives inhibit GSTP1-1 irreversibly mainly by binding to the cysteine residues, especially cysteine 47. This covalent inactivation might in particular be important, when glutathione concentrations are low.

The potential relevance of stereoselective formation of glutathione conjugates of α,β-unsaturated carbonyl compounds for the actual effects of these compounds led to the investigation of the relative formation of the two diastereoisomers of model compound ethacrynic acid. Ethacrynic acid has become a thoroughly and widely studied compound, lately especially with regard to multidrug resistance (Schultz *et al.*, 1997; Shen *et al.*, 1997). Glutathione conjugation of ethacrynic acid leads to the formation of two diastereoisomers, chemically in almost equal amounts (48:52). Although it has been shown that rat GST mu did not catalyse glutathione conjugation stereoselectively (Ploemen *et al.*, 1993b), human GSTP1-1 is stereospecific for the formation of one diastereoisomer, in this thesis designated as diastereoisomer A. GSTA1-1, but not GSTA1-2 and GSTA2-2 is stereoselective for the same diastereoisomer. No significant deconjugation of the diastereoisomeric mixture or of diastereoisomer A alone could be detected, chemically or upon addition of GSTP1-1; the latter probably due to inhibition of the enzyme. Clearly the equilibrium for glutathione conjugation

of ethacrynic acid is strongly in favour of product formation. As a first step to study the role of conjugation in relation to the other glutathione-related aspects, IGR-39 human melanoma cells, containing high levels of GSTP1-1, were exposed to ethacrynic acid. Diastereoisomer A was preferentially produced in the medium. Although this has not yet been proven definitively, this was probably due to the stereoselective formation of diastereoisomer A by GSTP1-1 catalysis rather than stereoselective transport of the conjugates (*chapter 3*).

From this chapter it is clear that the chemical and enzyme catalysed equilibria for the reaction between ethacrynic acid and glutathione are strongly in favour of product formation. GSTP1-1 stereospecifically catalyses the glutathione conjugation of ethacrynic acid GSTA1-1 is stereoselective for the same diastereoisomer. Furthermore it was shown that stereoselectivity plays a role in cellular systems.

As glutathione conjugation and inhibition of GST activity have mainly been studied using cytosol or purified enzymes, a method was developed to investigate modulation of glutathione conjugation in intact IGR-39 human melanoma cells by the quantification of the excretion of S-(2,4-dinitrophenyl)glutathione (DNPSG), the glutathione conjugate of the standard substrate 1-chloro-2,4-dinitrobenzene (CDNB). By investigating intracellular glutathione levels, GST activity and intra- and extracellular DNPSG concentration, some determinants involved in the mechanisms of inhibition of DNPSG excretion could be identified (*chapter 4*). These mechanisms include depletion of glutathione levels, reversible and irreversible inhibition of glutathione S-transferase activity, and modulation of the efflux of glutathione conjugates by an effect on the multidrug resistance associated protein (MRP) pump.

Using this assay, a series of α,β-unsaturated carbonyl compounds were tested for their inhibiting properties of DNPSG excretion. Curcumin, an antioxidant and anticarcinogenic compound, was the most potent inhibitor of DNPSG excretion in these cells, followed by ethacrynic acid. Citral did not show any effect of DNPSG excretion up to 100 μM and acrolein was too toxic to get any effect (*chapter 4*). The mechanisms of inhibition differed between the various α,β-unsaturated carbonyl compounds. For curcumin and ethacrynic acid, glutathione depletion, reversible inhibition of GSTs and covalent modification of GSTP1-1 all three play a role in the inhibition of DNPSG excretion. However for *trans*-2-hexenal and cinnamaldehyde, reversible GST inhibition seems to be the major determinant for its effect. Crotonaldehyde mainly inhibits DNPSG excretion by depleting glutathione, but reversible inhibition does presumably plays a role as well. Curcumin and ethacrynic acid also inhibit the efflux of DNPSG by an effect on the transport of the glutathione conjugate out of the cells, probably by the multidrug resistance associated protein (MRP) pump. Indeed, it has been shown that the glutathione conjugate of EA has an equal efficiency for transport by the MRP pump compared to DNPSG (Zaman *et al.*, 1996).

Some of the α,β-unsaturated carbonyl compounds, used in this study, thus inhibit GST avtivity in human melanoma cells. They modulate the glutathione related biotransformation system in these cells in different ways, i.e. glutathione depletion, reversible and irreversible inhibition of GST activity and/or influence on the efflux of glutathione conjugates.

For the endogenous α,β-unsaturated ketone prostaglandin A_2 a more complete picture of its metabolism and the influence on glutathione-related biotransformation could be obtained, as it was also possible to analyse PGA_2-glutathione conjugate excretion into the medium during exposure of the cells to PGA_2. After loading IGR-39 human melanoma cells with [^{3}H] glycine and subsequent exposure to PGA_2, both diastereoisomers of the PGA_2-glutathione conjugate are excreted into the medium, however with a clear excess of the S-form. Previous work indicates that this is the result of the preferential formation of the S-form by GSTP1-1 that is present in the cells and not from a stereoselectivity in transport (Bogaards *et al.*, 1997; Evers *et al.*, 1997). Incubation of IGR-39 human melanoma cells with PGA_2 during 1 or 4 hours clearly influenced the glutathione-related metabolism. After 1 hour exposure, DNPSG excretion was reduced mainly due to inhibition of the efflux of the conjugate. Indeed, it has recently been shown that the glutathione conjugates of PGA_2 have a higher affinity for MRP compared to DNPSG (Evers *et al.*, 1997). After 4 hours, total DNPSG formation was reduced markedly, resulting from depletion of glutathione and reversible and irreversible inhibition of GSTs; inhibition of efflux then only played a minor role. Although irreversible inhibition already accounted for about 25% of the GST inhibition by PGA_2, depletion of intracellular GSH with BSO resulted in an even higher level of covalent inactivation. Experiments with purified GSTP1-1 and mutants missing one or two cysteine residues, revealed that this covalent inhibition of GSTP1-1 resulted from the binding of PGA_2 to mainly the cysteine 47 moiety of the enzyme. This inactivation could be totally reversed by an excess of glutathione, indicative of a retro-Michael reaction (*chapter 5*).

The results of this chapter again prove that GSTP1-1 might play a role in scavenging alkylating agents especially when GSH concentrations are low, or conversely might serve as a storing or transport protein for physiologically important compounds such as PGA_2.

Perspectives

Both endogenous and exogenous α,β-unsaturated carbonyl compounds thus appear to influence several aspects of the glutathione-related biotransformation system. They are conjugated to glutathione, thereby depleting glutathione and thus influencing the redox status of the cells, which plays a role in regulation of Phase II enzymes (Talalay *et al.*, 1995; Primiano *et al.*, 1997). These conjugates usually are less toxic than their parent compounds, but it is also

possible that they undergo retro-Michael cleavage, releasing the reactive compound under different circumstances, then also influencing the redox status. The glutathione conjugates themselves are inhibitors of GST activity (product binding) and probably by binding the enzyme, they are transported to efflux pumps such as MRP. Stereoselectivity in the formation of GSH conjugates might influence function and toxicity of α,β-unsaturated carbonyl compounds. Future studies should be performed to further elucidate the conjugation mechanism and the relevance of stereoselectivity *in vivo*. One can only speculate about the physiological importance of this phenomenon. Studies with the recently developed GST pi knock-out mice (Henderson *et al.*, 1996) and MRP knock-out mice (Wijnholds *et al.*, 1997) could give some useful information. α,β-Unsaturated carbonyl compounds can inhibit GST activity both by competitive inhibition on the active site as well as covalent inactivation on the cysteine residues of the enzyme. This covalent binding is reversible due to retro-Michael reaction and most likely constitutes a functional role as well.

Man is exposed to substantial amounts of these α,β-unsaturated carbonyl compounds every day, dependent on life style factors, such as diet, smoking, contact with traffic exhaust. Therefore these findings are of considerable relevance. Especially when considering the total daily exposure to the various α,β-unsaturated carbonyl compounds, concentrations reached in the body might equal the concentrations used in this study. For instance: acrolein is present in wine up to about 3.8 ppm (70 μM) (Feron *et al.*,1991); curcumin, the major component of the spice curry, is widely used and consumption for adult Indians is estimated on about 125 mg/day (Opdyke and Letizia, 1983); cinnamaldehyde is present in food up to 700ppm (4.7 mM) (Feron *et al.*, 1991); adding the additional endogenously produced α,β-unsaturated carbonyl compounds, the combined exposure very likely influences the glutathione-related biotransformation system.

The fact that endogenous α,β-unsaturated carbonyl compounds as 4-hydroxy-2-nonenal, *trans*-2-hexenal and prostaglandin A_2 are good covalent inhibitors of GSTP1-1 and that covalent modification occurs intracellularly, supports the assumption, that GSTP1-1 might not only play a role in glutathione conjugation but also has other cellular functions. In this respect one can think of GSTP1-1 as a transport or storage protein for endogenous compounds and/or as a general intracellular scavenging protein for electrophilic agents.

The suggestion that GSTP1-1 might function as a storage for endogenous compounds, is a commonly accepted function of GSTs in general (Listowsky, 1988). GSTP1-1 is known to have a hydrophobic pocket, which binds fatty acids (Nishihira *et al.*, 1992). Experiments with fatty acids and human GSTP1-1 revealed that linolenic acid is capable of inhibiting GSTP1-1 activity thereby not affecting covalent modification of GSTP1-1 by ethacrynic acid (unpublished results). This means that it should be possible to bind covalently modified GSTP1-1 on the fatty acid binding site. Interesting possibilities arise when this would be

possible with regard to biomonitoring exposure to electrophilic compounds as GSTP1-1 is a major GST present in erythrocytes.

Closely linked to this storage function, is the possible function of GSTP1-1 as a transport protein. As indicated for prostaglandin A_2 in *chapter 5*, GSTP1-1 can transport this compound intracellularly to the nucleus by binding it. Localization of GSTP1-1 in human tissue, using immunohistochemical techniques, indeed show the presence of this isoenzyme in the nucleus (Terrier *et al.*, 1990). Compounds that are delivered in the nucleus can for instance change thiols, from GST to a transcription factor or other protein and accordingly trigger all sorts of events. It becomes now more and more accepted that genes, involved in protection against carcinogens are regulated by the redox status of cells (Talalay *et al.*, 1995; Primiano *et al.*, 1997; Itoh *et al.*, 1997). Another aspect in the role of GSTP1-1 as a transport protein is the capability of the enzyme to bind products. For instance the glutathione conjugate of ethacrynic acid is an even better inhibitor of GSTP1-1 than the parent compound. One could clearly think of a role of GSTP1-1 in transporting glutathione conjugates from the site of formation to efflux pumps in the plasma membrane, for instance MRP.

The third notion, that GSTP1-1 might function as a general scavenging protein, might especially be apparent when glutathione levels are low. Lipid peroxidation products as HNE and reactive oxygen species can thus be neutralized, but also other electrophilic compounds. The inactivation of GSTP1-1 by 4-hydroxy-2-nonenal (HNE) and the only partial recovery of activity after incubation with a molar excess of glutathione (*chapter 2*) are in line with previous findings with H_2O_2 (Sluis-Cremer *et al.*, 1996). The ability of other α,β-unsaturated carbonyl compounds to inactivate purified GSTP1-1 as well as GSTP1-1 in cells (*chapter 2, 4 and 5*), together with previous results (Berhane and Mannervik, 1990; Terada *et al.*, 1995) also support a general scavenging role of GSTP1-1.

The significance of MRP in the maintenance of intracellular concentrations of both functional and toxic or carcinogenic agents is under current investigation. However, α,β-unsaturated carbonyl compounds may influence its transport activity, for a start by depletion of glutathione, which seems to be essential for MRP. Furthermore, as the glutathione conjugates of both EA and PGA_2 are substrates, the glutathione conjugates of other α,β-unsaturated carbonyl derivates might be substrates as well. Future research should focus on structure activity relationships for MRP substrates. The importance of stereoselectivity in the transport of these conjugates by MRP also merits further investigation (Evers *et al.*, 1997, Loe *et al.*, 1997).

Conclusion

In conclusion, the results in this thesis demonstrate for the first time that GST activity is inhibited in cells exposed to α,β-unsaturated carbonyl compounds. It also became clear that GST activity should not be studied on its own, but, as it is a part of a glutathione-mediated biotransformation system, it should be investigated in conjunction with glutathione levels and the multidrug resistance associated protein (MRP). Moreover, the apparent involvement of GSTP1-1 in the metabolism of the endogenous compound prostaglandin A_2, indicates a possible role of this isoenzyme in regulation of cell proliferation. Most α,β-unsaturated carbonyl compounds, studied in this thesis, interact with the glutathione-related biotransformation system (i.e. glutathione conjugation, glutathione depletion, both reversible and irreversible inhibition of GST activity, modulation of MRP); some with three aspects, some only with one or two. In view of the multiple roles of this system in cellular physiology, cell proliferation, gene regulation, anticarcinogenicity and multidrug resistance, α,β-unsaturated carbonyl compounds indeed seem very important, especially as man is exposed to this class of compounds in everyday life. The results open further perspectives for the development of therapeutic agents regarding multidrug resistance and anticarcinogenicity. The potential effect of these compounds on vital processes emphasise the need for future research on the total exposure of people to these compounds, especially via diet and environment.

References

Adang, A.E.P., Brussee, J., Van der Gen, A., and Mulder, G.J. (1990) The glutathione-binding site in glutathione S-transferases. Investigation of the cysteinyl, glycyl and gamma-glutamyl domains. *Biochem. J.* **269**, 47-54.

Ahokas, J.T., Nicholls, F.A., Ravenscroft, P.J., and Emmerson, B.T. (1985) Inhibition of purified rat liver glutathione S-transferase isoenzymes by diuretic drugs. *Biochem. Pharmacol.* **34**, 2157-2161.

Akerboom, T.P.M., Narayanaswami, V., Kunst, M., and Sies, H. (1991) ATP-dependent S-(2,4-dinitrophenyl)glutathione transport in canalicular plasma membrane vesicles from rat liver. *J. Biol. Chem.* **266**, 13147-13152.

Ali-Osman, F., Akande, O., Antoun, G., Mao, J., and Buolamwini, J. (1997) Molecular cloning, characterization, and expression in Escherichia coli of full-length cDNAs of three human glutathione S-transferase pi gene variants. *J.Biol.Chem.* **272**, 10004-10012.

Ålin, P., Jensson, H., Guthenberg, C., Danielson, U.H., Tahir, M.K., and Mannervik, B. (1985) Purification of major basic glutathione transferase isoenzymes from rat liver by the use of affinity chromatography and fast liquid chromatofocusing. *Anal. Biochem.* **146**, 313-320.

Alper, T. (1956) The modification of damage caused by primary ionization of biological targets. *Radiat. Res.* **5**, 573-586.

Armstrong, R.N. (1991) Glutathione S-transferases: reaction mechanism, structure, and function. *Chem. Res. Toxicol.* **4**, 131-140.

Armstrong, R.N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem. Res. Toxicol.* **10**, 2-18.

Arrick, B.A. and Nathan, C.F. (1984) Glutathione metabolism as a determinant of therapeutic efficacy: a review. *Cancer Res.* **44**, 4224-4232.

Awasthi, S., Srivastava, S.K., Ahmad, F., Ahmad, H., and Ansari, G.A.S. (1993) Interactions of glutathione S-transferase pi with ethacrynic acid and its glutathione conjugate. *Biochim. Biophys. Acta* **1164**, 173-178.

Awasthi, S., Singhal, S.S., He, N., Chaubey, M., Zimniak, P., Srivasava, S.K., Singh, S.V., and Awasthi, Y.C. (1996) Modulation of doxorubicin cytotoxicity by ethacrynic acid. *Int. J. Cancer* **68**, 333-339.

Baillie, T.A. and Slatter, J.G. (1991) Glutathione: A vehicle for the transport of chemically reactive metabolites in vivo. *Acc. Chem. Res.* **24**, 264-270.

Bates, S.E., Regis, J.I., Robey, R.W., Zhan, Z., Scala, S., and Meadows, B.J. (1994) Chemoresistance in the clinic: overview 1994. *Bull.Cancer* **9**, Suppl 2, 55s-61s.

Berhane, K. and Mannervik B. (1990) Inactivation of the genotoxic aldehyde acrolein by human glutathione transferases of classes alpha, mu, and pi. *Mol. Pharmacol.* **37**, 251-254.

Berhane, K., Widersten, M., Engstrom, A., Kozarich, J.W., and Mannervik, B. (1994) Detoxication of base propenals and other α, ß-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. *Proc. Natl. Acad. Sci. USA* **91**, 1480-1484.

Bhuyan, B.K., Adams, E.G., Badiner, G.J., Li, L.H., and Barden, K. (1986) Cell cycle effects of prostaglandin A_1, A_2 and D_2 in human and murine melanoma cells in culture. *Cancer Res.* **46**, 1688-1693.

Black, S.M. and Wolf, C.R. (1991) The role of glutathione-dependent enzymes in drug resistance. *Pharmacol. Therap.* **51**, 139-154.

Boehlert, C.C. and Armstrong, R.N. (1984) Investigation of the kinetic and stereochemical recognition of arene and azarene oxides by isoenzymes A_2 and C_2 of glutathione S-transferase. *Biochem. Biophys. Res. Comm.* **121**, 980-986.

Bogaards, J.J.P., Van Ommen, B., and Van Bladeren, P.J. (1989) An improved method for the separation and quantification of glutathione S-transferase subunits in rat tissue using high-performance liquid chromatography. *J. Chromatogr.* **474**, 435-440.

Bogaards, J.J.P., Venekamp, J.C. and Van Bladeren, P.J. (1997) Stereoselective conjugation of prostaglandin A_2 and prostaglandin J_2 with glutathione, catalyzed by the human glutathione S-transferases A1-1, A2-2, M1a-1a and P1-1. *Chem. Res.Toxicol.* **10**, 310-317.

Boyland, E. and Chasseaud, L.F. (1967) Enzyme-catalysed conjugation of glutathione with unsaturated compounds. *Biochem. J.* **104**, 95-102.

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brambilla, G., Sciabà, L., Faggin, P., Maura, A., Marinari, U.M., Ferro, M., and Esterbauer, H. (1986) Cytotoxicity, DNA fragmentation and sister-chromatid exchange in Chinese hamster ovary cells exposed to the lipid peroxidation product 4-hydroxynonenal and homologous aldehydes. *Mutat. Res.* **171**, 169-176.

Bregman, M.D. and Meyskens, F.L. (1983) Inhibition of human malignant melanoma colony-forming cells *in vitro* by prostaglandin A_1. *Cancer Res.* **43**, 1642-1645.

Bruggeman, I.M., Temmink, J.H.M., and Van Bladeren P.J. (1986) Glutathione- and cysteine-mediated cytotoxicity of allyl and benzyl isothiocyanate. *Toxicol. Appl. Pharmacol.* **83**, 349-359.

Caccuri, A.M., Petruzzelli, R., Polizio, F., Federici, G., and Desideri, A. (1992) Inhibition of glutathione transferase pi from human placenta by 1-chloro-2,4-dinitrobenzene occurs because of covalent reaction with cysteine 47. *Arch. Biochem. Biophys.* **297**, 119-122.

Cagen, L.M., Pisano, J.J., Ketley, J.N., Habig, W.H., and Jakoby, W.B. (1975) The conjugation of prostaglandin A1 and glutathione catalyzed by homogeneous glutathione S-transferases from human and rat liver. *Biochim. Biophys. Acta* **298**, 205-208.

Cameron, A.D., Sinning, I., L'Hermite, G., Olin, B., Board, P.G., Mannervik, B., and Jones, T.A. (1995) Structural analysis of human alpha-class glutathione transferase A1-1 in the apo-form and in complexes with ethacrynic acid and its glutathione conjugate. *Structure* **3**, 717-727.

Chasseaud, L.F. (1979) The role of glutathione and glutathione S-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.* **29**, 175-273.

Chen, G. and Waxman, D.J. (1994) Role of cellular glutathione and glutathione S-transferase in the expression of alkylating agent cytotoxicity in human breast cancer cells. *Biochem. Pharmacol.* **47**, 1079-1087.

Chen, J. and Armstrong, R.N. (1995) Stereoselective catalysis of a retro-Micheal reaction by class mu glutathione transferases. Consequences for the internal distribution of products in the active site. *Chem. Res. Toxicol.* **8**, 580-585.

Chien, C.I., Kirollos, K.S., Linderman, R.J., and Dauterman, W.C. (1994) α,β-Unsaturated carbonyl compounds: inhibition of rat liver glutathione S-transferase isozymes and chemical reaction with reduced glutathione. *Biochim. Biophys. Acta* **1204**, 175-180.

Ciaccio, P.J., Tew, K.D., and LaCreta, F.P. (1991) Enzymatic conjugation of chlorambucil with glutathione by human glutathione S-transferases and inhibition by ethacrynic acid. *Biochem. Pharmacol.* **42**, 1504-1507.

Ciaccio, P.J., Shen, H., Kruh, G.D., and Tew, K.D. (1996) Effects of chronic ethacrynic acid exposure on glutathione conjugation and MRP expression in human colon tumor cells. *Biochem. Biophys. Res. Comm.* **222**, 111-115.

Ciaccio, P.J. and Tew, K.D. (1996) Adaptive response to glutathione S-transferase inhibitors. *British J.Cancer* **74**, S93-S98.

Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M.V., and Deeley, R.G. (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**, 1650-1654.

Coles, B. and Ketterer, B. (1990) The role of glutathione and glutathione transferases in chemical carcinogenesis. *Crit.Rev.Biochem.Molec.Biol.* **25**, 47-70

Commandeur, J.N.M., Stijntjes, G.J., Vermeulen, N.P.E. (1995) Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. *Pharmacol. Rev.* **47**, 271-330.

Commandeur, J.N.M. and Vermeulen, N.P.E. (1996) Cytotoxicity and chemoprotective activities of natural compounds. The case of curcumin. *Xenobiotica* **26**, 667-680.

Cooper, K.O., Witz, G., and Witmer, C. (1992) The effects of α, ß-unsaturated aldehydes on hepatic thiols and thiol-containing enzymes. *Fundam. Appl. Toxicol.* **19**, 343-349.

Desideri, A., Caccuri, A.M., Polizio, F., Bastoni, R., and Federici, G. (1991) Electron paramagnetic resonance identification of a highly reactive thiol group in the proximity of the catalytic site of human placenta glutathione transferase. *J. Biol. Chem.* **266**, 2063-2066.

Diliberto, J.J., Srinivas, P., Overstreet, D., Usha, G., Burka, L.T., and Birnbaum, L.S. (1990) Metabolism of citral, an α,β-unsaturated aldehyde, in male F344 rats. *Drug metabolism and Disposition* **18**, 866-875.

Dirr, H., Reinemer, P., and Huber, R. (1994) X-ray crystal structures of cytosolic glutathione S-transferases. Implications for protein architecture, substrate recognition and catalytic function. *Eur. J. Biochem.* **220**, 645-661.

Dirven, H.A.A.M., Van Ommen, B., and Van Bladeren, P.J. (1994) Involvement of human glutathione S-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. *Cancer Res.* **54**, 6215-6220.

Dirven, H.A.A.M., Dictus, E.L.J.T., Broeders, N.L.H.L., Van Ommen, B., and Van Bladeren, P.J. (1995) The role of human glutathione S-transferase isoenzymes in the formation of glutathione conjugates of the alkylating cytostatic drug thiotepa. *Cancer Res.* **55**, 1701-1706.

Dittberner, U., Eisenbrand, G., and Zankl, H. (1995) Genotoxic effects of the α, ß-unsaturated aldehydes 2-*trans*-butenal, 2-*trans*-hexenal and 2-*trans*,6-*cis*-nonadienal. *Mutat. Res.* **335**, 259-265.

Dohn, D.R., Quebbeman, A.J., Borch, R.F., and Anders, M.W. (1985) Enzymatic reaction of chlorotrifluoroethene with glutathione: ^{19}F NMR evidence for stereochemical control of the reaction. *Biochemistry* **24**, 5137-5143.

Dulik, D.M., Fenselau, C., and Hilton, J. (1986) Characterization of melphalan-glutathione adducts whose formation is catalysed by glutathione transferases. *Biochem. Pharmacol.* **35**, 3405-3409.

Eder, E., Scheckenbach, S., Deininger, C., and Hoffman, C. (1993) The possible role of α, ß-unsaturated carbonyl compounds in mutagenesis and carcinogenesis. *Toxicol. Lett.* **67**, 87-103.

Esterbauer, H., Zollner, H., and Scholz, N. (1975) Reaction of glutathione with conjugated carbonyls. *Z. Naturforsch.* **30c**, 466-473.

Esterbauer, H., Schaur, R.J., and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. & Med.* **11**, 81-128.

Evers, R., Cnubben, N.H.P., Wijnholds, J., Van Deemter, L., Van Bladeren P.J., and Borst P. (1997) Transport of glutathione prostaglandin A conjugates by the multidrug resistance protein 1. *FEBS Letters* **419**, 112-116.

Feron, V.J., Til, H.P., De Vrijer, F., Woutersen, R.A., Cassee, F.R., and Van Bladeren, P.J. (1991) Aldehydes: occurrence, carcinogenic potential, mechanism of action and risk assessment. *Mutat. Res.* **259**, 363-385.

Flatgaard, J.E., Bauer, K.E., and Kauvar, L.M. (1993) Isozyme specificity of novel glutathione-S-transferase inhibitors. *Cancer Chemother. Pharmacol.* **33**, 63-70.

Garcia-Saez, I., Parraga, A., Phillips, M.F., Mantle, T.J., and Coll, M. (1994) Molecular structure at 1.8 Å of mouse liver class pi glutathione S-transferase complexed with S-(p-nitrobenzyl)glutathione and other inhibitors. *J. Mol. Biol.* **237**, 298-314.

Griffith, O.W. (1982) Buthionine sulfoximine and its higher homologs. *J. Biol. Chem.* **257**, 13704-13712.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974) Glutathione S-transferases, the first step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130-7139.

Hansson, J., Berhane, K., Castro, V.M., Jungnelius, U., Mannervik, B., and Ringborg U. (1991) Sensitization of human melanoma cells to the cytotoxic effect of melphalan by the glutathione transferase inhibitor ethacrynic acid. *Cancer Res.* **51**, 94-98.

Harries, L.W., Stubbins, M.J., Forman, D., Howard, G.C.W., and Wolf, C.R. (1997) Identification of genetic polymorphisms at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis* **18**, 641-644..

Hayes, J.D. and Wolf, C.R. (1990) Molecular mechanisms of drug resistance. *Biochem. J.* **272**, 281-295.

Hayes, J.D. and Pulford, D.J. (1995) The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit.Rev.Biochem.Molec.Biol.* **30**, 445-600.

Hayes, P.C., Harrison, D.J., Bouchier, I.A.D., McLellan, L.I., and Hayes, J.D. (1989) Cytosolic and microsomal glutathione S-transferase isoenzymes in normal human liver and intestinal epithelium. *Gut* **30**, 854-859.

Henderson, C.J., Bacon, E.J., Smith, A.G., and Wolf, C.R. (1996) Abstract P175, XIth International Symposium on microsomes and drug oxidations, 21-24 july, Los Angeles.

Hissin, P.J. and Hilf, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74**, 214-226.

Hohn, K.V., Dunn, J.R., Morgan, L.R., Bienkowski, M., and Marnett, L.J. (1979) Inhibition of DNA synthesis in harding-passey melanoma cells by prostaglandin A_1 and A_2: comparison with chemotherapeutic agents. *Biochem. Biophys. Res. Comm.* **87**, 795-801.

Hohn, K.V. and Marnett, L.J. (1985) Requirement of a reactive α,β-unsaturated carbonyl for inhibition of tumor growth and induction of differentiation by 'A' series prostaglandins. *Biochem. Biophys. Res. Comm.* **129**, 34-40.

Hu, X., O'Donnell, R., Srivastava, S.K., Xia, H., Zimniak, P., Nanduri, B., Bleicher, R.J., Awasthi, S., Awasthi, Y.C., Ji, X., and Singh, S.V. (1997) Active site architecture of polymorphic forms of human glutathione S-transferase P1-1 accounts for their enantioselectivity and disparate activity in the glutathione conjugation of 7β, 8α-dihydroxy-9α, 10α-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene. *Biochem.Biophys.Res.Comm.* **235**, 424-428.

Huang, H.C., Jan, T.R., and Yeh, S.F. (1992) Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. *Eur. J. Pharm.* **221**, 381-384.

Ishikawa, T. (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem. Sciences. **17** 463-468.

Ishikawa, T., Akimaru, K., Kuo, M.T., Priebe, W., and Suzuki, M. (1995) How does the MRP/GS-X pump export doxorubicin? *J. Natl. Cancer Inst.* **87**, 1639-1640.

Ishioka, C., Kanamaru, R., Sato, T., Dei, T., Konishi, Y., Asamura, M., and Wakui, A. (1988) Inhibitory effects of prostaglandin A_2 on c-*myc* expression and cell cycle progression in human leukemia cell line HL-60. *Cancer Res.* **48**, 2813-2818.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf/small Maf heterodimer mediates the induction of Phase II detoxifying enzyme genes through antioxidant response elements. *Biochem.Biophys.Res.Comm.* **236**, 313-322.

Jaffe, B.M. and Santoro, M.G. (1977) Prostaglandins and cancer. *Prostaglandins* **3**, 329-351.

Jakoby, W.B. (1978) The glutathione S-transferases: a group of multifunctional detoxification proteins. *Adv. Enzymol. Rel. Areas Mol. Biol.* **46**, 383-414.

Jansen, P.L.M. and Oude Elferink, R.P.J. (1993) In: Tavoloni, N. and Berk, P.D. (Eds.), Hepatic transport and bile secretion: physiology and pathophysiology, Raven Press, New York, 721-731.

Jedlitschky, G., Leier, I., Buchholz, U., Center, M., and Keppler, D. (1994) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res.* **54**, 4833-4836.

Jedlitschky, G., Leier, I., Buchholz, U., Barouin, K., Kurz, G., and Keppler, D. (1996) Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res.* **56**, 988-994.

Jungnelius, U., Hao, X., Skog, S., Castro, V.M., Mannervik, B., and Ringborg, U. (1994) Cell cycle dependent sensitivity of human melanoma cells to melphalan is correlated with the activity and cellular concentration of glutathione transferases. *Carcinogenesis* **15**, 99-103.

Kashiwada, M., Kitada, M., Shimada, T., Itahashi, K., Sato, K., and Kamataki, T. (1991) Purification and characterization of acidic form of glutathione S-transferase in human fetal livers: High similarity to placental form. *J. Biochem.* **110**, 743-747.

Kato, T., Fukushima, M., Kurozumi, S., and Noyori, R. (1986) Antitumor activity of Δ^7-prostaglandin A_1 and Δ^{12}-prostaglandin J_2 in vitro and in vivo. *Cancer Res.* **46**, 3538-3542.

Keppler, D. (1992) Leukotrienes: biosynthesis, transport, inactivation, and analysis. *Rev.Physiol.Biochem.Pharmacol.* **121**, 2-30.

Kim, I-K., Lee, J-H., Sohn, H-W., Kim, H-S., and Kim, S-H. (1993) Prostaglandin A_2 and Δ^{12}-prostaglandin J_2 induce apoptosis in L1210 cells. *FEBS Letters* **321**, 209-214.

Kobayashi, K., Sogame, Y., Hara, H., and Hayashi, K. (1985) Mechanism of glutathione S-conjugates transport in canalicular and basolateral rat liver plasma membranes. *J. Biol. Chem.* **265**, 7737-7741.

Koechel, D.A. and Cafruny, E.J. (1973) Synthesis and structure-activity relationship of some thiol adducts of ethacrynic acid. *J.Med.Chem.* **16**, 1147-1152.

Kong, K.H., Inoue, H., and Takahashi, K. (1991) Non-essentiality of cysteine and histidine residues for the activity of human class pi glutathione S-transferase. *Biochem. Biophys. Res. Comm.* **181**, 748-755.

Kubal, G., Meyer, D.J., Norman, R.E., and Sadler, P.J. (1995) Investigations of glutathione conjugation in vitro by 1H NMR spectroscopy. Uncatalysed and glutathione transferase-catalysed reactions. *Chem. Res. Toxicol.* **8**, 780-791.

Kubo, Y. and Armstrong, R.N. (1989) Observation of a substituent effect on the stereoselectivity of glutathione S-transferase toward para-substituted 4-phenyl-3-buten-2-ones. *Chem. Res. Toxicol.* **2**, 144-145.

Kuzmich, S., Vanderveer, L.A., Walsh, E.S., LaCreta, F.P., and Tew, K.D. (1992) Increased levels of glutathione S-transferase π transcript as a mechanism of resistance to ethacrynic acid. *Biochem. J.* **281**, 219-224.

Laing. N.M. and Tew, K.D. (1997) Drug resistance to chemotherapy: mechanisms. In: Bertine, J.R. (Ed.), Encyclopedia of cancer, volume 1, Academic Press, New York, 560-570.

Lamotte, P.J., Campsteyn, H., Dupont, L., and Vermeire, M. (1978) Acide ethacrynique. *Acta Crystallogr.* **B34**, 2636-2638.

Lautier, D., Canitrot, Y., Deeley, R.G., and Cole, S.P.C. (1996) Multidrug resistance mediated by the multidrug resistance protein(MRP) gene. *Biochem.Pharmacol.* **52**, 967-977.

Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P.C., Deeley, R.G., and Keppler, D. (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J. Biol. Chem.* **269**, 27807-27810.

Linderman, R.J., Jamois, E.A., and Tennyson, S.D. (1994) Synthesis and analysis of thiol additions to β-alkyl-α,β-unsaturated trifluoromethyl ketones. *J. Org. Chem.* **59**, 957-962.

Lindwall, G. and Boyer, T.D. (1987) Excretion of glutathione conjugates by primary cultured rat hepatocytes. *J. Biol. Chem.* **262**, 5151-5158.

Listowsky, I., Abramovitz, M., Homma, H., and Niitsu, Y. (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione-S-transferases. *Drug Metabol. Rev.* **19**, 305-318.

LoBello, M., Petruzzelli, R., De Stefano, E., Tenedini, C., Barra, D., and Federici, G. (1990) Identification of a highly reactive sulphydryl group in human placental glutathione transferase by a site-directed fluorescent reagent. *FEBS Lett.* **263**, 389-391.

LoBello, M., Battistoni, A., Mazzetti, A.P., Board, P.G., Marumatsu, M., Federici, G., and Ricci, G. (1995) Site-directed mutagenesis of human glutathione transferase P1-1. *J. Biol. Chem.* **270**, 1249-1253.

LoBello, M., Oakley, A.J., Battistoni, A., Mazzetti, A.P., Nuccetelli, M. Mazzarese, G., Rossjohn, J., Parker, M.W., and Ricci, G. (1997) Multifunctional role of Tyr 108 in the catalytic mechanism of human glutathione transferase P1-1. Crystallographic and kinetic studies on the Y108F mutant enzyme. *Biochemistry* **36**, 6207-6217.

Loe, D.W., Almquist, K.C., Deeley, R.G., and Cole, S.P.C. (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J. Biol. Chem.* **271**, 9675-9682.

Loe, D.W., Stewart, R.K., Massey, T.E., Deeley, R.G., and Cole, S.P.C. (1997) ATP-dependent transport of aflatoxin B-1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. *Mol. Pharmacol.* **51**, 1034-1041.

Lyttle, M.H., Satyam, A., Hocker, M.D., Bauer, K.E., Caldwell, C.G., Hui, H.C., Morgan, A.S., Mergia, A., and Kauvar, L.M. (1994) Glutathione S-transferase activates novel alkylating agents. *J. Med. Chem.* **37**, 1501-1507.

Mannervik, B. and Guthenberg, C. (1981) Glutathione S-transferase (human placenta), *Methods Enzymol.* **77**, 231-235.

Mannervik, B. (1985) The isoenzymes of glutathione S-transferase. *Adv. Enzym. Rel. Areas Mol. Biol.* **57**, 357-417.

Mannervik, B. and Danielson, U.H. (1988) Glutathione S-transferases-Structure and catalytic activity. *Crit. Rev. Biochem. Mol. Biol.* **23**, 283-337.

Mathews, S. and Rao, M.N.A. (1991) Interaction of curcumin with glutathione. *Int. J. Pharmaceut.* **76**, 257-259.

McCaughan, F.M., Brown, A.L., and Harrison, D.J. (1994) The effect of inhibition of glutathione S-transferase P on the growth of the Jurkat human T cell line. *J. Pathol.* **172**, 357-362.

Meister, A. (1983a) Metabolism and transport of glutathione and other δ-glutamyl compounds. In: Larsson, A., Orrenius, S., Holmgrenand, A., and Mannervik, B. (Eds.), Functions of glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects, Raven Press, New York, 1-22.

Meister, A. (1983b) Selective modification of glutathione metabolism. *Science* **220**, 472-477.

Meister, A. (1994) Glutathione, ascorbate, and cellular protection. *Cancer Res. Suppl.* **54**, 1969s-1975s.

Meyer, D.J., Coles, B., Pemble, S.E., Gilmore, K.S., Fraser, G.M., and Ketterer, B. (1991) Theta, a new class of glutathione transferases purified from rat and man. *Biochem. J.* **274**, 409-414.

Mitchell, D.B., Santone, K.S., and Acosta, D. (1980) Evaluation of cytotoxicity in cultured cells by enzyme leakage. J. Tissue Culture Methods. **6**, 113-116.

Monks, T.J., Anders, M.W., Dekant, W., Stevens, J.L., Lau, S.S., and Van Bladeren, P.J. (1990) Glutathione conjugate mediated toxicities. *Toxicol. Appl. Pharmacol.* **106**, 1-19.

Monks, T.J. and Lau, S.S. (1997) Biological reactivity of polyphenolic-glutathione conjugates. *Chem.Res.Toxicol.***10**, 1296-1313.

Morel, F., Schulz, W.A., and Sies, H. (1994) Gene structure and regulation of expression of human glutathione S-transferases alpha. *Biol. Chem. Hoppe-Seyler* **375**, 641-649.

Morgan, A.S., Ciaccio, P.J., Tew, K.D., and Kauvar, L.M. (1996) Isoenzyme-specific glutathione S-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. *Cancer Chemother. Pharmacol.* **37**, 363-370.

Morrow, C.S., Diah, S., Smitherman, P.K., Schneider, E., and Townsend, A.J. (1998) Multidrug resistance protein and glutathione S-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. *Carcinogenesis* **19**, 109-115.

Moscow, J.A., Townsend, A.J., and Conwan, K.H. (1989) Elevation of pi class glutathione S-transferase activity in human breast cancer cells by transfection of the GST pi gene and its effect on sensitivity to toxins. *Mol. Pharmacol.* **36**, 22-28.

Müller, M., Meijer, C., Zaman, G.J.R., Borst, P., Scheper, R.J., Mulder, N., de Vries, E.G.E., and Jansen, P.L.M. (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc. Natl. Acad .Sci. USA* **91**, 13033-13037.

Naidu, A.K., Wiranowska, M., Kori, S.H., Prockop, L.D., and Kulkarni, A.P. (1993) Inhibition of human glioma cell proliferation and glutathione S-transferase by ascorbyl esters and interferon. *Anticancer Res.* **13**, 1469-1476.

Narumiya, S. and Fukushima, M. (1986) Site and mechanism of growth inhibition by prostglandins. I Active transport and intracellular accumulation of cyclopentenone prostglandins, a reaction leading to growth inhibition. *J. Pharmacol. Exp. Ther.* **239**, 500-505.

Narumiya, S., Ohno, K., Fukushima, M., and Fujiwara, M. (1987) Site and mechanism of growth inhibition by prostaglandins. III. Distribution and binding of prostaglandin A_2 and Δ^{12}-prostaglandin J_2 in nuclei. *J. Pharmacol. Exp. Ther.* **242**, 306-311.

Neudecker, T. (1992) The genetic toxicology of cinnamaldehyde. *Mutat. Res.* **277**, 173-185.

Nishihira, T., Maeda, H., Okamoto, K., Oshida, T., Mizoguchi, T., and Terada, T. (1991) Inactivation of human placenta glutathione S-transferase by SH/SS exchange reaction with biological disulfides. *Biochem. Biophys. Res. Comm.* **174**, 580-585.

Nishihira, T., Ishibashi, T., Sakai, M., Nishi, S., Kondo, H., and Makita, A. (1992) Identification of the fatty acid binding site on glutathione S-transferase P. *Biochem. Biophys. Res. Comm.* **189**, 197-205.

Nooter, K. and Stoter, G. (1996) Molecular mechanisms of multidrug resistance in cancer chemotherapy. *Path.Res.Pract.* **192**, 768-780.

O'Dwyer, P.J., LaCreta, F., Nash, S., Tinsley, P.W., Schilder, R., Clapper, M.L., Tew, K.D., Panting, L., Liwin, S., Comis, R.L., and Ozols, R.F. (1991) Phase I study of thiotepa in combination with glutathione transferase inhibitor ethacrynic acid. *Cancer Res.* **51**, 6059-6065.

O'Dwyer, P.J., Hamilton, T.C., Yao, K., Tew, K.D., and Ozols, R.F. (1995) Modulation of glutathione and related enzymes in reversal of resistance to anticancer drugs. *Drug resistance in clinical oncology and hematology* **9**, 383-396.

O'Dwyer, P.J., Hamilton, T.C., LaCreta, F.P., Gallo, J.M., Kilpatrick, D., Halbherr, T., Brennan, J., Bookman, M.A., Hoffman, J., Young, R.C., Comis, R.L., and Ozols, R.F. (1996) Phase I trial of buthionine sulfoximine in combination with mephalan in patients with cancer. *J. Clin. Oncology* **14**, 249-256.

Oakley, A.J., Rossjohn, J., Lo Bello, M., Caccuri, A.M., Federici, G., and Parker, M.W. (1997) The three dimensional structure of the human Pi class glutathione transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. *Biochemistry* **36**, 576-585.

Oetari, S., Sudibyo, M., Commandeur, J.N.M., Samhoedi, R., and Vermeulen, N.P.E. (1996) Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. *Biochem. Pharmacol.* **51**, 39-45.

Ohno, K., Fukushima, M., Fujiwara, M., and Naruyima, S. (1988a) Induction of 68,000-dalton heat shock proteins by cyclopentenone prostaglandins. *J. Biol. Chem.* **263**, 19764-19770.

Ohno, K., Sakai, T., Fukushima, M., Naruyima, S., and Fujiwara, M. (1988b) Site and mechanism of growth inibition by prostaglandins. IV Effect of cyclopentenone prostaglandins on cell cycle progression of G_1-enriched HeLa S3 cells. *J. Pharmacol. Exp. Ther.* **245**, 294-298.

Ohno, K. and Hirata, M. (1990) Induction of γ-glutamylcysteine synthetase by prostaglandin A2 in L-1210 cells. *Biochem. Biophys. Res. Comm.* **168**, 551-557.

Ohno, K., Hirata, M., Narumiya, S., and Fukushima, M. (1992) Effect of glutathione content on cellular uptake and growth inhibitory activity of prostaglandin A_2 in L-1210 cells. *Eicosanoids* **5**, 81-85.

Ohno, K. and Hirata, M. (1993) Characterization of the transport system of prostaglandin A_2 in L-1210 murine leukemia cells. *Biochem. Pharmacol.* **46**, 661-670.

Ohta, T. (1993) Modification of genotoxicity by naturally occurring flavorings and their derivatives. *Crit. Rev. Toxicol.* **23**, 127-146

Olive, C. and Board, P. (1994) Glutathione S-conjugate transport by cultured cells. *Biochim. Biophys. Acta* **1224**, 264-268.

Opdyke, D.L.J. and Letizia, C. (1983) Curcuma oil. *Food Chem. Techn.* **21**, 839-841.

Osawa, T., Sugiyama, Y., Inayoshi, M., and Kawakishi, S. (1995) Antioxidative activity of tetrahydrocurcuminoids. *Biosci. Biotech. Biochem.* **59**, 1609-1612.

Oude Elferink, R.P.J., Ottenhoff, R., Liefting, W., De Haan, J., and Jansen, P.L.M. (1989) Hepatobiliary transport of glutathione and glutathione conjugate in rats with hereditary hyperbilirubinemia. *J. Clin. Invest.* **84**, 476-483.

Ouwerkerk-Mahadevan, S., Van Boom, J.H., Dreef-Tromp M.C., Ploemen, J.H.T.M., Meyer, D.J. and Mulder, G.J. (1995) Glutathione analogues as novel inhibitors of rat and human glutathione S-transferase isoenzymes, as well as of glutathione conjugation in isolated rat hepatocytes and the rat in vivo. *Biochem. J.* **308**, 283-290.

Parker, J. (1995) Prostaglandin A_2 protein interactions and inhibition of cellular proliferation. *Prostaglandins* **50**, 359-375.

Parker, J. and Ankel, H. (1992) Formation of a prostaglandin A_2-glutathione conjugate in L1210 mouse leukemia cells. *Biochem. Pharmacol.* **43**, 1053-1060.

Pemble, S., Schroeder, K.R., Spencer, S.R., Meyer, D.J., Hallier, E., Bolt, H.M., Ketterer, B., and Taylor, J.B. (1994) Human glutathione S-transferase Theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem. J.* **300**, 271-276.

Petrini, M., Conte, A., Caracciolo, F., Sabbatini, A., Grassi, B., and Ronca, G. (1993) Reversing of chlorambucil resistance by ethacrynic acid in a B-CLL patient. *Br.J. Haematology* **85**, 409-410.

Ploemen, J.H.T.M., Van Ommen, B., and Van Bladeren, P.J. (1990) Inhibition of the rat and human glutathione S-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem. Pharmacol.* **40**, 1631-1635

Ploemen, J.H.T.M., Bogaards, J.J.P., Veldink, G.A., Van Ommen, B., Janssen, D.H.M., and Van Bladeren, P.J. (1993a) Isoenzyme selective irreversible inhibition of rat and human glutathione S-transferases by ethacrynic acid and two brominated derivatives. *Biochem. Pharmacol.* **45**, 633-639.

Ploemen, J.H.T.M., Van Ommen, B., Bogaards, J.J.P., and Van Bladeren, P.J. (1993b) Ethacrynic acid and its glutathione conjugate as inhibitors of glutathione S-transferases. *Xenobiotica* **23**, 913-923.

Ploemen, J.H.T.M., Johnson, W.W., Jespersen, S., Vanderwall, D., Van Ommen, B., Van der Greef, J., Van Bladeren, P.J., and Armstrong, R.N. (1994a) Active-site tyrosyl residues are targets in the irreversible inhibition of a class mu glutathione transferase by 2-(S-glutathionyl)-3,5,6-trichloro-1,4-benzoquinone. *J. Biol. Chem.* **269**, 26890-26897.

Ploemen, J.H.T.M., Van Schanke, A., Van Ommen, B., and Van Bladeren, P.J. (1994b) Reversible conjugation of ethacrynic acid with glutathione and human glutathione S-transferase P1-1. *Cancer Res.* **54**, 915-919.

Ploemen, J.H.T.M., Van Ommen, B., Van Iersel, M.L.P.S., Rompelberg, C.J.M., Verhagen, H., and Van Bladeren, P.J. (1996) Irreversible inhibition of cytosolic glutathione S-transferase. In: Vermeulen, N.P.E., Mulder, G.J., Nieuwenhuyse, H., Peters, W.H.M., and Van Bladeren P. J. (Eds.) Glutathione S-transferases: Structure, Functions and Clinical Applications, Taylor & Francis, London, UK., 143-153.

Primiano, T., Sutter, T.R., and Kensler, T.W. (1997) Redox regulation of genes that protect against carcinogens. *Comp. Biochem. Physiol.* **118B**, 487-497.

Ramachandran, C., Yuan, Z.K., Huang, X.L., and Krishan, A. (1993) Doxorubicin resistance in human melanoma cells: MDR-1 and glutathione S-transferase π gene expression. *Biochem. Pharmacol.* **45**, 743-751.

Ramachandran, C., Mead, D., Wellham, L.L., Sauerteig, A., and Krishan, A. (1995) Expression of drug resistance-associated *mdr-1*, GST π and topoisomerase II genes during cell cycle traverse. *Biochem. Pharmacol.* **49**, 545-552.

Ramu, K., Fraiser, L.H., Mamiya, B., Ahmed, T., and Kehrer, J.P. (1995) Acrolein mercapturates: synthesis, characterization, and assessment of their role in the bladder toxicity of cyclophosphamide. *Chem. Res. Toxicol.* **8**, 515-524.

Rhodes, T. and Twentyman, P.R. (1992) A study of ethacrynic acid as a potential modifier of melphalan and cisplatin sensitivity in human lung cancer parental and drug-resistant cell lines. *Br. J. Cancer.* **65**, 684-690.

Ricci, G., Del Boccio, G., Pennelli, A., LoBello, M., Petruzzelli, R., Caccuri, A.M., Barra, D., and Federici, G. (1991) Redox forms of human placenta glutathione transferase. *J. Biol. Chem.* **266**, 21409-21415.

Ricci, G., LoBello, M., Caccuri, A.M., Pastore, A., Nuccetelli, M., Parker, M.W., and Federici, G. (1995) Site-directed mutagenesis of human glutathione transferase P1-1. Mutation of Cys-47 induces a positive cooperativity in glutathione transferase P1-1. *J. Biol. Chem.* **270**, 1243-1248.

Rompelberg, C.J.M., Ploemen, J.H.T.M., Jespersen, S., Van der Greef, J., Verhagen, H., and Van Bladeren, P.J. (1996) Inhibition of rat, mouse, and human glutathione S-transferase by eugenol and its oxidation products. *Chem.-Biol. Interact.* **99**, 85-97.

Rossi, A., Elia, G., and Santoro, G. (1997) Inhibition of nuclear factor κB by prostaglandin A_1: An effect associated with heat shock transcription factor activation. *Proc. Natl. Acad. Sci. USA.* **94**, 746-750.

Santoro, M.G., Carruba, G., Garaci, E., Jaffe, B.M., and Benedetto, A. (1981) Prostaglandins of the A series inhibit Sendai virus replication in cultured cells. *J.Gen.Virol.* **53**, 75-83.

Santoro, M.G., Crisari, A., Benedetto, A., and Amici, C. (1986) Modulation of the growth of a human erythroleukemic cell line (K562) by prostaglandins: antiproliferative action of prostaglandin A. *Cancer Res.* **46**, 6073-6077.

Santoro, M.G., Garaci, E., and Amici, C. (1989) Prostaglandins with antiproliferative activity induce the synthesis of a heat shock protein in human cells. *Proc.Natl.Acad.Sci. USA* **86**, 8407-8411.

Sato, K. (1989) Glutathione transferases as markers of preneoplasia and neoplasia. *Adv. Cancer Res.* **52**, 205-255.

Sato, Y., Fujii, S., Jufii, Y., and Kaneko, T. (1990) Antiproliferative effects of glutathione S-transferase inhibitors on the K562 cell line. *Biochem. Pharmacol.* **39**, 1263-126.

Schäffer, J., Gallay, O., and Ladenstein, R. (1988) Glutathione transferase from bovine placenta. Preparation, biochemical characterization, crystallization and preliminary crystallographic analysis of a neutral class pi enzyme. *J. Biol. Chem.* **263**, 17405-17411.

Schauenstein, E., Taufer, M., Esterbauer, H., Kylianek, A., and Seelich, Th. (1971) The reaction of protein-SH-groups with 4-hydroxy-pentenal. *Monatsh. Chem.* **102**, 517-529.

Schneider, E., Yamazaki, H., Sinha, B.K., and Cowan, K.H. (1995) Buthionine sulphoximine-mediated sensitization of etoposide-resistant human breast cancer MCF7 cells overexpressing the multidrug resistance-associated protein involves increased drug accumulation. *Br. J. Cancer* **71**, 738-743.

Schultz, M., Dutta, S., and Tew, K.D. (1997) Inhibitors of glutathione S-transferases as therapeutic agents. *Adv. Drug Delivery Rev.* **26**, 91-104.

Senjo, M. and Ishibashi, T. (1988) Possible involvement of glutathione S-transferases in the cell growth of C6 astroglioma cells. *J-Neurochem.* **50**, 163-166.

Shea, T.C., Kelley, S.L., and Henner, W.D. (1988) Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines. *Cancer Res.* **48**, 527-533.

Shen, H., Tamai, K., Satoh, K., Hatayma, I., Tsuchida, S., and Sato, K. (1991) Modulation of class pi glutathione transferase activity by sulfhydryl group modification. *Arch. Biochem. Biophys.* **286**, 178-182.

Shen, H., Ranganathan, S., Kuzmich, S., and Tew, K.D. (1995) Influence of ethacrynic acid on glutathione S-transferase π transcript and protein half-lives in human colon cancer cells. *Biochem. Pharmacol.* **50**, 1233-1238.

Shen, H., Kauvar, L., and Tew, K.D. (1997) Importance of glutathione and associated enzymes in drug response. *Oncology Res.* **9**, 295-302.

Singh, S.V., Xu, B.H., Maurya, A.K., and Mian, A.M. (1992) Modulation of mitomycin C resistance by glutathione transferase inhibitor ethacrynic acid. *Biochim. Biophys. Acta* **1137**, 257-263.

Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G., Olin, B., Mannervik, B., and Jones, A. (1993) Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the mu and pi class enzymes. *J. Mol. Biol.* **232**, 192-212.

Sluis-Cremer, N., Naidoo, N., and Dirr, H. (1996) Class-pi glutathione S-transferase is unable to regain its native conformation after oxidative inactivation by hydrogen peroxide. *Eur. J. Biochem.* **242**, 301-307.

Smith, M.T., Evans, C.G., Doane-Setzer, P., Castro, V.M., Tahir, M.K., and Mannervik, B. (1989) Denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosurea by class mu glutathione transferases and its role in cellular resistance in rat brain tumor cells. *Cancer Res.* **49**, 2621-2625.

Sokolovsky, M., Sadeh, T., and Patchornik, A. (1964) Specific chemical cleavage of cysteinyl peptides. *J. Am. Chem. Soc.* **86**, 1212-1217.

Stoner, G.D. and Mukhtar, H. (1995) Polyphenols as cancer chemopreventive agents. *J. Cell. Biochem. suppl.* **22**, 169-180.

Takamatsu, Y. and Inaba, T. (1992) Inhibition of human hepatic glutathione S-transferases by ethacrynic acid and its metabolites. *Toxicol. Lett.* **62**, 241-245.

Talalay, P., DeLong, M.J., and Prochaska, H.J. (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc.Natl.Acad.Sci.USA* **85**, 8261-8265.

Talalay, P., Fahey, J.W., Holtzclaw, W.D., Prestera, T., and Zhang, Y. (1995) Chemoprotection against cancer by Phase 2 enzyme induction. *Toxicol. Lett.* **82/83**, 173-179.

Tamai, K., Satoh, H., Tsuchida, S., Hatayama, I., Maki, T., and Sato, H. (1990) Specific inactivation of glutathione S-transferases in class pi by SH-modifiers. *Biochem. Biophys. Res. Comm.* **167**, 331-338.

Terada, T., Matsumura, M., Abe, A., Morita, Y., Adachi, H., and Nanjo, H. (1995) Irreversible inactivation of glutathione S-transefarse-π by low concentration of naphtoquinones. *Redox Report* **1**, 125-130.

Terrier, P., Townsend, A.J., Coindre, J.M., Triche, T.J., and Cowan, K.H. (1990) An immunohistochemical study of pi class glutathione S-transferase expression in normal human tissue. *Am.J. Pathol.* **137**, 845-853.

Tew, K.D., Bomber, A.M. and Hoffman, S.J. (1988) Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res.* **48**, 3622-3625.

Tew, K.D. (1994) Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res.* **54**, 4313-4320.

Tew, K.D., Monks, A., Barone, L., Rosser, D., Akerman, G., Montali, J.A., Wheatley, J.B., and Schmidt, D.E., Jr. (1996) Glutathione-associated enzymes in the human cell lines of the National Cancer Institute Drug Screening Program. *Mol. Pharmacol.* **50**, 149-159.

Tsuchida, S. and Sato, K. (1992) Glutathione transferases and cancer. *Crit.Rev.Biochem. Molec.Biol.* **27**, 337-384.

Twentyman, P.R. (1997) Transport proteins in drug resistance: biology and approaches to circumvention. *J.Intern. Med.* **242** (suppl 740), 133-137.

Van Bladeren, P.J. (1988) Formation of toxic metabolites from drugs and other xenobiotics by glutathione conjugation. *Trends in Pharmacol. Sci.* **9**, 295-298

Van Bladeren, P.J. and Van Ommen, B.(1991) The inhibition of glutathione S-transferases, mechanisms, toxic consequences and therapeutic benefits. *Pharmac. Ther.* **51**, 35-46.

Van Iersel, M.L.P.S., Ploemen, J.H.T.M., Struik, I., Van Amersfoort, C., Keyzer, A., Schefferlie, J.G., and Van Bladeren, P.J. (1996) Inhibition of glutathione S-transferase activity in human melanoma cells by α,β-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid and *trans*-2-hexenal. *Chem.-Biol. Interact.* **102**, 117-132.

Van Iersel, M.L.P.S., Ploemen, J.H.T.M., LoBello, M., Federici, G., and Van Bladeren, P.J. (1997) Interactions of α,β-unsaturated adehydes and ketones with human glutathione S-transferase P1-1. *Chem.-*

Biol. Interact. **108**, 67-78.

Van Ommen, B., Bogaards, J.J.P., Peters, W.H.M., Blaauboer, B., and Van Bladeren, P.J. (1990) Quantification of human hepatic glutathione S-transferases. *Biochem. J.* **269**, 609-613.

Versantvoort, C.H.M., Broxterman, H.J., Bagrij, T., Scheper, R.J., and Twentyman, P.R. (1995) Regulation by glutathione of drug transport in multidrug-resistant human lung tumour cell lines overexpressing multidrug resistance-associated protein. *Br. J. Cancer* **72**, 82-89.

Vos, R.M.E. and Van Bladeren, P.J. (1990) Glutathione S-transferases in relation to their role in the bioransformation of xenobiotics. *Chem.-Biol. Interactions* **75**, 241-265.

Vroomen, L.H.M., Berghmans, M.C.J., Groten, J.P., Koeman, J.H., and Van Bladeren, P.J. (1988) Reversible interaction of a reactive intermediate derived from furazolidone with glutathione and protein. *Toxicol. Appl. Pharmacol.* **95**, 53-60.

Waxman, D.J. (1990) Glutathione S-transferases: role in alkylating agent resistance and possible target for modulation chemotherapy- a review. *Cancer Res.* **50**, 6449-6554.

Wijnholds, J., Evers, R., Van Leusden, M.R., Mol, C.A.A.M., Zaman, G.J.R., Mayer, U., Beijnen, J.H., Van der Valk, M., Krimpenfort, P., and Borst, P. (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nature medicine* **3**, 1275-1279.

Wilson, V.L., Foiles, P.G., Chung, F.L., Povey, A.C., Frank, A.A., and Harris, C.C. (1991) Detection of acrolein and crotonaldehyde DNA adducts in cultured human cells and canine peripheral blood lymphocytes by 32P-postlabeling and nucleotide chromatography. *Carcinogenesis* **12**, 1483-1490.

Witz, G. (1989) Biological interactions of α,β-unsaturated aldehydes. *Free Radical biology & Medicine* **7**, 333-349.

Yuan, J.H., Dieter, M.P., Bucher, J.R., and Jameson, C.W. (1992) Toxicokinetics of cinnamaldehyde in F344 rats. *Food Chem. Toxicol.* **30**, 997-1004.

Zaman, G.J.R., Cnubben, N.H.P., Van Bladeren, P.J., Evers, R., and Borst, P. (1996) Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP. *FEBS Lett.s* **391**, 126-130.

Abbreviations

ACR	acrolein
ARE	antioxidant-responsive element
ATP	adenosine triphosphate
BSO	D,L-buthionine-S,R-sulfoximine
C101S	cysteine 101 serine mutant of GSTP1-1
C47S	cysteine 47 serine mutant of GSTP1-1
C47S/C101S	cysteine 47 and 101 serine mutant of GSTP1-1
CA	cinnamaldehyde
CDNB	1-chloro-2,4-dinitrobenzene
CRA	crotonaldehyde
CUR	curcumin
DNA	deoxyribonucleic acid
DNPSG	S-(2,4-dinitrophenyl)glutathione
EA	ethacrynic acid
EASG	ethacrynic acid-glutathione conjugate
EDTA	ethylene diamine-tetracetic acid
EpRE	electrophile-responsive element
γ-GCS	γ-glutamylcysteine synthetase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
GST (A1-1, P1-1 etc)	glutathione S-transferase (A1-1, P1-1 etc)
GS-X	glutathione conjugate
HBSS	Hanks' balanced salt solution
HEX	*trans*-2-hexenal
HNE	4-hydroxy-2-nonenal
(RP)-HPLC	(reversed phase)-high performance liquid chromatrography
LDH	lactate dehydrogenase
LTC_4	leukotrieneC_4
MDR	multidrug resistance
MOAT	multispecific organic anion transporter
MRP pump	multidrug resistance-associated protein pump
NADH	nicotinamide adenine dinucleotide
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear Factor-κB binding sites
NMR	nuclear magnetic resonance
PBO	phenyl-3-buten-2-one
PBS	phosphate buffered saline
PG	prostaglandin
PGA_2-SG	prostaglandin A_2-glutathione conjugate
Pgp	P-glycoprotein
RNA	ribonucleic acid
ROS	reactive oxygen species
S.E.	standard error of the mean
TCA	trichloro acetic acid
TFA	trifluoro acetic acid
XRE	xenobiotic-responsive element

Samenvatting

Inleiding

Beïnvloeding van het glutathion gerelateerde biotransformatiesysteem kan een rol spelen bij belangrijke phenomenen als anticarcinogeniteit en multidrug resistentie. Glutathion gerelateerde biotransformatie kent drie belangrijke aspecten nl. glutathion (GSH), glutathion S-transferases (GSTs) en de multidrug resistance associated protein (MRP) pomp. Deze drie mechanismen werken samen en beïnvloeden elkaar. Glutathion is een belangrijke intracellulaire thiol dat aanwezig is in bijna alle cellen. Het beschermt cellen tegen reactieve zuurstofdeeltjes en electrofiele verbindingen, inclusief carcinogenen. Variaties in de concentratie glutathion in tumorcellen, wijzen op betrokkenheid van glutathion bij de bescherming van die cellen tegen cytostatica. Glutathion S-transferases zijn een groep enzymen, die een rol spelen bij de detoxificatie van een groot aantal electrofiele verbindingen, door de glutathionconjugatie van deze verbindingen te katalyseren. Vooral GST van de π-klasse is interessant aangezien dit isoenzym in verhoogde mate aanwezig is in bepaalde tumoren en daarom een belangrijke factor kan zijn in drug resistentie tegen alkylerende cytostatica. De derde factor is de MRP pomp, die voor het eerst beschreven is in een multidrug resistente cellijn en die een groot aantal, voornamelijk geconjugeerde, verbindingen van endogene en exogene aard uit de cel transporteert. De verhoogde aanwezigheid van dit eiwit in verschillende humane tumoren en in drug resistente tumorcellijnen doen een rol in multidrug resistentie vermoeden. Beïnvloeding van elk van deze processen -depletie van glutathion, inhibitie van GST activiteit en/of inhibitie van MRP activiteit- opent mogelijkheden om drug resistentie te overwinnen.

Een van de stoffen, die veel bestudeerd is in verband met beïnvloeding van deze processen, is het α,β-onverzadigde keton ethacrynezuur (EA). Ethacrynezuur wordt geconjugeerd met glutathion door een proces dat Michael additie genoemd wordt, zowel chemisch als gekatalyseerd door GSTs. Deze Michael additie kan reversibel zijn. EA inhibeert GST activiteit zowel competitief als door covalente binding en het glutathion conjugaat is zelf een GST inhibitor en een goed substraat en inhibitor voor MRP. Deze waarnemingen leidden tot de hypothese dat andere α,β-onverzadigde carbonyl verbindingen vergelijkbare eigenschappen zouden kunnen hebben. Aan deze klasse van verbindingen wordt de mens continu blootgesteld, zowel via het voedsel en milieu als endogeen. Ze worden met glutathion geconjugeerd, chemisch en gekatalyseerd door GSTs, wat hen een interessante groep maakt om te bestuderen in relatie tot het glutathion gerelateerde biotransformatiesysteem.

In dit proefschrift is onderzoek beschreven naar de effecten van een aantal van deze α,β-onverzadigde carbonylverbindingen op de glutathion gerelateerde processen, voornamelijk glutathion S-transferase P1-1. Een ander doel was om meer inzicht te verkrijgen in het metabolisme van deze verbindingen.

Als eerste is geprobeerd meer duidelijkheid te krijgen in de mechanismen van zowel GST inhibitie als glutathion conjugatie. Ten tweede is het effect van een serie exogene α,β-onverzadigde carbonylverbindingen (namelijk acroleïne, crotonaldehyde, cinnamaldehyde, citral, curcumine, ethacrynezuur en *trans*-2-hexenal) op glutathion gerelateerde biotransformatie in een celsysteem bekeken, omdat alledrie de processen in zo'n systeem geïntegreerd zijn en de relatieve rol van de verschillende stappen bekeken kan worden. Als laatste zijn het metabolisme en de effecten van een belangrijk endogeen α,β-onverzadigde keton, prostaglandine A_2, bestudeerd om het belang van glutathion gerelateerd metabolisme voor endogene verbindingen aan te tonen en om inzicht te verkrijgen in de rol van GST inhibitie in de regulatie van fysiologische processen.

Samenvatting

Om duidelijkheid te verschaffen in de mechanismen die ten grondslag liggen aan covalente interactie van α,β-onverzadigde carbonylverbindingen en GSTP1-1 en om de rol van cysteïne residuen hierin te bestuderen, zijn gezuiverd GSTP1-1 en drie mutanten daarvan gebruikt (*hoofdstuk 2*). Bij deze mutanten zijn cysteïne 47 en/of 101 vervangen door een serine. De gebruikte α,β-onverzadigde carbonylverbindingen remden de activiteit van GSTP1-1, maar als beide cysteïnes gemuteerd waren kon bijna geen remming aangetoond worden. Mutatie van alleen cysteïne 47 had bijna hetzelfde effect. Bij hoge concentratie van α,β-onverzadigde carbonyl verbindingen werd de activiteit van de mutanten toch enigszins geremd, implicerend dat andere plaatsen in het enzym ook gemodificeerd kunnen worden. Van de gebruikte verbindingen zijn ethacrynezuur, acroleïne, curcumine en 4-hydroxy-2-nonenal de meest potente covalente remmers. Omdat de verbindingen Michael acceptors zijn, is het herstel van activiteit door een overmaat glutathion onderzocht. Alleen de inhibitie door ethacrynezuur en crotonaldehyde kon volledig opgeheven worden. Maar bijvoorbeeld inhibitie door acroleïne kon niet opgeheven worden; experimenten met matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) toonden aan dat er een covalente cross linking tussen de twee subeenheden plaatsvond door acroleïne (ongepubliceerde resultaten). Voor de andere gebruikte verbindingen kon de GSTP1-1 activiteit gedeeltelijk hersteld worden, wat duidt op reacties met andere aminozuren of bindingsplaatsen in het enzym.

De resultaten van dit hoofdstuk tonen aan dat α,β-onverzadigde carbonylverbindingen GSTP1-1 irreversibel kunnen remmen voornamelijk door binding aan cysteïne 47 van GSTP1-

1. Deze covalente binding zou vooral fysiologisch een rol kunnen spelen als glutathionconcentraties laag zijn.

De potentiële relevantie van stereoselectieve vorming van glutathion conjugaten van α,β-onverzadigde carbonylverbindingen voor de fysiologische en/of toxische werking van deze verbindingen, heeft geleid tot onderzoek naar de relatieve vorming van de twee diastereoisomeren van het modelsubstraat ethacrynezuur. Ethacrynezuur is een uitgebreid bestudeerde verbinding die de laatste tijd voornamelijk aandacht heeft gekregen in relatie tot multidrug resistentie. Glutathion conjugatie van ethacrynezuur leidt tot de vorming van twee diastereoisomeren, chemisch in bijna gelijke hoeveelheden (ratio van 48:52). Katalyse door humaan GSTP1-1 resulteerde in een stereospecifieke vorming van een van de diastereoisomeren, in dit proefschrift aangegeven als diastereoisomeer A. Van de α-klasse, was alleen GSTA1-1 stereoselectief voor de vorming van diastereoisomeer A; GSTA1-2 en GSTA2-2 vertoonden geen stereoselectiviteit, ondanks dat ze wel de conjugatiereactie katalyseerden. GST-klasse μ is niet onderzocht, vanwege lage specifieke activiteit en eerdere bevindingen dat rat GST μ geen stereoselectiviteit vertoonde. Er kon geen significante deconjugatie van het diastereoisomere mengsel of van diastereoisomeer A alleen aangetoond worden, noch chemisch noch na toevoeging van GSTP1-1; dit laatste waarschijnlijk ten gevolge van enzyminhibitie. Het is duidelijk dat het evenwicht van de reactie aan de kant van productvorming ligt. Als een eerste stap om de rol van conjugatie in relatie tot andere glutathion gerelateerde aspecten te bestuderen, zijn IGR-39 humane melanoma cellen, die een hoog gehalte aan GSTP1-1 bevatten, blootgesteld aan ethacrynezuur. Na twee uur was voornamelijk diastereoisomeer A aanwezig in het medium. Dit is zeer waarschijnlijk het gevolg van stereoselectieve vorming van het conjugaat, en niet van stereoselectief transport, maar definitief bewijs ontbreekt nog (*hoofdstuk 3*).

Uit dit hoofdstuk blijkt dat het evenwicht van de reactie tussen ethacrynezuur en glutathion aan de productkant ligt. Verder katalyseren GSTP1-1 en GSTA1-1 stereospecifiek respectievelijk stereoselectief de glutathionconjugatie van ethacrynezuur en deze stereoselectiviteit speelt ook een rol in celsystemen.

Omdat glutathionconjugatie en inhibitie van GST-activiteit voornamelijk in cytosol of met gezuiverde enzymen is bekeken, is er een methode ontwikkeld om verandering in glutathionconjugatie in intacte IGR-39 humane melanoma cellen te meten. Dit is gedaan door kwantificering van de excretie in het medium van S-(2,4-dinitrofenyl)glutathion (DNPSG), het glutathionconjugaat van het standaard substraat 1-chloor-2,4-dinitrobenzeen (CDNB). Door bepaling van intracellulaire glutathionconcentraties, GST-activiteit en intra-en extracellulaire DNPSG-concentraties, zijn bepaalde determinanten in het mechanisme van inhibitie van DNPSG excretie geïdentificeerd (*hoofdstuk 4*). Deze mechanismen zijn glutathion depletie,

reversibele en irreversibele inhibitie van GST activiteit en beïnvloeding van de efflux van glutathionconjugaten door een effect op de 'multidrug resistance associated protein' (MRP) pomp. Met behulp van deze methode is een serie van α,β-onverzadigde carbonylverbindingen getest op hun vermogen om DNPSG-excretie te remmen. Curcumine, een antioxidant en anticarcinogeen, was de meest potente remmer van deze DNPSG-excretie in deze cellen, gevolgd door ethacrynezuur. Citral had geen effect op de DNPSG-excretie tot 100 μM en acroleïne was te toxisch om een effect te verkrijgen (*hoofdstuk 4*). Het mechanisme van inhibitie verschilde voor de verschillende verbindingen. Voor curcumine en ethacrynezuur spelen een aantal factoren een rol: glutathion depletie, reversibele inhibitie van GSTs en covalente modificatie van GSTP1-1. Maar voor *trans*-2-hexenal en cinnamaldehyde is reversibele GST inhibitie waarschijnlijk de belangrijkste factor voor het effect. Crotonaldehyde remt DNPSG excretie voornamelijk door depletie van glutathion, maar reversibele GST inhibitie speelt waarschijnlijk ook wel een rol. Curcumine en ethacrynezuur remmen daarnaast ook het transport van DNPSG uit de cel, misschien door MRP. Van het glutathionconjugaat van ethacrynezuur is het bekend dat het een goed substraat en inhibitor is van deze pomp.

Een aantal van de α,β-onverzadigde carbonylverbindingen, die gebruikt zijn in deze studie, remmen GST activiteit in humane melanoma cellen. Ze beïnvloeden op verschillende manieren het glutathion gerelateerde biotransformatiesysteem in deze cellen door glutathion depletie, reversibele en irreversibele remming van GST activiteit en/of beïnvloeding van efflux van glutathionconjugaten.

Voor het endogene α,β-onverzadigde keton prostaglandine A_2 (PGA_2) kon een meer compleet beeld van het metabolisme en de invloed op glutathion gerelateerde biotransformatie worden verkregen, omdat het mogelijk was om de excretie van PGA_2-glutathion conjugaten te meten in het medium tijdens blootstelling van cellen aan PGA_2. Nadat IGR-39 humane melanoma cellen opgeladen waren met [^{3}H] gelabeld glycine en vervolgens blootgesteld waren aan PGA_2, werden beide diastereoisomeren van het glutathionconjugaat uitgescheiden in het medium, maar een grotere hoeveelheid van de S-diastereoisomeer. Eerder onderzoek wijst uit, dat dit het gevolg is van stereoselectieve vorming van de S-vorm door katalyse door GSTP1-1 en minder waarschijnlijk van een stereoselectiviteit in het transport. Incubatie van humane melanoma cellen met PGA_2 gedurende 1 of 4 uur beïnvloedde duidelijk het glutathion gerelateerd metabolisme. Na blootstelling van 1 uur was de DNPSG-excretie gereduceerd voornamelijk ten gevolge van inhibitie van DNPSG-efflux uit de cel. Na 4 uur was de totale DNPSG-vorming afgenomen, ten gevolge van GSH depletie en reversibele en irreversibele inhibitie van GST activiteit; inhibitie van de efflux speelde na 4 uur nog maar een kleine rol. Ondanks dat irreversibele inhibitie al zorgde voor 25% van de totale GST-inhibitie, resulteerde depletie van GSH door middel van buthionine sulfoximine (BSO) in een sterkere covalente inactivatie. Deze covalente modificatie van GSTP1-1 was het gevolg van binding van PGA_2

voornamelijk aan cysteine 47. Deze inactivatie kon helemaal hersteld worden door een overmaat aan glutathion, wat duidt op een retro-Michael reactie (*hoofdstuk 5*).

De resultaten van dit hoofdstuk leveren aanvullend bewijs dat GSTP1-1 een rol kan spelen als algemene scavenger voor alkylerende verbindingen, vooral als de glutathionconcentratie laag is. Bovendien wijzen de resultaten op een rol voor GSTP1-1 als opslag of transport eiwit voor fysiologisch belangrijke verbindingen zoals PGA_2.

Conclusies

Uit de resultaten van het beschreven onderzoek blijkt dat GST activiteit in cellen inderdaad geremd kan worden en dat α,β-onverzadigde carbonyl verbindingen intracellulair GST activiteit kunnen remmen. Verder is het duidelijk geworden dat GST activiteit niet apart bestudeerd moet worden, maar, als onderdeel van een glutathion gerelateerd biotransformatie systeem, in samenhang met glutathionconcentraties en de multidrug resistance associated protein (MRP). De evidente betrokkenheid van GSTP1-1 in het metabolisme van de endogene verbinding prostaglandine A_2, onderschrijft de mogelijke rol van dit isoenzym in de regulatie van celproliferatie. De meeste α,β-onverzadigde carbonyl verbindingen, die in dit proefschrift gebruikt zijn, beïnvloeden het glutathion gerelateerde biotransformatiesysteem (glutathion depletie, zowel reversibele als irreversibele inhibitie van GST activiteit, invloed op MRP); sommige op alle aspecten, andere alleen op een of twee onderdelen. Gezien de verscheidene functies waar dit syteem in de cel bij betrokken is, i.e. celproliferatie, genregulatie, anticarcinogeniteit en multidrug resistentie, zijn α,β-onverzadigde carbonylverbindingen inderdaad een erg belangrijke groep stoffen, zeker omdat mensen iedere dag blootgesteld worden aan deze klasse van verbindingen. Het opent perspectieven voor verder onderzoek naar therapeutisch efficiënte stoffen in het kader van multidrug resistentie en anticarcinogeniteit. Het potentiële effect van deze stoffen op de verschillende fysiologische processen benadrukt dat meer onderzoek gedaan moet worden naar de totale blootstelling van mensen aan deze stoffen, vooral via dieet en milieu.

Uitleg voor familie en vrienden

Kanker is op dit moment een van de belangrijkste ziektes, waaraan mensen vooral in de westerse wereld overlijden. Door blootstelling aan stoffen, die voorkomen in de lucht en in voedsel, wordt het lichaam beinvloed. Het lichaam kan reageren op deze blootstelling met behulp van verdedigingsmechanismen, die zorgen dat de stoffen onschadelijk worden gemaakt. Normaal gesproken is het goed dat lichaamsvreemde stoffen op deze manier geen schadelijk effect kunnen uitvoeren, maar in het geval van geneesmiddelen die gegeven worden om kanker te bestrijden (cytostatica, chemotherapie) is het juist de bedoeling dat deze stoffen hun schadelijke effect op de kankercel uitoefenen en deze cellen doodmaken. De verdedigingsmechanismen zijn dan juist onhandig en als deze uitgeschakeld kunnen worden zouden middelen tegen kanker beter werken.

Een van deze verdedigingsmechanismen is het zogenoemde glutathion gerelateerde biotransformatiesysteem. Dit systeem bestaat uit drie belangrijke aspecten nl. glutathion (GSH), glutathion S-transferases (GSTs) en de 'multidrug resistance associated protein' (MRP) pomp.

Het tripeptide (klein eiwit van 3 aminozuren) glutathion is een belangrijke intracellulaire stof (thiol) die aanwezig is in bijna alle cellen. Het beschermt cellen tegen reactieve zuurstofdeeltjes en schadelijke verbindingen, bijvoorbeeld kankerverwekkende stoffen, door ermee te reageren.

Glutathion S-transferases vormen een belangrijke groep enzymen (grote eiwitten), die een rol spelen bij de detoxificatie (ontgiftiging) van een grote groep van schadelijke verbindingen. Dit gebeurt doordat ze de conjugatie (reactie) van deze stoffen met glutathion katalyseren (versnellen).Het is een familie van verschillende klassen isoenzymen. Vooral het isoenzym van de π-klasse is in verhoogde mate aanwezig in bepaalde soorten kanker. Daarom ook wordt dit isoenzym in verband gebracht met het onschadelijk maken van cytostatica in kankercellen, wat kan leiden tot resistentie tegen deze cytostatica.

Het derde aspect is de MRP pomp. Deze pomp zit in membranen van cellen en transporteert voornamelijk geconjugeerde verbindingen uit de cel. Deze pomp is in verhoogde mate aanwezig in tumorweefsel en speelt ook een rol bij het onschadelijk maken van cytostatica in kankercellen.

Wanneer een of meer van de bovengenoemde processen beïnvloed zouden kunnen worden, bijvoorbeeld verlaging van de hoeveelheid glutathion, remming van de activiteit van GSTs en/of remming van de pomp, dan zou dat mogelijkheden bieden voor het beter laten werken van cytostatica.

Een van de remmers van GST activiteit, die al vaker bestudeerd is, is ethacrynezuur (EA), een medicijn dat gebruikt werd als middel om meer te kunnen plassen, maar waarvan later de

remmende effecten duidelijk werden. Het is een lid van de familie van α,β-onverzadigde carbonylverbindingen. Dit zijn verbindingen met een bepaalde molecuul structuur, die daardoor reactief zijn. Dit ethacrynezuur kan rechtstreeks reageren met glutathion en deze reactie kan versneld worden door die enzymen (GSTs). Maar het is ook in staat om deze enzymen te remmen op twee verschillende manieren, zowel door op het actieve centrum te gaan zitten als door een hele sterke (covalente) binding aan te gaan vlakbij dit actieve centrum. Het glutathionconjugaat van ethacrynezuur zelf remt ook de enzymactiviteit. En dit conjugaat wordt daarnaast uit de cel gepompt door de MRP pomp. Kortom, het is een stof die alledrie de delen van dat glutathion gerelateerd verdedigingsmechanisme beïnvloedt. Zoals gezegd is er een grote groep van dit soort verbindingen en we vroegen ons af of die andere stoffen dezelfde soort effecten zouden kunnen hebben. Mensen worden elke dag blootgesteld aan deze verbindingen via hun eten en via sigarettenrook en inademing van uitlaatgassen, maar andere verbindingen worden ook gewoon in het lichaam gevormd.

In dit proefschrift is onderzoek beschreven naar de effecten van een aantal van deze α,β-onverzadigde carbonylverbindingen op glutathion gerelateerde processen. Een tweede doel was om meer inzicht te krijgen in de verwerking (metabolisme) van deze stoffen.

We hebben gekozen voor stoffen waaraan mensen iedere dag worden blootgesteld; een aantal voorbeelden zijn: curcumine, een hoofdbestanddeel van gele kerriepoeder; cinnamaldehyde, een hoofdbestanddeel van kaneel (beide stoffen worden ook gebruikt als smaakmaker in bijvoorbeeld ijs); acroleïne en crotonaldehyde, beide aanwezig in sigarettenrook en uitlaatgassen.

Als eerste is geprobeerd om meer duidelijkheid te krijgen in de mechanismen van de directe remming van de enzymen door binding van die specifieke groep stoffen. Daarnaast is van een van de stoffen (ethacrynezuur) wat dieper gekeken naar het mechanisme van de versnelde glutathionconjugatie door GSTs. Hiervoor is gebruikgemaakt van gezuiverde enzymen. Ten tweede is het effect van een aantal α,β-onverzadigde carbonylverbindingen op glutathion gerelateerde verdedigingsmechanismen in cellen bekeken. Deze cellen kunnen buiten het lichaam gekweekt worden en het voordeel is dat daarin de verschillende processen samenwerken. Als laatste is er onderzoek gedaan naar zo'n α,β-onverzadigde carbonylverbinding die van nature in het lichaam voorkomt, prostaglandine A_2. Hierdoor konden we een idee krijgen over het belang van die glutathion gerelateerde verdedigingsmechanismen voor de werking van lichaamseigen stoffen.

Uit het onderzoek dat beschreven wordt in dit proefschrift blijkt een aantal van de onderzochte stoffen heel goed, sterk te kunnen binden aan de gezuiverde GST enzymen waardoor de activiteit van deze enzymen geremd wordt. Bijvoorbeeld curcumine, acroleïne en ethacrynezuur remmen dit enzym al in hele lage concentraties, maar cinnamaldehyde doet dat veel minder goed. Ook in cellen bleken deze stoffen in staat te zijn om deze enzymen direct

te binden, maar daarnaast hadden een aantal van de gebruikte stoffen ook invloed op andere onderdelen van het totale verdedigingsmechanisme. Bijvoorbeeld konden ze de hoeveelheid glutathion in de cellen uitputten, omdat ze daarmee reageren in de cel. En doordat ze daarna de cel uit moeten, remden ze de pomp die daarvoor moet zorgen. Op pagina 72 staat een plaatje waarin alledrie deze onderdelen uitgebeeld zijn. Vooral curcumin en ethacrynezuur lijken alledrie de onderdelen te beïnvloeden. Door de lichaamseigen stof, prostaglandine A_2 te bestuderen zijn we erachter gekomen dat deze stof in ieder geval dit glutathion gerelateerde verdedigingsmechanisme gebruikt in de cel. Het reageert met glutathion tot een conjugaat en ook direct en sterk met het enzym GST π. Waarschijnlijk vervult het enzym in dit geval ook een transportfunctie voor deze stof en/of zijn glutathionconjugaat binnen in de cel. Wie weet ook wel voor andere stoffen, maar dat hebben we verder niet bestudeerd. Daarnaast wordt het conjugaat uit de cel gepompt en kan dus competern met andere stoffen die ook door dezelfde pomp worden uitgescheiden.

Wat kunnen we nu met deze resultaten?

Omdat het besproken verdedigingsmechanisme een belangrijke rol vervult in het functioneren van cellen en in de verdediging van de cellen tegen schadelijke stoffen, zijn de bestudeerde groep stoffen belangrijk vooral omdat mensen er iedere dag aan blootgesteld worden. Het waarschijnlijke effect van deze stoffen op allerlei celfuncties benadrukt dus dat er verder onderzoek moet gebeuren naar de totale blootstelling van de mens via dieet en milieu, vooral omdat mensen verschillend blootgesteld worden en allemaal anders gevoelig zijn.

Daarnaast opent het perspectieven voor verder onderzoek naar stoffen die kunnen helpen om anti-kanker middelen beter te laten werken.

Natuurlijk kunnen we niet zeggen op basis van deze resultaten: eet veel van het een of ander. We hebben in dit onderzoek alleen maar naar een klein onderdeeltje gekeken en alleen maar in buisjes en cellen, niet in een lichaam. Wie weet hebben de stoffen bij langdurige blootstelling hele andere effecten. Daarom is een belangrijke conclusie zoals zo vaak: er kan nog veel onderzoek gedaan worden.

Curriculum Vitae

Marlou van Iersel was born on April 30, 1969, in Schoonhoven, The Netherlands. In 1987 she completed secondary school (Gymnasium) at the Henric van Veldeke College in Maastricht and started to study Health Science at the State University of Limburg (RL) (now: University of Maastricht), with specialisation in Biological Health Science, Toxicology. As part of this study she conducted research projects at the Department of Health Risk Analysis and Toxicology (RL) (supervisor: Dr. T.M.C.M. de Kok), the Toxicology Group, Department of Food Technology and Nutritional Sciences (Wageningen Agricultural University, WAU) (supervisor: Dr. Ir. C. Den Besten) and the British Industrial and Biological Research Association (BIBRA), Surrey, United Kingdom (supervisor Dr. B.G. Lake and Prof. Dr. J.H. Koeman (WAU)). In 1993 she graduated and started as a PhD student at theToxicology Group, Department of Food Technology and Nutritional Sciences (WAU) on the research described in the present thesis, in association with TNO Nutrition and Food Research, Zeist under supervision of Prof. Dr. P.J. van Bladeren and Prof. Dr. J.H. Koeman. From October 1998 she is appointed as a post-doc in the Centre for Nutrition and Food Safety, Toxicology (collaboration between WAU and TNO), at the Toxicology Group, Department of Food Technology and Nutritional Sciences, WAU.

List of publications

1. T.M.C.M. de Kok, M.L.P.S. van Iersel, F. ten Hoor and J.C. Kleinjans. (1993) In vitro study on the effects of fecal composition on fecapentaene kinetics in the large bowel. *Mutation Research* **302(2)**, 103-108.
2. T.M.C.M. de Kok, D. Pachen, M.L.P.S. van Iersel, C.G. Baeten, L.G. Engels, F. ten Hoor and J.C. Kleinjans. (1993) Case-control study on fecapentaene excretion and adenomatous polyps in the colon end rectum. *Journal of the National Cancer Institute* **85(15)**, 1241-1244.
3. C. den Besten, M.M. Bennik, M.L.P.S. van Iersel, M.A. Peters, C. Teunis and P.J. van Bladeren. (1994) Comparison of the urinary metabolite profiles of hexachlorobenzene and pentachlorobenzene in the rat. *Chemico-Biological Interactions* **90(2)**, 121-137.
4. M.L.P.S. van Iersel, D.G. Walters, R.J. Price, D.P. Lovell and B.G. Lake. (1994) Sex and strain differences in mouse hepatic microsomal coumarin 7-hydroxylase activity. *Food and Chemical Toxicology*, **32 (4)**, 387-390.
5. M.L.P.S. van Iersel, C.J. Henderson, D.G. Walters, R.J. Price, C.R. Wolf and B.G. Lake. (1994) Metabolism of [^{14}C] coumarin by human liver microsomes. *Xenobiotica*, **24(8)**, 795-803.
6. J.H.T.M. Ploemen, M.L.P.S. van Iersel, L.W. Wormhoudt, J.N. Commandeur, N.P. Vermeulen and P.J. van Bladeren. (1996) In vitro inhibition of rat and human glutathione S-transeferase isoenzymes by disulfiram and diethyldithiocarbamate. *Biochemical Pharmacology*, **52(2)**, 197-204.
7. J.H.T.M. Ploemen, B. van Ommen, M.L.P.S. van Iersel, C.J.M. Rompelberg, H. Verhagen and P.J. van Bladeren. (1996) Irreversible inhibition of cytosolic glutathione S-transferase, in: N.P.E. Vermeulen, G.J. Mulder, H. Nieuwenhuyse, W.H.M. Peters and P.J. van Bladeren (Eds.), Glutathione S-transferases: Structure, Functions adn Clinical Applications, Taylor and Francis, London, 143-153.
8. M.L.P.S. van Iersel, J.H.T.M. Ploemen, I. Struik, C. van Amersfoort, A.E. Keyzer, J.G. Schefferlie and P.J. van Bladeren. (1996) Inhibition of glutathione S-transferase activity in human melanoma cells by α,β-unsaturated carbonyl derivatives. Effects of acrolein, cinnamaldehyde, citral, crotonaldehyde, curcumin, ethacrynic acid, and trans-2-hexenal. *Chemico-Biological Interactions*, **102**, 117-132.
9. M.L.P.S. van Iersel, J.H.T.M. Ploemen, M. LoBello, G. Federici and P.J. van Bladeren. (1997) Interactions of alpha, beta-unsaturated aldehydes and ketones with human glutathione S-transferase P1-1. *Chemico-Biological Interactions*, **108**, 67-78.

10. M.L.P.S. van Iersel, M.M.H. van Lipzig, I.M.C.M. Rietjens, J. Vervoort and P.J. van Bladeren. GSTP1-1 stereospecifically catalyzes glutathione conjugation of ethacrynic acid. (*Submitted to FEBS Letters*)
11. M.L.P.S. van Iersel, N.H.P. Cnubben, N. Smink, J.H. Koeman and P.J. van Bladeren. Interactions of prostaglandin A_2 with the glutathione-mediated biotransformation system. *(Submitted to Biochemical Pharmacology)*

Abstracts

The role of glutathione conjugation in the toxicity of α,β-unsaturated aldehydes. M.L.P.S. van Iersel and P.J. van Bladeren. International ISSX-workshop on Glutathione S-transferases, Noordwijkerhout, 22-25 april, 1995.

Inhibition of glutathione S-transferase activity in human melanoma cells by α,β-unsaturated carbonyl derivatives. M.L.P.S. van Iersel, J.H.T.M. Ploemen, I. Struik, C. Van Amersfoort, A.E. Keyzer, J.G. Schefferlie and P.J. van Bladeren. 7th North American ISSX Meeting, San Diego, 20-24 october, 1996.

Glutathione-related metabolism of prostaglandin A_2 and J_2. M.L.P.S. van Iersel, N.H.P. Cnubben, J.J.P. Bogaards, R. Evers, P. Borst and P.J. van Bladeren.International Workshop on glutathione transferases, Rome, 7-10 november, 1997.

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