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Divergent Activities of Human Glutathione Transferases in the Bioactivation of Azathioprine

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ABSTRACT

Azathioprine is a thiopurine prodrug clinically used for immunosuppression in the treatment of inflammatory diseases and in pharmacological regimens of organ transplantations. Its pharmacological action is based on the release of 6-mercaptopurine, but the biochemical processes underlying this biotransformation have remained obscure. In this investigation, human glutathione transferases (GSTs) from seven distinct classes were assayed with azathioprine. GSTs A1-1, A2-2, and M1-1, all abundantly expressed in human liver, displayed the highest activity among the 14 GSTs tested. The uncatalyzed reaction of azathioprine with glutathione was estimated to be less than 1% of the GST-catalyzed biotransformation. GST M1-1 is polymorphic with a frequently occurring null allele, and GSTs A1-1 and A2-2 show variable expression levels in human subjects, implying significant differences in the rate of 6-mercaptopurine release from azathioprine. Individuals expressing high GST activity are apparently predisposed for adverse reactions to azathioprine treatment, both by promoting excessively high concentrations of free 6-mercaptopurine and its toxic metabolites and by depleting cellular glutathione. These novel aspects of GST-dependent azathioprine biotransformation have not been considered previously.

The thiopurine drugs 6-mercaptopurine and 6-thioguanine were synthesized in the 1950s by George Hitchings and Gertrude B. Elion (Giner-Sorolla, 1988). The compounds act as antimetabolites that interfere with DNA synthesis. Nucleotide metabolites of the thiopurines are incorporated into replicating nucleic acids and compromise their chemical and physical stability. In particular, 6-mercaptopurine blocks the de novo pathway of purine biosynthesis by formation of 6-thioinosine 5′-monophosphate (6-TIMP) and ultimately 6-thioguanine nucleotides (6-TGN) (Lennard, 1992). 6-Mercaptopurine has found use in the chemotherapy of childhood acute lymphoblastic leukemia.

Azathioprine is a 1-methyl-4-nitroimidazol-5-yl derivative of 6-mercaptopurine (van Scoik et al., 1985). Since its introduction, the clinical use of azathioprine has been dominated by its ability to suppress the immune system. Soon after its first synthesis, azathioprine was introduced as an immunosuppressant for the transplantation of kidneys by Sir Roy Calne (Calne et al., 1962; Elion, 1989), thereby replacing 6-mercaptopurine. Azathioprine is still used for immunosuppression in connection with organ transplantation and in the treatment of autoimmune diseases (Cara et al., 2004).

The pharmacological action of azathioprine is based on the release of 6-mercaptopurine (Fig. 1), which can be effected by the elimination of the imidazole moiety by a chemical nucleophile such as a thiol. Thus, any enzyme selectively promoting the displacement reaction between a potential activator and azathioprine would play a pivotal role in the biotransformation of the drug. Because glutathione is the most abundant low-molecular-mass thiol in the cell (Josephy and Mannervik, 2006), it is a primary candidate as the activator of azathioprine. Glutathione *S*-transferases (GSTs) are enzymes catalyzing the conjugation of glutathione with a very wide range of electrophilic compounds and are highly specific for the thiol substrate (Josephy and Mannervik, 2006). Therefore, no other thiol group, in proteins or in any other cellular component known, could substitute for glutathione as a GST substrate. Given a sufficiently high GST activity, the imidazole portion of azathioprine will be exclusively transferred to glutathione and not to other intracellular thiols in the biotransformation of the drug.

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ABBREVIATIONS: 6-TIMP, 6-thioinosine 5′-monophosphate; 6-TGN, 6-thioguanine nucleotide; GST, glutathione transferase; GSH, glutathione; GS, glutathionyl; CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl-1-thio-β-D-galactopyranoside; TPMT, thiopurine *S*-methyltransferase; 6-MMPR, 6-methylmercaptopurine ribonucleotide; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PCR, polymerase chain reaction; buffer C, formic acid; buffer D, formic acid and acetonitrile.

Despite several decades of clinical use, the biochemistry of azathioprine has not been fully elucidated. Crude preparations of rat liver (Kaplowitz, 1976) and human liver (von Bahr et al., 1980) demonstrate GST activity with azathioprine, but experiments with purified enzymes have not been performed. To fully understand the pharmacological effect of azathioprine in humans, it is consequently important to elucidate the catalytic activities with azathioprine contributed by the numerous human GSTs. Human cytosolic GSTs are encoded by 17 genes, and the corresponding proteins can be divided into seven distinct classes based on their amino acid sequences (Mannervik et al., 2005). A distantly related enzyme called GST K1-1, occurring in mitochondria and peroxisomes, has also been characterized (Pemble et al., 1996; Morel et al., 2004). The members of the Alpha, Mu, and Pi classes are the most abundant GSTs and are the enzymes most likely to be involved in the metabolism of xenobiotics. The soluble GSTs are dimeric proteins composed of two identical or closely related subunits from the same class. GST subunits are differentially expressed in mammalian tissues (Mannervik et al., 1983). For example, GST A1-1 and GST A2-2 are highly abundant in human liver (van Ommen et al., 1990; Rowe et al., 1997), whereas the homologous Alpha class enzyme GST A3-3 is expressed primarily in tissues such as placenta, adrenal gland, and gonads. GST P1-1 of the Pi class is a principal enzyme in most tissues but is undetectable in normal hepatocytes (Johansson and Mannervik, 2001; Dhanani and Awasthi, 2006). Although GSTs have partly overlapping substrate specificities, there are marked differences in their substrate-selectivity profiles (Josephy and Mannervik, 2006). The differential expression of the GSTs may therefore have profound consequences for tissue-selective metabolism and organ-specific toxicity caused by drugs and other xenobiotics.

This investigation shows that three different GSTs, A1-1, A2-2, and M1-1, are the main contributors to the release of 6-mercaptopurine from azathioprine by the nucleophilic substitution involving glutathione. Our study also suggests that unexplained adverse drug reactions observed in the clinical use of azathioprine could be related not only to the thiopurine *S*-methyltransferase (TPMT) phenotype, as previously assumed, but also to excessive liberation of 6-mercaptopurine and depletion of glutathione as a result of high GST activities.

Materials and Methods

Reagents and Enzymes. Azathioprine, 6-mercaptopurine, glutathione, NADPH, glutathione reductase, 1-chloro-2,4-dinitrobenzene (CDNB), isopropyl-1-thio-β-D-galactopyranoside (IPTG), β-mercaptoethanol, and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO). Cumene hydroperoxide was purchased from Merck Schuchardt (Darmstadt, Germany). Recombinant human GSTs A1-1, A2-2, A3-3, A4-4, M1-1, M2-2, M3-3, M4-4, M5-5, P1-1, T1-1, and Z1-1 were expressed and purified by methods published previously (Blackburn et al., 2000; Lien et al., 2002). GST K1-1 was expressed in fusion with a calmodulin-binding peptide (Morel et al., 2004) and was used in unpurified form. GST O1-1 was cloned, expressed, and purified to homogeneity as described below.

Spectrophotometric Assays. All activity measurements were performed on a SPECTRAmax PLUS^{384} microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Measurements with azathioprine were performed in 0.1 M sodium phosphate buffer, pH 7.4, with 1 mM EDTA at 30°C. Concentrations of catalytically active GSTs were determined by measuring activities with 1 mM CDNB and 1 mM glutathione in 0.1 M sodium phosphate buffer, pH 6.5, at 30°C and comparing them with specific activities determined previously: GST A1-1 (82 $\mu mol \cdot min^{-1} \cdot mg^{-1}$), A2-2 (80), A3-3 (23), A4-4 (5), M1-1 (190), M2-2 (400), M3-3 (15), M4-4 (1.4), M5-5 (170), K1-1 (1.05), O1-1 (0.1), P1-1 (103), and T1-1 (0.005). GST Z1-1 activity with CDNB is undetectable and was assayed with 1.5 mM cumene hydroperoxide and 1 mM glutathione coupled with 0.2 mM NADPH and 1.0 U glutathione reductase in 0.1 M sodium phosphate buffer, pH 7.0, at 30°C (0.16 $\mu mol \cdot min^{-1} \cdot mg^{-1}$).

Determination of Extinction Coefficient. The net extinction coefficient for the reaction of azathioprine with glutathione was determined experimentally as 16,000 $M^{-1} \cdot cm^{-1}$ at 320 nm; the reaction of 0.1 mM azathioprine with 10 mM glutathione was followed to completion in the presence of enzyme.

Kinetic Constants. Specific activities of the GSTs were determined by measurements at 320 nm in 0.1 M sodium phosphate buffer, 1 mM EDTA, pH 7.4, at 30°C in a quartz cuvette with a 0.5-cm light path using 0.2 mM azathioprine and 1 mM GSH. Steady-state kinetic constants were determined by measurements with a constant glutathione concentration of 1 mM, and azathioprine

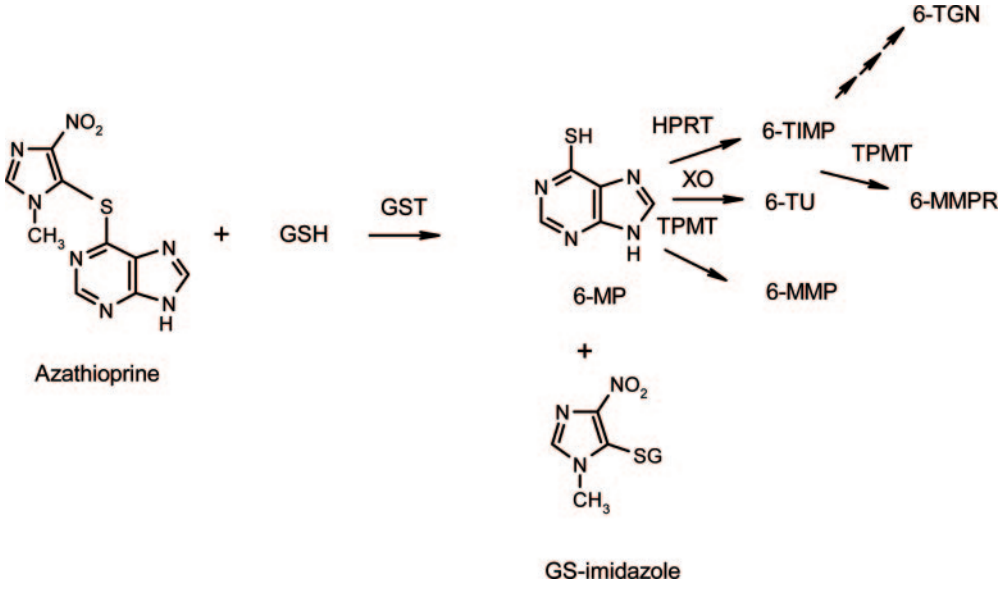

Fig. 1. Scheme of azathioprine and 6-mercaptopurine metabolism. 6-MP, 6-mercaptopurine; 6-MMP, 6-methylmercaptopurine; XO, xanthine oxidase; 6-TU, 6-thiouric acid; HPRT, hypoxanthine-guanine phosphoribosyl transferase. GS-Imidazole, glutathionyl derivative of 1-methyl-4-nitroimidazole.

concentrations varied between 0.05 and 1.0 mM. Nonlinear regression analyses were made with Prism (GraphPad Software Inc., San Diego, CA).

HPLC Analysis. Separations were performed on a 250 mm × 4.6 mm internal diameter Nucleosil 100-5 C18 column at 30°C using Merck Hitachi LaChrom Elite chromatograph (Darmstadt, Germany). The flow rate was 1 ml/min with a mobile phase of 50 mM sodium phosphate buffer and 1 mM EDTA, pH 7.4. Elution was made with a gradient of 0 to 27.5% (v/v) acetonitrile during 27 min, followed by re-equilibration with the mobile phase for 8 min. Analyses were made by injection of 1 μl of pure substrates of 0.1 mM azathioprine or 0.1 mM 6-mercaptopurine to determine their retention time. To follow the consumption of azathioprine and the formation of the products 6-mercaptopurine and GS-imidazole, 1-μl aliquots of the reaction mixture were taken every 35 min and analyzed. The reaction medium consisted of mobile phase (without acetonitrile) containing 0.1 mM azathioprine and 1 mM glutathione. The HPLC analysis of enzymatic reactions were carried out with 10 μg of GSTs A1-1, A2-2, M1-1, or M2-2.

Cloning of cDNA Encoding GST O1-1. cDNA encoding GST O1-1 was PCR amplified from a human cDNA library of normal human placenta (Invitrogen, Groningen, The Netherlands) using a sense primer with EcoR1 and NdeI restriction sites: AATAATGAATTCATATGTCCGGGGAGTCAGCAG; and antisense primer with a SalI restriction site: AATGTCGACTCAGAGCCCATAGTCACAG. The oligonucleotides were custom-synthesized by Interactiva (Ulm, Germany). The EcoR1 and SalI sites were used for cloning into the pGEM-3Z (Promega, Madison, WI) vector and the NdeI and SalI sites for the cloning into expression pET21a(+) vector (Novagen, Madison, WI). The PCR reaction mixture contained 0.5 μl of placenta cDNA, 0.2 mM dNTPs, 0.2 μM concentration of sense and antisense primer, respectively, 2 mM $MgCl_2$, 35 μl of H_2O, 10% Pfu buffer, and 0.5 μl of Pfu DNA polymerase (Stratagene, La Jolla, CA) in a total volume of 50 μl. The PCR program was started by denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 65°C for 2 min, and 72°C for 2 min. The PCR was terminated at 72°C for 10 min. The PCR fragment was electrophoresed on a 1% (w/v) agarose gel and purified with QIAGEN gel kit (QIAGEN, Hilden, Germany). The restricted fragment was ligated into a pGEM-3Z vector for transformation of *Escherichia coli* XL1 Blue cells (Stratagene) and subsequent analysis of the sequence. The *GSTO1* gene sequence is polymorphic at codon 140. The cDNA clone isolated corresponds to *GSTO1*D140,* where base number 2 in the codon is adenine, which makes the amino acid in position 140 an aspartic acid instead of an alanine as in *GSTO1*A140* (Whitbread et al., 2003).

Purification of GST O1-1. GST O1-1 in the pET21a(+) vector was used to transform *E. coli* BL-21 (DE3) (Novagen), and the cells were grown in 500 ml of LB medium with 100 μg/ml ampicillin. At optical density at 600 nm of 0.6, enzyme production was induced with IPTG to a final concentration of 1 mM. The cells were harvested 2 h 45 min after the induction by centrifugation and lysed by ultrasonication. The protein was purified on an *S*-hexylglutathione affinity gel (Mannervik and Guthenberg, 1981) and eluted with 5 mM *S*-hexylglutathione. The pooled fractions was dialyzed against 10 mM Tris-HCl buffer, pH 7.8, 0.02% (w/v) sodium azide, and 1 mM β-mercaptoethanol. The enzymatic activity of GST O1-1, measured with CDNB, was in agreement with the value reported previously.

Mass Spectrometric Analyses. HPLC/MS separations were performed on a Zorbax SB-C18 column (150 × 0.5 mm, 5 μm; Agilent Technologies, Palo Alto, CA) using gradient elution accomplished with a Rheos 2000 pump (Flux Instruments AG, Basel, Switzerland) at a total flow rate of 120 μl/min. The flow was split, giving a flow rate of approximately 10 μl/min through the separation column. Buffer C consisted of 10 mM formic acid, and buffer D was a mixture of 10 mM formic acid and acetonitrile, 5:95 (v/v). The programmed gradient was 0% D for 10 min followed by an increase of 0 to 50% D in 30 min. The HPLC column was coupled online to a Q TRAP linear ion-trap mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, ON, Canada) equipped with a pneumatically assisted electrospray ionization interface. Before HPLC/MS analysis, the reaction mixture (without EDTA) was diluted 1/10 in 10 mM formic acid, and subsequently, aliquots of 0.45 μl were injected onto the separation column. The effluent was directed away from the mass spectrometer for the first 2 min of the gradient to reduce the risk of contamination. The electrospray ionization and declustering potentials were 5000 and 70 V, respectively, and the curtain and nebulizer gas flows were 15 and 25 psi, respectively. Enhanced MS scanning from 150 to 1000 atomic mass units was performed using an ion-trap fill time of 50 ms and a scanning rate of 1000 atomic mass units per second. Concurrent Q0 trapping was also used during HPLC/MS analysis. The software Analyst 1.4.1 (MDS Sciex, Concord, ON, Canada) was used for data acquisition.

Results

The UV spectra of azathioprine and 6-mercaptopurine show a maximal difference at 320 nm (Fig. 2), which was used for monitoring the formation of 6-mercaptopurine from azathioprine in the presence of glutathione. The sulfur of glutathione forms a link to the imidazole moiety of azathioprine, and the glutathione conjugate contributes to the absorbance at the same wavelength. The net extinction coefficient for the reaction was determined as 16,000 $M^{-1} \cdot cm^{-1}$.

HPLC/UV. The reaction between azathioprine and glutathione is expected to yield 6-mercaptopurine and GS-imidazole (Fig. 1). This chemical transformation was indeed observed by following the disappearance of azathioprine and the formation of the products as a function of time. HPLC demonstrated that the reaction occurred with the expected stoichiometry (Figs. 3 and 4). This was basically true for both the nonenzymatic and all GST-catalyzed reactions. However, in the presence of GST M2-2, a very small peak of an unidentified product emerged in parallel with 6-mercaptopurine and GS-imidazole (Fig. 3). Mass spectrometric analysis verified the nature of the other reactants (Fig. 5), but the nature of the minor product could not be established. Because none of the other GSTs tested gave rise to a similar minor product, and given the low activity of GST M2-2 with azathioprine (see below), the nature of this component was not further investigated.

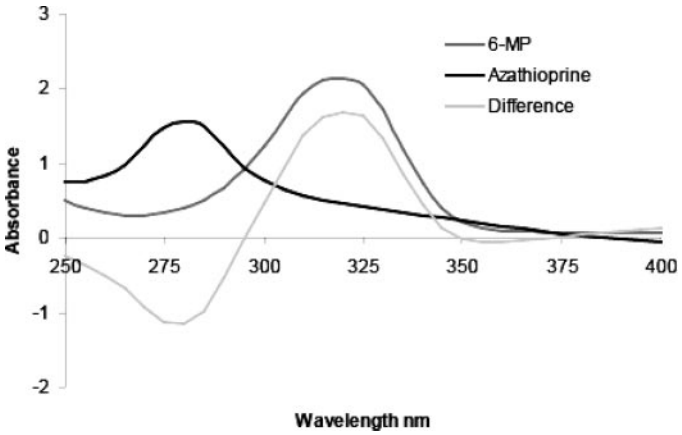

Fig. 2. Ultraviolet spectra of azathioprine (black) and 6-mercaptopurine (dark gray). Spectra of azathioprine and glutathione reaction in 0.1 M sodium phosphate buffer and 1 mM EDTA, pH 7.4, at 30°C. A difference spectrum (light gray) demonstrates the regions of maximal sensitivity for monitoring the reaction.

HPLC/MS. Through direct infusion experiments of different standards, each containing one analyte of interest, the mass-to-charge ratios (*m/z*) of the most abundant ions in each solution were determined. The protonated molecular ions of azathioprine and 6-mercaptopurine corresponded to *m/z* 278.0 and 153.1, respectively. Due to the absence of a standard, the *m/z* of the protonated molecular ion of GS-imidazole was calculated to 433.4. Injection of diluted reaction mixture onto the HPLC/MS system was performed 30 min and 3 h after the reaction initiation. Comparison of the extracted ion chromatograms, verified the bioactivation of azathioprine through the increased signals of 6-mercaptopurine and GS-imidazole and the accompanying decreased signal of azathioprine (Fig. 5, a–c). In Fig. 5d, a summed mass spectrum for the chromatographic peak of GS-imidazole in Fig. 5c is depicted. The most abundant peak has a *m/z* of 433.3, in perfect agreement with the calculated *m/z* for the protonated molecule of GS-imidazole. Due to partial fragmentation of the protonated molecule (attained up front or in the ion trap), some additional breakage of the peptide backbone was also observed. The similar fragmentation behavior, as established for glutathione alone (results not shown), further proves the identity of the reaction product. Cleavage of the peptide backbone yielded the b_2 and y_2 fragment ion, with *m/z* 358.2 and 304.2, respectively. The identity of fragment x (*m/z* 257.2) was not established.

Specific Activities with Azathioprine. Specific activities were measured for 14 members of 7 GST classes known to be expressed in human tissues. The proteins represent the Alpha, Mu, Omega, Pi, Theta, and Zeta class GSTs and the distantly related Kappa class. The enzymes are known to differ widely in their substrate selectivity profiles with alternative substrates. The divergent catalytic activities were also evident with azathioprine; the different GSTs were showing diverse activities in between classes and within a class (Table 1). All representatives of the Alpha class GSTs A1-1, A2-2, A3-3, and A4-4 displayed measurable activity. GST A2-2 had the highest value (0.51 μmol · min^{-1} · mg^{-1}) in the Alpha class and, in fact, the highest value of all 14 GSTs investigated. The specific activity of GST A1-1, the second best in the Alpha class, was 2.5 times lower than that of GST A2-2. The GST A3-3 and GST A4-4 activities were 50 times lower than the GST A2-2 value. With other substrates, GST A2-2

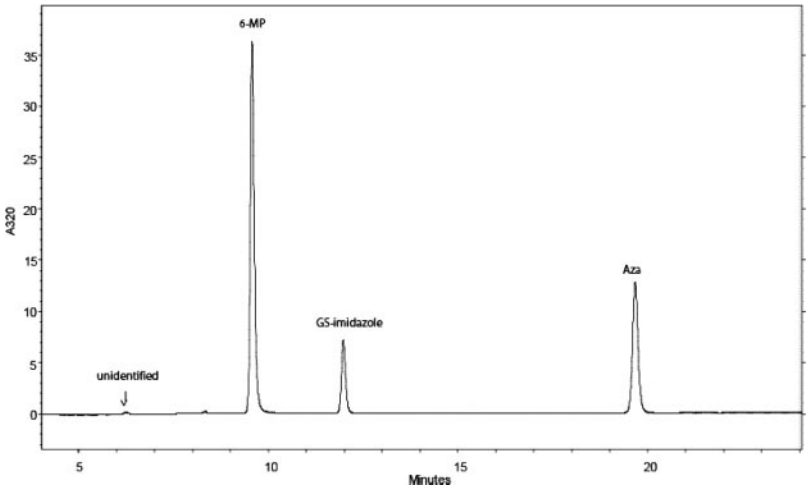

Fig. 3. HPLC analysis of the reaction between azathioprine (Aza) and glutathione. Chromatogram showing the substrate azathioprine and both products 6-mercaptopurine (6-MP) and GS-imidazole of the GST M2-2-catalyzed reaction with glutathione monitored at 320 nm. The reaction mixture contained 0.1 mM azathioprine and 1 mM glutathione in 50 mM sodium phosphate buffer, pH 7.4, and 10 μg of GST M2-2. In the chromatogram, there is also an unidentified peak increasing with time found in the presence of GST M2-2, but not with the other GSTs tested. The run was made on a reverse-phase C18 column at 30°C with a flow rate of 1 ml/min of 50 mM sodium phosphate buffer, pH 7.4, and an acetonitrile gradient (0% to 27%, v/v).

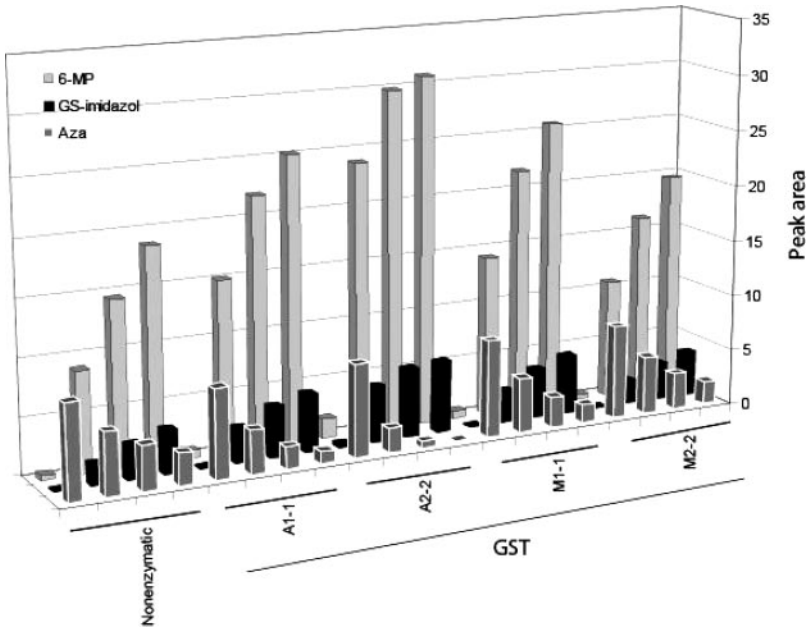

Fig. 4. Progress of reactions between azathioprine and glutathione. HPLC peak areas of azathioprine and products obtained from assay mixtures of nonenzymatic and GST-catalyzed reactions. For each reaction, consecutive HPLC runs were made on samples taken every 35 min. Separations were made as described in the legend to Fig. 3. The reaction mixtures contained 0.1 mM azathioprine and 1 mM glutathione in 50 mM sodium phosphate buffer, pH 7.4, at 30°C. GST-catalyzed reactions were monitored in the presence of 10 μg of enzyme.

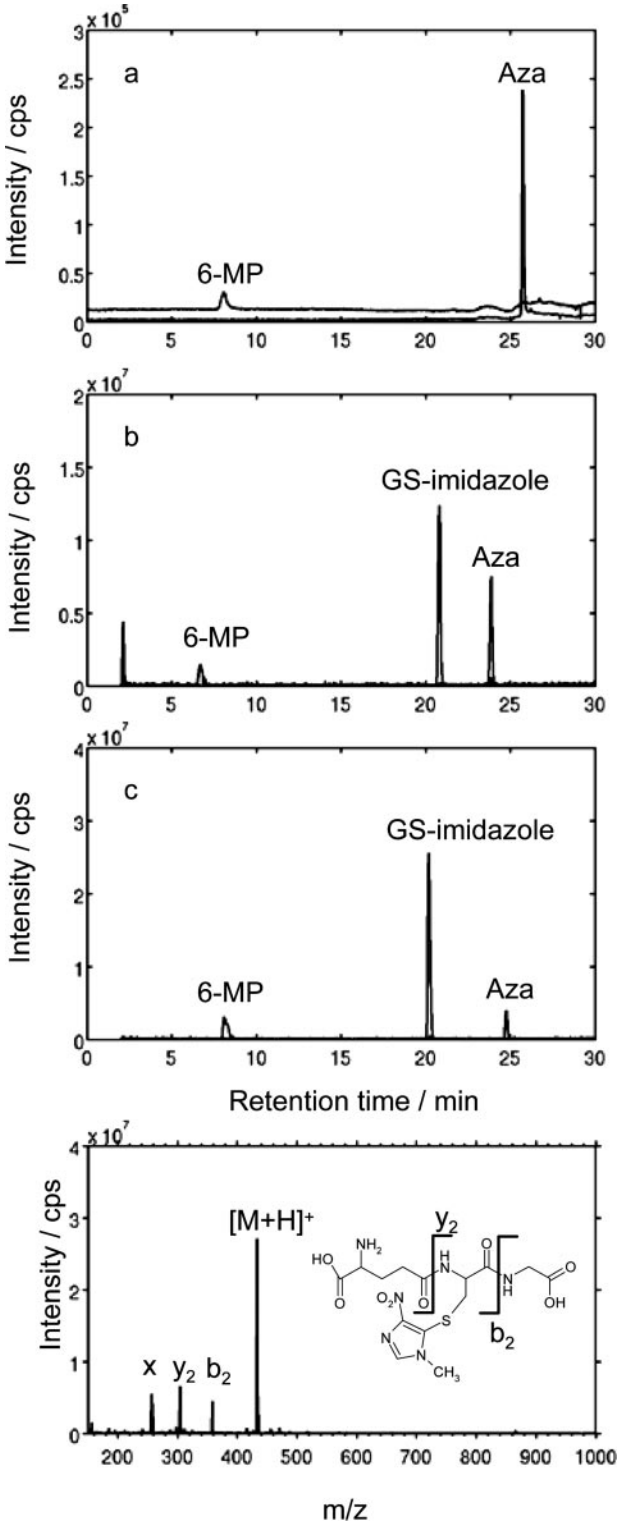

Fig. 5. Verification of the chemical transformation by use of HPLC/MS Injection of a standard solution containing 3 μM 6-mercaptopurine and azathioprine in 10 mM formic acid (a), a reaction mixture with GST M2-2 after 30 min diluted 1:10 in 10 mM formic acid (b), and a reaction mixture with GST M2-2 after 3 h diluted 1/10 in 10 mM formic acid onto the HPLC/MS-system (c and d). In a, selected ion chromatograms of *m/z* 153.1 and *m/z* 278.0, and in b and c, extracted ion chromatograms of *m/z* 432.8 to 433.7, *m/z* 277.7 to 279.5, and *m/z* 152.3 to 154.1 are shown. d, the summed mass spectrum for the chromatographic peak marked GS-imidazole is displayed, $[M+H]^+$ corresponds to the protonated molecule of GS-imidazole. The fragments b_2 and y_2 correspond to the cleavage of the peptide backbone of GS-imidazole as depicted in the inserted structure.

usually has a lower or equal activity compared with GST A1-1 catalyzing the same reaction.

In the Mu class, GST M1-1 had the highest specific activity with azathioprine among the five known enzymes, and the value is similar to that of GST A1-1. GST M1-1 is expressed in the liver, as are GST A1-1 and GST A2-2. The Mu class GST M2-2 also displays a clear activity with azathioprine, but the specific activity is 3 times lower than the GST M1-1 activity, and 10 times lower than the GST A2-2 activity. Activities of GST M3-3, GST M4-4, and GST M5-5 were barely detectable. None of representatives of the Omega, Pi, Theta, and Zeta classes had any detectable activity with azathioprine. Particularly noteworthy is the finding that no significant activity was detected with GST P1-1, which is the most widely distributed GST in the human body. The more distantly related mitochondrial and peroxisomal GST K1-1 displayed a low activity in the assay with azathioprine, comparable with the activity of GST M2-2.

The fact that GST K1-1 contained a peptide tag necessitated control experiments to ascertain that the enzyme was catalytically fully competent. Assays with CDNB demonstrated an activity in agreement with that reported for unmodified GST K1-1 (Morel et al., 2004), suggesting that the peptide had negligible influence and that the low activity with azathioprine in our experiments was truly representative of the GST K1-1 activity with this latter substrate.

An important aspect of azathioprine metabolism is the depletion of glutathione in hepatocytes, which eventually leads to mitochondrial injury and consequent ATP depletion (Lee and Farrell, 2001). In this context, the activity of GST K1-1 could possibly contribute to the depletion of glutathione in the mitochondrial matrix, even if the specific activity of the enzyme is relatively low. In isolated hepatocytes, the loss of ATP leads to cell necrosis rather than to apoptosis, which requires ATP (Leist et al., 1997). In these in vitro studies, the toxicity was not caused by the released 6-mercaptopurine, which when tested by itself was found to be nontoxic to the hepatocytes (Lee and Farrell, 2001). It can therefore be concluded that a high hepatic GST activity in combination with

TABLE 1

Tissue expression and specific activities with azathioprine of human GSTs

Values are based on means on replicates from separate ($n \geq 3$) measurements ± S.D. The tissue distributions are based on previous publications (Juronen et al., 1996; Rowe et al., 1997; Johansson and Mannervik, 2001; Coles and Kadlubar, 2005; M. H. Edalat, B. Mannervik, R. Löfberg, Å. Öst, S. Pettersson, L.-G. Axelsson, unpublished results).

GST	Small Intestine	Erythrocytes	Liver	Specific Activity	S.D.
				μmol/mg/min	
A1-1	+	−	+	0.21	0.060
A2-2	+	−	+	0.53	0.085
A3-3	−	−	−	0.01	0.003
A4-4	+	−	+	0.01	0.004
M1-1	(+)[a]	−	(+)[a]	0.17	0.003
M2-2	−	−	−	0.05	0.001
M3-3	+	−	+	<0.001	
M4-4	−	−	+	<0.001	
M5-5	−	−	-	<0.003	
K1-1	+	−	+	0.05	0.001
O1-1	+	−	+	0	
P1-1	+	+	−	0	
T1-1	(+)[a]	(+)[a]	(+)[a]	0	
Z1-1	+	−	+	0	

−, no detection.
[a] If not a null individual.

a high dose of azathioprine can lead to glutathione depletion and cause necrotic lesions. This hepatotoxicity is apparently independent of the TPMT activity, which does not influence glutathione metabolism.

Steady-State Kinetics. GST A1-1, A2-2, and M1-1 were investigated kinetically at the physiological pH value of 7.4. The K_m value for glutathione is approximately 0.1 mM for all enzymes, which is a concentration lower than the millimolar concentrations of the thiol in most tissues. The azathioprine concentration was therefore varied in the presence of 1 mM glutathione to determine the parameters of the Michaelis-Menten equation. The physiologically most important factor is the k_{cat}/K_m, which governs the substrate selectivity. This parameter is also a measure of catalytic efficiency and the rate constant for the enzymatic reaction at low substrate concentrations (in comparison with the K_m value). Table 2 shows that the apparent K_m values for azathioprine ranged between 0.5 and 1 mM, concentrations, considerably greater than those expected in pharmacological applications. The catalytically most efficient enzyme is GST A2-2. The efficiencies of GSTs A1-1 and M1-1 are similar but correspond only to half of the GST A2-2 value. The ratios of the apparent k_{cat} values reflect those of the catalytic efficiencies, with GST A2-2 as the most active among the enzymes. For experimental reasons, azathioprine concentrations could not reach enzyme saturation. The k_{cat}/K_m values were therefore estimated directly by regression analysis of the initial part of the saturation curve, which gives higher precision of the quotient than dividing the k_{cat} by the K_m value.

Discussion

The majority of the reactions catalyzed by GSTs are accompanied by a parallel nonenzymatic reaction. In the case of azathioprine, a significant nonenzymatic reaction with glutathione is observed (Fig. 4). However, GSTs are among the most abundant soluble proteins in liver and therefore play a prominent role in the biotransformation of electrophiles even when the compounds have high intrinsic reactivity. The cytoplasmic concentration of soluble proteins in hepatocytes has been determined as approximately 100 mg/ml (Moron et al., 1979), and GSTs may account for several percent of these proteins. For example, human GST A1-1 has been determined as approximately 1% of the human hepatic cytosolic proteins (Coles and Kadlubar, 2005). This value corresponds to 1 mg of GST A1-1 per milliliter of cytoplasm. Other studies have reported even higher cytosolic concentrations of GSTs (van Ommen et al., 1990; Rowe et al., 1997), making all values for the enzymes in Table 3 conservative estimates.

To assess the importance of the GST-dependent activation of azathioprine compared with the uncatalyzed reaction, an estimation was made on the basis of the determined k_{cat}/K_m values for GSTs A1-1, A2-2, and M1-1 (Table 2) and the reported values of the hepatic expression of these enzymes (Table 3). The nonenzymatic reaction is bimolecular and governed by the second-order rate constant (0.008 $mM^{-1} \cdot min^{-1}$). Assuming a glutathione concentration of 1 mM, the pseudo first-order rate constant of the nonenzymatic reaction is 0.008 min^{-1}. The calculated first-order rate constants for the GST-catalyzed reactions (Table 3) are based on the k_{cat}/K_m values multiplied by the GST expression levels reported by Coles and Kadlubar (2005). Obviously, the sum of the GST-catalyzed contributions exceeds the uncatalyzed by approximately 2 orders of magnitude. Assuming a 10-fold higher glutathione concentration in the relevant cells would preferentially increase the nonenzymatic rat, but would still make the GST-catalyzed reaction responsible for >90% of the biotransformation of azathioprine. The kinetic parameters were determined at 30°C, but control experiments at 37°C (data not shown) did not markedly change the relative contributions of GST-catalyzed and nonenzymatic reactions. In the small intestine, the relative rates would be similar to those in liver, but in the erythrocytes the GSTs probably make a negligible contribution (see below).

A given GST has a differential expression in human tissues (Coles and Kadlubar, 2005). Considering an oral administration of azathioprine, the expression of the various GSTs in the small intestine, erythrocytes, and liver is of particular interest. Table 1 summarizes information about the distribution of GSTs in these tissues. The liver is normally regarded as playing a dominant role in the biotransformation of xenobiotics, and in the case of azathioprine, it is noteworthy that the most efficient enzymes, GST A2-2, GST A1-1, and GST M1-1, are all expressed at high levels in this tissue. The small intestine has similar high expression levels of the same GSTs (Gibbs et al., 1998; Coles and Kadlubar, 2005), whereas erythrocytes are lacking detectable amounts of these same enzymes (Dhanani and Awasthi, 2006). Instead, erythrocytes are dominated by GST P1-1 and, to a smaller extent also contain GST T1-1 (Juronen et al., 1996; Schröder et al., 1996), which both have negligible activity with azathioprine. GST A1-1 and GST A2-2 are also expressed at high levels in the proximal tubules of the kidney (Sundberg et al., 1993) and are, like GST M1-1, present in lower concentrations in other tissues as well. However, it can still be concluded that the major fraction of azathioprine is bioactivated by the GSTs in the liver, considering the size of the organ and the abundance of the enzymes that are most active with azathioprine.

TABLE 2

Kinetic parameters of human GSTs with azathioprine and glutathione as substrates

The experimental conditions were 1 mM GSH in 0.1 M sodium phosphate buffer, pH 7.4, and 1 mM EDTA at 30°C. The parameter values are means from separate ($n \geq 3$) substrate-activity curves (± S.D.).

GST	K_m (app.)	k_{cat} (app.)	k_{cat}/K_m
	mM	s^{-1}	$mM^{-1} \cdot s^{-1}$
A1-1	0.56 ± 0.26	0.37 ± 0.20	0.48 ± 0.04
A2-2	0.95 ± 0.22	1.31 ± 0.70	1.17 ± 0.07
M1-1	0.91 ± 0.55	0.59 ± 0.28	0.53 ± 0.04

app., apparent.

TABLE 3

Relative contributions to the bioactivation of azathioprine

Apparent rate constant in liver is based on a total soluble protein concentration of 100 mg per milliliter of cytoplasm (Moron et al., 1979), the GST expression levels (Coles and Kadlubar, 2005), and the k_{cat}/K_m values (given in Table 2). The values are calculated for 1 mM GSH, which is close to a saturating concentration for the GSTs and in the (lower) range of estimated intracellular concentration.

GST	Expression			Apparent Rate Constant in Liver
	Small Intestine	Erythrocytes	Liver	
	μg/mg cytosolic protein			min^{-1}
A1-1	3–10	0	3–10	0.17–0.56
A2-2	1–2	0	1–7	0.14–0.96
M1-1	Unknown	0	9	0.56
Nonenzymatic				0.008

The expression of the mitochondrial and peroxisomal GST K1-1 is relatively high in liver, kidney, and adrenals (Morel et al., 2004), but its low specific activity (Table 1) suggests that its contribution to overall azathioprine bioactivation is marginal.

It should also be noted that a fraction of the administered drug may actually undergo extracellular metabolism in the gut, because both glutathione (Smith et al., 1996) and GSTs (Howie et al., 1989) are present in the bile and secreted into the duodenum. However, the quantitative importance of this extracellular glutathione-dependent biotransformation of xenobiotics is unknown.

Important interindividual differences in the expression of human GSTs have been discovered. The first report of phenotypic differences of functional importance was that some human livers contained a distinct GST, characterized by relatively high activity with *trans*-phenylbutenone. This previously unrecognized GST is present in some individuals, whereas the same enzyme is absent in other individuals. The same enzyme, first called GST μ and now known as GST M1-1, was subsequently shown to be active with mutagenic and carcinogenic epoxides such as benzo(*a*)pyrene 4,5-oxide (Warholm et al., 1981). The absence of GST M1-1 was later shown to be caused by the absence of the corresponding gene on human chromosome 1 (Seidegård et al., 1988). An additional null genotype was later demonstrated for human GST T1-1 (Pemble et al., 1994). Other allelic variants of GSTs have also been discovered and may influence the level of protein expression (Coles and Kadlubar, 2005; Holley et al., 2006). The latter genetic variability contributes to differences among individuals in the amounts of GSTs expressed. This is reflected in the initial studies of azathioprine conjugation in crude hepatic cytosol preparations from eight human subjects, which demonstrated up to 5-fold differences in activity (von Bahr et al., 1980).

Table 2 displays the steady-state kinetic parameters of the GSTs found to have the highest activity with azathioprine. Based on the k_{cat} values, GST A2-2 is clearly the most active enzyme. Based on the normal dosage of azathioprine and assuming an even distribution in the body, it can be estimated that the intracellular concentration could not increase to more than 0.1 mM. In fact, plasma concentrations are in the submicromolar range after oral administration (Odlind et al., 1986). These azathioprine concentrations are considerably lower than the K_m values of all GSTs in Table 2, suggesting that the enzymes act in a range of substrate concentrations far less than the saturation of their active sites. Under such conditions the rate is governed by the k_{cat}/K_m values, and also here, GST A2-2 is the most efficient catalyst (Table 2). The values for GSTs A1-1 and M1-1 are both approximately 50% of the GST A2-2 value. Considering the lower expression level of GST A2-2 in comparison with GST A1-1 (van Ommen et al., 1990; Rowe et al., 1997; Coles and Kadlubar, 2005), their contributions to the biotransformation of azathioprine will be quantitatively similar (Table 3).

The clinical use of azathioprine is associated with frequent cases of adverse drug reactions (Cara et al., 2004; Bajaj et al., 2005). This undesired toxicity is related to higher-than-expected rates of 6-mercaptopurine liberation from azathioprine. The portion of 6-mercaptopurine that is not consumed as an antimetabolite in the biosynthesis of nucleic acids is normally inactivated by *S*-methylation catalyzed by TPMT. Insufficient TPMT activity may cause an overshoot in 6-mercaptopurine production, resulting in enhanced toxicity. TPMT is genetically polymorphic, and certain genotypes lead to low enzyme activity (Weinshilboum, 2001). Some clinical centers therefore subject patients to genotyping before the administration of azathioprine to avoid adverse drug reactions (Cara et al., 2004). However, not all of the idiosyncratic reactions to azathioprine are related to the TPMT genotype. For example, in a recent study (Bajaj et al., 2005), early adverse effects have been noted in 29% of the patients with Crohn's disease compared with 5% of the patients with autoimmune hepatitis, but no explanation for this clinically important difference in hypersensitivity response rates could be offered. Adding to the complexity, TPMT can also methylate the proximal 6-mercaptopurine metabolite 6-TIMP (Fig. 1) to form 6-methylmercaptopurine ribonucleotide (6-MMPR), and the ratio of 6-TGN/6-MMPR has been found to influence the balance between therapeutic effect and hepatotoxicity in some patients with inflammatory bowel disease (Dubinsky et al., 2002).

The concentrations of 6-mercaptopurine and its metabolites attained in the tissues of a patient are obviously governed not only by the reactions that consume 6-mercaptopurine, but also by the rate of 6-mercaptopurine formation from azathioprine (Fig. 1). In view of the predominance of GST-catalyzed bioactivation of azathioprine over the nonenzymatic reaction with glutathione (Table 3), it is clear that individual differences in GST expression will have an impact on the concentration of free, unconjugated 6-mercaptopurine and its metabolites. The combination of high GST activity and low TPMT activity is expected to give the highest formation rate of 6-mercaptopurine and its consecutive inhibitory nucleotide derivatives. The polymorphism of GST M1-1 could be highly influential to azathioprine metabolism. On the one hand, some individuals are GST M1-1-null, and at the other extreme, cases have been reported in which the gene has been duplicated (McLellan et al., 1997). Allelic variants in the Alpha class have been characterized, which differ in the expression level (Rowe et al., 1997; Johansson and Mannervik, 2001; Coles and Kadlubar, 2005).

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References

Bajaj JS, Saeian K, Varma RR, Franco J, Knox JF, Podoll J, Emmons J, Levy M, and Binion DG (2005) Increased rates of early adverse reaction to azathioprine in patients with Crohn's disease compared to autoimmune hepatitis: A tertiary referral center experience. *Am J Gastroenterol* **100:**1121–1125.

Blackburn AC, Tzeng HF, Anders MW, and Board PG (2000) Discovery of a functional polymorphism in human glutathione transferase zeta by expressed sequence tag database analysis. *Pharmacogenetics* **10:**49–57.

Calne RY, Alexandre GP, and Murray JE (1962) A study of the effects of drugs in prolonging survival of homologous renal transplants in dogs. *Ann NY Acad Sci* **99:**743–761.

Cara CJ, Pena AS, Sans M, Rodrigo L, Guerrero-Esteo M, Hinojosa J, Garcia-Paredes J, and Guijarro LG (2004) Reviewing the mechanism of action of thiopurine drugs: towards a new paradigm in clinical practice. *Med Sci Monit* **10:**247–254.

Coles BF and Kadlubar FF (2005) Human alpha class glutathione S-transferases: genetic polymorphism, expression, and susceptibility to disease. *Methods Enzymol* **401:**9–42.

Dhanani S and Awasthi YC (2006) Glutathione S-transferase isozyme composition of human tissues, in *Toxicology of Glutathione Transferases* (Awasthi YC ed) pp 321–338, CRC Press, Boca Raton.

Dubinsky MC, Yang H, Hassard PV, Seidman EG, Kam LY, Abreu MT, Targan SR, and Vasiliauskas EA (2002) 6-MP metabolite profiles provide a biochemical explanation for 6-MP resistance in patients with inflammatory bowel disease. *Gastroenterology* **122:**904–915.

Elion GB (1989) The purine path to chemotherapy. *Science (Wash DC)* **244:**41–47.

Gibbs JP, Yang JS, and Slattery JT (1998) Comparison of human liver and small intestinal glutathione *S*-transferase-catalyzed busulfan conjugation *in vitro*. *Drug Metab Dispos* **26:**52–55.

Giner-Sorolla A (1988) The excitement of a suspense story, the beauty of a poem: the work of Hitchings and Elion. *Trends Pharmacol Sci* **9:**437–438.

Holley SL, Fryer AA, Carrol PR, and Strange RC (2006) GST polymorphism: where to now? Clinical applications and functional analysis, in *Toxicology of Glutathione Transferases* (Awasthi YC ed), CRC Press, Boca Raton.

Howie AF, Hayes PC, Bouchier IAD, Hayes JD, and Beckett GJ (1989) Glutathione S-transferase in human bile. *Clin Chim Acta* **184:**269–278.

Johansson AS and Mannervik B (2001) Interindividual variability of glutathione transferase expression in man, in *Interindividual Variability in Human Drug Metabolism* (Pacifici GC and Pelkonen P eds) pp 460–519, Taylor & Francis, London.

Josephy DP and Mannervik B (2006) *Molecular Toxicology*. Oxford University Press, Inc, New York.

Juronen E, Tasa G, Uuskula M, Pooga M, and Mikelsaar AV (1996) Purification, characterization and tissue distribution of human class theta glutathione S-transferase T1–1. *Biochem Mol Biol Int* **39:**21–29.

Kaplowitz N (1976) Enzymatic thiolysis of azathioprine in vitro. *Biochem Pharmacol* **25:**2421–2426.

Lee AU and Farrell GC (2001) Mechanism of azathioprine-induced injury to hepatocytes: roles of glutathione depletion and mitochondrial injury. *J Hepatol* **35:**756–764.

Leist M, Single B, Castoldi AF, Kuhnle S, and Nicotera P (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* **185:**1481–1486.

Lennard L (1992) The clinical pharmacology of 6-mercaptopurine. *Eur J Clin Pharmacol* **43:**329–339.

Lien S, Larsson A-K, and Mannervik B (2002) The polymorphic human glutathione transferase T1–1, the most efficient glutathione transferase in the denitrosation and inactivation of the anticancer drug 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochem Pharmacol* **63:**191–197.

Mannervik B, Board PG, Hayes J, Listowsky I, and Pearson WR (2005) Nomenclature for mammalian soluble glutathione transferases. *Methods Enzymol* **401:**1–8.

Mannervik B and Guthenberg C (1981) Glutathione transferase (human placenta). *Methods Enzymol* **77:**231–235.

Mannervik B, Guthenberg C, Jensson H, Warholm M, and and Ålin P (1983) Isozymes of glutathione *S*-transferases in rat and human tissues, in *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects* (Larsson A, Orrenius S, Holmgren A, and Mannervik B eds) pp 75–88, Raven Press, New York.

McLellan RA, Oscarson M, Alexandrie A-K, Seidegård J, Evans DAP, Rannug A, and Ingelman-Sundberg M (1997) Characterization of a human glutathione *S*-transferase μ cluster containing a duplicated *GSTM1* gene that causes ultrarapid enzyme activity. *Mol Pharmacol* **52:**958–965.

Morel F, Rauch C, Petit E, Piton A, Theret N, Coles B, and Guillouzo A (2004) Gene and protein characterization of the human glutathione *S*-transferase κ and evidence for a peroxisomal localization. *J Biol Chem* **279:**16246–16253.

Moron MS, DePierre JW, and Mannervik B (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta* **582:**67–78.

Odlind B, Hartvig P, Lindström B, Lönnerholm G, Tufveson G, and Grefberg N (1986) Serum azathioprine and 6-mercaptopurine levels and immunosuppressive activity after azathioprine in uremic patients. *Int J Immunopharmacol* **8:**1–11.

Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, Ketterer B, and Taylor JB (1994) Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem J* **300:**271–276.

Pemble SE, Wardle AF, and Taylor JB (1996) Glutathione S-transferase class Kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. *Biochem J* **319:**749–754.

Rowe JD, Nieves E, and Listowsky I (1997) Subunit diversity and tissue distribution of human glutathione *S*-transferases: interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem J* **325:**481–486.

Schröder KR, Hallier E, Meyer DJ, Wiebel FA, Muller AMF, and Bolt HM (1996) Purification and characterization of a new glutathione *S*-transferase, class theta, from human erythrocytes. *Arch Toxicol* **70:**559–566.

Seidegård J, Vorachek WR, Pero RW, and Pearson WR (1988) Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. *Proc Natl Acad Sci USA* **85:**7293–7297.

Smith CV, Jones DP, Guenthner TM, Lash LH, and Lauterburg BH (1996) Compartmentation of glutathione: Implications for the study of toxicity and disease. *Toxicol Appl Pharmacol* **140:**1–12.

Sundberg AG, Nilsson R, Appelkvist EL, and Dallner G (1993) Immunohistochemical localization of alpha-class and pi-class glutathione transferases in normal human tissues. *Pharmacol Toxicol* **72:**321–331.

van Ommen B, Bogaards JJ, Peters WH, Blaauboer B, and van Bladeren PJ (1990) Quantification of human hepatic glutathione S-transferases. *Biochem J* **269:**609–613.

van Scoik KG, Johnson CA, and Porter WR (1985) The pharmacology and metabolism of the thiopurine drugs 6-mercaptopurine and azathioprine. *Drug Metab Rev* **16:**157–174.

von Bahr C, Glaumann H, Gudas J, and Kaplowitz N (1980) Inhibition of hepatic metabolism of azathioprine by furosemide in human liver in vitro. *Biochem Pharmacol* **29:**1439–1441.

Warholm M, Guthenberg C, Mannervik B, and von Bahr C (1981) Purification of a new glutathione S-transferase (transferase mu) from human liver having high activity with benzo(alpha)pyrene-4,5-oxide. *Biochem Biophys Res Commun* **98:**512–519.

Weinshilboum R (2001) Thiopurine pharmacogenetics: clinical and molecular studies of thiopurine methyltransferase. *Drug Metab Dispos* **29:**601–605.

Whitbread AK, Tetlow N, Eyre HJ, Sutherland GR, and Board PG (2003) Characterization of the human Omega class glutathione transferase genes and associated polymorphisms. *Pharmacogenetics* **13:**131–144.

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