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Human glutathione transferases catalyzing the bioactivation of anticancer thiopurine prodrugs

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ABSTRACT

cis-6-(2-Acetylvinylthio)purine (cAVTP) and *trans*-6-(2-acetylvinylthio)guanine (tAVTG) are thiopurine prodrugs provisionally inactivated by an α,β-unsaturated substituent on the sulfur of the parental thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG). The active thiopurines are liberated intracellularly by glutathione (GSH) in reactions catalyzed by glutathione transferases (GSTs) (EC 2.5.1.18). Catalytic activities of 13 human GSTs representing seven distinct classes of soluble GSTs have been determined. The bioactivation of cAVTP and tAVTG occurs via a transient addition of GSH to the activated double bond of the S-substituent of the prodrug, followed by elimination of the thiopurine. The first of these consecutive reactions is rate-limiting for thiopurine release, but GST-activation of this first addition is shifting the rate limitation to the subsequent elimination. Highly active GSTs reveal the transient intermediate, which is detectable by UV spectroscopy and HPLC analysis. LC/MS analysis of the reaction products demonstrates that the primary GSH conjugate, 4-glutathionylbuten-2-one, can react with a second GSH molecule to form the 4-(bis-glutathionyl)butan-2-one. GST M1-1 and GST A4-4 were the most efficient enzymes with tAVTG, and GST M1-1 and GST M2-2 had highest activity with cAVTP. The highly efficient GST M1-1 is polymorphic and is absent in approximately half of the human population. GST P1-1, which is overexpressed in many cancer cells, had no detectable activity with cAVTP and only minor activity with tAVTG. Other GST-activated prodrugs have targeted GST P1-1-expressing cancer cells. Tumors expressing high levels of GST M1-1 or GST A4-4 can be predicted to be particularly vulnerable to chemotherapy with cAVTP or tAVTG.

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1. Introduction

Glutathione transferases (GSTs) (EC 2.5.1.18) are a superfamily of detoxication enzymes with members in several structural classes [1]. A major task of GSTs is to catalyze the conjugation of glutathione with a wide variety of electrophilic compounds and thereby facilitate their elimination from the body. GSTs have also been implicated in cellular resistance to chemotherapeutic agents, since exposure to these drugs is often associated with induction of GSTs, especially GST P1-1 [2–5]. Overexpression of GSTs could provide protection to the targeted cell and thereby compromise the chemotherapy [6].

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An approach to solving this problem is to design inhibitors for the GSTs that cause drug resistance [7]. An alternative is to take advantage of the GST overexpression and develop prodrugs that are activated by GSTs, and thus also gain the benefit of delivering the effect where it is desired and thereby attenuate adverse side effects. A few prodrugs have been developed to become bioactivated by GST P1-1.They are metabolized to turn into nitrogen mustards [8,9], alkylating exocyclic enones [10], or cytolytic nitric oxide [11]. In general prodrugs are biologically inactive molecules that are designed to become active locally, thereby minimizing undesired systemic toxicity.

cis-6-(2-Acetylvinylthio)purine (cAVTP) and *trans*-6-(2-acetylvinylthio)guanine (tAVTG) (Fig. 1) are thiopurine prodrugs provisionally inactivated by an α,β-unsaturated substituent on the sulfur of the parental thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG), respectively [12]. 6-MP and 6-TG are analogs of the natural purines hypoxanthine and guanine, and they are cytotoxic agents commonly used in the treatment of acute lymphoblastic leukemia (ALL) [13]. Their biotransformation leads to metabolites that interfere with DNA synthesis. 6-MP and 6-TG are both substrates of hypoxanthine guanine phosphoribosyltransferase (HPGRT) and are converted into the ribonucleotides 6-thioinosine monophosphate (6-TIMP), which is also an antimetabolite that inhibits de novo purine synthesis [14], and 6-thioguanosine monophosphate (6-TGMP), respectively (Fig. 1). The nucleotides are subsequently incorporated into RNA and DNA [13] and inhibit several processes of significance for cell proliferation. The prodrugs cAVTP and tAVTG were synthesized in 1982 by Anufriev et al. [15], and their bioactivation seems exerted by a nucleophilic attack of the cellular thiol glutathione (GSH) on the butenone moiety, followed by an elimination reaction releasing 6-MP or 6-TG, respectively.

cAVTP and tAVTG rapidly enter human renal carcinoma cells in culture, as shown by HPLC analysis [12].Their metabolism produce the parental drugs 6-MP and 6-TG and is accompanied by GSH consumption. Intracellular concentrations of 6-TG reached a higher level after incubation with tAVTG than with 6-TG itself [12]. In vivo studies of mice treated with tAVTG have shown no reduction of white blood cells, whereas mice treated with same or lower concentrations of 6-TG, demonstrated significant leukopenia [16]. On the other hand, using cell lines from the US National Cancer Institute's anticancer-drug screening program demonstrated that both prodrugs were more cytotoxic than 6-MP and 6-TG, respectively, for many tumor cells. Both prodrugs exerted similar high inhibitory activity on the growth of leukemic and melanoma cells. However, differential effects were also noted; cAVTP showed a distinct efficacy with renal cancer cells and tAVTG showed highest effectiveness with ovarian cancer cells [17]. Cell lines are known to differ qualitatively and quantitatively in the expression of various GSTs [18,19], and similar diversity characterizes human tumors treated clinically. It is therefore important to clarify the potential of the individual human GSTs for the activation of cAVTP and tAVTG.

The present study has examined the diverging catalytic activities of 13 human cytosolic glutathione transferases (GSTs) acting on cAVTP and tAVTG in vitro. GSTs participate in the first step of the bioactivation of cAVTP and tAVTG by catalyzing the addition of glutathione to the butenone moiety (Fig. 1), whereas the subsequent elimination step of the reaction appears to be independent of GST activity. We have provided evidence for this bioactivation mechanism and

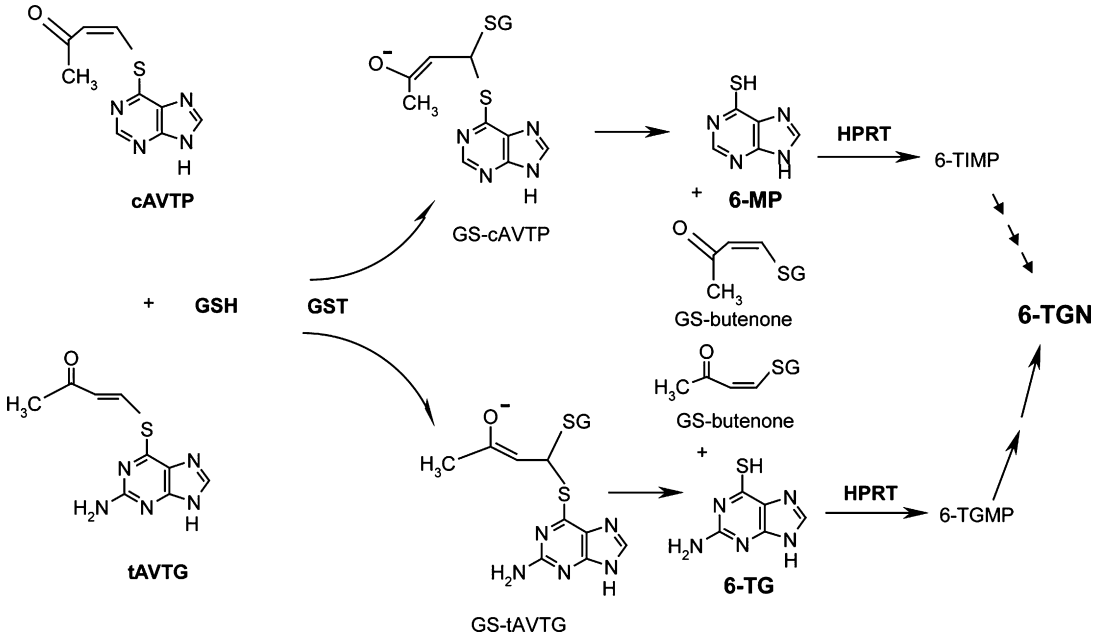

Fig. 1 – Scheme of cAVTP and tAVTG metabolism. Abbreviations are GSH, glutathione; GST, glutathione transferase; GS-butenone, 4-glutathionyl-buten-2-one, 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; HPRT, hypoxanthine-guanine phosphoribosyl transferase; 6-TIMP, 6-thioinosine 5′-monophosphate; 6-TGN, 6-thioguanine nucleotides.

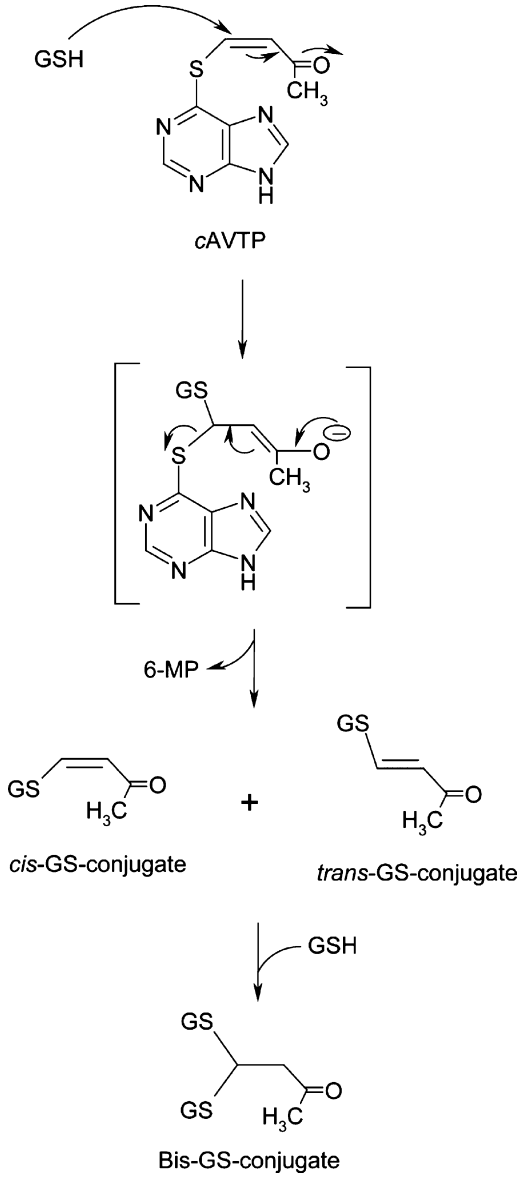

Fig. 2 – Proposed scheme for the formation of the mono- and bis-glutathionyl conjugates of butenone derived from cAVTP.

furthermore investigated the multiple GSH conjugates derived from the butanone moiety of cAVTP (Fig. 2).

2. Materials and methods

2.1. Reagents and enzymes

cis-6-(2-Acetylvinylthio)purine and *trans*-6-(2-acetylvinylthio)-guanine were synthesized as previously described [12] and dissolved in DMSO. 6-Mercaptopurine, 6-thioguanine and glutathione were purchased from Sigma–Aldrich. Acetonitrile was purchased from Merck. Recombinant human GSTs A1-1, A2-2, A3-3, A4-4, M1-1, M2-2, M4-4, M5-5, P1-1, O1-1, T1-1 and Z1-1 were expressed and purified by published methods [20–31]. GST K1-1 was expressed in fusion with a calmodulin-binding peptide [32] and was used in unpurified form.

2.2. Determination of extinction coefficients

The net extinction coefficient for the reaction of cAVTP, or of tAVTG, with glutathione was determined experimentally as 2170 $M^{-1}\,cm^{-1}$ at 350 nm and 1180 $M^{-1}\,cm^{-1}$ at 370 nm, respectively. The reaction of 0.1 mM of the thiopurine prodrug with 10 mM glutathione was followed to completion in the assay buffer containing active enzyme.

2.3. Spectrophotometric assays

Activity measurements were performed on a SPECTRAmax PLUS^{384} microplate spectrophotometer (Molecular Devices, Sunnyvale,CA). Specific activities of the GSTs were determined by measurements at 350 nm with cAVTP and at 370 nm with tAVTG 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 of either prodrug and 5 mM glutathione. Steady-state kinetic constants were determined by measurements with thiopurine prodrug concentrations varied between 0.04 and 1.0 mM at a constant glutathione concentration of 5 mM. Nonlinear regression analyses of the kinetic data were made with GraphPad Prism.

2.4. HPLC analyses

Separations were performed on a 250 mm × 4.6 mm i.d. Nucleosil 100-5 C18 column at 30 °C using a Merck Hitachi LaChrom Elite chromatograph (Darmstadt, Germany). The flow rate was 1 ml min^{-1} with a mobile phase of 50 mM sodium phosphate buffer, 1 mM EDTA, pH 7.4. Elution was made with a gradient of zero to 27.5% (v/v) acetonitrile during 40 min, followed by re-equilibration with the mobile phase for 16 min. Analyses were made by injection of 20 μl of 0.1 mM the pure substances cAVTP, tAVTG, 6-TG, or 6-MP to determine their retention times. In order to follow the consumption of cAVTP or tAVTG and the formation of the products 6-MP, 6-TG, and GS-butanone, 20 μl or 40 μl aliquots of the reaction mixture were taken every 56 min and analyzed. The reaction medium consisting of mobile phase (without acetonitrile) containing 0.1 mM cAVTP and 1 mM GSH or 0.1 mM tAVTG and 1 mM GSH. The HPLC analyses of enzymatic reactions were carried out with GSTs A2-2, A4-4, M1-1, M4-4 and M5-5.

2.5. HPLC fractionation for further analysis with MS

Separations for HPLC/MS were performed on a 250 × 4.6 mm semi-preparative reversed-phase C_{18} Ultrasphere-ODS 5 μm column (Beckman Instruments, Fullerton, CA) with a 4.6 × 30 mm, spheri-5 ODS 5 μm Brownlee guard column (Perkin Elmer, Norwalk, CT) using a HPLC system consisted of two Gilson 306 pumps, a Gilson 119 UV/Visible detector, and a Gilson 234 autoinjector (Gilson, Middleton, WI). The flow rate was 1 ml min^{-1} with a mobile phase for pump A consisting of 0.015 M H_3PO_4 whereas the mobile phase for pump B was 1:1 acetonitrile:0.015 M H_3PO_4 mixture. The gradient used for analyses was initially at 0% B for 2 min then increased to 15% B

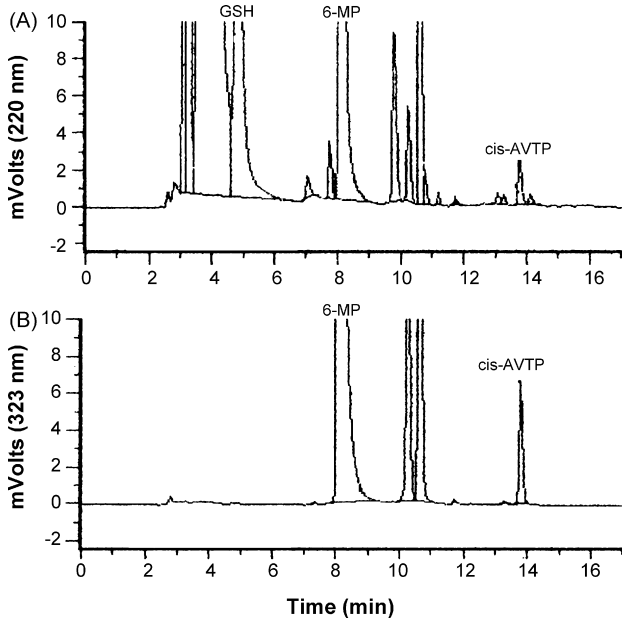

Fig. 3 – Representative chromatogram of products of cAVTP and GSH after a 10 min reaction as monitored by HPLC as 220 nm (A) and 323 nm (B). The region collected for LC/MS was between the 6-MP and cAVTP peaks.

over 1 min, constant at 12% B for 3 min, increased to 75% B over 3 min, constant at 75% B for 3 min, decreased to 0% B over 3 min, and constant at 0% B for 5 min for a total run time of 20 min. The detection wavelengths were 220 and 323 nm. The reaction mixture was prepared of 150 μl cAVTP 40 mM and 150 μl GSH 200 mM in 0.1 M phosphate, 0.1 M KCl buffer, 5 mM EDTA, pH 7.4 that were incubated in the presence of 200 μl diluted rat liver cytosol and 835 μl 0.1 M phosphate buffer, (pH 7.4) in a shaking water bath for 10 min at 37 °C. After the incubation 165 μl 10% trifluoroacetic acid (TFA) was added, and the samples were centrifuged and filtered before injection. Retention times obtained were as follows: GSH, 4.7 min; GSSG, 7.7 min; 6-MP, 8.1 min, cAVTP 13.9 min (Fig. 3A and B). Peaks eluting with retention times in between 8.1 min (6-MP) and 13.9 min (cAVTP) were collected lyophilized, and desalted with a prep-sep C_{18} column (the material was eluted with a 1:1 acetonitrile:water with the pH adjusted to 2.5 using TFA). The resulting eluate was concentrated on a SpeedVac to as-close-to-dryness as possible before it was submitted for analysis by LC/MS and MS/MS.

2.6. LC/MS/MS analysis

The characterization of the additional HPLC peaks described above was carried out on a Perkin Elmer Sciex API 365 triple quadrupole LC/MS/MS (Perkin Elmer Instruments, Norwalk, CT) coupled to an Agilent 1100 HPLC. The separation of the peaks was achieved on a 1 mm × 150 mm, 5 μm particle size, 300 Å pore size Vydac C_{18} column. Solvent A consisted of 0.05% TFA in water and solvent B contained 95% acetonitrile and 0.05% TFA. The column was equilibrated at 100% solvent A and then with a linear gradient to 100% B over 60 min. The flow rate was 0.02 ml min^{-1} and the peaks of interest were eluted between 22 and 28 min in both the total ion chromatogram (Fig. 4A) and the UV-absorbance chromatogram at 215 nm (Fig. 4B). Masses between 50 and 1500 Da were measured using a 0.2 Da step and a dwell time of 0.4 ms.

3. Results

3.1. UV-spectra

The UV-spectra of cAVTP and 6-MP show a difference in absorbance at 350 nm (Fig. 5A), which was used for monitoring

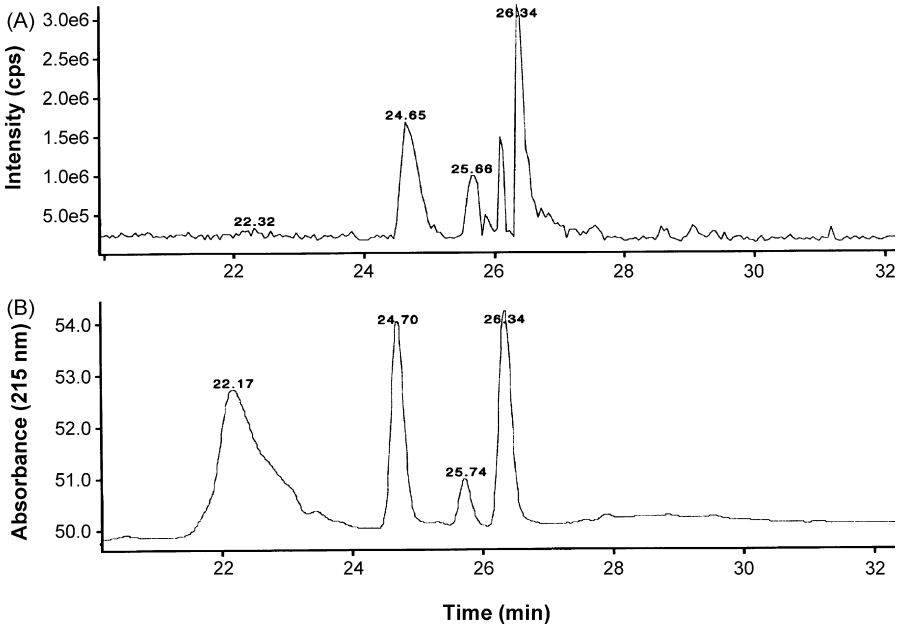

Fig. 4 – LC/MS chromatogram as the total ion chromatogram (A) and the UV spectrum at 215 nm (B) showing the additional products formed in the reaction of cAVTP and GSH.

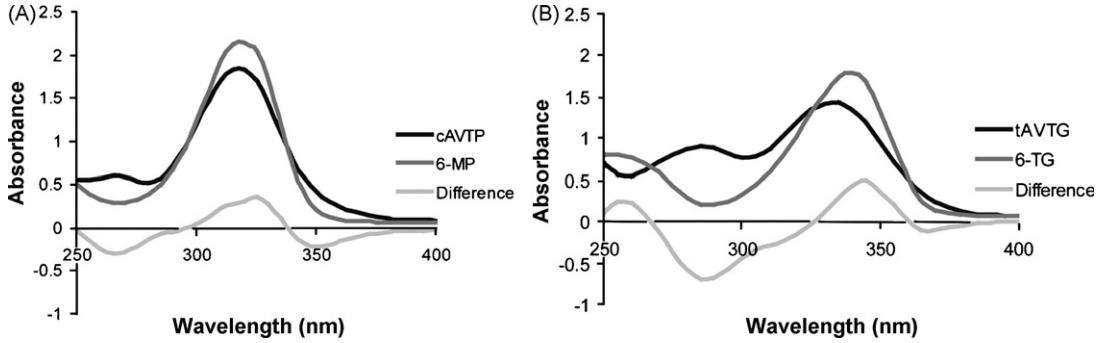

Fig. 5 – Ultraviolet spectra of the prodrugs and their parental thiopurines. (A) cAVTP (black) and 6-mercaptopurine (dark gray); (B) tAVTG (black) and 6-thioguanine (dark gray). Difference spectra (light gray) demonstrate the regions of maximal sensitivity for monitoring the reaction. The spectra were recorded with each prodrug and GSH in 0.1 M sodium phosphate buffer, 1 mM EDTA, pH 7.4 at 30 °C.

the formation of 6-MP in the presence of glutathione. The UV-spectra of tAVTG and 6-TG show a difference at 370 nm (Fig. 5B), which was used for monitoring the formation of 6-TG in the presence of glutathione. The sulfur of glutathione forms a link to the butenone moiety of both cAVTP and tAVTG. The net extinction coefficients for the reactions were determined as 2170 M^{-1} cm^{-1} and 1180 M^{-1} cm^{-1}, respectively.

3.2. *HPLC/UV*

The reaction between cAVTP and glutathione is expected to yield 6-MP and 4-glutathionyl-butenone (GS-butenone) (Figs. 1 and 2). The chemical transformation of 6-MP was observed by following the disappearance of cAVTP and the formation of the products as a function of time. The reaction was monitored at two wavelengths, 320 and 350 nm (Figs. 6A and B, respectively). The UV-spectra (Fig. 5A) show a maximal difference between cAVTP and 6-MP at 320 nm; whereas the wavelength 350 nm was more suitable for the spectrophotometric measurements, owing to a lower absolute absorbance. There were no differences in the number of products obtained in the nonenzymatic or enzymatic reactions visible in the HPLC profiles, based on the two wavelengths (Fig. 6A and B).

The reaction between tAVTG and glutathione is expected to yield 6-TG and GS-butenone (Fig. 1), and the formation of 6-TG was also monitored at two wavelengths, 335 and 370 nm, respectively (Fig. 6C and D). Fig. 6C shows the enzymatic reaction catalyzed by GST A4-4, and in the presence of this enzyme, a broad peak of an unidentified intermediate absorbing at 335 nm emerged in parallel with 6-TG. This transient peak disappeared with time, as shown by two consecutive runs from HPLC analyses of the same reaction mixture (Fig. 6E) that contained GST A4-4. For the catalytic activity measurements the wavelength 370 nm was chosen, where only tAVTG and 6-TG contributed to the absorbance (Fig. 6D), in order to avoid any interference of the intermediate in the measurements. GSTs M1-1 and M4-4, like GST A4-4, showed an intermediate peak at 335 nm that disappeared with time, but the intermediate was not detectable with the less active GSTs A2-2 or M5-5 under the conditions investigated.

3.3. *Demonstration of several GSH conjugates by LC/MS and MS/MS analyses*

An HPLC analysis of the products of cAVTP incubated with GSH and rat liver cytosol showed, consistent with our previous results [33], that 6-MP was the major product and revealed a few unidentified peaks in addition to the expected GSH adduct (Fig. 3A and B). These components prompted further analysis with LC/MS/MS. The total ion chromatogram of the lyophilized HPLC fractions showed four major product peaks in between 24 and 27 min (Fig. 4A), and three of them corresponded to three peaks in the UV chromatogram at 215 nm (Fig. 4B). The two peaks with retention times of 25.7 and 26.3 min, respectively gave similar mass spectra (M + 1 ion at 376.2, Fig. 7A and B), which corresponded to the *cis*- and *trans*-GS-conjugates of butanone derived from cAVTP depicted in Fig. 2. The MS/MS spectra of the M + 1 ions (Fig. 7C) exhibited fragmented ions at *m/z* 301.2, 247.2 and 101.0, corresponding to the loss of the glycine (−75) and the γ-glutamyl (−129) moieties, and the breakage of the cysteine C-S bond (−275), respectively. The MS/MS results provided additional evidence for the formation of two isomeric GS-conjugates of cAVTP (Fig. 2).

The LC/MS component eluting at 24.7 min (Fig. 4A and B) exhibited an M + 1 ion at 683.4 (Fig. 8A), corresponding to the bis-GS-conjugate of cAVTP (Fig. 2). The MS/MS spectrum of this ion (Fig. 8B), exhibited fragment ions at *m/z* 665.2, 608.2, 554.2, and 376.2, corresponding to loss of water (−18), glycine (−75) and γ-glutamyl (−129) moieties, as well as loss of glutathione (−307). The fragment ions at *m/z* 536, 479, and 425 corresponded to loss of water (−18) glycine (−75) and γ-glutamyl (−129) moieties from the ion at *m/z* 554, respectively. Thus, the LC/MS and MS/MS results were consistent with the proposed bis-glutathionyl conjugate of cAVTP (Fig. 2). The peak eluting at 22.17 min (Fig. 4B), was a minor peak in the total ion chromatogram (Fig. 4A), which exhibited major ions at 613.4 and 307.4, corresponding to the M + 1 ion of GSSG and GSH, respectively. These results suggest the presence of GSSG in the LC/MS sample.

3.4. *Specific activities with cAVTP and tAVTG*

The GSTs are known to differ widely, but also to overlap, in their substrate selectivity profiles with alternative substrates

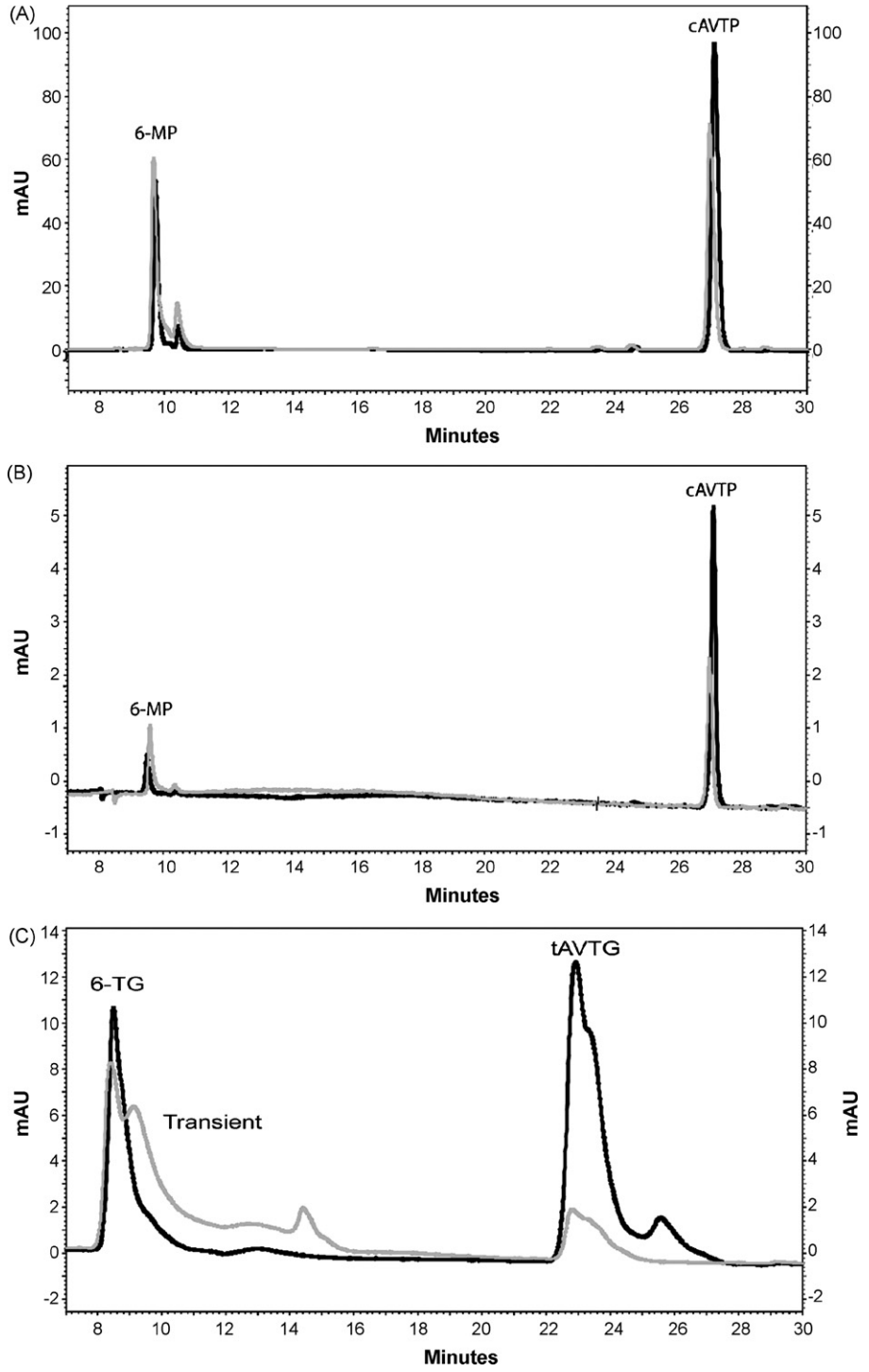

Fig. 6 – HPLC analysis of the reactions between glutathione and cAVTP and tAVTG. Chromatogram showing (A) cAVTP and the product 6-mercaptopurine following the reaction with GSH in the absence (black) and the presence of GST M1-1 (gray) monitored at 320 nm. (B) Same reaction as in (A) monitored at 350 nm. The reaction mixture contained 0.1 mM cAVTP and 1 mM glutathione in 50 mM sodium phosphate buffer pH 7.4 and 3 μg GST M1-1. (C) tAVTG and the product 6-thioguanine following the reaction with GSH in the absence (black) and the presence of GST A4-4 (gray) monitored at 335 nm. (D) Same conditions as in (C) monitored at 370 nm. The reaction mixture contained 0.1 mM tAVTG and 1 mM glutathione in 50 mM sodium phosphate buffer pH 7.4 and 15 μg GST A4-4. In chromatogram (C) a transient intermediate; found in the presence of GSTs A4-4, M1-1 and M4-4, but not with A2-2 or M5-5, of the GSTs tested. (E) The disappearance of the transient peak (in C) illustrated with two consecutive run HPLC profiles, monitored at 335 nm, from the same reaction mixture containing 150 μg GST A4-4. The retention times in (E) are shifted in comparison to (C) and (D), since the column was replaced. The HPLC was made at 30 °C on a reversed phase C_{18} column, with a flow rate of 1 ml min^{-1} of 50 mM sodium phosphate buffer pH 7.4 and an acetonitrile gradient (zero to 27%, v/v).

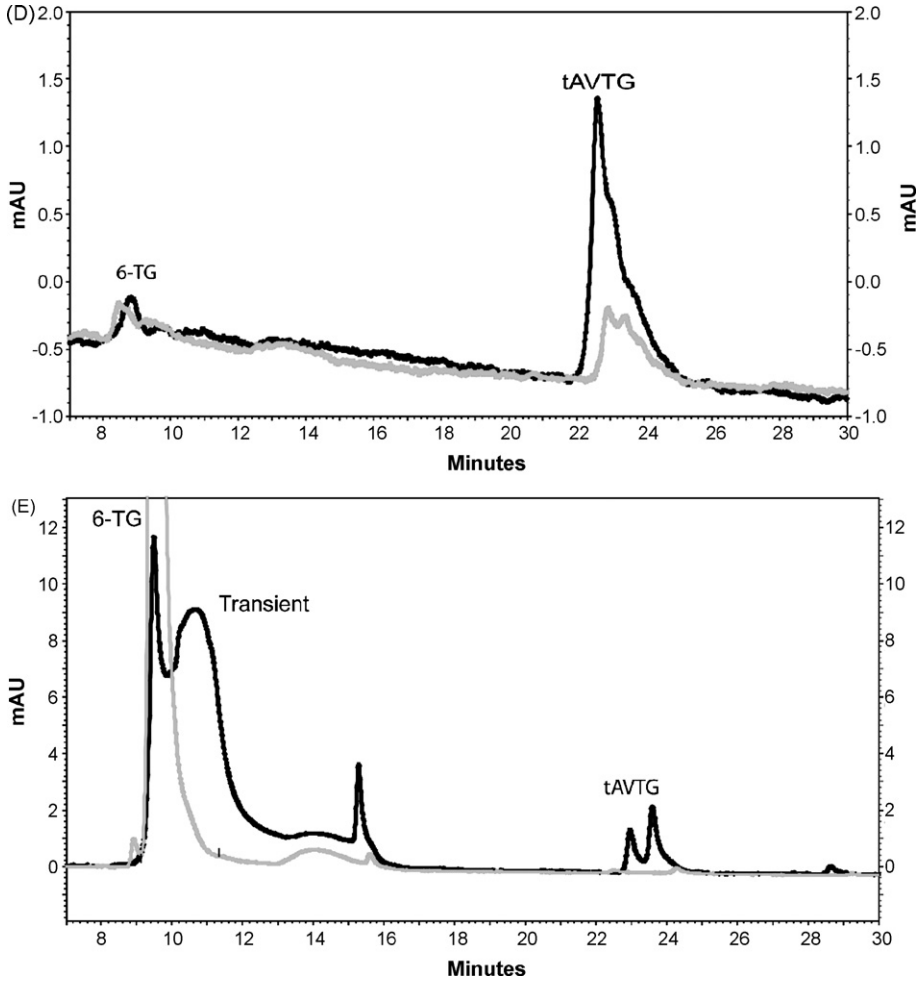

Fig. 6. (Continued).

[6]. The divergent catalytic activities were evident also with cAVTP and tAVTG. Specific activities were measured for 13 members of seven GST classes (Table 1) known to be expressed in human tissues. The proteins represent GSTs from the Alpha, Mu, Omega, Pi, Theta, and Zeta classes, as well as from the distantly related Kappa class. All representatives of the Alpha class, GSTs A1-1, A2-2, A3-3, and A4-4, displayed measurable activity with both cAVTP and tAVTG. GST A4-4 had the highest value in the Alpha class with tAVTG (55 μmol min^{-1} mg^{-1}), and in fact the highest specific activity of all 13 GSTs investigated with either tAVTG or cAVTP. GST A4-4 also had the highest specific activity with cAVTP among the Alpha class members, even though the activity was 32 times lower than with tAVTG. The specific activity of GST A1-1 was equal with tAVTG and cAVTP, but was lower than those obtained with GST A4-4 by 55- and 1.7–fold, respectively. GST A3-3 was similar to GST A1-1 in the activity with tAVTG (approximately 1 μmol min^{-1} mg^{-1}) but showed only half the value with cAVTP. GST A2-2 displayed the lowest activities in the Alpha class with both prodrugs. In the Mu class, GST M1-1 had the highest specific activities with both prodrugs among the four enzymes investigated. cAVTP gave the highest activity (31 μmol min^{-1} mg^{-1}), and the activity was similar with tAVTG (24 μmol min^{-1} mg^{-1}). The Mu-class GST M2-2 also displayed a clear activity with cAVTP (10 μmol min^{-1} mg^{-1}). This value was 20 times higher than with tAVTG as a substrate for the same enzyme, but 3 times lower than the GST M1-1 activity with cAVTP. Activities of GST M4-4, and GST M5-5 with tAVTG were equal (9 μmol min^{-1} mg^{-1}), but were lower with cAVTP (1 and 3 μmol min^{-1} mg^{-1}, respectively). GST P1-1 displayed a small but measurable activity with tAVTG, but that of the more distantly related mitochondrial and peroxisomal GST K1-1 was barely detectable. However, neither of these two enzymes had any measurable activity with cAVTP. GST K1-1 contained a peptide tag, but control experiments demonstrated that the enzyme was catalytically fully competent [29]. None of the representatives of the Omega, Theta, and Zeta classes had any detectable activity with either cAVTP or tAVTG.

3.5. *Steady-state kinetic studies*

Eight enzymes out of the13 GSTs had readily measurable activity and were investigated kinetically (Table 2). The steady-state parameter k_{cat}/K_m was determined for the following GSTs in the Alpha class, A1-1, A2-2, A3-3 and A4-4, and in the Mu

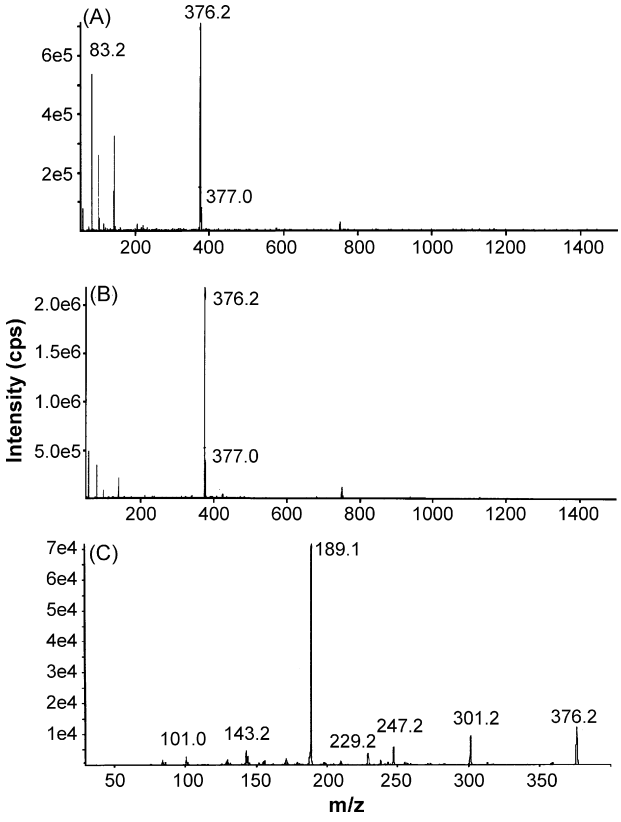

Fig. 7 – Mass spectra of the peaks from Fig. 4 at 25.74 (A) and 26.34 min (B). The lower spectrum is the MS/MS of the 376.2 ion (C).

class, M1-1, M2-2, M4-4 and M5-5. The prodrug concentrations were varied in the presence of 5 mM glutathione at the physiological pH value of 7.4 to determine the parameters of the Michaelis–Menten equation. The K_m value for GSH is generally <0.5 mM for GSTs, but was determined as equal to 1 mM with GST M5-5; the higher GSH concentration of 5 mM

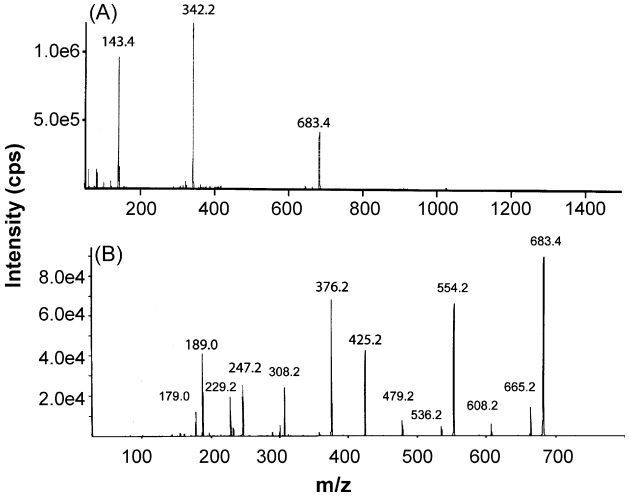

Fig. 8 – Mass spectrum (A) of the di-GS-conjugate (24.65 min). The lower spectrum (B) is the MS/MS of the 683.4 ion.

Table 1 – Specific activity of human GSTs with thiopurine prodrugs and GSH

GST	Specific activity[a]	
	cAVTP	tAVTG
	μmol min^{-1} mg^{-1}	
A1-1	1.0 ± 0.01	1.1 ± 0.1
A2-2	0.2 ± 0.04	0.5 ± 0.06
A3-3	0.4 ± 0.03	0.8 ± 0.1
A4-4	1.7 ± 0.10	55.3 ± 3.7
M1-1	30.8 ± 1.0	23.6 ± 1.3
M2-2	10.2 ± 0.3	0.5 ± 0.02
M4-4	1.2 ± 0.03	8.6 ± 0.1
M5-5	3.4 ± 0.1	9.0 ± 0.2
O1-1	nd	nd
K1-1	nd	<0.001
P1-1	nd	0.3 ± 0.03
T1-1	nd	nd
Z1-1	nd	nd

[a] Data represents means (S.D.) of assays conducted in triplicate; nd designates non detectable.

was therefore used in the kinetic measurements. For experimental reasons thiopurine concentrations could not reach enzyme saturation. The k_{cat}/K_m values were consequently estimated directly by regression analysis of the initial part of the saturation curve. The k_{cat}/K_m parameter is the rate constant for the enzymatic reaction at low substrate concentrations and a measure of catalytic efficiency. It is also an important physiological factor that governs the substrate selectivity [34]. Table 2 shows that with cAVTP the catalytically most efficient enzyme is GST M1-1, and that the second best is GST M2-2, with a 5 times lower catalytic efficiency. Third in rank is GST M5-5, with a value 16 times lower than that of GST M1-1. The remaining enzymes investigated, i.e., GST M4-4 as well as all four enzymes in the Alpha class had 30 to 115 times lower efficiencies than GST M1-1. With tAVTG the most efficient enzyme in the Alpha class was GST A4-4 (130 mM^{-1} s^{-1}). This enzyme was also the most efficient of all GSTs investigated. The three additional members of the Alpha class, GSTs A1-1, A2-2 and A3-3, had between 130 and 65 times lower values than GST

Table 2 – Catalytic efficiencies (k_{cat}/K_m values) of human GSTs catalyzing the reaction of thiopurine prodrugs with GSH

GST	k_{cat}/K_m[a]	
	cAVTP	tAVTG
	mM^{-1} s^{-1}	
A1-1	2.2 ± 0.1	2.4 ± 0.2
A2-2	0.5 ± 0.03	1.4 ± 0.1
A3-3	1.3 ± 0.1	2.2 ± 0.2
A4-4	3.7 ± 0.3	129.9 ± 5.6
M1-1	114.6 ± 4.8	51.8 ± 3.1
M2-2	22.4 ± 1.2	1.0 ± 0.1
M4-4	3.0 ± 0.1	15.8 ± 1.0
M5-5	7.3 ± 0.3	14.8 ± 0.6

[a] Values are based on triplicates of initial rates and calculated with graphpad software.

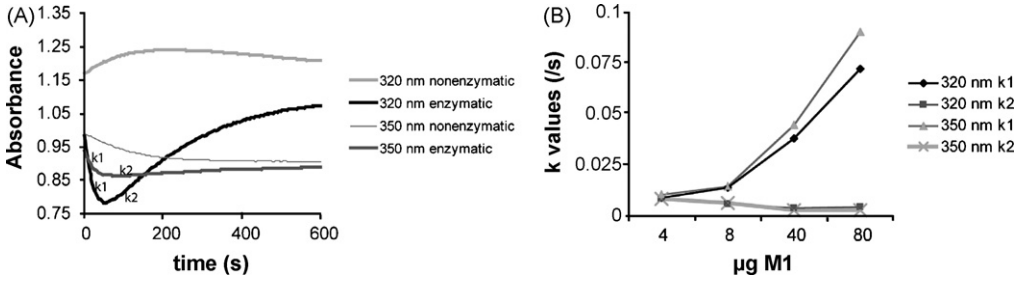

Fig. 9 – Progress of the GST-catalyzed reaction GSH and cAVTP monitored spectrophotometrically at 320 and 350 nm. Spectral changes recorded at 320 nm (black) and 350 nm (gray). The reaction was carried out with 0.1 mM cAVTP and 5 mM GSH in 0.1 M sodium phosphate buffer pH 7.4 at 30 °C in the presence of 40 μg GST M1-1 ml^{-1}. (B) Rate constants, k_1 and k_2, were determined by fitting a bi-exponential equation to the progress curves in (A) as well as to corresponding curves for different GST M1-1 concentrations; the y-axis is logarithmic and the units of the rate constants is s^{-1}.

A4-4. In the Mu, class GST M1-1 was the most efficient enzyme with a 2.5 lower k_{cat}/K_m than GST A4-4. GST M2-2 was 52 times less efficient than GST M1-1, showing a larger difference between the enzymes with tAVTG than with cAVTP. With GST M4-4 and M5-5 the k_{cat}/K_m values were similar (16 and 15 mM^{-1} s^{-1}, respectively), sharing a third place in efficiency with tAVTG.

The most active GSTs demonstrated a transient intermediate that disappeared in the time scale of minutes. Fig. 9A shows progress curves for the GST M1-1 catalyzed reaction between GSH and cAVTP monitored at two wavelengths, 320 and 350 nm. The curves could be fitted by a bi-exponential rate equation characteristic of consecutive reactions:

$$A_{tot} = A_{(1)}e^{-k_1 t} + A_{(2)}e^{k_2 t} + C$$

Measurements with different amounts of GST M1-1 (4–80 μg per ml) demonstrated that the first rate constant (k_1) increased with enzyme concentration, whereas the second (k_2) decreased to a smaller degree with enzyme concentration (Fig. 9B). This effect is consistent with a catalyzed reversible reaction followed by an uncatalyzed reaction. Extrapolating to the intracellular concentrations of GSTs (cf. [29]), it can be calculated that the nonenzymatic of both cAVTP and tAVTG with GSH is negligible in the comparison with the enzyme-catalyzed biotransformation (see below).

4. Discussion

The ideal design of an anticancer prodrug would bring forth a molecule that selectively becomes activated at the desired location but remains totally unaffected in other tissues or cells, thereby avoiding systemic side effects. Members of the GST superfamily, in particular GST P1-1, are often over-expressed in tumor cells [35,5], and selected GSTs have consequently been targeted for prodrug activation [9]. A latent phosphoramide mustard TER286, preferentially activated by GST P1-1 [8], is currently in clinical trials under the name Telcyta® [36], and cycloalkenones [10] as well as a NO-releasing compound [11] are other promising GST-activated prodrugs. The effectiveness of the cAVTP and tAVTG prodrugs against certain tumor cell lines [17] suggests that these compounds may serve as valuable complements to this category of pharmaceuticals. Their differential effects indicate that the particular expression profile of GSTs in a tumor could make one or the other of them more efficacious. It was therefore important to investigate their activation with purified GSTs to find out if the most efficient combination of prodrug and GST. This information is also applicable for understanding the possible side effects that can arise when the activating GST is present in a normal tissue.

Our results demonstrate that the 13 soluble human GSTs examined differ widely in their catalytic activities with the two prodrugs cAVTP and tAVTG. These differences could be utilized for optimized targeting of the chemotherapeutic effect in relation to the profile of GST expression in tumors and normal tissues. It is noteworthy that in distinction from TER286 [8], neither cAVTP nor tAVTG is activated especially well by GST P1-1 (Table 1). This has particular significance for the treatment of cancer in prostate, liver and breast, neoplasias that often do not express GST P1-1 because of down-regulation of the corresponding gene by hypermethylation of CpG islands in the DNA [37].

Three human GSTs showed particularly high activities with cAVTP and tAVTG (Table 1). The catalytic efficiency is best described by the kinetic parameter k_{cat}/K_m, which (at equal enzyme concentrations) quantifies the relative contributions of GSTs acting simultaneously on the same substrate [34]. GST M1-1 displayed high efficiency with both cAVTP and tAVTG, although the value with cAVTP was 2 times higher. GST M2-2 had a 5-fold lower efficiency with cAVTP, while it had lower activity with tAVTG. GST A4-4 showed the highest k_{cat}/K_m value for tAVTG of all investigated enzymes, but a 35 times lower efficiency with cAVTP.

The chemical transformation of cAVTP and tAVTG catalyzed by GSTs produce 6-MP and 6-TG, respectively, in accord with Fig. 1, as demonstrated by HPLC (Fig. 6A–D). The primary reaction is a Michael addition of the thiol group of GSH to the α,β-unsaturated ketone of the thiopurine prodrug (Figs. 1 and 2). This is one of the typical GST reactions that protect cells from reactive products of lipid peroxidation and radical reactions [38]. GST A4-4 has particularly prominent activity with this class of substrates [23], and it has been suggested that *trans*-4-hydroxyalkenals, in particular *trans*-4-hydroxy-nonenal, are endogenous substrate for this GST. The structure

of the enzyme displays a narrow elongated binding site for the electrophilic substrate [39], apparently fit to accommodate tAVTG readily, like other *trans*-configured substrates. The specific activity of GST A4-4 with tAVTG (55 μmol min^{-1} mg^{-1}) is only 3-fold lower than that with *trans*-4-hydroxynonenal (189 μmol min^{-1} mg^{-1}). However, the k_{cat}/K_m value (130 mM^{-1} s^{-1}) is 24-fold lower with tAVTG (Table 2) than with *trans*-4-hydroxynonenal [23], signifying a higher barrier to reach the transition state of the enzyme-catalyzed transformation of tAVTG. The approximately 30-fold lower specific activity and k_{cat}/K_m values of GST A4-4 with cAVTP are presumably due to its *cis*-configuration, which is not optimal for catalysis to occur.

The second enzyme with high activity is GST M1-1, which in contrast to GST A4-4 is efficient with both cAVTP and tAVTG (Tables 1 and 2). GST M1-1 is not generally known to be highly active with enones, but it was in fact discovered in human liver as the most active GST with *trans*-4-phenyl-3-buten-2-one [40]. The specific activity of GST M1-1 with *trans*-4-phenyl-3-buten-2-one (0.36 μmol min^{-1} mg^{-1}) is similar to that with 13-oxooctadeca-9,11-dienoate (0.32), a naturally occurring linoleic acid oxidation product [41], but the activities are lower by two orders of magnitude than those with the two thiopurine prodrugs, which are intrinsically more reactive. It would thus appear that GST M1-1 is relatively insensitive to the *cis*/*trans* isomerism.

The closely related Mu class enzyme, GST M2-2, is also efficient with cAVTP (Tables 1 and 2). The k_{cat}/K_m value of GST M2-2 with cAVTP (22 mM^{-1} s^{-1}) is close to its value with prostaglandin A_2 (32 mM^{-1} s^{-1}), another *cis*-configured enone [42]. However, GST M2-2 displays low activity with tAVTG, in distinction from GST M1-1. This selectivity of GST M2-2 for *cis*- or *trans*-configured substrates is second only to that of GST A4-4, which however has the reverse preference (Table 2). The additional Mu class GSTs, M4-4 and M5-5, have relatively high activities with tAVTG and lower activities with cAVTP, but with a less marked selectivity.

The available evidence indicates that the GSTs primarily catalyze the addition of GSH to the enone structure, whereas the release of the thiopurine from the initial adduct is uncatalyzed (Fig. 1). In the presence of high enzyme activity a transient decrease in absorbance is observed with 0.1 mM cAVTP and 5 mM GSH. The rate constant for the decrease is enhanced by increasing the enzyme concentration, in contrast to the subsequent increase in absorbance. In the absence of enzyme the absorbance increases monotonically. The explanation is that 6-MP has the highest absorbance at 320 nm, followed in turn by cAVTP and the GSH-cAVTP adduct. The release of 6-MP from the adduct is facile and not rate-limiting in the absence of enzyme, and consequently does not lead to accumulation of the intermediate. However, in the presence of a sufficiently high GST activity the intermediate builds up, and the 6-MP release become rate-limiting. In a similar manner a transient intermediate can be observed at 335 nm with tAVTG. Fig. 6C shows the adduct of GSH and tAVTG as a peak in between 6-TG and tAVTG. In view of these results, the GST activities are a measure of GSH addition rather than thiopurine release from the prodrugs. From a pharmacological viewpoint this is of minimal consequence, since the transient phase is completed in a time span of a few minutes.

Another aspect of the chemical reactions accompanying the bioactivation of the cAVTP and tAVTG is the possibility of adding a second GSH molecule to the 4-glutathionylbutenone adduct, which like the prodrugs themselves feature the character (α,β-unsaturated ketone) of a Michael acceptor. It was previously shown that the cAVTP readily reacts with GSH in the presence of rat liver cytosol to yield 6-MP, which is also the major in vivo metabolite of cAVTP [17,33,12,16]. Therefore, in the present study we used HPLC, LC/MS and MS/MS to characterize the GSH conjugates produced in the cytosol fraction. The purpose was only to demonstrate the possibility of forming more than one end product of GSH, and a rat liver preparation previously characterized was therefore adequate.

The HPLC chromatograms of the incubation mixture, obtained at both 220 and 323 nm (Fig. 3A and B), showed 6-MP as the major product of cAVTP after its incubation with GSH and rat liver cytosol, consistent with the previous results. Additional peaks, however, were consistently detected in between the 6-MP peak and the cAVTP peak, suggesting formation of additional products. Therefore, these peaks were fraction collected, lyophilized, and characterized using LC/MS and MS/MS analyses. Two of the peaks were assigned as *cis*- and *trans*-isomers of the GS-conjugate as evidenced by their different retention times and identical mass fragmentation patterns. Both of these conjugates would react further with GSH to yield the bis-glutathionyl-conjugate, 4-bis-(glutathionyl)butan-2-one, which was clearly demonstrated as a product. The latter compound has the character of a thioacetal, and may have partially decomposed during processing of the sample to yield GSH and the *cis*- and *trans*-glutathione-conjugate. Evidence for this transformation is provided by the detection of GSSG in the LC/MS sample.

So far, we have no evidence for GST catalysis of the bis-glutathionyl conjugate formation, and any such reaction would not directly influence the rate of thiopurine release from the prodrug. On the other hand, the stoichiometry of GSH consumption would be increased such that cellular depletion of GSH may become a more serious consequence.

Two pathways of biotransformation contribute to the toxicity and the mode of action of the thiopurine prodrugs. The first involves the well-studied metabolism of 6-MP and 6-TG leading to nucleotide derivatives that inhibit nucleic acid biosynthesis, de novo purine production, and other processes of significance for cell proliferation [43]. The second pathway concerns depletion of glutathione and the consequent disruption of the cellular redox homeostasis [44]. Obviously, GSTs that catalyze the activation of cAVTP and tAVTG promote both pathways by releasing the thiopurines at the expense of conjugating GSH (Fig. 1). Clinical data indicate that the rate of thiopurine production should be moderated, since deficiency of the thiopurine methyltransferase leads to undesired toxicity [45]. Similarly, high GST activity may lead to harmful GSH consumption. In particular, depletion of the mitochondrial GSH pool will eventually cause necrotic cell death [46].

We have recently examined the catalytic activity of 14 human GSTs with azathioprine as a substrate [29]. Like cAVTP, azathioprine is a derivative of 6-MP, which releases 6-MP from the prodrug by displacement of its S-substituent by GSH. Azathioprine has been used clinically for more than 40 years

and serves as an immunosuppressant in the control of inflammatory bowel diseases and in preventing rejection of organ transplants. The highest catalytic efficiencies with azathioprine were displayed by GSTs A2-2, M1-1, and A1-1, ranked in this order. The k_{cat}/K_m values were 1.17, 0.53, and 0.48 $mM^{-1}\,s^{-1}$, respectively [29]. The azathioprine k_{cat}/K_m value for GST A2-2 is similar to those obtained with cAVTP and tAVTG, whereas the value for GST A1-1 is 5-fold lower than those with cAVTP and tAVTG (Table 2). With most substrates investigated GST A2-2 has a lower catalytic activity than that of GST A1-1 [47] and the reactions with cAVTP and tAVTG follow this rule, whereas azathioprine is an exception. The high specific activity of GST A4-4 with tAVTG (55 $\mu mol\,min^{-1}\,mg^{-1}$) is particularly noteworthy in comparison with its more than 5000-fold lower specific activity with azathioprine (0.01 $\mu mol\,min^{-1}\,mg^{-1}$). Thus, GSTs A1-1 and A2-2 have approximately the same catalytic activity with all three prodrugs, whereas GST A4-4 has markedly divergent activities with the same three substrates. The third enzyme with high azathioprine activity, GST M1-1, has k_{cat}/K_m values with cAVTP and tAVTG that are approximately 200- and 100-fold higher, respectively (Table 2). These higher values reflect the higher chemical reactivity of the latter compounds; the second-order rate constants for the reaction with GSH are 0.06, 0.17, and 0.008 $mM^{-1}\,min^{-1}$ for cAVTP, tAVTG, and azathioprine, respectively.

For use of cAVTP and tAVTG as cytostatic agents the target cells should obviously have high GST activity with the drugs. Tissues that are not targeted should ideally have zero or low such GST activity. Cancer cells often express elevated concentrations of both GSH and GSTs [6], which may be favorable for bioactivation of cAVTP and tAVTG. However, the GST most commonly overexpressed is GST P1-1, which has zero or low activity with these prodrugs (Table 1). Cells characterized by high levels of GSTs A4-4 or M1-1 would appear to be those with highest sensitivity to cAVTP and tAVTG. Based on estimates of GSH and GST concentrations in human liver, it has been calculated that the nonenzymatic reaction of azathioprine is negligible in comparison with the GST-catalyzed bioactivation [29]. Using the same liver parameters and the nonenzymatic rate constants for cAVTP and tAVTG in combination with the k_{cat}/K_m values (Table 2), the rate of GST-catalyzed reaction with GSH is at least two orders of magnitude higher than the uncatalyzed reactions for both prodrugs, provided that GST M1-1 is present. For GST M1-1 deficient cells accurate calculations cannot be made, because quantitative values of GST A4-4 expression are not available.

The other aspect of possible clinical applications of cAVTP and tAVTG is the avoidance of undesired side effects. Cells to be spared should not express high levels of the most active GSTs. The major hepatic enzymes GSTs A1-1 and A2-2 have modest activities, but the third hepatic enzyme GST M1-1 displays prominent activity with both prodrugs. Hepatotoxicity is therefore a possible side effect. However, most ethnic groups have a frequent null genotype such that approximately half the population does not express GST M1-1 due to the absence of the gene [48]. This fraction could be less susceptible to hepatotoxicity. Another concern is the high activity of GST A4-4, which appears to be ubiquitous in mammalian tissues [49]. GST A4-4 is present in mitochondria [50], which are particularly susceptible to GSH depletion. On the other hand, tumors overexpressing this enzyme could be expected to be particularly sensitive to the prodrugs.

Animal model studies suggest that cAVTP and tAVTG are at least as effective chemotherapeutic agents and display equal or less toxicity in comparison with the parent thiopurine drugs [12]. These promising results are accompanied by a lower bone marrow toxicity. In the mouse, the prodrugs did not significantly alter the GSH (or GSSG) concentration in plasma, erythrocytes, liver, or intestine [33]. However, it is not yet clear how these findings translate into human tumors and normal tissues, since GST expression profiles and the properties of individual enzymes differ noticeably between rodents and humans [51]. Nevertheless, experiments with human cell lines show that cAVTP and tAVTG are generally more cytotoxic than 6-MP and 6-TG and that the prodrugs have divergent efficacies with certain cell lines [17]. Further information is lacking about possible relationships between GST activities and cytotoxicities.

For possible clinical applications, two major issues should be further investigated. First, to what extent is toxicity to normal tissues limiting the use of cAVTP and tAVTG? In particular, is GST M1-1 an enzyme causing undesired effects in any of the many organs in which it is expressed [48]. Such toxicity may be absent in the half of the population lacking the enzyme. Secondly, does overexpression of particular GSTs in tumors, in particular GSTs M1-1 or A4-4, make the cancer cells more vulnerable to the prodrugs? Affirmative answers to these questions could lead to optimized chemotherapy based on genotyping of patients and proteomics of tumor tissues. Assays for analysis of the *GSTM1* genotype have been designed [52], and antibodies could be used for monitoring GST protein expression [53,54]. Development of these tools for clinical use and their application in cancer chemotherapy would be beneficial to patients and in line with current advances in clinical pharmacology towards individualized treatment.

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