The Glutathione-activated Thiopurine Prodrugs *trans*-6-(2-Acetylvinylthio)guanine and *cis*-6-(2-Acetylvinylthio)purine Cause Less *in Vivo* Toxicity than 6-Thioguanine after Single- and Multiple-Dose Regimens[1]

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Abstract

***trans*-6-(2-Acetylvinylthio)guanine (*trans*-AVTG) and *cis*-6-(2-acetylvinylthio)purine (*cis*-AVTP) are glutathione-activated prodrugs of 6-thioguanine (6-TG) and 6-mercaptopurine, respectively. In tumor cell lines, these prodrugs exhibit similar IC_{50} values that are comparable to or lower than those of 6-TG and 6-mercaptopurine, respectively. In this study, the *in vivo* toxicity and metabolism of the prodrugs were assessed. Mice given multiple treatments of 6-TG and, to a lesser extent, *trans*-AVTG exhibited decreased peripheral WBC and RBC counts and increased myeloid:erythroid ratios in bone marrow; no change was observed in mice given *cis*-AVTP. Similarly, intestinal epithelial crypt cell apoptosis was more extensive in mice treated with 6-TG than in those treated with *trans*-AVTG, whereas mice given *cis*-AVTP had little apoptosis. Epithelial crypt cell apoptosis was more extensive in the small intestine than in the large intestine in all treatment groups. Histopathological examination detected no kidney or liver toxicity, whereas mild increases in the activities of hepatocellular leakage enzymes were observed in mice treated with *trans*-AVTG. Only metabolites of *trans*-AVTG and *cis*-AVTP were recovered in urine. A higher fraction of the dose was recovered in urine as the parent thiopurine and the metabolites thiopurine riboside, thioxanthine, and thiouric acid after 6-TG treatment than after *trans*-AVTG treatment; *cis*-AVTP recovery was slightly less than that of 6-TG. Thioxanthine and thiouric acid comprised a higher fraction of the recovered dose after *cis*-AVTP treatment than after *trans*-AVTG or 6-TG treatment. Overall, the results suggest that the prodrugs exhibit less *in vivo* toxicity than 6-TG. Thus, investigations into their antitumor efficacy are warranted.**

Introduction

The widely used antileukemic thiopurine drugs 6-TG[3] and 6-MP (Fig. 1) are structural analogues of the endogenous purines hypoxanthine and guanine, respectively. 6-TG and 6-MP were initially synthesized to explore whether neoplasia might be controlled with compounds acting as antimetabolites in nucleic acid synthesis (1). Research into the mechanism of action of the thiopurines revealed that they are metabolized by three competing pathways. In the first pathway, 6-TG and 6-MP are anabolized by hypoxanthine-guanine phosphoribosyltransferase to form the nucleotides 6-thioguanylate and 6-thioinosinate, respectively. The latter nucleotide can be further metabolized to 6-thioguanylate. 6-Thioinosinate and 6-thioguanylate can inhibit *de novo* biosynthesis and interconversion of normal purines. These biochemical effects have been implicated in cytotoxicity of the thiopurines (2). However, a key step in the cytotoxicity of the thiopurines is the further metabolism of 6-thioguanylate resulting in the incorporation of 6-TG nucleotides into nucleic acids (2, 3). The second pathway of thiopurine metabolism constitutes the methylation of the sulfhydryl moiety of the thiopurines and their nucleosides and nucleotides by thiopurine methyltransferase (4, 5). Methylated thiopurines may be demethylated to form the parent thiopurine (6). Methylated 6-TG and 6-MP are pharmacologically inactive and are not substrates for hypoxanthine-guanine phosphoribosyltransferase (4). In contrast, methylated 6-TG nucleotides and methylated 6-thioinosinate monophosphate can inhibit *de novo* purine biosynthesis, with methylated 6-thioinosinate monophosphate being more active (2). In the last thiopurine metabolic pathway, 6-TG is deaminated to form TX, which is then further oxidized to TU, whereas 6-MP is oxidized to TX and subsequently to TU. Both TX and TU are inactive metabolites that are readily excreted into urine and do not contribute to the biological effects of the thiopurines (5).

The cytotoxic potency of 6-TG *in vitro* and *in vivo* is greater than that of 6-MP (7, 8). Likewise, the systemic toxicity of 6-TG is greater than that of 6-MP. The most severe and

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[3] The abbreviations used are: 6-TG, 6-thioguanine; 6-MP, 6-mercaptopurine; TX, thioxanthine; TU, thiouric acid; GSH, glutathione; *trans*-AVTG, *trans*-6-(2-acetylvinylthio)guanine; *cis*-AVTP, *cis*-6-(2-acetylvinylthio)purine; TFA, trifluoroacetic acid; HPLC, high pressure liquid chromatography; M:E, myeloid:erythroid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AZA, 6-[(1-methyl-4-nitro-5-imidazolyl)thio]purine.

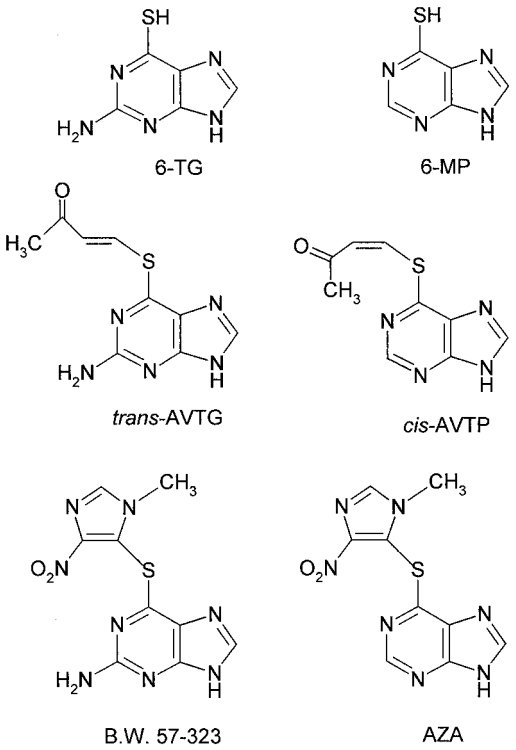

Fig. 1. Chemical structures of 6-TG, 6-MP, *trans*-AVTG, *cis*-AVTP, B.W. 57-323, and AZA.

commonly observed adverse effect associated with clinical use of the thiopurines is bone marrow suppression, primarily leukopenia and thrombocytopenia but also anemia at higher doses (9). Other side effects include vomiting, nausea, stomatitis, and jaundice (9–13). This systemic toxicity may be dose-limiting in clinical treatment of solid tumors (11, 14, 15). Therefore, to increase the clinical utility of the thiopurines, it is imperative to decrease their systemic toxicity. An approach frequently attempted to decrease the systemic toxicity of anticancer drugs is the use of prodrugs that may be preferentially activated in tumor cells for more selective delivery of the drug to tumor cells. Thus, selective bioactivation of thiopurine prodrugs by tumor cells may improve the targeting of the thiopurines, decrease their systemic toxicity, and increase their clinical utility.

A biochemical difference that exists between normal and malignant tissues is associated with GSH metabolism (16–18). GSH is a thiol-containing tripeptide that plays a crucial role in the detoxification of many chemotherapeutics (19). Furthermore, GSH is important in the regulation of the intracellular redox state whose disruption has been implicated in apoptosis (20–22). Elevated GSH levels have been detected in many tumors compared with normal surrounding tissue (16, 17, 23, 24). Up-regulation of GSH has also been associated with drug resistance and poor patient prognosis (25–27). Thus, thiopurine prodrugs activated by GSH may be metabolized in greater quantities in the tumor than in the normal surrounding tissue and thereby more selectively deliver the thiopurine drugs to the tumor. Furthermore, thiopurine prodrugs activated by GSH may also deliver the thiopurine to a tumor that has become drug resistant because of up-regulated levels of GSH. Additionally, it is possible that the metabolism of GSH-activated prodrugs will disrupt the tumor redox state, further enhancing the effects of the thiopurines.

Of late, we have been characterizing the novel GSH-activated thiopurine prodrugs *trans*-AVTG and *cis*-AVTP (Fig. 1). We demonstrated that bioactivation of *trans*-AVTG and *cis*-AVTP occurs via an addition-elimination reaction with cellular thiols to yield 6-TG and 6-MP, respectively (28). Furthermore, the intracellular metabolism of *trans*-AVTG was shown to deplete intracellular GSH. Additionally, the intracellular concentrations of 6-TG after *trans*-AVTG treatment were significantly higher than those after equimolar concentrations of 6-TG itself, suggesting that *trans*-AVTG delivers more of the parent drug to the cell than 6-TG itself. Furthermore, *in vitro* cytotoxicity studies using human renal cell carcinoma cells revealed that the cytotoxicities of the *cis* and *trans* isomers of either 6-(2-acetylvinylthio)guanine or 6-(2-acetylvinylthio)purine were comparable and similar or better than the cytotoxicities of the parent thiopurines.

In preliminary *in vivo* experiments, it was observed that mice given 8.5 or 21.25 μmol/kg 6-TG for 3 consecutive days exhibited a significant reduction in peripheral WBC counts 24 h after the last treatment, whereas no such reduction was observed in mice treated with 21.25 μmol/kg *trans*-AVTG or *cis*-AVTP (28). These results suggested that the prodrugs exhibit bone marrow sparing properties as compared with equimolar doses of 6-TG. Therefore, studies were carried out to examine in more detail the differences in systemic toxicity observed after treatment of mice with single or multiple treatments of the prodrugs or 6-TG. Additionally, to assess the *in vivo* metabolism of the prodrugs, urinary metabolites were characterized and quantitated. A preliminary report of the results obtained from these experiments has been published (29).

Materials and Methods

Chemicals. 6-TG, 6-MP, 6-TG riboside, 6-MP riboside, TX, and TFA were purchased from Sigma-Aldrich Research (St. Louis, MO). HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ). *trans*-AVTG and *cis*-AVTP were synthesized as described previously (28). Reference TU was synthesized by the enzymatic oxidation of TX by xanthine oxidase in buffer at 37°C according to the method of Bergmann and Ungar (30). The TU formed in the reaction was purified on a Sephadex LH-20 column (Amersham Pharmacia, Piscataway, NJ) using 5% acetonitrile adjusted to pH 2.5 with TFA. Fractions containing TU were combined and lyophilized. The purity of the final product was >96% as assessed by HPLC. All other chemicals were of the highest grade commercially available.

Assessment of the *in Vivo* Toxicity and Metabolism of 6-TG, *trans*-AVTG, and *cis*-AVTP. The *in vivo* toxicity and metabolism of a single dose of 6-TG or *trans*-AVTG and multiple doses of 6-TG, *trans*-AVTG, or *cis*-AVTP were assessed in CD-1 heterozygous nude male mice (Charles River, Wilmington, MA). All experiments were carried out in accord-

ance with the Guide for the Care and Use of Laboratory Animals as recommended by the United States NIH and the University of Wisconsin-Madison Animal Care Committee. As indicated earlier, the prodrug regioisomers were equally cytotoxic *in vitro*. Therefore, the regioisomer formed in higher quantities during prodrug synthesis was used to assess the *in vivo* toxicity (28). The mice were kept on a 12-h light/dark schedule and were allowed food and water *ad libitum*. To minimize the number of mice used in these experiments, we chose to evaluate the toxicity of a single dose of *trans*-AVTG before assessing *cis*-AVTP toxicity because *trans*-AVTG is a prodrug of the more toxic thiopurine 6-TG. Therefore, mice were treated with a single i.p. dose of vehicle alone, 6-TG (42.5 μmol/kg), or *trans*-AVTG (8.5, 21.25, 42.5 or 85 μmol/kg). The *in vivo* toxicity and metabolism of multiple treatments with 6-TG, *trans*-AVTG, or *cis*-AVTP were assessed by treating mice (i.p.) either with vehicle alone, 6-TG (8.5 or 21.25 μmol/kg), *trans*-AVTG (21.25 μmol/kg), or *cis*-AVTP (21.25 μmol/kg) once daily for 3 consecutive days (one cycle) or with vehicle alone, 6-TG (8.5 or 21.25 μmol/kg), *trans*-AVTG (8.5 or 21.25 μmol/kg), or *cis*-AVTP (8.5 or 21.25 μmol/kg) for 3 consecutive days, repeated again 5 days later (two cycles). A dose of 8.5 μmol/kg corresponds to 1.4, 2, and 1.9 mg/kg for 6-TG, *trans*-AVTG, and *cis*-AVTP, respectively, whereas a dose of 21.25 μmol/kg corresponds to 3.6, 5, and 4.7 mg/kg for 6-TG, *trans*-AVTG, and *cis*-AVTP, respectively. These doses are similar to doses of 6-TG that had previously been shown to have antitumor activity against sarcoma 180 in mice (1). All drugs were dissolved in buffer (0.78 mM phosphate, 137 mM NaCl, 5.4 mM KCl, and 4.2 mM $NaHCO_3$) with a few drops of dilute NaOH. The pH of the final solution was between 8 and 8.5. Fresh drug solutions were made up daily. Injection volume was 0.5 ml. All treatments were carried out between 9 and 11 a.m. During each experiment, the mice were housed individually in metabolic cages. Four mice were used per treatment group. The mice were sacrificed 24 h after the last treatment. Urine was collected for 24 h before treatment initiation and every 24 h thereafter until the mice were sacrificed. Approximately 1 ml of blood was collected from all animals by cardiac puncture and put into tubes containing K-EDTA. Approximately 500 μl of the whole blood were centrifuged, and the plasma was used for assessment of kidney and liver function or integrity as described below. The remaining 500 μl of whole blood were used for hematology analysis using the Advia 120 Hematology Analyzer (Bayer, Leverkusen, Germany). Bone marrow smears from the right femur were prepared using fine paint brushes, stained with modified Wright's stain, and examined by light microscopy. M:E ratios were determined by counting 500 cells and calculating the ratio of myeloid (granulocytic) to erythroid precursors. Pieces of liver, kidney, stomach, and large intestine were harvested from all treated mice; pieces of small intestine were also harvested from mice given multiple treatments. The tissue specimens were fixed in 10% neutral buffered formalin, and glass slides containing 4-μm-thick sections of paraffin-embedded tissues were stained with H&E. The prepared slides were analyzed blindly and given a score on a scale of 0–4 where sequentially increasing scores reflected increasing degrees of the characteristic being evaluated (*e.g.,* necrosis, inflammation). A score of 0 was assigned to normal tissue, and a score of 4 was assigned to diffuse severe necrosis or inflammation resulting in loss of normal architecture. Hepatocellular integrity was assessed by measuring ALT and AST activities in plasma using kits from Sigma Diagnostics (St. Louis, MO). Kidney integrity and function were evaluated by measuring blood urea nitrogen in plasma and γ-glutamyltransferase activity and glucose levels in urine using kits from Sigma Diagnostics.

Metabolite Characterization and Quantitation. Urinary metabolites were characterized and quantitated by HPLC. Briefly, into 50 μl of urine were added 50 μl of 10% TFA to deproteinize the samples. The samples were centrifuged at 20,000 × *g* for 20 min, left standing for 5 min, and then recentrifuged at 20,000 × *g* for 2 min. The supernatant was filtered through Acrodisc LC13 0.2 μm filters (Gelman Sciences, Ann Arbor, MI) and analyzed by HPLC. The HPLC system used consisted of two Gilson 306 pumps, a Gilson 117 UV/vis detector, and a Gilson 234 autoinjector (Gilson, Middleton, WI). The column used was a Beckman ultrasphere ODS 5 μm reversed-phase C_{18} (4.6 × 250 mm; Beckman Instruments, Fullerton, CA) with a Brownlee spheri-5 ODS 5 μm (4.6 × 30 mm) guard column (Perkin-Elmer, Norwalk, CT). The mobile phase for pump A consisted of water adjusted to pH 2.5 with TFA, and the mobile phase for pump B was 1:1 acetonitrile-water mixture adjusted to pH 2.5 with TFA. Injection volume was 20 μl, and the flow rate was 0.9 ml/min. The gradient used for analysis of metabolites excreted in urine was as follows: initially at 5% B, constant at 5% B for 10 min, increased to 25% B over 4 min, constant at 25% B for 3 min, increased to 35% B over 2 min, constant at 35% B for 2 min, increased to 60% B for 5 min, constant at 60% B for 5 min, decreased to 5% B over 5 min, and constant at 5% B for 4 min for a total run time of 40 min. Detection wavelengths were 323 and 342 nm. Retention times of metabolites under these conditions was as follows: TU, 8.7 min; TG, 9.2 min; 6-MP, 9.4 min; TX, 12.8 min; 6-MP riboside, 16.4 min; and 6-TG riboside, 16.8 min. All metabolites were quantitated using standard curves that were generated by linear regression of peak area *versus* concentration of a standard solution. The standard solutions were made up 10-fold concentrated in water and diluted to the appropriate concentration by adding 5 μl of the standard solution to 45 μl of urine. The standards were then processed in a manner identical to the samples. The limits of detection were between 1 and 2 nmol/ml for the metabolites. All standard curves were linear ($r > 0.99$) over the concentration range observed for metabolites in urine.

Statistical Analysis. All values are reported as the mean ± SD with the number (*n*) of experiments or animals as indicated in figure or table legends. Statistical analysis was carried out using Sigma Stat (SPSS Inc., Chicago, IL). Comparison of means was assessed using a *t* test or ANOVA. Occasionally, it was necessary to log transform the data before ANOVA analysis to obtain normal distribution of the data. *Post hoc* comparisons were carried out using Dunnett's test for the comparisons of *in vivo* treatments to control. α was set at 0.05.

Table 1 Hematology analysis for mice treated with vehicle alone (control), 6-TG, *trans*-AVTG, or *cis*-AVTP for two cycles as described in "Materials and Methods"

Values presented are the means ± SD (n = 3–4). PLT, platelets.

Drug	Dose (μmol/kg)	WBC ($\times 10^3$ cells/μl)	RBC ($\times 10^6$ cells/μl)	PLT ($\times 10^3$ cells/μl)
Control		7.8 ± 1.1	9.3 ± 0.4	1167 ± 204
6-TG	8.5	5.3 ± 1.6	8.3 ± 0.2	906 ± 140
	21.25	3.6 ± 0.1[a]	8.0 ± 0.3[a]	1351 ± 126
trans-AVTG	8.5	5.4 ± 1.6	8.9 ± 0.5	1054 ± 61
	21.25	2.3 ± 1.2[a]	7.6 ± 1.0[a]	828 ± 144
cis-AVTP	8.5	5.9 ± 2.3	9.5 ± 0.3	1160 ± 349
	21.25	8.4 ± 1.9	9.0 ± 0.5	1257 ± 335

[a] Significantly different from the corresponding control value ($P < 0.05$).

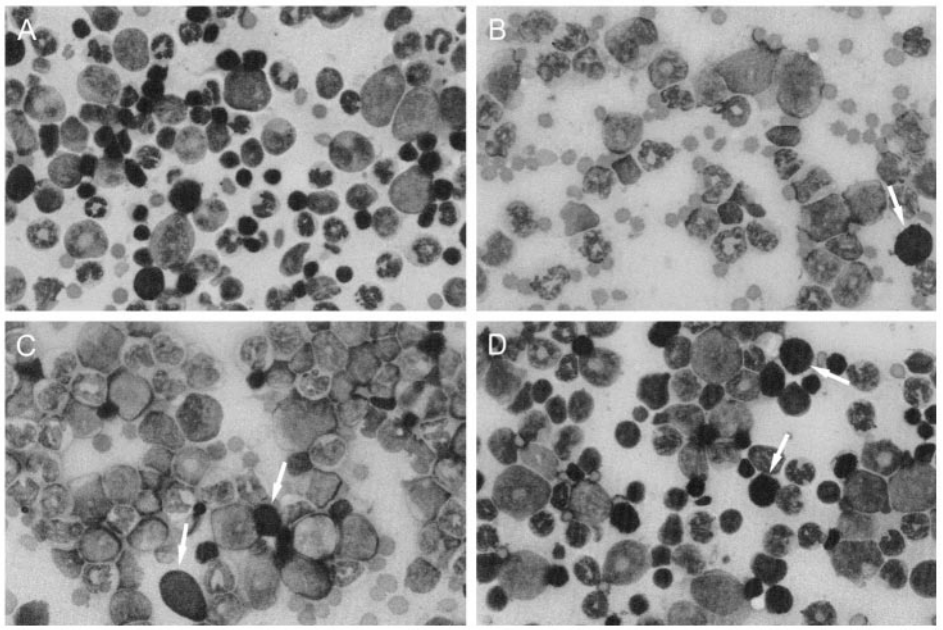

Fig. 2. Representative bone marrow smears from mice treated with vehicle alone (*A*) or 21.25 μmol/kg 6-TG (*B*), *trans*-AVTG (*C*), or *cis*-AVTP (*D*) for two cycles as described in "Materials and Methods." *Arrows* indicate the darkly stained erythroid precursors. Note the low numbers of erythroid cells in *C* and their rare presence in *B*. ×600.

Results

Analysis of peripheral blood obtained from mice treated with a single dose of 6-TG (42.5 μmol/kg) did not reveal any change in blood cell counts 24 h after treatment as compared with mice given a single dose of vehicle alone. Similarly, no change in peripheral blood cell counts was detected in mice given a single dose of *trans*-AVTG (8.5–85 μmol/kg; data not shown). No changes in kidney or liver integrity or function were detected in mice treated with 6-TG or *trans*-AVTG, and no histopathological lesions were evident upon examination of liver, kidney, stomach, or large intestine (data not shown).

Because we had previously determined that mice given 8.5 and 21.25 μmol/kg 6-TG for 3 consecutive days (one cycle) had significantly reduced peripheral WBC and RBC counts, whereas no such reduction was observed in mice treated with *trans*-AVTG or *cis*-AVTP, it was of interest to determine whether a longer treatment period would affect peripheral blood cell counts in drug-treated mice. Similar to what was observed after treatment of mice with 6-TG for one cycle, mice treated with 21.25 μmol/kg 6-TG for 3 consecutive days, repeated again 5 days later (two cycles), had reduced WBC counts compared with mice treated with vehicle alone (Table 1). Likewise, mice treated for two cycles with 21.25 μmol/kg *trans*-AVTG had significantly reduced peripheral WBC counts. Additionally, RBC counts were reduced in mice treated with 21.25 μmol/kg 6-TG or *trans*-AVTG for two cycles. In contrast, mice given 8.5 or 21.25 μmol/kg *cis*-AVTP for two cycles had no change in peripheral blood cell counts as compared with mice given vehicle alone.

Because bone marrow suppression is the most common adverse effect associated with use of 6-TG and 6-MP, we examined smears of femoral bone marrow from mice treated with vehicle alone for one and two cycles; 8.5 μmol/kg 6-TG, *trans*-AVTG, or *cis*-AVTP for two cycles; or 21.25 μmol/kg 6-TG, *trans*-AVTG, or *cis*-AVTP for one and two cycles (Fig. 2). All mice treated with 6-TG had M:E ratios that were substantially higher than the ratios obtained from mice treated with vehicle alone (Table 2). Mice treated with *trans*-AVTG had M:E ratios lower than mice treated with 6-TG but higher than mice treated with vehicle alone. In contrast, mice treated with *cis*-AVTP had M:E ratios that were comparable

Table 2 M:E ratio in bone marrow of mice treated with vehicle alone (control), 6-TG, *trans*-AVTG, or *cis*-AVTP as described in "Materials and Methods"

Values presented are the means and range ($n = 4$).

	Dose (μmol/kg)	M:E ratio (mean and range)	
		One cycle	Two cycles
Control		1.7:1 (1.5:1–2.3:1)	1.6:1 (1.2:1–1.8:1)
6-TG	8.5	ND[a]	11.8:1 (8.1:1–15.7:1)
	21.25	44.8:1 (32.3:1–49:1)	86.5:1 (49:1–99:1)
trans-AVTG	8.5	ND	3.2:1 (2.0:1–5.3:1)
	21.25	6.6:1 (3.0:1–10.1:1)	5.7:1 (4.6:1–7.3:1)
cis-AVTP	8.5	ND	1.4:1 (1.2:1–1.6:1)
	21.25	1.8:1 (1.5:1–2.3:1)	1.8:1 (1.3:1–2.2:1)

[a] ND, not done.

to those of mice treated with vehicle alone. Interestingly, a time- and dose-dependent effect on M:E ratios was observed in mice treated with 6-TG; mice treated with 21.25 μmol/kg 6-TG for two cycles had approximately 2- and 7-fold higher M:E ratios than mice treated with 21.25 μmol/kg for one cycle and 8.5 μmol/kg for two cycles, respectively. No time-dependent effect was observed for the M:E ratios in mice treated with *trans*-AVTG, whereas an approximately 2-fold increase was observed in the M:E ratios obtained after treatment of mice with 21.25 μmol/kg for two cycles compared with 8.5 μmol/kg for two cycles. No time- or dose-dependent effects were observed for the M:E ratios obtained after treatment of mice with *cis*-AVTP.

Because the observed reduction in the numbers of peripheral WBCs might also result in part from the sequestration of leukocytes in tissues secondary to inflammation, histopathological examination of liver, kidney, stomach, and large and small intestine was carried out. Inflammation was not detected in any of the tissues examined (data not shown). However, histopathological examination of the small intestine from mice treated with 6-TG, *trans*-AVTG, or *cis*-AVTP revealed the presence of condensed pyknotic nuclei within shrunken globular hypereosinophilic crypt epithelial cells, which were occasionally phagocytosed by adjacent viable neighboring epithelial cells (Fig. 3). Inflammation was absent in these foci. These findings suggest that 6-TG, *trans*-AVTG, and *cis*-AVTP induce apoptosis in crypt cells of the small intestinal epithelium. No signs of apoptosis were detected in mature cells of the intestinal villi or in gut-associated lymphoid tissue.

Assessment of the extent of epithelial crypt cell apoptosis in the small intestine of mice treated for one cycle revealed that mice given either 8.5 or 21.25 μmol/kg 6-TG had moderate or extensive crypt cell apoptosis as compared with mice given vehicle alone (Table 3). Mice treated with 21.25 μmol/kg *trans*-AVTG had a moderate increase in epithelial crypt cell apoptosis, whereas mice given 21.25 μmol/kg *cis*-AVTP had a slight increase in crypt cell apoptosis compared with mice treated with vehicle alone. Similar results were obtained from mice treated for two cycles (Table 3). A trend toward increasing severity of epithelial crypt cell apoptosis was observed with increasing dose, whereas inconsistent changes in the extent of apoptosis were observed with treatment length. Epithelial crypt cell apoptosis was also detected in the large intestine of mice treated with 6-TG or *trans*-AVTG but to a lesser extent than was observed in the small intestine (Table 3).

No histopathological lesions were detected in the stomach of any of the mice treated for either one or two cycles. Similarly, no kidney lesions or changes in kidney function or integrity were detected in any of the treatment groups compared with vehicle alone-treated mice (data not shown). In contrast, mice treated with 21.25 μmol/kg *trans*-AVTG for one and two cycles had a slight elevation of ALT activity in plasma, and mice treated for two cycles with 21.25 μmol/kg *trans*-AVTG had a slight elevation of AST activity in plasma (Table 4). However, by histopathological examination, livers from mice given 21.25 μmol/kg *trans*-AVTG had no lesions and were similar to livers from mice given vehicle alone.

To assess the *in vivo* metabolism of the prodrugs compared with 6-TG, urine from mice treated with single or multiple doses of 6-TG or the prodrugs was analyzed for the presence of the prodrug, the parent thiopurines, the corresponding thiopurine riboside, and the inactive metabolites, TU and TX. Mice treated with a single 42.5 μmol/kg dose of 6-TG excreted approximately 33% of the administered dose as 6-TG, 6-TG riboside, and TU in urine (Fig. 4). Approximately 26% of the dose was excreted as 6-TG, whereas 6-TG riboside and TU each accounted for approximately 3% of the dose. Analysis of urine from mice given a single dose of *trans*-AVTG revealed no unmetabolized prodrug at any of the dose levels examined. Less than 10% of the administered *trans*-AVTG dose was recovered as urinary metabolites. Furthermore, the fraction of *trans*-AVTG dose recovered in urine did not change significantly with increasing dose (Fig. 4). 6-TG and the bioactivation metabolite 6-TG riboside were readily detected in urine from all treated mice and accounted for approximately 4.5% and 1% of the dose, respectively. Whereas the inactive metabolite TU accounted for approximately 0.7% of the dose in urine from mice given 42.5 and 85 μmol/kg *trans*-AVTG, negligible amounts of TU were found in urine from mice given 8.5 and 21.25 μmol/kg *trans*-AVTG. It is likely that the concentrations of TX and TU present in the urine of mice given 8.5 or 21.25 μmol/kg *trans*-AVTG were below the limits of detection of the analysis method used in these studies. These results show that a much smaller fraction of the administered dose is recovered in urine over 24 h after treatment with a single *trans*-AVTG dose than after a single dose of 6-TG.

Mice given multiple treatments with 8.5 μmol/kg 6-TG excreted approximately 25% of the dose in urine as 6-TG and its metabolites; however, this fraction was highly variable both between mice and between days and ranged from 4% to 66% after individual treatments (Fig. 5*A*). Similar to what was observed after a single-dose treatment, 6-TG accounted for the majority of the metabolites excreted into urine after multiple treatments. Little change was observed between the average fraction of dose recovered in urine from mice treated for one or two cycles. Similar high variability in the fraction of dose excreted in urine, as was obtained after multiple 8.5 μmol/kg treatments, was also observed when mice were treated with multiple treatments of 21.25 μmol/kg 6-TG; on

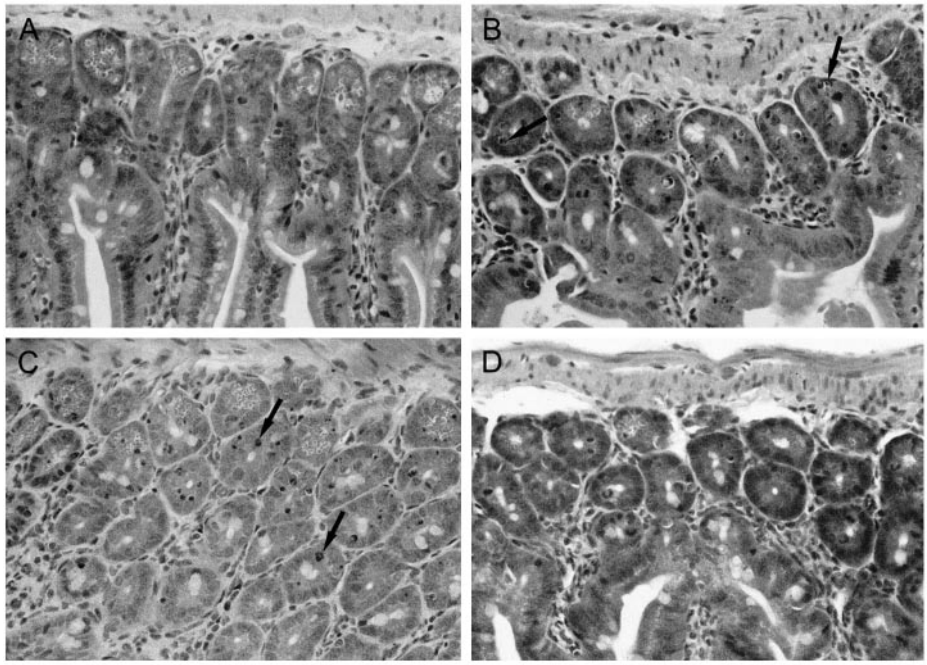

Fig. 3. Representative figures of H&E-stained sections of small intestine from mice treated with vehicle alone (*A*) or 21.25 μmol/kg 6-TG (*B*), *trans*-AVTG (*C*), or *cis*-AVTP (*D*) for two cycles as described in "Materials and Methods." *Arrows* indicate apoptotic cells. ×400.

Table 3 Summary of morphological analysis of sections of small or large intestine from mice treated with vehicle alone (control), 6-TG, *trans*-AVTG, or *cis*-AVTP

Values presented are the median scores and range ($n = 4$). A score of 0 was assigned to normal tissue, and a score of 4 was assigned to tissues with diffuse severe apoptosis or inflammation resulting in loss of normal architecture.

	Dose (μmol/kg)	Small intestine		Large intestine	
		One cycle	Two cycles	One cycle	Two cycles
Control		0 (0)	0 (0–0.5)	0 (0)	0 (0)
6-TG	8.5	2 (2–3)	3 (2–3)	1 (1)	2 (0.5–2)
	21.25	3 (2–3)	3 (3–4)	2 (1–2)	1 (0.5–1)
trans-AVTG	8.5	ND[a]	1.5 (1–2)	ND	0 (0–0.5)
	21.25	2 (2–3)	2 (2)	0.5 (0–1)	0.25 (0–1)
cis-AVTP	8.5	ND	0 (0–1)	ND	0 (0)
	21.25	0.75 (0.5–1)	0.25 (0–1)	0 (0)	0 (0)

[a] ND, not done.

average, about 28% of the administered dose was recovered in urine of mice given 21.25 μmol/kg 6-TG.

Similar to what was observed for mice given a single dose of *trans*-AVTG, no unchanged prodrug, TX, or TU was detected in urine of mice given multiple treatments with 8.5 μmol/kg *trans*-AVTG, whereas 6-TG and 6-TG riboside were readily detected. Likewise, the percentage of administered dose recovered as urinary metabolites was only about 1–5% (Fig. 5*A*). Little change was detected in the amount of metabolites excreted between one- and two-cycle treatments. Higher amounts of metabolites were obtained in urine after multiple treatments of mice with 21.25 μmol/kg *trans*-AVTG (Fig. 5*B*) than were obtained after treatment with multiple 8.5 μmol/kg doses, whereas the percentage of dose recovered in urine was comparable with that obtained after treatment with multiple 8.5 μmol/kg doses or 2–10%.

When urine obtained from mice treated with multiple doses of 8.5 μmol/kg *cis*-AVTP was analyzed, no unchanged prodrug was detected. On the other hand, the bioactivation metabolites 6-MP and 6-MP riboside, as well as the inactive metabolites TX and TU, were easily detected. Contrary to what was observed after treatments with 6-TG or *trans*-AVTG, where most of the metabolites identified in urine were 6-TG and 6-TG riboside, the metabolites 6-MP and 6-MP riboside comprised only about half of the amount recovered in urine after *cis*-AVTP treatment. The remaining 50% consisted of the oxidative metabolites TX and TU. Approximately 17% of the administered dose was recovered as urinary metabolites after 8.5 μmol/kg *cis*-AVTP, with little difference detected between the average amount excreted after one- or two-cycle treatments (Fig. 5*A*). Whereas approximately 2.8-fold higher amounts of urinary metabolites were recovered after multiple treatments with 21.25 μmol/kg *cis*-AVTP, the percentage of dose recovered was similar to that obtained after 8.5 μmol/kg treatments or 18% (Fig. 5*B*); again, approximately half of the amount recovered consisted of TX and TU.

Table 4 Plasma AST and ALT activities in mice treated with vehicle alone (control), 6-TG, *trans*-AVTG, or *cis*-AVTP for one or two cycles

Values presented are the means ± SD (n = 3–4).

Drug	Dose (μmol/kg)	AST (SF U/ml)[a]		ALT (SF U/ml)	
		One cycle	Two cycles	One cycle	Two cycles
Control		71.4 ± 44.9	40.2 ± 26.9	6.1 ± 2.5	8.1 ± 4.4
6-TG	8.5	43.5 ± 24.8	37.9 ± 12.2	9.2 ± 3.2	10.5 ± 6.6
	21.25	44.4 ± 5.4	68.2 ± 54.5	15.0 ± 2.5	12.6 ± 4.8
trans-AVTG	8.5	ND[a]	43.6 ± 26.8	ND	14.5 ± 5.0
	21.25	132.5 ± 40.7	236.9 ± 40.4[b,c]	46.3 ± 41.6[c]	20.7 ± 1.3[c]
cis-AVTP	8.5	ND	55.1 ± 37.5	ND	7.8 ± 4.0
	21.25	25.5 ± 4.3	60.2 ± 30.8	2.0 ± 2.0[c]	16.0 ± 4.3[b]

[a] One Sigma-Frankel (SF) unit of activity will form 4.82×10^{-4} μmol glutamate/min at pH 7.5 and 25° C. ND, not done.
[b] Significantly different from the corresponding one cycle value.
[c] Significantly different from the corresponding control value.

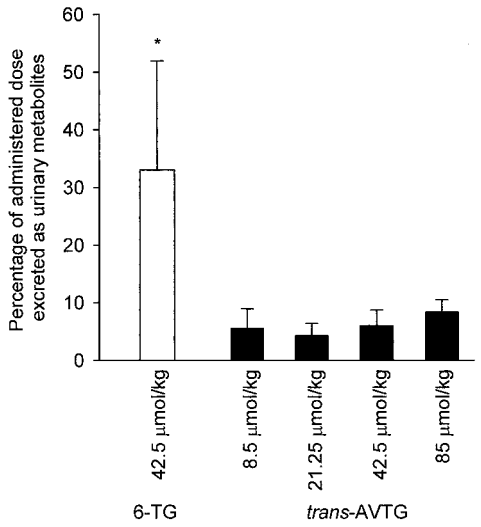

Fig. 4. Percentage of administered dose excreted as 6-TG and its metabolites into urine over 24 h after a single treatment of mice with 6-TG or *trans*-AVTG as described in "Materials and Methods." Values presented are the means ± SD (n = 4). *, significantly different from an equimolar dose of *trans*-AVTG ($P < 0.05$).

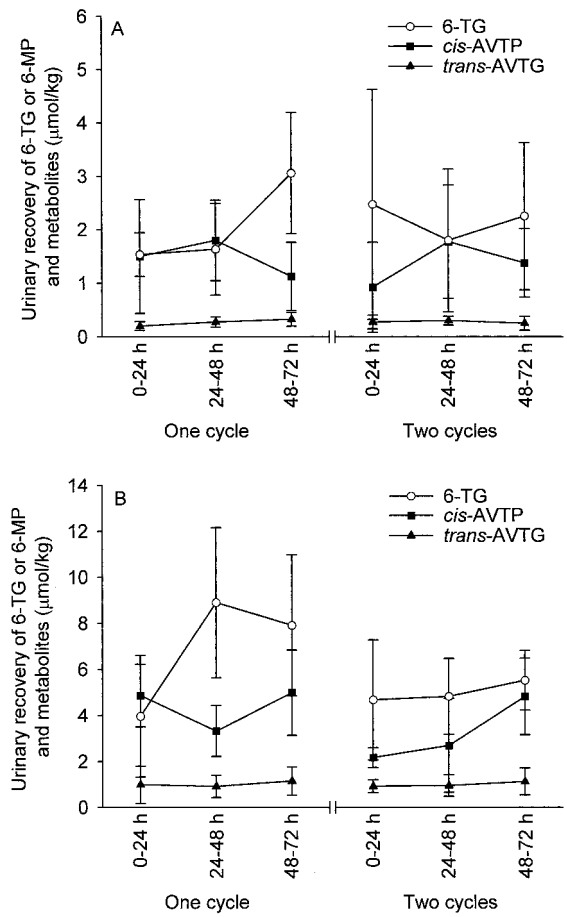

Fig. 5. Amount of metabolites excreted into urine every 24 h after treatment of mice with 8.5 (*A*) or 21.25 μmol/kg (*B*) 6-TG, *trans*-AVTG, or *cis*-AVTP for one and two cycles. Values presented are the means ± SD (n = 4–8).

Discussion

In this study, we have examined the *in vivo* toxicity and metabolism of the prodrugs *trans*-AVTG and *cis*-AVTP as compared with 6-TG. We have shown that a single treatment of 6-TG or *trans*-AVTG is not toxic to mice up to 24 h after administration. Additionally, the decreased bone marrow and intestinal toxicity observed after multiple treatments with *trans*-AVTG and *cis*-AVTP suggests that the prodrugs are less toxic to these tissues than equimolar doses of 6-TG. However, the increase in hepatocellular permeability observed after high doses of *trans*-AVTG may suggest a minor impairment in liver function. Lastly, the differences observed in the amount of administered dose recovered in urine after treatment with 6-TG, *trans*-AVTG, and *cis*-AVTP may suggest pharmacokinetic and metabolic differences between these compounds.

It is well known that the thiopurines markedly affect the bone marrow. Treatment with either 6-TG or 6-MP causes a reduction in peripheral leukocyte counts in many species, including mice, rats, dogs, and humans. Similarly, reduction in peripheral RBC counts has been observed after treatment with 6-TG or 6-MP (9, 31–33). This decrease in peripheral blood cell counts is likely to be caused by thiopurine-associated bone marrow hypoplasia (32, 34) affecting cells of the erythroid lineage more severely than cells of the myeloid

lineage (31, 35). This bone marrow damage can be reversible. Dogs treated with 6-TG (25 mg/kg/day, i.v.) for 4 days exhibited a marked decrease in WBC counts 1–4 days after discontinuation of treatment, after which the counts increased again (32). Similarly, examination of peripheral blood from mice treated with 6-MP (125 mg/kg) every other day for a total of eight treatments showed that the numbers of leukocytes, neutrophils, and erythrocytes were rebounding 5 days after the end of treatment (31). Likewise, bone marrow in mice given a single 6-MP treatment (2.5–25 mg/kg, s.c.) was found to be rebounding within 7 days after treatment (36). Our findings that treatment of mice with 6-TG causes a reduction in peripheral WBC and RBC counts agree well with known effects of 6-TG treatment on peripheral blood cell counts. Similarly, the increased M:E ratio in 6-TG-treated mice likely results from severe erythroid hypoplasia rather than granulocytic hyperplasia in bone marrow because erythroid cells were virtually absent from bone marrow, and there was no evidence of tissue inflammation with sequestered leukocytes.

In light of the known toxic effects of the thiopurines on bone marrow, it is of considerable interest to note the less pronounced effect of *trans*-AVTG on peripheral blood cell counts and on the bone marrow M:E ratio as compared with equimolar treatments with 6-TG, as well as the apparent lack of bone marrow toxicity after comparable *cis*-AVTP treatment. However, because mice given prodrugs were sacrificed 24 h after the last injection, any potential delayed or irreversible toxic effect caused by the prodrugs would not have been detected in our experiments. On the other hand, in an experiment determining the toxic effects of B.W. 57-323 (2-amino-6-[(1-methyl-4-nitro-5-imidazolyl)thio]purine; Fig. 1), a GSH-activated prodrug of 6-TG and a structural analogue of *trans*-AVTG, mice that were given 13 treatments of B.W. 57-323 (25 mg/kg, p.o.) over a 17-day period had developed a marked depression in peripheral WBC and RBC counts 2 days after the last dose but recovered fully a week later (37). Because B.W. 57-323 and *trans*-AVTG seem to have similar structural features and mechanisms of bioactivation, these findings suggest that whereas a more pronounced decrease in peripheral blood cell counts might have been observed in mice treated with *trans*-AVTG at a later time point, the bone marrow suppressive effects observed after *trans*-AVTG treatment are likely to be reversible. Similarly, whereas rats treated with the compound AZA (Fig. 1), a 6-MP analogue of B.W. 57-323, developed agranulocytic bone marrow at high doses, no histological abnormalities were observed in surviving animals after 6 months (38).

Our results further show that mice given multiple treatments of 6-TG have more extensive apoptosis in epithelial crypt cells in the large and small intestines compared with mice treated with the prodrugs or vehicle alone. These findings are in agreement with previously published results demonstrating necrosis of the intestinal epithelial crypt cells in mice given 6-TG (5 mg/kg, i.p.) once a day for 9 consecutive days (35). The increased treatment length in this latter experiment may explain why the mice exhibited intestinal epithelial crypt cell necrosis rather than apoptosis as was observed in our experiments. In the same way, 6-MP causes damage to intestinal epithelium (34). Therefore, it is of considerable interest that less extensive intestinal epithelial crypt cell apoptosis was observed after multiple treatments with the prodrugs as compared with 6-TG.

The observed differences in the fraction of administered dose recovered in urine as the parent thiopurine 6-TG or 6-MP and their corresponding metabolites together with the distinct metabolite profile for each drug suggest that 6-TG, *trans*-AVTG, and *cis*-AVTP exhibit different pharmacokinetic properties. The fact that no unmetabolized prodrug was recovered in the urine of mice treated with *trans*-AVTG or *cis*-AVTP suggests that the prodrugs are rapidly converted to the parent thiopurine *in vivo*. These results agree well with previous *in vitro* experiments that demonstrated rapid conversion of the prodrugs to the parent thiopurines when the prodrugs were incubated with cellular thiols (28).

In our experiments, we observed a trend toward a higher fraction of the administered dose recovered as metabolites in urine after *cis*-AVTP than after *trans*-AVTG treatments (Fig. 5). Interestingly, a positive correlation has been found between the amount of 6-MP excreted in urine over 24 h and the area under the curve obtained for 6-MP in plasma after treatment of children with 6-MP (39). Furthermore, it has been demonstrated that children given identical doses of 6-MP and 6-TG had 4-fold higher peak plasma drug levels after 6-MP treatment than after 6-TG treatment (40). Therefore, it is conceivable that the difference in urinary excretion observed in our experiments reflects that mice given *cis*-AVTP have higher plasma concentrations of the drug and its metabolites than do mice given *trans*-AVTG.

After treatment with both 6-TG and *trans*-AVTG, 6-TG was the major compound recovered in urine, whereas lower amounts of 6-TG riboside and TU were recovered. However, a much lower percentage of the administered dose was recovered after *trans*-AVTG treatment than after equimolar treatments with 6-TG, suggesting that the *in vivo* pharmacokinetic properties of *trans*-AVTG differ from those of 6-TG. These findings differ from those obtained in experiments examining the recovery of B.W. 57-323, where about 20% of the administered oral dose could be recovered in urine as the prodrug and its metabolites 6-TG and TU, whereas about 14% of an oral dose of 6-TG could be recovered in urine as 6-TG and TU (37). The difference in recovery of urinary metabolites observed between *trans*-AVTG and 6-TG likely results from a combination of several factors. We have previously shown that the *in vitro* cellular uptake of *trans*-AVTG is more efficient than that of 6-TG (28). Similarly, uptake of the 6-TG prodrug B.W. 57-323 after oral administration in mice was better than that of 6-TG (37). Therefore, it is plausible that the *in vivo* cellular uptake of *trans*-AVTG is also more efficient than that of 6-TG. Intracellularly, *trans*-AVTG may be metabolized to 6-TG and its further metabolites, as was previously demonstrated to occur *in vitro* (28). However, the fact that more severe toxicity was observed in the intestines and bone marrow of mice given multiple treatments with 6-TG than in mice given *trans*-AVTG suggests that the cellular burden of potentially toxic compounds is lower in these tissues after *trans*-AVTG treatment than after equimolar treatments with 6-TG. Therefore, if the lower urinary recovery

is the result of more efficient uptake of *trans*-AVTG than of 6-TG, then *trans*-AVTG is metabolized to a larger extent than 6-TG to nontoxic metabolites, to compounds not detected in these studies such as inorganic sulfate or *S*-methylated 6-TG, or to metabolites that bind more extensively to cellular macromolecules (4, 37). Alternatively, it is possible that the volume of distribution for *trans*-AVTG is greater than that observed for 6-TG. Furthermore, it is also possible that biliary excretion of *trans*-AVTG and its metabolites is greater than that of 6-TG and its metabolites. Such difference in the route of elimination might explain in part the lower amount of the dose recovered in urine after *trans*-AVTG treatment than after 6-TG treatment. Differences in metabolite composition or enhanced biliary excretion for *trans*-AVTG compared with 6-TG might also explain in part why slight hepatocellular damage may occur in mice given 21.25 μmol/kg *trans*-AVTG but not in mice given comparable doses of 6-TG.

Similar to what was observed for *trans*-AVTG, the GSH-activated prodrug B.W. 57-323 was less toxic than the parent compound 6-TG and was less active when administered i.p. for the treatment of adenocarcinoma 755 in mice. In contrast, B.W. 57-323 had a superior chemotherapeutic index compared with 6-TG after oral administration (37). Likewise, the GSH-activated 6-MP prodrug AZA had a better chemotherapeutic index when given p.o. than i.p. for adenocarcinoma 755, whereas the reverse was true for the parent compound 6-MP (38). These results, demonstrating that pharmacokinetic properties and the efficacy of drugs can be significantly affected by their route of administration, are of special interest because B.W. 57-323 and AZA are structural analogues of AVTG and AVTP, respectively. In light of these findings, it is imperative to examine the effects of route of administration on the systemic toxicity of *cis*-AVTP and *trans*-AVTG.

In summary, we have demonstrated that multiple doses of *cis*-AVTP and *trans*-AVTG are less toxic to the bone marrow and intestine than multiple equimolar doses of 6-TG. However, because a lower fraction of the dose was recovered in urine after *trans*-AVTG treatment than after 6-TG treatment, and because *cis*-AVTP is a prodrug of the less toxic thiopurine, it is possible that the decrease in systemic toxicity is due to decreased systemic exposure to potentially toxic compounds. We are currently carrying out experiments examining in detail the tissue distribution of 6-TG, *trans*-AVTG, and *cis*-AVTP and their metabolites to determine whether differences in metabolite composition and tissue accumulation of potentially toxic metabolites can provide an explanation for the differences in toxicity and urinary metabolite recovery observed in the studies described here. Additionally, because *trans*-AVTG and *cis*-AVTP have been shown to deplete cellular GSH *in vitro* (28), studies are also underway to examine whether treatment of mice with the prodrugs alters tissue GSH homeostasis. Such disruption may potentially play a role in the hepatocellular membrane damage observed after treatment with *trans*-AVTG.

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