Novel Glutathione-Dependent Thiopurine Prodrugs: Evidence for Enhanced Cytotoxicity in Tumor Cells and for Decreased Bone Marrow Toxicity in Mice

SJOFN GUNNARSDOTTIR, MARIAN RUCKI, and ADNAN A. ELFARRA

Environmental Toxicology Center (S.G., A.A.E.) and Department of Comparative Biosciences (S.G., M.R., A.A.E.), University of Wisconsin, Madison, Wisconsin

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ABSTRACT

Elevated glutathione (GSH) levels have been detected in many tumors compared with the healthy, surrounding tissue. Often, this GSH up-regulation is associated with drug resistance. The prodrugs 6-(2-acetylvinylthio)guanine (AVTG) and 6-(2-acetylvinylnithio)purine (AVTP) contain a novel butenone moiety that allows the prodrugs to react selectively with sulfhydryl nucleophiles to release the chemotherapeutic drug 6-thioguanine (6-TG) or 6-mercaptopurine (6-MP), respectively. The cellular uptake and metabolism of trans-AVTG in two human renal carcinoma cell lines that were used as models were rapid and associated with depletion of intracellular GSH. Formation of 6-TG from trans-AVTG correlated positively with intracellular GSH concentrations, and was significantly reduced by diethyl maleate pretreatment. Intracellular concentrations of 6-TG after incubations with trans-AVTG were significantly higher than the 6-TG concentrations obtained after incubations with equimolar concentrations of 6-TG; thus, the prodrug delivered more 6-TG to the cell than did 6-TG itself. Cytotoxicity studies demonstrated that AVTG and AVTP had similar IC₅₀ values that were comparable with those of 6-TG, but were significantly lower than those of 6-MP. Furthermore, after in vivo treatment of mice with the prodrugs, no reduction was observed in circulating white blood cell counts, whereas white blood cell counts of mice treated with equimolar or 60% lower doses of 6-TG were reduced by 50 to 60%. Collectively, the results show that AVTG and AVTP are novel potential chemotherapeutic agents that may target tumors with up-regulated levels of GSH, and may exhibit less systemic toxicity than the parent thiopurines.

The narrow therapeutic index associated with most chemotherapeutic agents often leads to toxicity in nontarget tissues (Hoekman et al., 1999). An approach commonly used to decrease systemic toxicity of chemotherapeutics is prodrug design. Prodrugs are pharmacologically inactive compounds that are metabolized in vivo to yield active drugs. Because quantitative and/or qualitative biochemical differences exist between tumors and the healthy surrounding tissue, it may be possible to selectively deliver drugs to the tumor by designing prodrugs that are metabolized more rapidly or in greater quantities in the tumor than in the surrounding tissue (Dubowchik and Walker, 1999). Thus, prodrugs may decrease adverse drug reactions and systemic toxicity, and increase tissue selectivity and therapeutic efficacy of the parent drug.

Glutathione (GSH) plays a major role in the detoxification of numerous chemotherapeutics (Schroeder et al., 1996). Changes in GSH content and levels of glutathione S-transferases (GSTs), GSH peroxidase, and GSH reductase (GR) have been detected in tumors (Di Ilio et al., 1991, 1995; Gajewska et al., 1995; Institoris et al., 1995; Blair et al., 1997). Moreover, increased levels of GSH have been linked with drug resistance (Mickisch et al., 1990; Yang et al., 1992), and up-regulation of the π isoform of GST is often associated with drug resistance and poor patient prognosis (Hamada et al., 1994; Ali-Osman et al., 1997). Thus, an excellent opportunity exists for the design of prodrugs that specifically target tumors with abnormal GSH metabolism. Recently, a novel alkylating agent prodrug designed to target tumors with up-regulated levels of GST π was characterized (Lyttle...
et al., 1994). The prodrug, TER286, is a GSH analog that is activated after binding in place of GSH in the active sites of GST isoforms α and π. Response to TER286 in murine xenografts of colon carcinoma cells was positively correlated with the level of GST π expression (Morgan et al., 1998). Other GST-activated chemotherapeutic prodrugs have been characterized. The 6-mercaptopurine (6-MP) prodrug 6-chloropurine can be conjugated to GSH to yield S-(9H-purin-6-yl)glutathione (PG; Hwang and Elfarra, 1993), which can be further metabolized in vivo to yield 6-MP (Hwang and Elfarra, 1991; Elfarra and Hwang, 1993; Lash et al., 1997). Similarly, S-(guanin-6-yl)-L-cysteine, the cysteine conjugate of 6-chloroguanine, has been shown to be metabolized in vivo to yield 6-TG (Elfarra et al., 1995). 6-MP and its analog 6-TG are antimitabolites that have long been used in treatment of leukemias (Adamson et al., 1994; Erb et al., 1998). Although 6-MP and 6-TG are effective against several solid tumor cell lines in vitro, severe bone marrow toxicity has limited their utility (van Scoik et al., 1985; Hayden et al., 1989; Lennard, 1992). Development of suitable prodrugs that would decrease the systemic toxicity by improving the targeting of the thiopurines to tumor cells is likely to increase clinical use of these chemotherapeutics.

Recently, we characterized the metabolism of cis-3-(9H-purin-6-ylthio)acrylic acid (PTA; Fig. 1; Gunnarsdottir and Elfarra, 1999), a potential 6-MP prodrug targeting tumors with up-regulated GSH levels. Structurally, PTA is a butenoic acid conjugate of 6-MP and a Michael acceptor that undergoes addition-elimination reaction with nucleophiles to yield 6-MP. We showed that PTA metabolism to 6-MP was GSH-dependent, and that 6-MP was formed via two distinct pathways. In the first pathway, 6-MP was formed indirectly via the formation and further metabolism of PG, the major metabolite formed in the reaction between PTA and GSH. In the second pathway, 6-MP was formed directly from PTA through an addition-elimination reaction with GSH. The in vitro and in vivo conversion of PTA to 6-MP was slow, possibly because the butenoic acid moiety of PTA was ionized at physiological pH. This ionization decreases the reactivity of PTA toward GSH. Therefore, we have investigated the properties of the PTA structural analogs 6-(2-acetylvinylthio)guanine (AVTG; Fig. 1) and 6-(2-acetylvinylthio)purine (AVTP; Fig. 1). AVTG and AVTP represent a class of structurally novel prodrugs in which a chemotherapeutic agent with a sulfur heteroatom is conjugated to a butenone moiety. Because AVTG and AVTP are α, β-unsaturated compounds without an ionizable carboxylic group, they were expected to efficiently undergo the addition-elimination reaction with GSH to yield 6-TG and 6-MP, respectively. Thus, these compounds might be potential candidates of prodrugs that target tumors with up-regulated levels of GSH.

In this study, we investigated the in vitro uptake, metabolism, and cytotoxicity of AVTG and AVTP by using two human renal cell carcinoma (RCC) cell lines as models. To investigate potential toxicity of the prodrugs, experiments were also carried out in mice and indicators of myelotoxicity, hepatotoxicity, and nephrotoxicity were assessed.

**Experimental Procedures**

**Materials.** 6-MP, 6-TG, azathioprine (AZA), trans-4-phenyl-3-buteno-2-one (PBO), dimethyl sulfoxide (DMSO), diethyl maleate (DEM), GSH, GSSG, GR, NADPH, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), sulfoalicylic acid (SSA), EDTA, 2-vinylpyridine, trimethanolamine, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). 3-Butyn-2-one was obtained from Lancaster (Windham, NH). Sephadex LH-20 column packing material was purchased from Amersham Biosciences, Inc. (Piscataway, NJ). HPLC-grade acetonitrile was purchased from EM Scientific (Gibbstown, NJ). Eagle’s minimum essential medium with Earle’s salts; Hanks’ balanced salt solution (HBSS) without calcium, magnesium, and phenol red; glucose; nonessential amino acids; penicillin/streptomycin; pyruvate; and vitamins were obtained from Mediatech (Herndon, VA). Fetal bovine serum and “fortified” bovine calf serum (cosmic calf serum) were purchased from Hyclone Laboratories (Logan, UT). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). PTA was synthesized as previously described (Gunnarsdottir and Elfarra, 1999). All other chemicals were of highest grade commercially available.

**Synthesis and Characterization of AVTG and AVTP.** AVTG and AVTP had previously been synthesized (Anufriev et al., 1982), but their biological properties had not been described. AVTG and AVTP were synthesized by dissolving 6-TG or 6-MP, respectively, in DMSO in a test tube at room temperature. Thereafter, approximately 3 equivalents of 3-butyn-2-one were added to the solution. The reaction was allowed to proceed for 10 min after which the reaction mixture was injected onto a Sephadex LH-20 column for separation and purification of geometrical isomers formed in the reaction. The products were eluted with 10% acetonitrile in water. The ratio of trans-to-cis isomers of AVTG formed was approximately 2:1, whereas the ratio of the cis-to-trans-isomers of AVTP formed was approximately 2:1. The isomer that was formed in greater quantity was primarily used in the studies described below because preli-

![Fig. 1. Chemical structures of 6-TG, 6-MP, trans-AVTG, cis-AVTP, cis-PTA, AZA, and PBO.](image-url)
nary experiments indicated similar chemical reactivities and biological properties for each of the geometrical isomers.

Proton nuclear magnetic resonance spectra of AVTG and AVTP were obtained at the National Magnetic Resonance Facility at Madison (Madison, WI), with a 500-MHz Bruker spectrometer by using DMSO as solvent. Chemical shifts are reported in parts per million, with water as the internal standard. cis-AVTG: 2.29 ppm (3H, s, methyl protons), 6.85 ppm (1H, J = 9.8 Hz, vinyl proton), 8.57 ppm (1H, s, purine ring carbon proton), 8.74 ppm (1H, d, J = 9.4 Hz, vinyl proton), 8.34 ppm (1H, s, purine ring carbon proton), 13.72 ppm (1H, s, purine ring carbon proton), 8.83 ppm (1H, s, purine ring nitrogen proton), 6.73 ppm (1H, d, J = 16.3 Hz, vinyl proton), 6.85 ppm (1H, d, J = 16.9 Hz, vinyl proton), and 13.8 ppm (1H, s, purine ring nitrogen proton). cis-AVTG: 2.37 ppm (3H, s, methyl protons), 6.87 ppm (1H, d, J = 9.8), 8.24 ppm (1H, s, purine ring carbon), 8.79 ppm (1H, d, J = 9.8 ppm). trans-AVTG: 2.39 ppm (3H, s, methyl protons), 6.74 ppm (1H, d, J = 16.4 Hz, vinyl proton), 8.19 ppm (1H, s, purine ring carbon proton), 8.98 ppm (1H, d, J = 16.4 Hz). The values obtained agree with those previously reported for these compounds (Anufriev et al., 1982).

Electrospray ionization mass spectra of AVTG and AVTP were obtained at The Mass Spectrometry/Bioanalytical Facility of the University of Wisconsin Biotechnology Center (Madison, WI) with an API365 PerkinElmer mass spectrometer. AVTG: 221 (M + 1), 119 (M – SCHCHCOCH3), 101 (SCHCHCOCH3), 69 (CH2COCH3), 43 (COCH3). AVTP: 236 (M + 1), 134 (M – SCHCHCOCH3), 101 (SCHCHCOCH3), 69 (CH2COCH3), 43 (COCH3).

Cell Culture. The two cell lines, A-498 and ACHN, used in the studies described below were purchased from the American Type Culture Collection (Manassas, VA). The A-498 line is derived from a human kidney carcinoma, whereas the ACHN line is derived from metastatic human renal adenocarcinoma. The cells were grown in Eagle’s minimum essential medium with Earle’s salts, supplemented with 5% fetal bovine serum and 5% cosmic calf serum, glutamine, nonessential amino acids, vitamins, pyruvate, and penicillin/streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO2 and maintained as recommended by the American Type Culture Collection. These two cell lines were chosen as models because of their different characteristics; the A-498 line has higher intracellular GSH levels than the ACHN line [84.1 ± 16.7 (mean ± S.D.) and 48.5 ± 2.3 nmol/mg of protein, respectively; Fig 4 at 0 min, open symbols]. In addition, the reported doubling time of A-498 cells is longer than that of ACHN cells (66.8 and 27.5 h, respectively; http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html).

Activity of Prodrugs toward Cellular Nucleophiles. The reactivity of AVTG, AVTP, PTA, and AZA toward cellular nucleophiles to yield the thiopurines was assessed. Briefly, each prodrug (1 μM) was incubated with GSH, cysteine, N-acetyl-cysteine, methionine, serine, histidine, or lysine (1 mM) in buffer (100 mM phosphate, 100 mM KCl, and 5 mM EDTA) at pH 7.4 in a shaking water bath at 37°C. A 5-min incubation, a 500-μl aliquot of the solution was removed and added to 50 μl of 50% (w/v) trichloroacetic acid. The samples were filtered through Acrodisc LC13 0.2-μm filters (Gelman Instrument Co., Ann Arbor, MI) and analyzed by HPLC. Further studies looking at the time course of the reaction between the prodrugs and GSH were carried out. The prodrugs (1 μM) were incubated with GSH (1 mM) in buffer at pH 7.4 in a shaking water bath at 37°C. At 0, 2, 4, 6, and 10 min, a 250-μl sample was removed and added to 25 μl of 50% (w/v) trichloroacetic acid for analysis by HPLC as described below.

Cellular Uptake and Metabolism of trans-AVTG and 6-TG. ACHN and A-498 renal cell carcinoma cells were plated at a density of 200,000 to 250,000 cells/dish in 60-mm plastic culture dishes and grown until 80 to 100% confluent. Confluent cells were used in these experiments because it has been suggested that the GSH status of confluent tumor cells in vitro more closely resembles that of tumors grown from the same cells in vivo (Allahum-Turner et al., 1988). Experiments were initiated by aspirating the culture medium and adding 3 ml of fresh medium with or without 2.5 mM DEM, which rapidly and efficiently depletes intracellular GSH by forming a DEM-GSH conjugate. After addition of the fresh medium, the cells were incubated for 1 h. Subsequently, 7.5 μl of trans-AVTG (400X solution in DMSO, final concentration 500 μM) were added to each of the dishes and incubated for 0, 1, 5, 10, 20, 60, and 120 min. At each of the specified time points, a dish was removed from the incubator, and a 450-μl aliquot of the medium was taken and added to 50 μl of 50% (w/v) SSA in a microcentrifuge tube. The sample was vortexed and put on ice. The rest of the medium was aspirated, the cells washed twice with 1 ml of cold HBSS, after which 300 μl of 5% (w/v) SSA were added to the dish to lyse the cells. The lysate was scraped off the dish and transferred to microcentrifuge tubes. To account for trans-AVTG breakdown that can occur due to the presence of thiols in medium, dishes containing medium with or without DEM but without cells were processed identically to dishes containing cells.

All samples were centrifuged for 10 min at 4°C at approximately 12,000g. A portion of the resulting supernatant was filtered for metabolite analysis by HPLC. The remainder of the supernatant, to be used for GSH and GSSG measurements, was transferred to clean microcentrifuge tubes and stored at −80°C until analyzed. The protein pellet was resuspended in 250 μl of 0.5 M NaOH for subsequent determination of protein content according to the method described by Lowry et al. (1951), with bovine serum albumin as the standard.

Measurements of GSH and GSSG. The method used for measurements of GSH and GSSG was based on the enzymatic recycling method of Tietze (1969), as described by Wild and Mulcahy (1999). In brief, for measurements of “total GSH” found intracellularly or in medium, the supernatant from the metabolism experiments was diluted up to 20-fold with 5% (w/v) SSA. Standards of GSH were also made up in 5% (w/v) SSA. Aliquots (10 μl) of the diluted supernatant and the standards were pipetted into a 96-well plate. A 100-μl volume of 143 mM phosphate buffer containing 6.3 mM EDTA, 1.05 mM DTNB, and 0.35 mM NADPH at pH 7.5 was added to each sample, followed by the addition of 50 μl of 5 U/ml GR. The plate was read several times over 5 min at 412 nm. Determination of GSSG present intracellularly or in medium was carried out by the assay described above, after the free GSH had been derivatized by 2-vinylpyridine (Griffith, 1980). Briefly, 100 μl of the supernatant or GSSG standards were transferred to microcentrifuge tubes into which were added 2 μl of 2-vinylpyridine and 6 μl of triethanolamine. The tubes were vortexed and incubated for 1 h at room temperature in the dark. An aliquot of the derivatized samples or standards (10 μl) was then transferred to a 96-well plate, followed by the addition of phosphate buffer containing DTNB and NADPH, and GR as described above. The reaction was monitored for 10 min at 412 nm. GSSG levels present intracellularly or in medium were calculated from the standard curve and expressed as nanomoles of GSSG per milligram of protein for intracellular GSSG or as nanomoles of GSSG per millilitre for GSSG in medium. The concentration of reduced GSH in a sample was determined by subtracting the molar amount of GSH equivalents coming from GSSG from the molar amount of total GSH calculated from the standard curve, and expressed as nanomoles of GSH per milligram of protein for intracellular GSH, and as nanomoles of GSH per millilitre for in medium.

HPLC Analyses. The HPLC system used consisted of two Gilson 306 pumps, a Gilson 119 UV/VIS detector, and a Gilson 234 autoinjector (Gilson Medical Electronics, Middleton, WI). The column used was a Beckman ultrasphere ODS 5-μm reversed phase C18 (4.6 × 250 mm; Beckman Coulter, Inc., Fullerton, CA) with a Brownleeressive 5-μm ODS 5-μm (4.6- × 30-mm) guard column (PerkinElmer Instruments, Norwalk, CT). Mobile phase for pump A consisted of water adjusted to pH 2.5 with TFA and for pump B 1:1 acetonitrile/water mixture adjusted to pH 2.5 with TFA. Injection volume was 20 μl and the flow rate was 1 ml/min.

The gradient used for analysis of samples generated in experiments with trans-AVTG or 6-TG was as follows: 0% B for 1.5 min,
increased to 10% B over 1 min, constant at 10% B for 6.5 min, increased to 90% B over 6 min, constant at 90% B for 5 min, decreased to 0% B over 5 min, constant at 0% B for 6 min, for a total run time of 30 min. This method gave the following retention times: 6-TG, 8.4 min, and trans-AVTG, 16.8 min. The detection wavelength was 343 nm. trans-AVTG and 6-TG were quantitated using standard curves that were generated by linear regression of peak area versus concentration of standard solutions made up in 5% (v/v) SSA or phosphate buffer. The limit of quantitation for 6-TG or trans-AVTG was 0.5 nmol/ml. Recovery of all analytes was quantitative.

The gradient used for analysis of samples generated in experiments with cis-AVTG or 6-MP was as follows: 0% B for 1.5 min, increased to 10% B over 1 min, constant at 10% B for 6.5 min, increased to 80% B over 5 min, constant at 80% B for 4 min, decreased to 0% B over 4 min, constant at 0% B for 7 min, for a total run time of 30 min. This method gave the following retention times: 6-MP, 8.8 min, and cis-AVTG 17.6 min. The wavelength of detection was 323 nm. Quantitation of 6-MP was achieved using a standard curve that was generated by linear regression of peak area versus concentration of standard solutions made up in phosphate buffer. The limit of quantitation was 0.5 nmol/ml.

**Cytotoxicity Assessment.** The cytotoxicity of cis- and trans-AVTG, cis- and trans-AVTG, PTA, 6-TG, 6-MP, AZA, and PBO was evaluated using the MTT cell proliferation factor I from Roche Molecular Biochemicals (Mannheim, Germany). ACHN and A-498 cells were plated in flat-bottomed 96-well plates at a density of 10,000 and 7,500 cells/well, respectively, and allowed to grow for 24 h. The medium was then aspirated and new medium containing drug was added. After 24, 48, 72, or 96 h, the cell viability was assessed as recommended by the manufacturer. Absorbance was measured at 595 nm. Eight drug concentrations ranging from 10 nM to 500 µM, and fresh medium control and solvent control were included in each experiment. All drug solutions, except PBO, were made up in HBSS, or a mixture of HBSS and medium, and diluted 10- or 100-fold by addition to medium to initiate cell treatment. PBO was made up in DMSO and diluted 1000-fold in medium before cell treatment. IC50 values, the concentrations at which 50% color formation obtained for the solvent controls were observed, were calculated by nonlinear curve fitting of the data to Hill's four-parameter equation by using SigmaPlot (version 5.0; SPSS Inc., Chicago, IL).

**In Vivo Toxicity Assessment.** Toxicity of trans-AVTG, cis-AVTG, and 6-TG was assessed in 6- to 10-week-old CD-1 heterozygous nude male mice (Charles River Laboratories, Inc., Wilmington, MA). The mice were kept on a 12-h light/dark schedule and allowed food and water ad libitum. During each experiment, the mice were housed individually in metabolic cages. Four mice were used per group and housed together in plastic cages. Food and water were provided ad libitum. During each experiment, the mice were housed individually in metabolic cages. Four mice were used per experiment. Toxicity of trans-AVTG, cis-AVTG, and 6-TG was assessed by injecting the mice i.p. once daily for three consecutive days with vehicle only, 21.25 µmol/kg trans-AVTG, 21.25 µmol/kg cis-AVTG, or 8.5 or 21.25 µmol/kg 6-TG dissolved in 0.5 ml of buffer (0.78 mM phosphate, 137 mM NaCl, 5.4 mM KCl, and 4.2 mM NaHCO3) with a few drops of dilute NaOH. The pH of the final solution was 343 nm. Quantitation of cis-AVTG and trans-AVTG was achieved using standard curves that were generated by linear regression of peak area versus concentration of standard solutions made up in phosphate buffer. The limit of quantitation was 0.5 nmol/ml.

**Results**

To test the hypothesis that replacement of the butenoic acid moiety of PTA with a butenone moiety enhances its reactivity with nucleophiles to yield 6-MP, the prodrugs PTA or cis-AVTG (1 mM) were incubated for 5 min with the cellular nucleophiles GSH, cysteine, N-acetyl-cysteine, methionine, histidine, lysine, or serine (1 mM) at pH 7.4 and 37°C. Similar experiments were carried out with trans-AVTG and the clinically used 6-MP prodrug AZA (Fig. 1). The results showed that both trans-AVTG and cis-AVTG reacted rapidly with the sulfhydryl nucleophiles GSH, cysteine, and N-acetyl-cysteine to yield the parent thiopurine as the major product; reactivity of trans-AVTG and cis-AVTG toward histidine, lysine, serine, and methionine was negligible. The butenoic acid analog PTA (Fig. 1) did not show any detectable reactivity with the nucleophiles examined under the experimental conditions used. The same pattern of reactivity was observed for AZA as for trans-AVTG and cis-AVTG (data not shown). However, examination of the rate of thiopurine formation when trans-AVTG, cis-AVTG, or AZA was incubated with GSH revealed that formation of the parent thiopurine became nonlinear very quickly in trans-AVTG and cis-AVTG incubations (Fig. 2) with comparable results obtained for the other geometrical isomer of the prodrugs (data not shown). On the other hand, formation of 6-MP from AZA was linear for at least 10 min (Fig. 2). When the reaction was allowed to

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**Fig. 2.** Formation of the parent thiopurine after reaction of the prodrugs cis-AVTG, trans-AVTG, and AZA with GSH. The prodrugs (1 mM) were incubated with 1 mM GSH in buffer at pH 7.4 and 37°C. Samples were analyzed for the parent thiopurine by HPLC as described under Experimental Procedures. Values presented are the mean ± S.D. (n = 3).
proceed to completion (less than 1 h), roughly 80% of AVTG and AVTP had been converted to the parent thiopurine (data not shown). These results provide evidence for higher reactivity of AVTP and AVTG in comparison with PTA and AZA.

To characterize the cellular uptake of the prodrugs AVTG and AVTP, cultures of A-498 cells were incubated with 500 μM trans-AVTG for 20 min, after which the prodrug was removed, the cells washed, scraped off the dish, and homogenized. HPLC analyses of the cell homogenate showed several peaks that were not detected when trans-AVTG was omitted from the incubations (Fig. 3). The first peak (peak I; Fig. 3B) coeluted with reference 6-TG, the peak with retention time 10.9 min (peak II; Fig. 3B) coeluted with reference mercaptoguanosine, whereas the peak with retention time 16.8 min (peak III; Fig. 3B) coeluted with trans-AVTG. Similar results were observed when cis-AVTP was incubated with A-498 cells. Figure 3D shows that both cis-AVTP (peak II; Fig. 3D) and the parent thiopurine 6-MP (peak I; Fig. 3D) were detected intracellularly after 20-min prodrug incubation.

Further studies were carried out to investigate in more detail how the structural difference between trans-AVTG and 6-TG influenced the uptake and metabolism of these two compounds. Moreover, because GSH mediates the conversion of trans-AVTG to 6-TG, it was examined how modulation of intracellular GSH by pretreatment of cells with DEM affected the extent of trans-AVTG and 6-TG uptake and metabolism. Preincubation of cells for 1 h with 2.5 mM DEM effectively depleted GSH from ACHN and A-498 cells, reducing their intracellular GSH concentrations to 25% of those detected in cells not treated with DEM (Fig. 4, at 0 min). The addition of 500 μM trans-AVTG to cells not treated with DEM lead to rapid time-dependent depletion of intracellular GSH (Fig. 4, A and B, open symbols). Further depletion of GSH was similarly detected in DEM-treated cells after trans-AVTG incubation (Fig. 4, A and B, closed symbols). In comparison, the addition of 500 μM 6-TG to cells not treated with DEM had no effect on the GSH status (Fig. 4C). No change was observed in intracellular GSSG concentration over the course of the experiment in both DEM-treated and untreated cells after trans-AVTG or 6-TG treatment. Furthermore, lev-
els of GSH and GSSG in medium were at the limits of the detection of the assay and did not increase during the experiment (data not shown). These results show that trans-AVTG rapidly depletes GSH from cells, without formation of GSSG or extrusion of GSH into the medium.

Intracellular accumulation of trans-AVTG plateaued after approximately 20 min incubation of ACHN and A-498 cells in the presence of 500 μM trans-AVTG with and without DEM pretreatment (Fig. 5, A and B). A significantly higher AUC value was obtained for intracellular trans-AVTG in A-498 cells pretreated with DEM than in cells without DEM pretreatment (Table 1). On the other hand, the AUC value for trans-AVTG in ACHN cells seems not to be affected by the DEM treatment, possibly because of higher cellular export of trans-AVTG back into the medium in the presence of DEM. Accumulation of 6-TG in ACHN and A-498 cells after incubation with 500 μM trans-AVTG reached maximum after 10 to 20 min in both DEM-treated and untreated cells, where after the concentration decreased significantly (Fig. 6, A and B). Higher levels of 6-TG were detected in cells not treated with DEM compared with cells treated with DEM; the AUC values obtained for cells not treated with DEM were 2.5- and 2.1-fold higher than those obtained for DEM-treated ACHN and A-498 cells, respectively (Table 1). In comparison, cellular accumulation of 6-TG after treatment with 500 μM 6-TG plateaued after only 1-min incubation and no difference was observed in intracellular 6-TG accumulation between DEM-treated and untreated cells (Fig. 6C; Table 1). More importantly, the AUC values obtained for intracellular 6-TG after 6-TG treatment in both DEM-treated and untreated A-498 cells were significantly lower than those obtained after trans-AVTG treatment.

It is conceivable that 6-TG formed by intracellular GSH-mediated metabolism of trans-AVTG can be passively or actively transported back into the medium. Therefore, we measured the concentration of 6-TG present in medium from the experiments described above. Furthermore, to account for 6-TG formation that occurs due to breakdown of trans-AVTG because of its reaction with thiols present in medium, trans-AVTG was incubated in medium without cells and formation of 6-TG measured. Significantly more 6-TG was detected in medium from cellular incubations compared with medium from incubations without cells (Fig. 6D), suggesting that at this high AVTG concentration, a portion of the 6-TG formed intracellularly diffuses or is transported into medium. Additionally, significantly more 6-TG was detected in medium from A-498 incubations than in medium from ACHN incubations.

The above-described results obtained from the uptake and metabolism studies prompted the investigation of the in vitro cytotoxicity of trans-AVTG and cis-AVTG. Furthermore, to assess whether the increase in intracellular concentration of the parent thiopurine after treatment with the prodrug compared with the parent thiopurine also led to an increase in cytotoxicity of the prodrugs compared with the parent thiopurines, the cytotoxicities of 6-TG and 6-MP were also examined. Moreover, to explore whether a relationship exists between cytotoxicity and reactivity of α, β-unsaturated thiopurine prodrugs toward GSH, the cytotoxicity of PTA and AZA was also studied. Finally, the role that GSH depletion may play in the cytotoxicity of the prodrugs was further assessed by investigating the cytotoxicity of the structural analog PBO, an α, β-unsaturated compound that reacts with GSH (Sauer et al., 1997) but does not release a thiopurine. Table 2 lists IC50 values that were obtained after ACHN and A-498 cells had been incubated for 72 h in the presence of 10 nM to 500 μM of these compounds. No statistical difference was observed between the IC50 values of trans-AVTG and cis-AVTG in each of the RCC cell lines tested. However, the IC50 values for both prodrugs were approximately 2.5-fold higher in the A-498 cell line than in the ACHN cell line that was more sensitive to all the compounds tested. The shorter doubling time of the ACHN cell line compared with the A-498 cell line may have contributed to the higher sensitivity of the ACHN cells to these compounds. Preliminary experiments revealed that comparable IC50 values were obtained for the geometric isomers cis-AVTG and trans-AVTG as for the major isomers trans-AVTG and cis-AVTG (data not shown). It is worth pointing out that the IC50 value for cis-AVTG was significantly lower than what was observed for the parent thiopurine 6-MP in the two RCC cell lines. On the other hand, no difference in potency was observed between trans-AVTG and 6-TG in either cell line at the time point tested (72 h). Interestingly, the IC50 value for

![Fig. 5. Intracellular concentration of trans-AVTG after treatment of human RCC cells with trans-AVTG. Confluent cells were preincubated for 1 h with or without DEM and then treated with 500 μM trans-AVTG. Intracellular trans-AVTG concentration was measured as described under Experimental Procedures. A, ACHN cells; B, A-498 cells. Closed symbols represent DEM-treated cells, whereas open symbols represent cells not treated with DEM. Values presented are the mean ± S.D. (n = 3).](image-url)
TABLE 1
Calculated AUC for trans-AVTG and 6-TG detected intracellularly after treatment of human RCC cells with trans-AVTG or 6-TG
Confluent cells were preincubated for 1 h with or without DEM and then treated with 500 μM trans-AVTG or 6-TG. Intracellular trans-AVTG or 6-TG were analyzed by HPLC as described under Experimental Procedures. Values presented are the mean ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Drug Treatment</th>
<th>DEM</th>
<th>AUC_{0-120 min} Intracellular trans-AVTG</th>
<th>Intracellular 6-TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACHN</td>
<td>trans-AVTG</td>
<td>+</td>
<td>842.4 ± 127.5</td>
<td>494.9 ± 69.5*</td>
</tr>
<tr>
<td>ACHN</td>
<td>trans-AVTG</td>
<td>−</td>
<td>687.2 ± 37.6</td>
<td>1286.4 ± 86.0</td>
</tr>
<tr>
<td>ACHN</td>
<td>6-TG</td>
<td></td>
<td>502.7 ± 36.4*</td>
<td>764.8 ± 26.8*</td>
</tr>
<tr>
<td>A-498</td>
<td>trans-AVTG</td>
<td>+</td>
<td>407.3 ± 43.7*</td>
<td>1575.5 ± 154.3*</td>
</tr>
<tr>
<td>A-498</td>
<td>trans-AVTG</td>
<td>−</td>
<td></td>
<td>316.4 ± 153.3*</td>
</tr>
<tr>
<td>A-498</td>
<td>6-TG</td>
<td>+</td>
<td></td>
<td>269.2 ± 40.4*</td>
</tr>
</tbody>
</table>

* Significantly different from the same cells incubated with the same prodrug/drug but not treated with DEM (p < 0.05).
+ Significantly different from the corresponding treatment in ACHN cells (p < 0.05).
+ Significantly different from intracellular 6-TG in A-498 cells after corresponding trans-AVTG/DEM treatment (p < 0.01).
+ Significantly different from intracellular trans-AVTG in A-498 cells treated with trans-AVTG but not with DEM (p < 0.05).

AZA was 5- to 6-fold higher than those observed for AVTG and AVTP, whereas the butenoic acid analog PTA was not cytotoxic within the concentration range examined (data not shown). The finding that PBO was significantly less cytotoxic within the concentration range examined (data not shown). Concentration of 6-TG intracellularly or in medium after treatment of human RCC cells with trans-AVTG or 6-TG. Intracellular 6-TG concentration was measured as described under Experimental Procedures. A, intracellular 6-TG in ACHN cells treated with trans-AVTG; B, intracellular 6-TG in A-498 cells treated with trans-AVTG; C, intracellular 6-TG in A-498 cells treated with 6-TG; and D, 6-TG accumulation after trans-AVTG treatment in medium of A-498 cells (△), ACHN cells (□), or medium incubated without cells (■). Closed symbols represent DEM-treated cells, whereas open symbols represent cells not treated with DEM. Values presented are the mean ± S.D. (n = 3).

Table 2
IC_{50} values obtained after incubation of human RCC cells with 10 nM to 500 μM concentration of several drugs for 72 h

<table>
<thead>
<tr>
<th>Drug</th>
<th>ACHN</th>
<th>A-498</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-TG</td>
<td>2.5</td>
<td>6.9</td>
</tr>
<tr>
<td>6-MP</td>
<td>6.3</td>
<td>60.8</td>
</tr>
<tr>
<td>trans-AVTG</td>
<td>4.1</td>
<td>9.7</td>
</tr>
<tr>
<td>cis-AVTG</td>
<td>3.7</td>
<td>8.0</td>
</tr>
<tr>
<td>AZA</td>
<td>22.1</td>
<td>50.2</td>
</tr>
<tr>
<td>PBO</td>
<td>62.3</td>
<td>252.5</td>
</tr>
</tbody>
</table>

In this article, we have presented evidence for the cellular uptake and GSH-mediated metabolism of the structurally novel prodrugs AVTG and AVTP to their parent thiopurines 6-TG and 6-MP, respectively. Furthermore, our results demonstrate that intracellular concentrations of 6-TG were higher after incubations with AVTG compared with cells incubated with 6-TG. Moreover, although the prodrugs exhibited cytotoxicity that was similar to or exceeded that of

Discussion
In this article, we have presented evidence for the cellular uptake and GSH-mediated metabolism of the structurally novel prodrugs AVTG and AVTP to their parent thiopurines 6-TG and 6-MP, respectively. Furthermore, our results demonstrate that intracellular concentrations of 6-TG were higher after incubations with AVTG compared with cells incubated with 6-TG. Moreover, although the prodrugs exhibited cytotoxicity that was similar to or exceeded that of

FIG. 6. Concentration of 6-TG intracellularly or in medium after treatment of human RCC cells with trans-AVTG or 6-TG. Confluent cells were preincubated for 1 h with or without DEM and then treated with 500 μM trans-AVTG or 6-TG. Intracellular 6-TG concentration was measured as described under Experimental Procedures. A, intracellular 6-TG in ACHN cells treated with trans-AVTG; B, intracellular 6-TG in A-498 cells treated with trans-AVTG; C, intracellular 6-TG in A-498 cells treated with 6-TG; and D, 6-TG accumulation after trans-AVTG treatment in medium of A-498 cells (△), ACHN cells (□), or medium incubated without cells (■). Closed symbols represent DEM-treated cells, whereas open symbols represent cells not treated with DEM. Values presented are the mean ± S.D. (n = 3).
Further studies were carried out to investigate the role that intracellular GSH plays in the uptake and metabolism of trans-AVTG and 6-TG in the two RCC cell lines. Consistent with the fact that A-498 cells have higher GSH levels compared with ACHN cells, both the AUC value and the maximum intracellular concentration of 6-TG were significantly higher in A-498 cells compared with ACHN cells (Table 1; Figs. 4 and 6). Similarly, when cells were depleted of intracellular GSH by preincubation with DEM, intracellular 6-TG accumulation was significantly decreased compared with cells not treated with DEM (Table 1; Fig. 6). Thus, the intracellular GSH status is a major determinant of the cellular burden of 6-TG after treatment with trans-AVTG. It is worth pointing out that the AUC values obtained for 6-TG after incubation of cells with trans-AVTG were significantly higher than those obtained after incubation of cells with 6-TG (Table 1; Fig. 6). Hence, the prodrug delivered more of the drug to the cell than was obtained after incubation with the drug itself. The decrease in intracellular 6-TG concentrations observed after 10 min of trans-AVTG incubations (Fig. 6) is presumably due to the reduced formation of intracellular 6-TG from trans-AVTG because of the rapid depletion of intracellular GSH (Fig. 4). Concurrently, intracellular 6-TG concentrations are reduced by further metabolism of 6-TG as was evidenced by the detection of 6-mercaptopurine as an intracellular metabolite of trans-AVTG (Fig. 3). Furthermore, 6-TG may diffuse or be transported out of the cell because increased 6-TG concentrations were detected in medium after incubations of a high concentration of trans-AVTG compared with cell free incubations (Fig. 6D).

Our results demonstrate that after 72-h drug incubation, 6-TG is more cytotoxic than 6-MP in RCC cells in vitro. This finding is consistent with what has been observed in other cell lines as well as in vivo (Elion, 1989; Adamson et al., 1994). However, the potency of the thiopurine prodrugs AVTG and AVTP was the same, and similar to the potency of 6-TG. Despite the fact that higher intracellular 6-TG levels were observed after incubation of cells with 500 μM trans-AVTG compared with an equimolar concentration of 6-TG, the cytotoxicity of the prodrug was not increased compared with that of the parent thiopurine after 72-h incubation (Table 2). A possible explanation for this finding may be that at low concentrations, trans-AVTG reacted preferentially with thiols present in medium to generate 6-TG extracellularly. This process reduces the effective prodrug concentration that is available for cellular uptake because mostly 6-TG instead of AVTG is present in medium. Consequently, in our assay, there may exist a concentration threshold below which AVTG is not readily available for cellular uptake due to formation of extracellular 6-TG. Because 6-TG is a potent cytotoxic agent, the limited cellular uptake of 6-TG is sufficient to cause substantial cytotoxicity. Contrary to what was observed with 6-TG and AVTG, the 6-MP prodrug cis-AVTG was more potent than the parent thiopurine in both cell lines and at 24, 48, and 72 h (Tables 2 and 3). Assuming that the uptake and metabolism of cis-AVTG and 6-MP are comparable with those observed for trans-AVTG and 6-TG, it is likely that the increased amount of 6-MP delivered to the cell played a significant role in the increased potency of the prodrug compared with 6-MP.

Mechanisms other than thiopurine release, such as reaction of the prodrugs with cellular protein thiols or increased

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**TABLE 3**

IC$_{50}$ values obtained after incubation of human RCC cells with 10 nM to 500 μM concentration of 6-MP or cis-AVTG for 24, 48, 72, or 96 h. Viability was measured using the MTT assay, and IC$_{50}$ values were calculated by nonlinear regression as described under Experimental Procedures. Values presented are the mean ± S.D. (n = 3–6). Statistical comparisons were made for each time point for both drug treatments within each cell line. Within each cell line and time course, the IC$_{50}$ values with different superscripts are significantly different (p < 0.05).

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>h</th>
<th>IC$_{50}$ 6-MP (μM)</th>
<th>IC$_{50}$ cis-AVTG (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACHN</td>
<td>24</td>
<td>N.D.</td>
<td>58.2 ± 1.7$^*$</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>44.8 ± 5.0$^{a,c}$</td>
<td>8.7 ± 0.9$^{b,c}$</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>6.3 ± 1.9$^{b,c}$</td>
<td>3.7 ± 0.7$^{b,c}$</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>0.9 ± 0.3$^{c}$</td>
<td>3.5 ± 0.4$^{c}$</td>
</tr>
<tr>
<td>A-498</td>
<td>24</td>
<td>N.D.</td>
<td>106.9 ± 14.7$^{a,c}$</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>N.D.</td>
<td>24.6 ± 4.0$^{b,c}$</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>60.6 ± 25.5$^{a,c}$</td>
<td>8.0 ± 1.2$^{b,c}$</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>14.9 ± 6.7$^{b,c}$</td>
<td>5.5 ± 0.9$^{b,c}$</td>
</tr>
</tbody>
</table>

N.D., value could not be determined due to limited cytotoxicity observed.

$^1$ Significantly different from the IC$_{50}$ value of 6-MP in ACHN cells at the corresponding time point (p < 0.01).

$^2$ Significantly different from the IC$_{50}$ value of 6-MP in A-498 cells at the corresponding time point (p < 0.05).
cellular stress due to depletion of intracellular GSH, may contribute to the cytotoxicity of the prodrugs. A growing body of evidence suggests that the onset of apoptosis is associated with intracellular redox imbalance (Coppola and Ghibelli, 2000; Davis et al., 2001). It has recently been shown that depletion of intracellular GSH by treatment with DEM (Coffey et al., 2000) or ethylene (Yang et al., 2000a,b) led to apoptosis in prostate carcinoma cells and HepG2 cells, respectively. As our results show, the prodrugs AVTG and AVTP react readily with GSH, and the incubation of RCC cells with trans-AVTG leads to rapid depletion of intracellular GSH. In light of these findings, it is tempting to speculate that GSH depletion may be a factor in the cytotoxicity of the prodrugs. GSH depletion may not in itself play a major role because the butenone analog PBO, which has been shown to react with and deplete GSH, was much less cytotoxic to RCC cells than AVTG and AVTP. However, intracellular 6-MP and GSH depletion may have synergistic effects; the additional stress of GSH depletion that the cell encounters when treated with cis-AVTG, compared with 6-MP, may be a factor in the increased cytotoxicity observed for the prodrug. Further studies aimed at clarifying the mechanism of the cytotoxicity of AVTG and AVTP, and the role that GSH depletion plays in the process, are under way.

The major limitation in the clinical use of 6-TG and 6-MP is their bone marrow toxicity (van Scoik et al., 1985; Hayder 2000; Davis et al., 2001). It has recently been shown that 6-TG exhibited more than 50% reduction in their circulating white blood cell counts, whereas no decrease was observed in mice treated with 6-TG for three consecutive days.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose μmol/kg</th>
<th>WBC × 10^6 cells/μl</th>
<th>RBC × 10^6 cells/μl</th>
<th>PLT × 10^6 cells/μl</th>
<th>HGB g/dl</th>
<th>HCT %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>9.63 ± 2.27</td>
<td>9.50 ± 0.44</td>
<td>1134 ± 111</td>
<td>15.9 ± 0.3</td>
<td>54.8 ± 1.8</td>
</tr>
<tr>
<td>cis-AVTG</td>
<td>21.25</td>
<td>7.98 ± 1.16</td>
<td>8.93 ± 0.56</td>
<td>1644 ± 253</td>
<td>15.2 ± 0.9</td>
<td>52.8 ± 1.7</td>
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<tr>
<td>trans-AVTG</td>
<td>21.25</td>
<td>7.44 ± 0.91</td>
<td>9.09 ± 0.72</td>
<td>1101 ± 383</td>
<td>15.6 ± 1.1</td>
<td>55.9 ± 3.8</td>
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<td>6-TG</td>
<td>8.5</td>
<td>4.84 ± 1.19</td>
<td>8.27 ± 0.40</td>
<td>1172 ± 161</td>
<td>14.6 ± 0.5</td>
<td>51.9 ± 1.1</td>
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<tr>
<td></td>
<td>21.25</td>
<td>3.84 ± 1.09</td>
<td>8.40 ± 0.58</td>
<td>752 ± 416</td>
<td>14.7 ± 0.7</td>
<td>49.8 ± 2.0</td>
</tr>
</tbody>
</table>

WBC, white blood cells; RBC, red blood cells; PLT, platelets; HGB, hemoglobin; HCT, hematocrit.

References


Hwang IY and Elfarra AA (1993) Detection and mechanism of formation of S-(6-


Address correspondence to: Dr. Adnan A. Elfarra, Department of Comparative Biosciences, School of Veterinary Medicine, 2015 Linden Dr., University of Wisconsin—Madison, Madison, WI 53706. E-mail: elfarraa@svm.vetmed.wisc.edu