Thioguanine Administered as a Continuous Intravenous Infusion to Pediatric Patients Is Metabolized to the Novel Metabolite 8-Hydroxy-Thioguanine

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ABSTRACT
Thiopurine antimetabolites have been in clinical use for more than 40 years, yet the metabolism of thiopurines remains only partially understood. Data from our previous pediatric phase 1 trial of continuous i.v. infusion of thioguanine (CIVI-TG) suggested that TG was eliminated by saturable mechanism, with conversion of the drug to an unknown metabolite. In this study we have identified this metabolite as 8-hydroxy-thioguanine (8-OH-TG). The metabolite coeluted with the 8-OH-TG standard on HPLC and had an identical UV spectrum, with a \( \lambda_{\text{max}} \) of 350 nm. On mass spectroscopy, the positive ion, single quad scan of 8-OH-TG yielded a protonated molecular ion at 184 Da and contained diagnostic ions at \( m/z \) 167, 156, 142, and 125 Da. Incubation of TG in vitro with partially purified aldehyde oxidase resulted in 8-OH-TG formation. 8-OH-TG is the predominant circulating metabolite found in patients receiving CIVI-TG and is likely generated by the action of aldehyde oxidase.

Thiopurine antimetabolites have been in clinical use for the treatment of acute leukemias for more than 40 years. The thiopurines are prodrugs that must be converted to nucleotides intracellularly to exert their cytotoxic effect. In addition to being activated by biotransformation, 6-mercaptopurine (MP) and 6-thioguanine (TG) are eliminated by biotransformation to inactive metabolites. MP is catabolized by xanthine oxidase to thiouric acid, which is the primary metabolite identified in plasma and urine following MP administration (Hamilton and Elion, 1954; Elion et al., 1961, 1963). The nucleoside metabolite MP-riboside is a minor circulating metabolite of MP (Zimm et al., 1984). TG is also metabolized to thiouric acid, but the metabolic pathway differs from that of MP. When the xanthine oxidase inhibitor allopurinol is administered with oral MP, plasma MP concentrations are 5-fold higher, and the toxicity of MP is enhanced (Rundles et al., 1963; Berns et al., 1972; Zimm et al., 1983a). TG, however, is not a substrate for xanthine oxidase (Krenitsky et al., 1972), and thus when allopurinol and TG are administered simultaneously, there is no known drug interaction (Hande and Garrow, 1996).

In our pediatric phase 1 trial and pharmacokinetic study of TG administered as a continuous i.v. infusion (CIVI) (Kitchen et al., 1997), similar to an earlier study of i.v. TG (Konits et al., 1982), we detected an unknown metabolite in plasma. Pharmacokinetic modeling of the data from our pediatric phase 1 trial suggested that TG was eliminated by a capacity-limited (saturable) elimination mechanism. The enzymatic conversion of TG to this unknown metabolite may be the rate-limiting step in the elimination of TG from plasma (Kitchen et al., 1997). We now report identification of this metabolite as 8-hydroxy-thioguanine (8-OH-TG) and provide data that suggest its formation is catabolized by aldehyde oxidase (AO).

Materials and Methods

Patient Plasma Samples. Eighteen pediatric patients with refractory cancer were enrolled in a phase 1 trial of CIVI-TG administered at a dose rate of 10 or 20 mg/m²/h for 24 to 36 h (Kitchen et al., 1997). Blood samples, which were drawn via a percutaneously placed i.v. catheter at a site distant from the infusion site, were obtained 18, 23, and 24 h following initiation of the infusion. Plasma

ABBREVIATIONS: MP, 6-mercaptopurine; TG, thioguanine; 8-OH-TG, 8-hydroxy-thioguanine; CIVI, continuous intravenous infusion; AO, aldehyde oxidase.
was rapidly separated by centrifugation and stored at −20°C until time of analysis.

**Chemicals.** All chemicals, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO). TG for i.v. administration was provided by the National Cancer Institute (Bethesda, MD). Glaxo Wellcome (Research Triangle Park, NC) kindly provided 8-OH-TG standard.

**HPLC/UV Spectroscopy.** Plasma TG concentration was measured with a modification of a previously described reverse-phase HPLC method (Adamson et al., 1991). Plasma samples (0.5 ml) were spiked with 10 μl of 0.5 M dithiothreitol and 10 μl of 200 μM MP riboside (internal standard) and extracted using Waters C-18 Sep-Pak solid-phase extraction cartridges (Waters Co., Milford, MA) that had previously been primed with 2 ml of methanol and washed with 5 ml of 0.2% glacial acetic acid. The cartridges were rinsed with 3 ml of 0.2% glacial acetic acid, and the samples were then eluted with 2 ml of methanol. Samples were evaporated to dryness under a gentle stream of nitrogen, reconstituted with 100 μl of mobile phase, and injected onto the HPLC system. The HPLC system included a Waters model 510 pump, a Waters WISP 712 automated sample injector, and a Beckman Ultrasphere ODS 4.6-mm × 25-cm column (5 μm particle size) (Beckman Instruments Inc., San Ramon, CA). The mobile phase consisted of 0.2% acetate buffer and 2.5% acetonitrile at a flow rate of 1.4 ml/min. The eluant was monitored with a Waters 490 multiwavelength detector at wavelengths of 320 and 340 nm or with a Waters photodiode array detector. Retention times under these conditions were approximately 4.9 min for thiouric acid, 5.1 min for TG, 5.7 min for the metabolite, 7.3 min for thioxanthine, and

**Fig. 1.** A representative chromatogram from a patient receiving CIVI-TG at 20 mg/m²/h, demonstrating the parent compound and metabolite.

**Fig. 2.** The UV spectra of the unknown metabolite in patient plasma (solid line) and an aqueous sample of 8-OH-TG standard (dashed line) obtained by HPLC/photodiode array.
8.9 min for MP riboside. The intra- and inter-day coefficients of variation for 1 μM TG were 3.8% and 3.9%. For 8-OH-TG (generated using AO), the intra- and interday coefficients of variation for approximately 0.3 μM were 4.6% and 13.8%.

**HPLC/Mass Spectroscopy.** Mass spectroscopy analysis was performed on a Finnigan model TSQ7000 (Finnigan Corporation, San Jose, CA) operated in positive ion electrospray mode. The spray voltage was set at 4.5 kV and the heated capillary was maintained at 225°C. Product ion scans were produced with 1.1 mT argon and 221eV offset in the collision cell. Extracted samples were introduced into the mass spectroscopy after injection onto an HP ODS 2.1 × 100-mm HPLC column (Hewlett-Packard, Palo Alto, CA). The metabolite was eluted with 10 mM ammonium acetate containing 6% acetonitrile at a flow rate of 0.2 ml/min. Retention times under these conditions were approximately 21 min for TG and 19 min for the metabolite.

**Reaction with AO.** AO was partially purified using a previously described method (Johns et al., 1966; Johns and Loo, 1967). In brief, six frozen white rabbit livers (585 g) were thawed overnight at 5°C. Two volumes of water (1000 ml) were added, and the mixture was homogenized and then centrifuged (27,500g) for 10 min at room temperature. The supernatant (no. 1) was separated from the semi-solid pellet with a sieve, heated in a 60°C water bath for 10 min, and centrifuged. This reddish-brown, clear supernatant (no. 2) was decanted and fractionated with a solution of saturated ammonium sulfate (940 ml) and ammonium hydroxide (60 ml) at a ratio of 59 ml ammonium sulfate solution/100 ml of supernatant. The opaque solution was centrifuged, and the supernatant (no. 3) was decanted into a 2-liter Erlenmeyer flask to which 23 ml of ammoniacal solution was added per 100 ml of supernatant. The solution again became opaque with stirring, was centrifuged, and the pellets were collected and dissolved in 35 ml of a diluted ammoniacal solution (10% in distilled water). The protein concentration was determined by UV spectrophotometry (Waddell and Hill, 1956).

As a positive control for AO activity, 20 µl of the partially purified AO (protein concentration 53 ± 2 mg/ml) was added to a mixture of 50 µl of a 1 mM solution of methotrexate and 930 µl of 50 µM potassium phosphate buffer (pH 7.8) with 0.005% EDTA, and the resultant product was analyzed by a previously described HPLC method (Widemann et al., 1995) for 7-OH-methotrexate formation. In duplicate experiments, 20 µl of the partially purified AO (protein concentration, 53 ± 2 mg/ml) was added to 980 µl of a 0 mM solution of TG in potassium phosphate buffer (pH 7.8, with 0.005% EDTA) and incubated in a 37°C water bath for 2.5 h. The resultant solution was analyzed by directly injecting onto the HPLC/UV system described above.

**Results**

**HPLC/UV Spectroscopy.** A representative chromatogram of a plasma sample obtained at steady state from a...
As analogs of hypoxanthine and guanine, MP and TG are anabolized and catabolized through many of the same enzymatic pathways as endogenous purines (Elion et al., 1963; Elion, 1969). One important difference between the thiopurines and the naturally occurring purines is that both TG and MP are substrates for thiopurine methyltransferase (Elion, 1967; Weinshilboum et al., 1978), which catabolizes MP and TG to 6-methyl-MP and 6-methyl-TG. Thiouric acid is the principal catabolite of MP and TG found in urine (Hamilton and Elion, 1954; Elion et al., 1961, 1963). Whereas MP is converted to thioxanthine and thiouric acid by xanthine oxidase (Elion, 1989), TG is not a substrate for xanthine oxidase (Gee et al., 1969; Krenitsky et al., 1972). Guanine deaminase converts TG to thioxanthine (Bronk et al., 1988), which is then converted to thiouric acid by xanthine oxidase.

Under our HPLC assay conditions, 8-OH-TG is the primary circulating metabolite in patients receiving CIVI-TG. Oxidation of thiopurinates at the 8-position renders the compounds inactive (Clarke et al., 1958). The metabolite was identified initially by its coelution with 8-OH-TG standard on HPLC and by its UV spectra. Identity was confirmed by mass spectroscopy. In vitro experiments demonstrated that TG is a substrate for AO and the reaction between the two results in formation of 8-OH-TG. Other potential oxidative pathways for TG include cytochrome P-450-mediated oxidation, but this was not evaluated in the current study. The AO pathway has also been found to be the predominant pathway for the generation of 8-hydroxy metabolites from other guanine analogs such as O6-benzylguanine (Hall and Krenitsky, 1986). There are thus two potential pathways for thiouric acid formation from TG, either via guanine deaminase followed by xanthine oxidase, or via AO followed by guanine deaminase (Fig. 5). The fact that significant concentrations of 8-OH-TG are found in plasma following CIVI-TG, and that thioxanthine is not detected, suggests that the latter pathway may predominate in humans.

Oxidation at the 8-position of thiopurinates has also recently been observed with CIVI-MP. In the case of MP, however, only the methylated derivative, 6-methylmercapto-8-hydroxypurine, was detected and found to be the predominant circulating metabolite during CIVI-MP administration (Keuzenkamp-Jansen et al., 1996). The differences in substrate specificity between the thiopurinues may have clinical relevance. As methylation is known to be the first step of a primary catabolic route of elimination for both thiopurinues, in patients who are heterozygote or homozygote thiopurine methyltransferase deficient, TG may prove to have a route of elimination that is not available to MP.

The bioavailability of MP is low in part because of first-pass metabolism by xanthine oxidase (Zimm et al., 1983a,b). The bioavailability of TG is also low (Brox et al., 1981), but the basis for this has not yet been defined. Data presented here suggest that first-pass metabolism to 8-OH-TG may be the primary mechanism responsible for low plasma TG exposure following oral administration. Studies are therefore under way to determine whether this metabolite is detected following oral TG administration.