Formation of 6-Thioguanine and 6-Mercaptopurine from Their 9-Alkyl Derivatives in Mice

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ABSTRACT

Several 9-alkyl, 6-thiopurines have been reported to have more favorable therapeutic indexes than do the parent drugs, 6-mercaptopurine (MP) and 6-thioguanine (TG). Some of these compounds were reported to be active against cells in culture resistant to 6-thiopurines, and it has been assumed that their mechanisms of action may differ from those of TG and MP. 9-(n-Butyl)-6-thioguanine was essentially inactive toward Chinese hamster ovary cells in vitro when compared with TG (50% effective dose, 250 and 1 μM, respectively). However, lethal doses of 9-(n-butyl)-6-thioguanine and TG in mice were similar when these agents were given i.p. daily for 9 consecutive days (50% lethal dose, 13 and 9 mg/kg/day). Similar organ toxicities were observed upon histopathological examination of dying animals. The cumulative, daily urinary excretion of TG was virtually identical in mice given 20- and 10-mg/kg/day of doses of 9-(n-butyl)-6-thioguanine or TG, respectively, for 9 days. The TG formed was identified by ultraviolet light (340 nm) detection following separation on a reverse phase high performance liquid chromatography system and by fluorescent detection of the permanganate oxidation product separated on a strong anion-exchange system. Dealkylation of 9-(n-butyl)-6-mercaptopurine and 9-ethyl-6-mercaptopurine also occurred in AKR mice. At near equitoxic doses, the daily cumulative urinary excretion of MP from 9-(n-butyl)-6-mercaptopurine and 9-ethyl-6-mercaptopurine was about 20-30% of that observed in mice receiving MP. The MP was confirmed in each case by enzymatic peak-shift of MP to 6-thiouric acid and ultraviolet light detection using the high performance liquid chromatography systems referred to above. The results suggest that these 9-alkyl derivatives serve as prodrugs for TG and MP, a finding that explains a number of their pharmacological and toxicological properties.

INTRODUCTION

A number of 9-substituted 6-thiopurines have been synthesized and evaluated as antitumor agents (1-4). Several members of this class of compounds exhibited activity in vitro against mutants selected for resistance to TG (15) and/or MP (5, 6), and some have been reported to have more favorable chemotherapeutic indexes than do TG or MP (7, 2-4). One 9-alkyl thiopurine, EMP, was found to have activity against adult chronic granulocytic leukemia (8) and to be as effective as MP in maintaining prednisone-induced remissions in childhood leukemia (9). Previous studies with BTG in mice (10, 11), with EMP in rats (12), and with BMP in humans (13, 14) have failed to indicate metabolism of these compounds to TG or MP. However, EMP has been reported to be significantly converted to MP in humans (15). Since N-dealkylation is commonly observed in drug metabolism (16), we have reinvestigated the possible in vivo dealkylation of BTG, BMP, and EMP in mice. The results suggest that significant dealkylation to the parent 6-thiopurines occurs. A preliminary report of this work has been presented (17).

MATERIALS AND METHODS

Animal and Tissue Culture Experiments. The lethality effects of TG and BTG to outbred ICR mice (Harlan-Sprague Dawley, Houston, TX) were determined following daily i.p. injection for 9 days. These and other thiopurines were prepared fresh every 3 days by dissolving them in 0.15 M NaOH, followed by neutralization with HCl. The animals were observed daily to record deaths and they were weighed at least three times weekly. The urinary excretion of TG and MP from mice treated with thiopurines was determined by collection of urine in metabolic cages (Econo-Metabolism Unit, E-1100; Maryland Plastics, Inc., Federalsburg, MD). The 9-alkyl derivatives were stable and not converted to the parent TG or MP in the presence of mouse urine during a 24-h collection interval. In other groups of animals, urine was collected from bladders in mice, in situ, as described previously (18). Chemotherapeutic activity of 6-thiopurines was evaluated in male AKR mice (The Jackson Laboratory, Bar Harbor, ME) bearing Riddsqay osteogenic sarcoma (19). The tumor growth rate was estimated by caliper measurement of s.c. implants three times weekly. The time required for the tumors to grow to a mass of 2 g was estimated for each mouse by semilogarithmic plots of the data (see Ref. 19, Fig. 1). The toxicity of the thiopurines toward Chinese hamster ovary and Hep. 2 cells was determined by colony assay as described previously (20).

Measurement of TG and MP in Urine. The urine collected from mice in metabolic cages or directly from the bladder was extracted with 1 N HCl and heated at 90°C for 30 min. Recovery of the thiopurines was 65 to 75% as determined by collecting the urine in metabolic cages to which the drugs were added 24 h prior to analysis. The values given are not corrected for this recovery. TG was measured by two different HPLC methods as follows. TG in the neutralized urine extracts was measured by absorbance at 340 nm (Spectro Monitor II; Laboratory Data Control, Riviera Beach, FL) following elution from a C_{18}Bondapak column (Waters Associates, Milford, MA). Also TG was measured by fluorescence detection following oxidation with alkaline potassium permanganate and elution of the oxidation product from a Partisol SAX column (Whatman, Inc., Clifton, NJ) by its absorbance at 320 nm following elution from the reverse phase column. The presence of MP in the urine samples was confirmed by enzymatic peak-shift (23) using xanthine oxidase. For this confirmation, MP in urine samples was purified by elution from two C_{18}Bondapak columns connected in series. The fractions containing MP were combined and concentrated by evaporation. The residue was dissolved in 1 ml of

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3 The abbreviations used are: BMP, 9-(n-butyl)-6-mercaptopurine; BTG, 9-(n-butyl)-6-thioguanine; EMP, 9-ethyl-6-mercaptopurine; MP, 6-mercaptopurine; TG, 6-thioguanine; HPLC, high performance liquid chromatography.
water and treated with xanthine oxidase, which converted the MP, quantitatively, to 6-thiouric acid. The 6-thiouric acid was then measured by its absorbance at 340 nm following elution from the reverse phase and Partisol SAX columns. The HPLC system used has been described previously (21, 24). Reverse phase chromatography was generally performed at a flow rate of 2 ml/min using a linear gradient (15 min) from water to 20% methanol. For some applications, the gradient was extended to 70% methanol to elute the more lipophilic 9-alkyl thiopurines. Anion-exchange chromatography was performed as described previously (24, 25).

Materials. The sources of materials were as follows: TG, MP, 6-thioguanine, and xanthine oxidase (1 unit/mg), Sigma Chemical Co., St. Louis, MO; BTG, Vega Biochemicals, Tucson, AZ, and the National Cancer Institute, Bethesda, MD; BMP and EMP, National Cancer Institute. The purity of these substances was estimated to be greater than 95% by HPLC analysis. Less than 0.05% (minimal detectable amount) of the parent MP or TG was present in BTG, BMP, or EMP as determined by reverse phase chromatography of 100 nmol of each sample. A standard 6-thioguanine acid solution was prepared by treatment of 6-thioguanine with xanthine oxidase.

RESULTS AND DISCUSSION

Although the potency of the 9-alkyl thiopurines toward Chinese hamster ovary cells in tissue culture are remarkably less than that of TG or MP, their toxicities in mice are quite similar to the parent thiopurines (Table 1). For example, BTG is approximately 250-fold less active on a molar basis than is TG in vitro (50% effective dose values versus Chinese hamster ovary cells); however, in intact mice the lethal doses after 9 consecutive days of treatment are about the same on a molar basis (Table 1). Similar results are also apparent when BMP and EMP are compared with MP. These data suggest that the 9-alkyl thiopurines in Table 1 may require metabolic activation in the intact animal. Histopathological examination of mice treated with TG and BTG revealed similar patterns of organ toxicities as follows. Groups of three to six mice each were sacrificed on Day 9 following administration of BTG (15 and 30 mg/kg/day) or TG (5 and 10 mg/kg/day). Both TG and BTG had their greatest effects on bone marrow. The most severe damage was to myeloid cells at all doses; however, the higher doses of both compounds caused damage to the myeloid components as well. All doses of these drugs also caused necrosis of intestinal epithelium in crypts. For each drug, two mice exhibited acute renal tubular necrosis. One mouse at each dose of BTG exhibited myocardial

![Chart 1. Formation of TG from BTG in mice. Groups of 6 to 8 AKR mice were treated daily with TG (10 mg/kg) or BTG (20 mg/kg). Urine was collected at 24-h intervals in metabolic cages and TG was measured by fluorescent detection of the pergam mate oxidation product as described in "Materials and Methods." Urine collected during the first 24-h interval prior to thiopurine administration (controls) contained less than 0.7 nmol/mouse (minimal detectable value for the assay conditions used). The values shown are the mean values for three separate experiments. The mice lost 24 and 15% of their original body weight during this period of treatment with TG and BTG, respectively.](chart1.png)

![Chart 2. Measurement of TG in the urine of mice treated with BTG. Urine was collected from the bladders, in situ (18), of mice treated for 3, 5, or 7 consecutive days with BTG (20 mg/kg/day). Samples were obtained during a 1-h interval following the last dose of BTG. TG was measured by UV absorbance (Y-axis) after separation on a reverse phase column or by fluorescence (X-axis) following separation of the permanganate oxidation product with an anion-exchange column (see "Materials and Methods"). The values given for each axis are nmol TG collected per mouse.](chart2.png)

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>In vitro (Chinese hamster ovary cells) ED₅₀ (μM)</th>
<th>In vivo LD₅₀ (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>1</td>
<td>10⁴</td>
</tr>
<tr>
<td>BTG</td>
<td>250</td>
<td>6⁵</td>
</tr>
<tr>
<td>MP</td>
<td>50</td>
<td>6⁴</td>
</tr>
<tr>
<td>BMP</td>
<td>400</td>
<td>6⁵</td>
</tr>
<tr>
<td>EMP</td>
<td>600</td>
<td>3¹⁷</td>
</tr>
</tbody>
</table>

* ED₅₀, 50% effective dose; LD₅₀, 10% lethal dose.
* Data from Chart 6. Drugs given daily for 9 days.
* Data from the Southern Research Institute obtained in C57BL x DBA/2 F₁ mice. Drugs were given daily for 11 days. LD₅₀ summary, Contract NIH-NCI-C-71-2098 (N01-CM-12059).
treated with BTG was confirmed by collecting urine directly from the bladder as described previously (18) and subsequent measurement of the TG by two HPLC methods. By collecting the urine from sterile bladders, the possible dealkylation of BTG by bacteria is obviated. Urine was collected from the bladders of 10 mice treated with BTG and TG was determined in the urine by two HPLC methods (Chart 2). TG was easily detected by both methods. Furthermore there was good agreement for the TG values estimated by the UV absorbance or fluorescent technique. An example of the measurements of TG in the urine of mice treated with BTG is given in Chart 3.

BMP and EMP also appear to be dealkylated in mice (Chart 4). The doses of MP, BMP, and EMP used for these experiments were approximately equitoxic (Table 1). The in vivo excretion of MP from mice treated with BMP or EMP appears to be about 20 to 30% of that observed in mice treated with MP at the doses shown (Chart 4). In each case, the presence of MP in the urine was confirmed by treatment of partially purified samples with xanthine oxidase to convert MP to 6-thiouric acid. 6-Thiouric acid was confirmed in each case by separation on the anion-exchange system described in “Materials and Methods” (data not shown). Urine samples without xanthine oxidase treatment; and chromatograms obtained after xanthine oxidase treatment.

Chart 3: Measurement of TG in the urine of mice treated with BTG. The two HPLC methods used to measure TG are illustrated using one of the urine samples from Chart 2. A, UV measurement of TG during elution from a reverse phase column; B, fluorescent detection of the same sample separated on an anion-exchange column after permanganate oxidation (see “Materials and Methods”). ---, urine obtained from control, untreated mice; ----, chromatogram of urine obtained from the bladder of a mouse treated for 3 days with BTG (20 mg/kg/day; see “Materials and Methods” and Chart 2).

Chart 5: Confirmation of MP in the urine of mice treated with MP, BMP, or EMP. The urine samples from the mice used for the experiments illustrated in Chart 4 were combined and partially purified by passage through two reverse phase columns. The peak corresponding to MP was collected and concentrated by evaporation. The sample was redissolved in 1 ml of distilled water and an aliquot was treated with xanthine oxidase to convert MP (retention time, 5–6 min) to 6-thiouric acid (retention time, 3 min). 6-Thiouric acid was confirmed in each case by separation on the anion-exchange system described in “Materials and Methods” (data not shown). ---, urine samples without xanthine oxidase treatment; and ----, chromatograms obtained after xanthine oxidase treatment.

Chart 6: Chemotherapeutic and lethal effects of TG and BTG in mice. Outbred and AKR mice were treated with TG or BTG for 9 consecutive days at the doses shown. Lethality was determined in non-tumor-bearing, outbred mice during a 30-day interval following drug administration. Therapeutic activity refers to the percentage of increase in time, over controls, required for Ridgway osteogenic sarcoma implants to grow to 2 g when treated with the drugs shown for 9 consecutive days (days 8–16 after tumor implantation). Numbers in parentheses, number of mice per group for the doses shown.
xanthine oxidase (Chart 5).

The chemotherapeutic activities of TG, BTG, MP, and EMP against Ridgway osteogenic sarcoma were determined by measuring the delay in tumor growth associated with drug treatment. Neither of the 9-alkyl derivatives appeared to offer a therapeutic advantage over the parent thiopurine, i.e., similar delays in tumor growth were observed at maximally tolerated doses (data not shown). As can be ascertained from Chart 6, the chemotherapeutic index of BTG against Ridgway osteogenic sarcoma is not superior to that of TG when the drugs are given daily for 9 days.

Indirect evidence from other laboratories has indicated previously that some of the compounds studied herein may be dealkylated to TG or MP in mice. For example, BTG was relatively inactive against Ehrlich carcinoma cells in mice unless treatment was combined with azaserine (26), a finding similar to that observed with TG or MP. Furthermore adenocarcinoma 755 cells resistant to MP were observed to be cross-resistant to several 9-alkyl, 6-thiopurines in vivo (4). As reported herein, significant dealkylation of BTG to TG (Chart 2) and of BMP and EMP to MP (Chart 4) occurs in AKR mice. Although the amounts of parent TG or MP excreted into urine are small compared with the dose administered, dealkylation may have pharmacological significance since the similar amounts of TG and MP excreted occurred at doses nearly equitoxic with those of the parent thiopurines.

Dealkylation is a more universal and well-studied processes in drug metabolism (16); therefore it is not surprising that dealkylation of these compounds occurs. This metabolism is characteristic of the cytochrome P-450 enzyme complex in mammalian liver.

In the Ridgway osteogenic sarcoma model, BTG does not appear to offer any advantages over TG; i.e., it does not appear to be more effective nor is it safer than TG (Chart 6). Since BTG, BMP, and EMP all appear to be dealkylated by mice (Charts 1 and 4), previously reported chemotherapeutic and toxic properties of these drugs in this species may be consequences of their conversion to MP and TG.

REFERENCES

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