Conversion of 6-Thioguanine to the Nucleoside Level by Purine Nucleoside Phosphorylase of Sarcoma 180 and Sarcoma 180/TG Ascites Cells

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ABSTRACT

Tumor sublines sensitive (Sarcoma 180) and resistant (Sarcoma 180/TG) to the cytotoxic action of 6-thioguanine (6TG) actively metabolize the 6-thiopurine to 6-thioguanosine (6TGuo). The conversion of 6TG to 6TGuo by intact cells was enhanced by p-ribosyl 1-phosphate but not by p-ribosyl 5-phosphate. This corresponded to the finding that p-ribosyl 1-phosphate supported the synthesis of 6TGuo from 6TG by cell-free extracts of Sarcoma 180/TG, whereas 5-phosphoribosyl 1-pyrophosphate was essentially inactive. 6-Methylmercaptopurine ribonucleoside and 6-nitrobenzylmercaptopurine ribonucleoside markedly inhibited the formation of 6TGuo from 6TG by intact cells; however, 6-methylmercaptopurine ribonucleoside did not alter 6TGuo synthesizing activity from 6TG and p-ribosyl 1-phosphate by cell-free extracts. The majority of the 6TGuo synthesized by intact cells was excreted into the medium. The findings support the concept that the metabolism of 6TG to 6TGuo in Sarcoma 180 and Sarcoma 180/TG cells is mediated by purine nucleoside phosphorylase and that the newly synthesized 6TGuo is readily effluxed into the cellular environment. The reduction of 6TGuo formation by 6-methylmercaptopurine ribonucleoside in intact cells is speculated to be the result of inhibition of the subsequent efflux of 6TGuo by the nucleoside transport system after its formation by purine nucleoside phosphorylase, a phenomenon that affects the equilibrium of this enzymatic reaction. It is further hypothesized that the formation of 6TGuo by purine nucleoside phosphorylase may be important to the expression of cellular sensitivity to 6TG, in that it decreases the availability of 6TG for direct conversion by hypoxanthine-guanine phosphorylsytransferase to the nucleotide level, a phenomenon critical to the expression of the antineoplastic activity of the 6-thiopurines.

INTRODUCTION

The 6-thiopurines (i.e., 6-mercaptopurine and 6TG) are effective agents in the treatment of the acute leukemias of humans. The metabolism of these purine antimetabolites by tumor and host tissues has been extensively studied in an effort to understand the mechanism by which they exert their antineoplastic properties. These investigations have demonstrated that 6TG is metabolized by various cells and tissues to 6TGM and higher nucleotides, 6-thioxanthine, 6-methylthioguanine, 6-thiouric acid, and 6TGuo (3, 4, 5, 13, 21).

The enzyme PNPase has been considered by cancer chemotherapists to be involved largely in degradative reactions that convert purine nucleosides to their respective purine base analogs. Although reports from other laboratories have described the conversion of physiological purines to the corresponding nucleoside level by isolated PNPase (7, 24), investigators designed to measure the impact of this enzyme on the generation of purine analog ribo- and deoxyribonucleosides in intact cells have been minimal. Studies with the 6-thiopurines has indicated that 6TGuo and 6-mercaptopurine ribonucleoside can be relatively rapidly cleaved by this enzymatic action from the free base in several tumor cell lines (11, 15).

The present study has investigated the role of PNPase in the formation of 6TGuo from 6TG by Sarcoma 180 cells sensitive and resistant to the growth-inhibitory effects of this purine antimetabolite. The findings demonstrate that neoplastic cells exposed to 6TG readily convert the 6-thiopurine to the nucleoside level, presumably by the direct action of PNPase. This conclusion was supported by the finding of enzymatic reactivity between 6TG and ribose-1-P to form 6TGuo by cell-free extracts of these tumors. Pronounced inhibition of the intracellular conversion of 6TG to 6TGuo was caused by MMPr and NBMPR. This action did not appear to be the result of direct inhibition of PNPase activity by these compounds or the metabolite(s). The blockage of efflux of synthesized 6TGuo by MMPr and NBMPR, a process which would affect the equilibrium of the PNPase reaction, is suggested as a mechanism by which these nucleosides diminish the intracellular accumulation of 6TGuo. The importance of the metabolic conversion of 6TG to the nucleoside level to the therapeutic efficacy of the thiopurine as an antineoplastic agent is discussed.

MATERIALS AND METHODS

6TG, 6TGMP, ribose-1-P, ribose-5-P, and PRPP we purchased from Sigma Chemical Co. (St. Louis, Mo.). NMMPR was the kind gift of Dr. T. S. Lin of the Department of Pharmacology, Yale University School of Medicine, and 6TGM was supplied by the Division of Cancer Treatment, National Cancer Institute.

Measurement of 6TGuo and 6TGMP Formation by Tum Cells. Sarcoma 180 and Sarcoma 180/TG cells (22) were serially maintained in female CD-1 mice (Charles River Breeding Farm, Portage, Mich.) by weekly i.p. inoculation of appro


imately 10^6 cells/mouse. The 6TG-resistant subline (Sarcoma 180/TG) contains normal levels of HGPRTase activity and markedly elevated levels of alkaline phosphatase; this latter enzyme has been implicated in the mechanism of resistance of this variant cell line. For experiments, tumor cells were collected from CD-1 mice 6 days after i.p. implantation and centrifuged at low speed in 0.9% NaCl solution to facilitate the removal of contaminating RBC, and cell numbers were determined with a Coulter Model ZBI particle counter after appropriate dilution with Krebs-Ringer phosphate medium (110 mM NaCl-4.9 mM KCl-1.2 mM MgCl2-6.4 mM sodium phosphate buffer, pH 7.4). Reactions were carried out with cell suspensions (10^7 cells/ml) in the presence of 5.5 mM glucose (unless otherwise specified) and terminated by the addition of 1.2 N perchloric acid, and the resulting acid-soluble fraction was neutralized with 1.0 N KOH. Separation of acid-soluble materials present in the resulting cell pellets was measured by high-pressure liquid chromatography as described by Wohlhueter ef al. (28). One ml of cell suspension was placed into an Eppendorf microcentrifuge tube containing 0.5 ml of an oil mixture consisting of 84 parts of silicone oil (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 16 parts of paraffin oil (Fisher Scientific Co., Fair Lawn, N. J.) by weight (final density, 1.034 g/ml). The tubes were centrifuged in an Eppendorf microcentrifuge for 15 sec (12,000 x g), and the supernatant solution was dialyzed for 20 hr against 4 liters of the buffer used in the disruption of cells. Tumor cells collected from CD-1 mice 6 days after i.p. implantation were centrifuged at 12,000 x g for 10 min at 4°, and the supernatant solution was dialyzed for 20 hr against 4 liters of the buffer used in the disruption of cells.

**RESULTS**

The quantity of 6TGuo generated by Sarcoma 180 and Sarcoma 180/TG cells incubated with 6TG is shown in Chart 1. The amount of 6TGuo formed was dependent upon the incubation time, and approximately 10 and 20% conversion of 6TG to 6TGuo occurred in 10 min when 10^5 Sarcoma 180 and Sarcoma 180/TG cells, respectively, were incubated with 0.1 mM 6TG at 37°. Total PNPase activity of homogenates of Sarcoma 180/TG cells was about 30 to 50% higher than was enzyme activity present in homogenates prepared from a comparable number of Sarcoma 180 cells.

To gain information on the metabolic pathway through which 6TGuo was generated, Sarcoma 180/TG cells suspended in Krebs-Ringer phosphate medium in the absence of glucose were incubated with 6TG and either ribose-1-P or ribose-5-P. As shown in Chart 2, a 10-fold greater rate of 6TGuo synthesis occurred in cells incubated with ribose-1-P than in control cells without a phosphorylated ribose derivative in the medium. In contrast, the rate of synthesis of 6TGuo from ribose-5-P by Sarcoma 180/TG cells was comparable to that of the control. Consistent with this result was the finding that the conversion of 6TG to 6TGuo by cell-free extracts prepared from Sarcoma 180/TG cells required the presence of ribose-1-P in the incubation medium; whereas, in contrast, the cosubstrate for HGPRTase, PRPP, did not lead to the formation of 6TGuo in this system (Chart 3). These results support the concept that 6TGuo was not generated through conversion of the 6-thiopurine to 6TGMP which then was dephosphorylated to form the nucleoside.

**Intracellular Concentration of 6TGuo.** Separation of cells from the incubation medium was carried out essentially as described by Wohlhueter et al. (28). One ml of cell suspension was placed into an Eppendorf microcentrifuge tube containing 0.5 ml of an oil mixture consisting of 84 parts of silicone oil (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 16 parts of paraffin oil (Fisher Scientific Co., Fair Lawn, N. J.) by weight (final density, 1.034 g/ml). The tubes were centrifuged in an Eppendorf microcentrifuge for 15 sec (12,000 x g), and the sedimented material present in the resulting cell pellets was analyzed by high-pressure liquid chromatography as described above.

**Assay of PNPase Activity.** Cell-free extracts were prepared for enzyme assays as described by Ullman et al. (25). Five % suspensions containing 1 g of cells (~10^7 cells/ml) from either Sarcoma 180 or Sarcoma 180/TG were made in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol and 10% (v/v) glycerol. The cells were lysed by 3 cycles of sonication of 15 sec each at 20% output of a Branson sonicator. The cell extracts were centrifuged at 12,000 x g for 10 min at 4°, and the supernatant solution was dialyzed for 20 hr against 4 liters of the buffer used in the disruption of cells. The assay mixture contained 0.1 mM 6TG, 0.1 mM ribose-1-P or PRPP, 0.5 mg protein, and Krebs-Ringer phosphate medium with or without an inhibitor in a final volume of 1.0 ml. Reactions were initiated by the addition of 6TG and were carried out at 37°. To terminate the reactions, 0.2 ml of 1.2 N perchloric acid was added, and acid-soluble fractions were assayed chromatographically for 6TGuo as described above. Protein concentra-

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**Chart 1.** Rate of synthesis of 6TGuo by Sarcoma 180 and Sarcoma 180/TG cells. Tumor cells collected from CD-1 mice 6 days after i.p. implantation were incubated at 37° with 0.1 mM 6TG in Krebs-Ringer phosphate medium containing 5.5 mM glucose. At various times thereafter, the concentration of 6TGuo in acid-soluble extracts was measured by high-pressure liquid chromatography as described in "Materials and Methods." •, Sarcoma 180; ○, Sarcoma 180/TG. Points, mean of 2 separate experiments each having 3 samples. Bars, S.E.
The effects of several purines and purine nucleosides on the conversion of 6TG to the nucleoside and nucleotide levels were measured. Among the analogs tested, MMPR and NBMPR were found to decrease the accumulation of 6TGuo by neoconversion of 6TG to the nucleoside and nucleotide levels were not significantly affected (Chart 4). These data add further support to the premise that 6TGuo is not generated from 6TGMP, since almost 25 times more nucleoside was present, and inhibition of the conversion of 6TGMP to 6TGuo would be expected to result in the accumulation of analog nucleotide. Due to the insolubility of NBMPR in aqueous solvents, the highest concentration of this agent tested was 1 μM; this level of NBMPR caused approximately 40% inhibition of the conversion of 6TG to 6TGuo (Table 1). Unlike the case of MMPR, a slight but significant increase in the levels of cellular 6TGMP was produced by the presence of NBMPR in the reaction medium.

In an effort to elucidate the mechanism(s) by which MMPR and NBMPR inhibit the formation of 6TGuo, the effects of these agents on PNPase activity from both Sarcoma 180 and Sarcoma 180/TG were measured. Enzymatic assays for PNPase with cell-free extracts were linear with protein concentration and time for at least 30 min, and the activity depended on the presence of ribose-1-P. Neither 2 mM MMPR nor 1 μM NBMPR (the highest concentrations tested) affected the formation of 6TGuo by cell-free extracts (data not shown). Since a metabolite of MMPR or NBMPR might be responsible for inhibition of the formation of 6TGuo, assays were also conducted with cell-free extracts prepared from cells treated with MMPR or NBMPR; these investigations demonstrated no difference in the capacity of cell-free extracts from control and drug-treated cells to convert 6TG to 6TGuo.

The intracellular concentration of 6TGuo was ascertained

![Chart 2. Influence of ribose-1-P and ribose-5-P on the synthesis of 6TGuo by Sarcoma 180/TG cells. Sarcoma 180/TG cells were incubated at 37° with 0.1 mM 6TG in the presence of either 0.5 mM ribose-1-P or 0.5 mM ribose-5-P in Krebs-Ringer phosphate medium without glucose. At various times thereafter, the concentration of 6TGuo in acid-soluble extracts was measured by high-pressure liquid chromatography. O, ribose-1-P; ●, ribose-5-P; △, control without ribose phosphate. Points, means of 2 experiments each having 2 to 3 samples. Bars, S.E.](chart2)

![Chart 3. Synthesis of 6TGuo by cell-free extracts from Sarcoma 180/TG. Cell-free extracts were prepared by sonication of Sarcoma 180/TG cells and were incubated (0.5 mg protein) with 0.1 mM 6TG in the presence of either 0.1 mM ribose-1-P or 0.1 mM PRPP in Krebs-Ringer phosphate medium without glucose (pH 7.4) at 37°. At various times thereafter, reaction mixtures were analyzed for the presence of 6TGuo by high-pressure liquid chromatography. O, ribose-1-P; ●, PRPP; △, control. Points, means of 2 separate experiments each having 2 samples; S.E.'s were no greater than 3% of each value.](chart3)

![Chart 4. Effects of MMPR on the synthesis of 6TGMP and 6TGuo by Sarcoma 180/TG cells. Sarcoma 180/TG cells were preincubated at 37° with various concentrations MMPR in Krebs-Ringer phosphate medium for 10 min at 37°. At this time, 0.1 mM 6TG was added, and incubation was continued for 20 min. Acid-soluble extracts were then prepared and analyzed by high-pressure liquid chromatography. The control sample synthesized 35 nmol 6TGuo and 1.4 nmol 6TGMP per 10⁷ cells in 20 min. O, 6TGMP; ●, 6TGuo. Points, means of 3 separate experiments each having 2 samples, with S.E.'s being 5% or less.](chart4)

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<th>Incubation time (min)</th>
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<tr>
<td></td>
<td>-NBMPR</td>
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after incubation of Sarcoma 180/TG cells with 0.1 mM 6TG for 20 min. This was accomplished by rapid separation of cells from incubation medium by centrifugation through a layer of an oil mixture. The results of this experiment, shown in Chart 5, demonstrate that less than 5% of the total 6TGuo produced remained as intracellular material. Furthermore, although the presence of 0.1 mM MOMP in the reaction mixture reduced by approximately 60% the total amount (i.e., medium and cellular) of 6TGuo, the intracellular concentration of the 6-thiopurine nucleoside was comparable to that of non-MOMP-treated control samples.

DISCUSSION

6TGMP has been shown to be a major metabolite of 6TG in normal tissues and tumors of mice (12, 13); however, there is a paucity of reports concerned with the direct ribosylation of this 6-thiopurine to its nucleoside. Investigations concerned with the action of PNPase on the 6-thiopurines have tended to stress the degradative importance of this enzyme to the antineoplastic efficacy of the 6-thiopurine nucleosides (11, 15); this paper is concerned with the potential impact of 6TGuo synthesis on the anticancer activity of 6TG. Since 6TG must be converted to 6TGMP to exert its cytotoxic action (2, 6, 10), and the activity of a kinase(s) which converts 6TGuo to 6TGMP appears to be at best minimal, the finding that neoplastic cells synthesize significant amounts of 6TGuo from 6TG suggests that this reaction might function to decrease the potency of 6TG by making it less readily available for conversion to 6TGMP by HGPRTase.

A significant rate of conversion of 6TG to the nucleoside level occurred in Sarcoma 180 cell lines, with a markedly greater accumulation of 6TGMP taking place in the 6-thiopurine-resistant subline. This observation is consistent with the concept that such conversion may be of importance to the sensitivity of cells to the cytotoxic action of this agent. In addition, the formation of 6TGuo was strongly inhibited by MOMP, a purine nucleoside analog which is synergistic with the 6-thiopurines in the treatment of murine neoplasms (14, 16, 23, 26), adding further support to the idea that the metabolic conversion of 6TG to 6TGuo may be involved in the expression of cellular sensitivity to the cytotoxic action of this drug.

Two obvious possibilities for the generation of 6TGuo exist. These are: (a) that 6TG is directly ribosylated to 6TGuo; or (b) that 6TG is initially converted to 6TGMP by HGPRTase, followed by dephosphorylation of the nucleotide to 6TGuo. The equilibrium of the enzymatic reaction catalyzed by phosphoribomutase (i.e., ribose-1-P ⇔ ribose-5-P) is significantly in favor (more than 90%) of ribose-5-P (9). Therefore, the observation that ribose-5-P in the reaction medium did not support the synthesis of 6TGuo by neoplastic cells, whereas ribose-1-P strongly stimulated the reaction, is consistent with the concept that a direct conversion of 6TG to 6TGuo by PNPase occurred. In support of this hypothesis, cell-free extracts from both Sarcoma 180 and Sarcoma 180/TG were found to have high levels of 6TGuo-forming activity which required the presence of ribose-1-P but not PRPP.

The marked inhibition of 6TGuo formation by MOMP in intact cells was not observed in cell-free extracts or in extracts prepared from cells pretreated with MOMP. These results imply that the reduced conversion of 6TG to 6TGuo caused by treatment of cells with MOMP is not due to direct inhibition of PNPase by MOMP or a metabolite(s) thereof. It is conceivable, however, that an inhibitory metabolite of MOMP was diluted to a noneffective level during preparation of cell-free extracts.

Nucleosides have been shown to be transported and countertransported through the cell membrane by a nucleoside carrier system with a broad spectrum of structural specificity (17, 27). Structure-activity studies have demonstrated that purine ribonucleosides with hydrophobic constituents connected via N, S, or O linkage at position 6 are potent inhibitors of nucleoside transport (19). Consistent with the findings presented in this report is the possibility that newly synthesized intracellular 6TGuo is rapidly removed by efflux into the medium through the nucleoside carrier mechanism. Since MOMP has the potential to occupy the transport mechanism, it is reasonable to speculate that such occupancy would interfere with the efflux of 6TGuo. PNPase has been considered to be the primary means by which animal tissue degrades purine ribo- and deoxyribonucleosides to their corresponding free base and pentose-1-phosphate (7, 24). The reaction, however, is readily reversible with the equilibrium favoring nucleoside synthesis (K_{eq} 54) (7, 8). Therefore, in the presence of a nucleoside transport inhibitor, the rate of nucleoside formation will be considerably reduced due to inhibition of the constant removal

Chart 5. Intracellular concentration of 6TG and its metabolites. Sarcoma 180/TG cells were exposed to 0.1 mM 6TG for 20 min following preincubation in the absence or presence of 0.1 mM MOMP for 10 min. Cells were separated from incubation medium by centrifugation through a layer of an oil mixture. Total and intracellular metabolites of 6TG were analyzed by high-pressure liquid chromatography. – – – – –, total 6TG metabolites produced by cells treated with MOMP; – – – – – –, total 6TG metabolites produced by cells not exposed to MOMP; – – – – – – –, intracellular 6TG metabolites present in cells treated with MOMP; – – – – – – – –, intracellular 6TG metabolites present in cells not exposed to MOMP. The chromatograms were obtained with extracts from about 10^6 cells. The profile represents the results from a representative experiment.

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of the nucleoside from the cellular compartment. Such a result was obtained with both MMPR and the strong inhibitor of nucleoside transport, NBMPR (20).

The generally accepted mechanism by which MMPR potentiates the antineoplastic effects of the 6-thiopurines is that MMPR, following conversion to the nucleotide level, increases the steady-state concentration of PRPP through inhibition of purine nucleotide biosynthesis de novo, thereby permitting increased formation of 6TGMP from 6TG by HGPRTase (14, 16). In addition, the nucleotide of MMPR serves as an alternate substrate for nucleotidase enzymes, thereby serving to protect the pool of 6TGMP from catabolic dephosphorylation (14). Since MMPR causes marked increase in PRPP concentrations only after 6 to 12 hr of exposure (18), and relatively short incubation periods were used in the present study, no major elevation in the levels of 6TGMP were demonstrated in these studies. It is conceivable that MMPR and other inhibitors of nucleoside transport function in a third way to cause synergism when used in combination with 6TG in the treatment of transplanted tumors; thus, MMPR enhances the formation of 6TGMP not only by increasing levels of PRPP and preventing its dephosphorylation but also by preventing the trapping of 6TG as 6TGuo, which is destined to be largely lost from the cell.

REFERENCES


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