Biochemical Mechanisms for the Synergism between 6-Thioguanine and 6-(Methylmercaptopo)purine Ribonucleoside in Sarcoma 180 Cells

J. Arly Nelson and R. E. Parks, Jr.

Section of Biochemical Pharmacology, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912

SUMMARY

The synergism between 6-(methylmercaptopo)purine ribonucleoside (MMPR) and 6-thioguanine was studied in the mouse Sarcoma 180 ascites tumor. The 5'-monophosphate nucleotide of MMPR forms rapidly to levels of 1 to 2 mM in the tumor cells, and the steady-state levels of 5-phosphoribosyl 1-pyrophosphate increase 4- to 5-fold in 6 to 12 hr. This permits a significantly greater synthesis of 6-thioguanosine monophosphate (6-thioGMP) after injection of 6-thioguanine. These findings are similar to those in previous reports concerning the synergism between 6-mercaptopurine and MMPR. In addition, MMPR increases the biological t1/2 of 6-thioGMP from about 7 hr to 10 hr. The concentrations of ATP and GTP decrease by 50% or greater after MMPR and the concentration of UTP in the cell doubles, probably as the result of greater 5-phosphoribosyl 1-pyrophosphate availability for pyrimidine biosynthesis. With a 6-thioguanine-resistant cell line, the t1/2 of 6-thioGMP is only 3 hr but increases to about 7 hr after MMPR pretreatment. Azaserine produces effects on endogenous nucleotide pools and 6-thioGMP formation similar to those of MMPR, but it is without effect on the t1/2 of 6-thioGMP. The synergism between MMPR and other thiopurines may involve effects of MMPR on catabolism as well as synthesis of the analog nucleotides and sequential blockade of purine biosynthesis.

INTRODUCTION

Striking synergism occurs in antitumor effects when MMPR is administered in combination with other thiopurines such as 6-MP or 6-TG. Several biochemical observations offer insights into the mechanism of this synergism. Treatment with MMPR causes a significant elevation of the steady-state levels of PRPP in tissues (25), probably as a result of pseudofeedback inhibition of PRPP amidotransferase by MMPR-5'-P (10, 11, 13, 17). The increased availability of PRPP results in enhanced synthesis of the analog nucleotide, 6-mercaptopurine ribonucleoside 5'-phosphate in cells treated with 6-MP (25, 33). A marked synergism was also observed when 6-MP and MMPR were examined for their effects on the steady-state levels of adenine nucleotides of S-180 cells (33).

Although an earlier report from this laboratory dealt primarily with the interaction of MMPR and 6-MP, it was reported that potentiation also occurred with MMPR and 6-TG in prolonging the survival time of mice bearing the S-180 tumor (33). The present study explores in greater detail the mechanism of this synergism, and the results indicate a mechanism that is similar to that seen with MMPR and 6-MP. In addition to enhanced formation of 6-thioGMP, it has been observed that pretreatment of S-180 cells with MMPR significantly increases the half-life of 6-thioGMP in the cells. Pretreatment with MMPR also causes a marked increase in the steady-state levels of UTP, which suggests that when the de novo pathway of purine biosynthesis is blocked by MMPR-5'-P, the pathway of pyrimidine nucleotide synthesis is stimulated. Preliminary reports of portions of this work have been presented (22, 23).

MATERIALS AND METHODS

Treatment of Cells. The S-180 and S-180/TG cells were kindly supplied by Dr. A. C. Sartorelli of Yale University. They were maintained by the i.p. transplantation of 5 to 6 X 10^6 tumor cells each week in female CD1 mice (Charles River Laboratories, North Wilmington, Mass.). Drugs, in 0.9% NaCl solution, were given i.p. to mice 4 to 6 days after implantation of the tumor cells. Nucleotide levels and 6-TG-35S metabolites were measured in acid-soluble extracts of the cells. For preparation of the extract, the cells were collected from the peritoneal cavity and washed by centrifugation at room temperature; approximately 3 X 10^7 tumor cells in a volume of 1 ml were then added to 0.5 ml of 12% perchloric acid at 0°. As determined by microscopic examination, the washed tumor cells were virtually free of other cells, such as erythrocytes and granulocytes. Any tumor cell samples that were visibly contaminated with blood were discarded. After...
 removal of the insoluble material by centrifugation, the acid-soluble extract was neutralized with KOH (for details, see Ref. 33). The wash medium was composed of 40 mM Tris-HCl, 20 mM KCl, 88 mM NaCl, 2 mM MgCl₂, 5.5 mM glucose, and 22 mM potassium phosphate buffer at a final pH of 7.4. This medium was also used in the in vitro incubation of S-180 cells.

**Chromatographic Procedures and Identification of 6-TG-3²S Metabolites.** Nucleotide levels were determined in the acid-soluble extracts by high pressure liquid chromatography, with a Varian Aerograph LCS-1000 instrument, exactly as described by Brown (5). Generally, 20 μl of the extract were placed on the column; therefore, the nucleotide content of about 4 X 10⁸ cells (approximately 2 mg, wet weight, of cells) was measured. The cells from each mouse were analyzed separately. Metabolites of 6-TG-3²S in the acid-soluble material were also determined with the LCS-1000. For this purpose, fractions of the eluting material were collected over consecutive 2-min intervals (about 0.4 ml/fraction), and the radioactivity in each fraction was measured. Identities of chromatographic peaks were determined by comparison of experimental retention times to those of authentic material, enzymatic peak shift (5), and/or thin-layer chromatography. Acid-soluble extracts from S-180 cells treated with 6-TG-3²S yielded a peak of radioactivity with a retention time of 35 min, a time that corresponds to that of authentic 6-thioGMP. The radioactivity of this peak also cochromatographed with 6-thioGMP when developed with 1 N NH₄OH (RF 0.62), twice with 1 M LiCl (RF 0.50), or with a stepwise formate buffer system (RF 8; RF 0.30). Furthermore, incubation of the acid-soluble extract with guanylate kinase (ATP:GMP phosphotransferase, EC 2.7.4.8) for 12 hr at room temperature shifted all the radioactivity with a retention time of 35 min to a peak with a retention time near 80 min (6-thioGTP; see below). The reaction did not stop at the nucleoside diphosphate level (6-thioGDP), probably because the conditions of incubation are favorable for nucleoside diphosphokinase activity, and small amounts of this enzyme would lead to the formation of 6-thioGTP. The incubation mixture contained 200 μl of concentrated acid-soluble extract, 4 mM ATP, 125 mM KCl, 12.5 mM MgCl₂, and 1 μM unit of guanylate kinase in a final volume of 262 μl. The analog nucleotides, 6-thioGDP and 6-thioGTP, had retention times of about 60 and 80 min, respectively. In extracts of S-180 cells treated with 6-TG-3²S, succinate thiokinase (succinate:CoA ligase-GTP, EC 6.2.1.4) shifted the radioactivity eluting at 80 min to a peak with a retention time of 60 min. It has previously been shown that 6-thioGTP can replace GTP in the succinate thiokinase reaction (7). The incubation was performed at room temperature for 30 min with 100 μl of concentrated extract, 50 mM Tris-succinate, 0.1 mM CoA, and 1 μM unit of succinate thiokinase in a final volume of 161 μl.

**Identification of UTP in S-180 Cell Extracts.** An enzymatic peak shift (5) was performed to verify the presence of UTP in acid-soluble extracts of S-180 cells. UTP, with a retention time of 50 min on the high-pressure liquid chromatograph, was quantitatively converted to UDP-glucose (retention time, 25 min) by the action of uridyl transferase in the presence of glucose-1-P and inorganic pyrophosphatase (Chart 1). Omission of glucose-1-P in the incubation was necessary to demonstrate specificity for UTP, since some hydrolysis of ATP and GTP occurred in the presence or absence of glucose-1-P, possibly due to ATPase activity in the enzyme preparations used. The incubation was performed at room temperature for 90 min with 0.5 ml of neutralized cell extract, 0.20 mM glucose-1-P, 5 mM MgCl₂, 0.15 μM unit of uridyl transferase (UDP-glucose-pyrophosphorylase, EC 2.7.7.9), 1 μM unit of inorganic pyrophosphatase (EC 3.6.1.1), and 80 mM Tris-HCl at pH 8.5 in a final volume of 1 ml.

**Other Procedures.** Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer with a dioxane scintillation solution (4). Tumor cells were counted with a Model B Coulter counter, Coulter Electronics, Inc., Hialeah, Fla. PRPP was determined as described by Henderson and Kho (12) with S-180 cells instead of Ehrlich ascites cells as a source of AMP pyrophosphorylase activity.

**Reagents and Chemicals.** Azaserine was purchased from Calbiochem, Los Angeles, Calif. MMPR, 6-TG, and 6-thiouric acid were obtained from Sigma Chemical Co., St. Louis, Mo. Dr. Shih-Hsi Chu of this laboratory prepared the 6-TG-3²S by an isotope exchange reaction (21) using rhombic 35S obtained from New England Nuclear, Boston, Mass. Dr. K. C. Agarwal synthesized the 6-thioGMP by an enzymatic method (19). Mr. David Baccanari provided the succinate thiokinase, which he had purified from pig heart to a specific activity of approximately 20 μM units/mg protein (through Stage I in Table 1 of Ref. 7). Dr. K. C. Agarwal supplied the partially purified guanylate kinase (specific activity, 1 μM unit/mg protein) from human erythrocytes (1). Uridyl transferase (Grade 3 from Baker’s yeast; specific activity, 45 μM units/mg protein) and inorganic pyrophosphatase (Type 3 from Baker’s yeast, 500 μM units/mg protein) were products of Sigma.

**Presentation of Data.** Results are expressed as mean value ± standard error of the mean unless otherwise specified. Comparison of sample means by Student’s t test and the regression analyses were performed as described by Goldstein (9). Values for metabolite concentrations are given per ml of packed tumor cells without correction for extracellular space. In these experiments, 1 ml of packed cells was equivalent to approximately 2.2 X 10⁸ cells.

**RESULTS**

**Synergistic Effects of MMPR and 6-TG on Cellular Growth.** In survival studies reported earlier, mice bearing S-180 cells were treated for 6-day periods with combinations of MMPR and 6-TG, and a marked synergism was observed with some long-term survivors (33). In survival studies in which daily treatments are given over a period of days, an element of pretreatment is introduced. This is especially true of a drug such as MMPR, since it has been shown that the high intracellular concentrations of the metabolite, MMPR-5'-P, are maintained in tumor cells for relatively long periods of time, i.e., t1/2 of 24 hr or more (6, 11, 16, 25). Therefore, substantial amounts of this analog nucleotide may remain at the end of each 24-hr period. For evaluation of the effect of
MMPR pretreatment of the action of 6-TG, mice bearing approximately $1 \times 10^6$ S-180 cells were given single treatments with 6-TG and MMPR alone or in combination. Six days later the numbers of tumor cells present in the peritoneal cavities of the animals were counted (Table 1). Treatment with MMPR or TG alone had little effect, a finding which is consistent with the results of earlier survival time studies (33), and single, simultaneous treatment with MMPR and 6-TG showed slight additive effects. However, when the mice were pretreated with a single dose of MMPR followed by an injection of 6-TG 6 hr later, a marked decrease in cell numbers was observed.

Effect of MMPR Pretreatment on 6-TG-35S Uptake. The effect of MMPR pretreatment on 6-TG uptake into S-180 cells was examined by administration of 6-TG-35S i.p. to mice bearing the ascites tumor. The amount of acid-soluble radioactivity in the tumor cells and the radioactivity in the peritoneal fluid was then determined over a 1-hr period. There was a rapid disappearance of radioactivity from the peritoneal fluid (Chart 2). Only 10 to 20% of the administered radioactivity remained after 5 min, and after 1 hr this level was reduced to only 1 to 2%. The disappearance of radioactivity from the peritoneal fluid appeared to be more rapid in animals pretreated with MMPR. The amount of acid-soluble radioactivity in the tumor cells and the radioactivity in the peritoneal fluid was then determined over a 1-hr period. There was a rapid disappearance of radioactivity from the peritoneal fluid was then determined over a 1-hr period.

from mice treated with MMPR in vivo. These results strongly suggest that the increased uptake of 6-TG is due to an effect of MMPR on the tumor cells rather than an effect on the host.

Effect on MMPR on PRPP, 6-thioGMP, and 6-thioGTP Levels

Earlier studies (25, 33) demonstrated that prior or concurrent treatment with MMPR causes a significant increase in the rate and quantity of 6-thioIMP synthesized after 6-MP administration. In studies with Ehrlich ascites cells, marked increases in the PRPP levels occurred after MMPR treatment (25). These PRPP increases became pronounced after a lag of about 6 hr and lasted for at least 96 hr. In experiments not shown, similar results were obtained with S-180 cells in response to treatment with MMPR alone or in combination with 6-TG. Administration of MMPR caused a 4- to 5-fold increase in PRPP concentrations to levels of about 2.5 mM.
hypoxanthine-guanine phosphoribosyltransferase (25, 33), which supports the hypothesis that the greater availability of PRPP increases the function of the purine salvage enzyme, synthesis occurred concurrently with the elevated PRPP levels, and the levels of UTP were increased more than 2-fold (Chart 4). These effects lasted as long as 48 hr after administration of the drug. The reductions in ATP and GTP were similar except for an apparently greater sensitivity of GTP to the drug seen at 1 hr. The effect of MMPR on ATP and GTP levels is much like the reduction in total adenine pools produced by azaserine, another inhibitor of purine biosynthesis de novo, in S-180 cells (see Fig. 1 in Ref. 30). It appears that the effect of MMPR (presumably via inhibition of de novo purine biosynthesis) is to lower total purine nucleotide pools rather than to shift the energy charge (2), since no marked increase in ADP, GDP, AMP, or GMP occurred in these experiments.

The effect of MMPR on UTP levels may be independent of the effect of the drug on ATP and GTP, since there is a partial recovery of UTP levels to control values between 24 and 48 hr, whereas the ATP and GTP levels are reduced to the same degree at these times after giving the drug (Chart 4).

Simultaneous administration of 6-TG added to the effect of MMPR on ATP and GTP levels and prevented the increase in UTP observed 1 hr after a dose of MMPR alone (Table 2). Unlike MMPR or azaserine, 6-TG did not increase UTP levels at any time studied. This might indicate that the effect of

Effect of MMPR on the Intracellular Half-life of 6-TG-35S

Earlier studies have shown that when S-180 cells are incubated with MMPR there is a marked accumulation of MMPR-5'-P to levels in the range of 1 to 2 mM in a period of 15 to 30 min. Also, MMPR pretreatment was shown to interfere with the conversion of 6-MP to MMPR-5'-P, and it was suggested that the high levels of MMPR-5'-P interfere with an S-methylation reaction (33). Because of this observation, it appeared likely that the high levels of MMPR-5'-P might also interfere with other enzymatic reactions in which 5'-monophosphate nucleotides are utilized. Evidence suggesting that this occurs was obtained when the intracellular half-life of 6-TG-35S in S-180 cells was determined in control animals and in animals pretreated with MMPR (Chart 3). The disappearance of radioactivity from the tumor cells followed 1st-order kinetics in both control and MMPR-pretreated animals, which implies that throughout the time course studied a single rate-limiting step predominated. The half-time of disappearance of the intracellular radioactivity in control animals was about 7 hr, whereas in MMPR-pretreated animals the half-time was increased about 38% to approximately 10 hr. Since the acid-soluble radioactivity consists predominantly of 6-thioGMP in these cells, the observed increase in half-life is consistent with the possibility that the high concentration of MMPR-5'-P impedes the metabolism of 6-thioGMP. The combination of enhanced synthesis and impeded degradation of 6-thioGMP results in an approximately 3-fold greater amount of this metabolite in the cells after a 24-hr period.

Effects of MMPR and 6-TG on Nucleotide Pools. After a single dose of MMPR, the levels of ATP and GTP in S-180 cells were reduced by as much as 50% of control values in 5 to 6 hr, and the levels of UTP were increased more than 2-fold (Chart 4). These effects lasted as long as 48 hr after administration of the drug. The reductions in ATP and GTP were similar except for an apparently greater sensitivity of GTP to the drug seen at 1 hr. The effect of MMPR on ATP and GTP levels is much like the reduction in total adenine pools produced by azaserine, another inhibitor of purine biosynthesis de novo, in S-180 cells (see Fig. 1 in Ref. 30). It appears that the effect of MMPR (presumably via inhibition of de novo purine biosynthesis) is to lower total purine nucleotide pools rather than to shift the energy charge (2), since no marked increase in ADP, GDP, AMP, or GMP occurred in these experiments.

The effect of MMPR on UTP levels may be independent of the effect of the drug on ATP and GTP, since there is a partial recovery of UTP levels to control values between 24 and 48 hr, whereas the ATP and GTP levels are reduced to the same degree at these times after giving the drug (Chart 4).

Simultaneous administration of 6-TG added to the effect of MMPR on ATP and GTP levels and prevented the increase in UTP observed 1 hr after a dose of MMPR alone (Table 2). Unlike MMPR or azaserine, 6-TG did not increase UTP levels at any time studied. This might indicate that the effect of

Chart 3. Effect of MMPR pretreatment on the biological half-life of 6-TG-35S metabolites in S-180 cells in vivo. Mice bearing 4-day implants of S-180 cells were given MMPR, 4 mg/kg, 12 hr before the administration of 6-TG-35S, 2.2 mg/kg (specific activity, 610 cpm/nmole). The cells were removed, and the radioactivity in the acid-soluble extracts was determined at the times shown. Each point represents the mean value determined from 5 mice. The slopes of the 2 lines are significantly different (p < 0.02).
6-TG on ATP and GTP levels is not due to inhibition of purine biosynthesis de novo via pseudofeedback inhibition of PRPP amidotransferase by 6-thioGMP (10, 13, 17, 31, 32). Also, 6-TG alone was without effect on ATP and GTP levels 0.5, 3, 6, or 24 hr after the drug, and the effect of a simultaneous dose of 6-TG and MMPR was the same as MMPR alone at these times (data not shown). A possible explanation for the effect of 6-TG at 1 hr is that partial inhibition of purine (and possibly pyrimidine) biosynthesis de novo occurs shortly after administration of 6-TG due to utilization of PRPP for 6-thioGMP formation. Since the drug disappears rapidly from the injection site (see Chart 2), the utilization of PRPP for 6-thioGMP formation may be transitory. Utilization of PRPP by 6-TG may also account for the prevention of the increase in UTP levels caused by MMPR. Specifically, UTP levels increased from 0.27 to 0.50 μmole/ml cells after MMPR alone, but increased only to 0.34 μmole/ml cells after MMPR and 6-TG (Table 2).

The effects of azaserine to lower ATP and GTP levels and to increase UTP levels are similar to those of MMPR seen 12 to 13 hr after the drugs (Table 2; Chart 4). Administration of a high dose of 6-TG 12 hr after azaserine had been given did not enhance the effects of azaserine on the nucleotide pools.

Since the decreased concentrations of the natural nucleotides in response to drug administration could have been an artifact resulting from the death of significant numbers of cells, the technique of fluorochromasia (28) was applied to determine whether the tumor cells were in a viable state at the time when the nucleotide measurements were made. Cells were examined 12 and 36 hr after treatment with MMPR alone and after simultaneous or sequential treatment with MMPR plus 6-TG. By the use of this technique, no significant loss of cellular viability was detected at the time of measurement. Approximately 90 to 95% of the cells in the treated and control groups appeared viable. Although a large percentage of these cells are destined to die in response to combined treatment with MMPR and 6-TG, a significant degree of cellular lysis had not occurred at the time of biochemical measurements performed in these studies.

6-TG-35S Metabolites and Nucleotide Pools in S-180 Cells

The nucleotide profile of S-180 cells is shown in the upper figure of Chart 5. The adenine and guanine nucleotides were present primarily in the form of ATP and GTP whether or not the cells were treated with MMPR, 6-TG, or the combination of 6-TG and MMPR. The effect of 6-TG on ATP, GTP, or UTP 1 hr after giving the drug was insignificant or less than the effect of MMPR at 13 hr (Chart 4; Table 2); therefore, the dramatic effect of MMPR to lower the ATP and GTP levels while increasing the UTP level is well demonstrated in Chart 5. The adenine and guanine nucleotides were measured in the acid-soluble extracts. Each point represents the mean of values determined separately for cells removed from each of 4 to 7 mice. All mean values 1 hr or later were significantly different (p < 0.05) from simultaneous controls, except for the ATP level measured 1 hr after MMPR. Control values are given in Table 2.

Chart 4. Effects of MMPR on nucleotide levels in S-180 cells. MMPR, 4 mg/kg, was administered to mice 4 to 5 days after implantation of the tumor cells. At the times shown the cells were removed, and the nucleotide levels were measured in the acid-soluble extracts. Each point represents the mean of values determined separately for cells removed from each of 4 to 7 mice. All mean values 1 hr or later were significantly different (p < 0.05) from simultaneous controls, except for the ATP level measured 1 hr after MMPR. Control values are given in Table 2.

<table>
<thead>
<tr>
<th>First treatment</th>
<th>Second treatment</th>
<th>ATP</th>
<th>GTP</th>
<th>UTP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>2.57 ± 0.18</td>
<td>0.95 ± 0.07</td>
<td>0.27 ± 0.04</td>
<td>a</td>
</tr>
<tr>
<td>None</td>
<td>6-TG, 1 mg/kg</td>
<td>1.98 ± 0.20</td>
<td>0.64 ± 0.05b</td>
<td>0.24 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>0.9% NaCl</td>
<td>6-TG, 6.5 mg/kg</td>
<td>1.92 ± 0.24</td>
<td>0.63 ± 0.13b</td>
<td>0.19 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>MMPR</td>
<td>2.32 ± 0.07</td>
<td>0.62 ± 0.05b</td>
<td>0.50 ± 0.04b</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>6-TG, 1 mg/kg + MMPR</td>
<td>1.56 ± 0.08b</td>
<td>0.42 ± 0.05b</td>
<td>0.34 ± 0.03</td>
<td>6</td>
</tr>
<tr>
<td>Azaserine</td>
<td>0.9% NaCl</td>
<td>1.11 ± 0.09b</td>
<td>0.43 ± 0.06b</td>
<td>0.50 ± 0.03b</td>
<td>3</td>
</tr>
<tr>
<td>Azaserine</td>
<td>6-TG, 6.5 mg/kg</td>
<td>1.07 ± 0.02b</td>
<td>0.49 ± 0.01b</td>
<td>0.57 ± 0.03b</td>
<td>3</td>
</tr>
</tbody>
</table>

a Value determined for 39 mice in 7 individual experiments. Other values of n refer to numbers of mice.
b Significantly different from no treatment controls (p < 0.05).
c Doses in mg/kg were MMPR, 4; azaserine, 2.
Chart 5. High-pressure liquid chromatograph of acid-soluble material from S-180 cells. Cells were removed from mice 1 hr after administration of 6-TG-$^{35}$S, 6.5 mg/kg (specific activity, 500 cpm/nmole). MMPR, 4 mg/kg, was given 12 hr before 6-TG-$^{35}$S to one group of 5 mice, and another group of 5 mice was not pretreated. The combined acid-soluble extracts of the tumor cells from each group were concentrated and applied to the column. Upper tracing, nucleotide profile of the extract of about 4.5 mg of cells; lower tracing, radioactivity eluting from the LCS-1000 instrument at 2-min intervals (see "Materials and Methods"), and representing the extract of about 9 mg of the same cells. TU, 6-thiouric acid; TGMP, TGDP, and TGTP, 6-thioGMP, 6-thioGDP, and 6-thioGTP, respectively.

form of 6-thioGMP and 6-thioGTP (Chart 5, lower tracing). Since the acid-soluble material from about 9 mg of cells is shown, the levels of 6-thioGMP were of the order of 0.45 and 0.10 μmole/ml cells in MMPR-pretreated and control samples, respectively. The corresponding levels of 6-thioGTP were of the order of 0.20 and 0.04 μmole/ml cells. The acid-soluble metabolites shown in Chart 4 were from cells of animals treated with a high dose of 6-TG. However, qualitatively similar results were obtained 1 to 24 hr after a dose of 6-TG of only 1 mg/kg (data not shown) and in previous studies of the metabolism of 6-TG (3, 20).

6-TG-$^{35}$S Metabolism by S-180 Cells Resistant to 6-TG. The prolongation of the biological $t_{1/2}$ of 6-TG-$^{35}$S by MMPR pretreatment (Chart 3) indicated that MMPR-5'-P may interfere with the catabolism of 6-thioGMP. Dr. A. C. Sartorelli and his colleagues have developed a S-180 cell line that is resistant to 6-TG due to an increase in the rate of catabolism of 6-thioGMP by a nonspecific phosphohydrolase (3, 34). This tumor provided a model to test whether or not MMPR-5'-P interferes with the breakdown of 6-thioGMP. The $t_{1/2}$ of 6-TG-$^{35}$S acid-soluble metabolites is only about 3 hr in the resistant cell line (Chart 6), compared to a $t_{1/2}$ of about 7 hr in the sensitive tumor (Chart 3). Pretreatment with MMPR increased the $t_{1/2}$ to greater than 6 hr in the resistant tumor. Although azaserine pretreatment, like MMPR, increased the uptake of 6-TG-$^{35}$S by the tumor cells, azaserine pretreatment did not increase the $t_{1/2}$. The $t_{1/2}$ of 6-thioGMP was the same as that for the total acid-soluble radioactivity in the control and MMPR-pretreated samples, i.e., 3.4 and 7.1 hr, respectively, by high-pressure liquid chromatography of the samples shown in Chart 6. These data strongly indicate that MMPR-5'-P interferes with the catabolism of 6-thioGMP via inhibition of a phosphohydrolase in the tumor cells.

The acid-soluble metabolites of 6-TG-$^{35}$S in the S-180/TG cells are shown in Table 3. There is a greater amount of 6-TG and 6-TGR in the resistant cells than in the sensitive tumor (Chart 5). This finding is consistent with a lower concentration of 6-thioGMP maintained by an increased phosphohydrolase activity in this cell line (34). MMPR pretreatment increased the level of 6-thioGMP and reduced the levels of 6-TG and 6-TGR in the cells, a result which would be expected from inhibition of the phosphohydrolase by MMPR-5'-P. As determined by thin-layer chromatography (polyethyleneimine-cellulose with water as the solvent), there were about equal
amounts of 6-TG and 6-TGR present in the samples shown in Table 3. MMPR pretreatment also increased the levels of 6-thioGTP about 3-fold in the S-180/TG tumor.

**DISCUSSION**

The mechanism by which MMPR potentiates the effect of 6-TG in S-180 cells may be similar to that from the MMPR and 6-MP synergism described previously (24, 25, 33). Specifically, MMPR increases the steady-state levels of PRPP, thereby permitting an increased formation of 6-thioGMP from 6-TG or of 6-thioIMP from 6-MP. In the present report it was also noted that MMPR protects 6-thioGMP from metabolic degradation. It appears likely that an alternative substrate effect occurs. The high concentrations of MMPR-5'-P found in the tumor cells may compete for a 5'-nucleotidase or phosphohydrolase that degrades 6-thioGMP. Since the product of MMPR-5'-P cleavage, MMPR, is a poor substrate for purine nucleoside phosphorylase (14), MMPR can recycle by reacting with adenosine kinase. This may account for the maintenance of high MMPR-5'-P levels in the cell for long periods of time.

There was a marked fall in purine nucleotides after MMPR and 6-TG treatment, with a greater effect on GTP than ATP (Table 2). The opposite was found with MMPR and 6-MP, i.e., a greater effect on ATP than GTP (33). The greater inhibitory effect of 6-TG on guanine metabolism and the accumulation of high levels of 6-thioGMP in S-180 cells (Chart 5) are consistent with a metabolic block at the level of guanylate kinase (18, 19). The concentrations of 6-thioGMP in the tumor cells, especially when the 6-TG and MMPR combination is given, exceed that of GMP by 1 to 2 orders of magnitude. The presence of 6-thioGTP in cells treated with 6-TG is in accord with the earlier observations that 6-thioGMP is an alternative substrate with a low maximal velocity for guanylate kinase (19) and that 6-TG is incorporated into the DNA of various tumors (15). Thus, the 6-thioGTP:6-thioGMP ratio strongly favors the 5'-monophosphate nucleoside, whereas the opposite is true with the GTP:GMP ratio (Chart 5). Also, the low level of 6-thioGDP implies that the 6-thioGDP to 6-thioGTP conversion is rapid relative to the 6-thioGMP to 6-thioGDP conversion.

Although a decrease in adenine and guanine nucleotides occurs as would be predicted from the postulated sites of enzymatic blockade by MMPR and 6-TG, it is not possible to conclude without further evidence that this decrease is directly related to lethal effects. This matter has been discussed in detail in an earlier report (33).

Azaserine, like MMPR-5'-P, is an inhibitor of the de novo pathway of purine biosynthesis and also increases the formation of 6-thioGMP from 6-TG (29). When tested in the S-180/TG tumor, a marked increase in 6-thioGMP synthesis occurred as expected, but azaserine did not increase the t1/2 of 6-thioGMP as did MMPR (Chart 6). Thus, it appears that azaserine made more PRPP available for 6-thioGMP synthesis but did not interfere with the degradation of 6-thioGMP. In contrast, MMPR treatment of the S-180/TG tumor increased both the synthesis of 6-thioGMP and its biological half-life. This observation is consistent with the hypothesis that MMPR-5'-P protects 6-thioGMP by competing with the phosphohydrolase that appears responsible for the rapid degradation of 6-thioGMP in this resistant tumor line (34).

The increase in UTP levels observed following MMPR treatment might be explained by an increased availability of PRPP for orotic acid phosphoribosyltransferase and resultant enhancement of pyrimidine nucleotide synthesis. The possibility exists that MMPR might also potentiate the action of 5-fluorouracil since a pyrimidine phosphoribosyltransferase has been shown to be responsible for 5-fluorouridine 5'-phosphate formation (26, 27). Furthermore, if MMPR-5'-P proves to be a potent alternative substrate for 5'-nucleotidases or phosphohydrolases as indicated above, perhaps the degradation of analog nucleotides other than 6-thioGMP will be inhibited. These speculations are the subject of continued investigation.

**REFERENCES**

Biochemical Mechanisms for the Synergism between 6-Thioguanine and 6-(Methylmercapto)purine Ribonucleoside in Sarcoma 180 Cells

J. Arly Nelson and R. E. Parks, Jr.