Synergistic Effect of 6-Mercaptopurine and 6-Methylmercaptopurine Ribonucleoside on the Levels of Adenine Nucleotides of Sarcoma 180 Cells

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SUMMARY

The synergistic antitumor action of 6-mercaptopurine (6-MP) and 6-methylmercaptopurine ribonucleoside (MMPR) is confirmed with Sarcoma 180 ascites cells. Striking decreases are seen in the adenine nucleotide concentrations in cells incubated for 1 hr with this drug combination, and lesser effects are seen on the guanine nucleotide concentrations. Still greater decreases in adenine nucleotide levels are observed when the cells are preincubated for 30 min with MMPR before addition of 6-MP.

The 5'-monophosphate nucleotides of 6-MP and MMPR were synthesized in Sarcoma 180 cells without mutual interference and, in addition, stimulation of 6-thiinosine 5'-phosphate formation occurred when the cells were preincubated with MMPR. The formation of MMPR-5'-P from 6-MP shown in other tumors was demonstrated in Sarcoma 180 cells. It was also shown that this conversion was inhibited in cells incubated with MMPR, suggesting a product inhibition of the enzyme system responsible for this S-methylation.

INTRODUCTION

The hypoxanthine analog, 6-MP, and its derivative, azathioprine, are active antileukemic and immunosuppressive agents in humans. An extensive literature exists on their use and mechanism of action (14-16, 19, 37). Recently, it has been shown that MMPR, a weak antitumor agent when administered alone, produces significant synergistic effects when administered in combination with 6-MP (6, 36, 39).

The metabolic pathways and the specific enzymes believed important in the antitumor actions of 6-MP and MMPR are presented in Chart 1. Here are shown the 2 different enzymes for mononucleotide formation (6-MP, Enzyme 3; MMPR, Enzyme 6) and the enzymes at which clear-cut metabolic blocks have been demonstrated (Enzymes 1, 2, 4, and 5) (5, 7, 10, 18, 20). It is significant that with this drug combination the formation of analog containing 5'-monophosphate nucleotides occurs by 2 distinct enzymes that do not mutually interact.

Using combinations of 6-MP and MMPR with Ehrlich ascites cells, Wang et al. (39) obtained results that indicate that the formation of both 6-thioIMP and MMPR-5'-P are necessary for synergism to occur and that EAC-R1 cells are resistant to this combination because of an impairment in the formation of 6-thioIMP. In intact tumor cells the presence of 6-MP does not interfere with formation of MMPR-5'-P; whereas the addition of MMPR actually enhances the formation of 6-thioIMP (39). These authors suggest that increased formation of 6-thioIMP may be the key factor in this drug synergism. In a related study, Paterson and Moriwaki (28) found similar results with L5178Y cells in culture and in vivo. Pretreatment with MMPR enhanced 6-thioIMP formation. It was suggested that this was the result of the diversion of PRPP to the hypoxanthine-guanine phosphoribosyl transferase reaction by which 6-MP is converted to 6-thioIMP. MMPR-5'-P is a potent inhibitor of PRPP amidotransferase and thus might block the utilization of PRPP by the de novo pathway of purine nucleotide synthesis. Since the formation of 6-thioIMP is an obligatory process in the mechanism of action of 6-MP, the hypothesis was offered that the enhancement of 6-thioIMP synthesis in tumor cells resulting from exposure to MMPR may be the mechanism of the synergism.

The studies reported here examine the dynamics of synthesis of the nucleotides of 6-MP and MMPR when incubated alone, or in combination, as well as the rate of formation of MMPR-5'-P from 6-MP. These studies confirm with isolated Sarcoma 180 cells a number of the observations made in other laboratories (1, 5, 28, 31, 36). If the hypothesis is valid that an inhibitory effect is produced by 6-thioIMP and MMPR-5'-P on the synthesis and/or interconversions of normal nucleotides, one would expect to find this inhibition reflected in effects on the steady-state concentrations of natural purine nucleotides of the cell. Therefore, the influence of the analogs alone or in combination on the nucleotide concentrations in Sarcoma 180 cells is examined.
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Chart 1. Schematic diagram of the sites of action of 6-MP and MMPR on nucleotide synthesis and interconversions. See text for discussion. The enzymes indicated by number are:

<table>
<thead>
<tr>
<th>Enzyme No.</th>
<th>Common name</th>
<th>EC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>En-1</td>
<td>PRPP amidotransferase</td>
<td>2.4.2.14</td>
</tr>
<tr>
<td>En-2</td>
<td>IMP dehydrogenase</td>
<td>1.2.1.14</td>
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<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
<td>2.4.2.8</td>
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<td>Adenylosuccinate synthetase</td>
<td>6.3.4.4</td>
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<td>Adenylosuccinate lyase</td>
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<tr>
<td>En-6</td>
<td>Adenosine kinase</td>
<td>2.7.1.20</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

MMPR-14CH3 was synthesized by Dr. Shih-Hsi Chu of this laboratory; 6-MP-8-14C (25 mCi/m mole) was obtained from Schwarz BioResearch, Inc. (Orangeburg, N. Y.); MMPR was purchased from Aldrich Chemical Co., Inc. (Cedar Knolls, N. J.); 6-MP and Tris (enzyme and buffer grade) were from Mann Research Laboratories (New York, N. Y.); 6-MPR, 6-TG, the barium salt of 6-thioIMP, the lithium salt of coenzyme A, and the sodium salts of GDP, GTP, and ATP were obtained from P-L Biochemicals (Milwaukee, Wis.). The sodium salts of NADP and phosphoenolpyruvate, 6-thioxanthine, pyruvate kinase (rabbit skeletal muscle, type II, crystalline suspension, 625 units/mg), hexokinase (yeast, type C-130, 130 units/mg), lactic acid dehydrogenase (rabbit muscle, 550 units/mg), and 5′-nucleotidase (*Crotalus adamanteus* venom, Grade II, 18 units/mg protein) were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Succinate thiokinase was prepared from pig heart by A. F. Ross of this laboratory according to a method described by Cha et al. (11, 13) and had a specific activity of about 15 enzyme units/mg of protein. The anion-exchange resin, AG-1-X10 (Cl−), 200 to 400 mesh, was purchased from Bio-Rad Laboratories (Richmond, Calif.) and was converted to the formate form by successive washing with 1.0 N NaOH and 1.0 N formic acid. Plastic sheets coated with PEI-cellulose for thin-layer chromatography [MN-Polygram-Cel-300-PEI (Machery-Nagel and Co., Duren, Germany)], and plastic sheets coated with cellulose (Cellulose MN 300) were supplied by Brinkmann Instruments, Inc. (Westbury, N. Y.). The PEI sheets were washed with 10% NaCl and water, followed by ascending irrigation with water (33) and stored at 0–4°.

Whatman No. 4 cellulose paper was obtained from H. Reeve Angel and Co. (Clifton, N. J.).

Tumor Strain. The initial inoculum of Sarcoma 180 cells was obtained from Dr. A. C. Sartorelli of Yale University and was maintained in female CF1 mice (Carworth, Inc., New City, N. Y.) by the i.p. transplantation of 5 to 6 × 106 tumor cells each week.

Survival Time Studies. Mice were inoculated, each with 1 million tumor cells (taken from a pool of tumor cells collected after 7 days growth in donor mice). Drug doses were based on the average weight of mice in each treatment group at the start of the experiment. Antitumor agents were administered by i.p. injection at 24-hr intervals starting 24 hr after tumor
transplantation, for a total of 5 doses. Drugs were administered in 0.9% NaCl solution. Control mice received injections of 0.9% NaCl solution. The time of death of the mice was recorded to the nearest 12 hr.

Preparation of Cell-free Extracts. Sarcoma 180 ascites cells, obtained from mice 5 to 6 days after implantation, were freed of ascitic fluid and erythrocytes by repeated low-speed centrifugation in an isotonic, buffered NaCl wash medium of Tris-HCl buffer, pH 7.4, 10 mM; potassium phosphate buffer, pH 7.4, 4 mM; NaCl, 140 mM; and glucose, 5.5 mM (40). Centrifugation and washing were repeated 3 to 4 times as above and finally with the incubation medium described below. Packed cell volume was determined in an Adams Autocrit centrifuge. The cells were suspended in an incubation medium of Tris-HCl buffer, pH 7.4, 40 mM; potassium phosphate buffer, pH 7.5, 22 mM; NaCl, 88 mM; KCl, 20 mM; MgCl₂·6H₂O, 2 mM; and glucose, 15 mM (40).

The cell suspension was preincubated at 37° in a Dubnoff metabolic incubator in an air atmosphere for 10 min, and each experiment was then initiated by the addition of the appropriate analog or combination of analogs. 6-MP was dissolved in the incubation medium without glucose with the aid of a few drops of 1 N NaOH (final pH was 8 to 8.5). When 6-MP was used, the incubation medium was such that upon dilution with the 6-MP solution the glucose concentration was 15 mM. In experiments with both radioactive and nonlabeled 6-MP, the final concentration of the 6-MP was 1.3 mM. When 6-MP-8-¹⁴C was used, there were approximately 3 × 10⁶ cpm/ml of incubation medium. MMPR was dissolved in water to a final concentration of 0.5 mM. With MMPR,¹⁴CH₃ there were approximately 2.5 × 10⁶ cpm/ml of incubation medium. The time of incubation was 60 min except where indicated in the results. Samples were removed at the indicated times and added with dropwise addition to rapidly mixed chilled tubes containing equal volumes of 8% perchloric acid to extract the acid-soluble components. After removal of the denatured protein, the extracts were neutralized with KOH to pH 6.7 to 7.0. The neutralized extracts were kept at 4° for at least 30 min, and the insoluble potassium perchlorate was removed by centrifugation and filtration. The extracts were stored at −80° for further study.

Determination of Adenine and Guanine Nucleotide Pools. Two different methods were used for the measurement of adenine and guanine nucleotides: the use of enzymatic assays and the use of high-speed liquid chromatography with the Varian Aerograph LCS-1000. ATP was measured by a coupled hexokinase-glucose 6-phosphate dehydrogenase assay in which the appearance of NADH was measured fluorometrically (22). The assay was slightly modified so that the reaction was stopped by the addition of an excess of EDTA. The ATP solutions used for determination of the standard curves were pretreated in a manner identical to that for the test solutions, i.e., stock ATP solutions were prepared in 4% perchloric acid and neutralized with KOH prior to dilution. GDP and GTP levels were measured by an enzyme cycling technique developed by Cha and Cha (12). In this system GTP and GDP are cycled by coupled succinyl thio kinase and pyruvate kinase reactions in which the pyruvate accumulated. The amount of pyruvate that accumulated is directly proportional to the concentration of GTP or GDP. The procedure was modified so that the NAD⁺ formed during the reaction of accumulated pyruvate with lactic acid dehydrogenase was measured fluorometrically after alkalization. The use of fluorimetry increased the sensitivity of the assay 10-fold so that 10⁻¹ⁱ mole of GDP or GTP could be measured in the reaction mixture. In these experiments the perchloric acid extracts were diluted sufficiently so that there was no interference with the enzyme-cycling procedures (12).

In most experiments the concentration of nucleotides in cellular extracts was estimated through the use of a liquid-chromatographic system (Varian Aerograph LCS-1000) which includes anion-exchange liquid chromatography under pressure (8). This system allows for determination of the nucleotide profile of the cell extracts both qualitatively and quantitatively in about 60 min. An aliquot of the neutralized cellular extracts (8 µl) was injected into the column. The nucleotides were eluted with a linear gradient system of 0.015 M KH₂PO₄ to 0.25 M KH₂PO₄ in 2.2 M KCl under the standardized conditions described by Brown (8). The nucleotide concentrations were estimated by determining the area under the peak by multiplying the height of the peak by the width at half height, since the peaks were symmetrical. In preliminary experiments, excellent agreement (within 2%) was demonstrated in the ATP concentrations determined by the enzymatic assays and by use of the Varian Aerograph LCS-1000 (8).

Adenylate Energy Charge. Adenylate energy charge was calculated by the following formula:

\[
\frac{(ATP + 1/2 ADP)}{(ATP + ADP + AMP)}
\]

as described by Atkinson (2).

Measurement of Analog Nucleotide Formation. Aliquots of neutralized cellular extracts were chromatographed in a descending system on Whatman No. 4 paper with the use of Solvent System A to develop the chromatogram (1). In each case the extracts were cochromatographed with authentic standards in order to locate the various metabolites by their fluorescence or absorption under UV light. For determination of radioactivities, the pertinent areas of the chromatograms were cut out and counted directly in a toluene scintillation solution (23). When the cells were incubated with radioactive MMPR either alone or in combination with 6-MP, only 2 radioactive areas were found, that of MMPR¹⁴CH₃ and MMPR-⁵'-P with Rᶠ values of 0.76 and 0.33, respectively. When 6-MP-8-¹⁴C was the substrate, the chromatogram was more complex since radioactive areas corresponding to 6-thioIMP, MMPR-⁵'-P, 6-MPR, and 6-MP were found with Rᶠ values of 0.10, 0.33, 0.40, and 0.53, respectively. In this case, the difficulty presented by the similar Rᶠ values of 6-MPR and MMPR-⁵'-P was overcome by allowing the chromatograms to run for 4 to 8 additional hours and permitting the solvent front to run off the paper. By this means nonoverlapping separations of 6-MPR and MMPR-⁵'-P of between 1 and 0.2 cm could be achieved. Recognition of the 2 spots was aided by the fact that the 2 components have different colors of fluorescence. MMPR-⁵'-P fluoresced bluish-white under UV light at 254 nm, and 6-MPR fluoresced greenish-yellow under UV light at 350 nm. In addition to the metabolites mentioned there is the possible occurrence of thioIMP which might be generated by IMP dehydrogenase. Since the 2 compounds appear to migrate closely, it is possible
that small amounts of thioXMP may occur in the thiolIMP spot. The original solutions of MMPPR-8-14C and 6-MP-8-14C were chromatographed and were found to be 99 and 95% pure, respectively, by recovery of added radioactivity in the appropriate spot.

Identification of the Analog Nucleotides. Spots tentatively identified as 6-thioIMP and MMPPR-5'-P were eluted from Whatman No. 4 paper with water. Recoveries close to 100% were obtained in the first 1 ml of eluent. In order to confirm the identity of these nucleotides the following methods were used: (a) spectrophotometry; (b) rechromatography in 3 or 4 solvent systems; (c) hydrolysis by snake venom (C. adamanteus) 5'-nucleotidase to the nucleoside and subsequent identification of the nucleoside by paper and thin-layer chromatography as described below. The enzymic hydrolysis with 5'-nucleotidase was conducted for 30 min at 37° in a solution containing Tris-acetate buffer, pH 8.6, 0.13 M; MgCl2, 0.001 M; 1 mg of enzyme; and nucleotide substrate (27); (d) conversion of the nucleotide upon hydrolysis in 1 N HCl for 1 hr to the purine base with further identification of the nucleoside base in at least 2 chromatographic systems.

Chromatography Systems. The following chromatography systems were used in identification of the nucleotide analogs: A, equal parts 93.8% aqueous 1-butanol and 44% aqueous propionic acid (1); B, equal volumes 5% Na2HPO4 and isoamyl alcohol (24); C, 0.5 M LiCl; D, 5% Na2HPO4/5% isopropyl alcohol (3/2) (21); E, equal volumes of 5% Na2HPO4 and 5% isoamyl alcohol; F, 1-butanol/methanol/H2O/NH4OH (60/20/20/1); G, 1-butanol/acetic acid/water (25/15/10) (21). A and B were used with Whatman No. 4 paper in a descending system; C was used with PEI thin-layer chromatography; and D, E, F, and G were used with cellulose thin-layer chromatography.

RESULTS

Survival Time Studies. The synergistic effects of 6-MP and MMPPR combinations in mice bearing Sarcoma 180 ascites tumor were studied in experiments designed to utilize the logarithmic kill principles elucidated by Skipper (38). As seen in Table 1, Experiment A, when the compounds were administered individually at doses of 4 mg/kg, no increase in life-span was seen with 6-MP alone and only a slight increase in survival time of questionable significance was produced by MMPPR alone. When both analogs were tested in combination at one-half the individual dose, a significant prolongation of survival time was observed which was the equivalent of about a 2 log cell-kill, and 2 mice survived more than 30 days. When the 2 drugs were given in full doses of 4 mg/kg each, a striking synergistic effect was observed with 5 of 20 mice surviving for more than 30 days. Thus it is seen that this drug combination brings about a marked synergism in the antitumor effectiveness of these 2 agents. Since 10⁶ cells/animal were administered in this experiment, this result represents a 4 to 6 logarithmic cell-kill. The selection of 30 days for long-term survival is based on the observation that the generation time of the Sarcoma 180 cells used in these studies is approximately 24 hr and on the assumption that death occurs when approximately 2 g or about 1 X 10⁹ tumor cells are present. From the equation 1 X 10⁹ = 2ⁿ, where n = no. of generations, death of the animals occurs after about 30 generations or 30 to 32 days (38). Therefore, if an animal that received 1 X 10⁶ tumor cells survived for 30 or more days, it is assumed that fewer than 10 tumor cells survived the therapeutic regimen.

Although biochemical studies on 6-TG are not the topic of the present communication and will be considered later, an antitumor experiment similar to that of Table 1 was performed with 6-TG for comparison (Table 1, Experiment B). Here it was seen that 6-TG in doses of 1 or 2 mg/kg administered alone did not increase the survival time of mice inoculated with 1 X 10⁶ Sarcoma 180 cells, whereas MMPPR in doses of 4 and 8 mg/kg gave slight increases in survival time equivalent to an approximately 1 to 2 logarithmic cell kills. However, when both drugs were given in combination, a striking synergism occurred. For example, at doses of 1 mg/kg of 6-TG and 8 mg/kg of MMPPR, the average survival time of the mice was approximately 23 days and in addition 2 mice of the 10 were long-term survivors. Similar evidence of multilog cell kill occurred at higher concentrations of this drug combination. It is apparent that a striking synergistic effect occurs although perhaps by a different mechanism than that with the combination of 6-MP and MMPPR.

Formation of Analog Nucleotides by Isolated Sarcoma 180 Ascites Cells

Effect of 6-MP-8-14C or 6-MP-8-14C + MMPPR on Analog Nucleotide Formation. Since 6-MP or MMPPR must be converted to their respective nucleotides in order to have antitumor or immunosuppressive activity, it is important that attempts be made to correlate intracellular concentrations of analog nucleotides with any biological or biochemical event observed. Therefore, Experiments A and B of Table 2 were performed. Sarcoma 180 ascites cells were incubated with 6-MP-8-14C or 6-MP-8-14C + MMPPR. As seen in Table 2, Experiment A, the concentration of 6-thioIMP formed was similar when 6-MP was added alone or when both drugs were added simultaneously. As will be discussed below, enhanced synthesis of 6-thioIMP occurred on preincubation with MMPPR. The concentrations of 6-thioIMP achieved were in the range of 10 to 100 times greater than the concentrations of GMP or IMP that occur in most tissues and are similar to the 6-thioIMP levels found in Ehrlich ascites cells by Paterson (27). Table 2 shows that under these experimental conditions a significant amount of MMPPR-5'-P-8-14C was formed from 6-MP-8-14C. This confirms the prior observations of Allan et al. (1) with human epidermoid carcinoma cells (H. Ep. 2) grown in suspension culture and of Caldwell (9) with Ehrlich ascites carcinoma cells that MMPPR-5'-P may be formed from 6-MP.

Effect of MMPPR-14CH₃ or MMPPR-14CH₃ + 6-MP on Analog Nucleotide Formation. As shown in Table 2, Experiment B, when MMPPR-14CH₃ was incubated for 1 hr alone or in combination with 6-MP, essentially identical amounts of the analog nucleotide, MMPPR-14CH₃-5'-P, were formed. Relatively high concentrations of MMPPR-5'-P accumulate under these conditions and reach levels that are similar to the ATP concentrations normally found in these
cells (about 1.5 to 3 mM). In the paper chromatograms used in these studies, no radioactivity was found in the position where the polyphosphate nucleotides would be located. This is consistent with the concept that MMPR-5'-P is not a substrate for 5'-monophosphate nucleotide kinases.

Time Course of Analog Nucleotide Formation after Incubation with 6-MP-8-14C or 6-MP-8-14C + MMPR. When the short time course of analog nucleotide formed from 6-MP-8-14C was examined (Chart 2), it was seen that 6-thiopolIMP synthesis was preceded by a lag period of about 20 min. This lag period occurred with either 6-MP alone or with 6-MP + MMPR but was shortened by about 50% in the combination treatment. In addition to the more rapid onset, the formation of MMPR-5'-P-8-14C from 6-MP-8-14C was reduced about 50%, whereas this reaction was almost entirely abolished when the cells were preincubated for 30 min with MMPR. This suggests that MMPR or its nucleotide is a potent inhibitor of the S-methylation of 6-MP or its nucleotide. Although the S-methylation of 6-MP and of 6-thiopolIMP have been shown with bacterial and mouse liver systems (34), the mechanism by which this methylation and its inhibition occurs in tumor tissue is obscure and deserves study. Since MMPR is not a substrate for the purine phosphoribosyl transferase (5) or for the polyphosphate nucleotides would be located. This is consistent with the concept that MMPR-5'-P is not a substrate for 5'-monophosphate nucleotide kinases.
nucleotide synthesis was observed. MMPR-5'-P accumulated rapidly in the cells for the 1st 10 min, reaching a level of about 1.2 mM. This was followed by a plateau lasting about 30 min, a highly significant decrease in the ATP concentration (averaging about 53%) occurred and even more profound decreases in ATP concentrations (averaging about 74%) were seen when cells were preincubated with MMPR for 30 min prior to addition of 6-MP.

In control experiments run under the conditions used in Table 3, the nucleotide levels remained constant over the 1-hr incubation period. In addition, on microscopic examination, there was no evidence of cellular swelling, shrinking, or breakdown, and drug-treated cells were indistinguishable in their appearance from control cells. Also, the pH of the incubation medium remained constant.

Effects on Nucleotide Profiles. Chart 4 shows a set of tracings obtained with the Varian Aerograph LCS-1000 when 8-μl aliquots of cell extracts were subjected to gradient elutions. The top graph is a tracing obtained with an extract of cells prior to incubation with drug. The middle and bottom graphs are from extracts of cells incubated for 30 and 60 min, respectively, with a combination of 6-MP and MMPR. Here the peak of MMPR-5'-P formation is clearly seen, and the marked decrease in the adenine nucleotide concentrations is also apparent. Since the LCS-1000 has a UV detector which operates at 254 nm, compounds such as 6-thioIMP (A_max = 323 nm) and MMPR-5'-P (A_max = 292 nm) do not give absorbance

Effects on ATP and GTP + GDP Levels. Table 3 summarizes the results of a number of experiments in which the effects on the ATP and GTP + GDP concentrations were examined in response to 1 hr of incubation with 6-MP, MMPR, or both drugs in combination. The concentrations of nucleotides in the control or zero-time samples varied from one experiment to the next, and this is explained by the fact that these experiments were performed over a 6-month period with Sarcoma 180 cells that differed slightly in conditions of isolation and in time after inoculation of the donor animals. However, in each experiment the cells incubated with drug were compared with an identical aliquot of cells incubated in the absence of drug. The effect on the guanine nucleotide concentration was relatively minor with decreases of 16 to 27% which are of questionable significance. Also, 6-MP and MMPR, when incubated alone, caused only slight decreases of questionable significance in the ATP concentrations. However, when 6-MP and MMPR were incubated in combination for 1 hr, a highly significant decrease in the ATP concentration (averaging about 53%) occurred and even more profound decreases in ATP concentrations (averaging about 74%) were seen when cells were preincubated with MMPR for 30 min prior to addition of 6-MP.

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Synergistic Effect of 6-MP and MMPR

Chart 3. Time course of MMPR-14CH3-5'-P formation in Sarcoma 180 cells after incubation with MMPR-14CH3 and MMPR-14CH3 + 6-MP. Two g of washed Sarcoma 180 cells were incubated for 60 min with MMPR-14CH3 (0.5 mM) or MMPR-14CH3 + 6-MP (1.2 mM). Final incubation volume was 12 ml. One-mi aliquots were removed at the indicated times and analyzed for MMPR-14CH3-5'-P on the neutralized extracts as described in “Materials and Methods.” Each point represents the average of 2 separate determinations.

**Table 3**

*Adenine and guanine nucleotide levels after incubation of Sarcoma 180 cells with 6-MP, MMPR or 6-MP + MMPR*

One g of washed Sarcoma 180 cells was incubated for 1 hr with either 6-MP, MMPR, or a combination of both. In 1 case the cells were preincubated with MMPR for 30 min before addition of 6-MP. Extracts were prepared as described in “Materials and Methods.” Adenine and guanine nucleotide levels were assayed by either enzymatic cycling or on the Varian analyzer.

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Estimated intracellular ATP concentration (mM)</th>
<th>% change</th>
<th>p</th>
<th>Estimated intracellular GDP + GTP concentration (mM)</th>
<th>% change</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.68 ± 0.53</td>
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<td>0.54 ± 0.02</td>
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<tr>
<td>6-MP</td>
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<td>0.05</td>
<td>0.39 ± 0.03</td>
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<tr>
<td>MMPR</td>
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<td>0.1</td>
<td>0.72 ± 0.07</td>
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<tr>
<td>Control</td>
<td>2.50 ± 0.42</td>
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<td></td>
<td>0.73 ± 0.10</td>
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<tr>
<td>6-MP + MMPR added simultaneously</td>
<td>1.15 ± 0.52</td>
<td>-53</td>
<td>0.01</td>
<td>0.54 ± 0.11</td>
<td>-25</td>
<td>0.1</td>
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<tr>
<td>Control</td>
<td>3.08 ± 0.21</td>
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<tr>
<td>MMPR added 30 min prior to 6-MP</td>
<td>0.80 ± 0.18</td>
<td>-74</td>
<td>0.01</td>
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</tbody>
</table>

*Mean ± S.E.

peaks as great as those of other compounds present in equivalent concentrations. It is possible, however, to identify the 6-thioIMP peak in the cell extracts when concentrations reach approximately 0.1 mM, but it is often seen only as a shoulder on the MMPR-5'-P peak. There has been little difficulty in identifying the formation of MMPR-5'-P in these extracts because of the relatively large amounts of this analog nucleotide formed (in the range of 1 mM). Of course, the value of this instrument in the study of analog nucleotides would be greatly increased if it could be operated at wavelengths closer to the absorbance maxima of these analog nucleotides.

**Time-Course Studies of Adenine Nucleotide Levels.** Since the decreases in ATP concentration seen in Table 3 could have resulted from decreases in the energy charge (2) of the cell that caused redistribution of the adenine nucleotides among ATP, ADP, and AMP, without significant effects on adenine nucleotide synthesis or breakdown, the experiments of Charts 5 and 6 were performed. In these experiments, the concentrations of AMP, ADP, and ATP were determined at various times during 1 hr of incubation following the initiation of drug treatment. Chart 5 (upper) shows the effects on the adenine nucleotide levels when Sarcoma 180 cells were treated with 6-MP alone. With MMPR alone similar results were obtained, but they are not presented for the sake of brevity. In these experiments slight decreases in the ATP concentrations were seen only at 30 to 35 min after the start of treatment, but no significant effect was seen on the total adenine nucleotide concentrations or any individual nucleotide. However, in Chart 5 (lower) where cells were incubated with 6-MP and MMPR simultaneously, there was a striking decrease in both the ATP concentration and the total adenine nucleotide concentration. In addition, in the period between 10 and 40 min, increases were observed in the concentrations of both AMP and ADP which suggests a decrease in the energy charge of the cell. This could be due to an effect on the generation or breakdown of high-energy phosphate bonds or to excessive consumption of high-energy phosphate resulting from the synthesis of large amounts of analog nucleotides.
Chart 4. Effect of 6-MP and MMPR on nucleotide patterns of Sarcoma 180 cells. Two g of washed Sarcoma 180 cells were incubated for 60 min with 6-MP plus MMPR. Final incubation volume was 12 ml. Extracts were prepared at 0, 30, and 60 min as described in "Materials and Methods." Aliquots of extracts made at each time period were analyzed on the Varian Aerograph LCS-1000. Top, tracing obtained with an extract of cells prior to incubation with drug; middle and bottom tracings from extracts of cells incubated for 30 and 60 min, respectively, with a combination of 6-MP and MMPR.

However, the decrease in the ATP concentration can be explained only in part by the decrease in the energy charge since there was also a decrease in the total adenine nucleotides of about 30%. In Chart 3 the most rapid synthesis of MMPR-5'-P occurred in the first 10 min of the incubation, whereas the greatest depletion of high-energy phosphate was not observed until 20 to 30 min of incubation. As seen in Chart 6, when Sarcoma 180 cells were preincubated for 30 min with MMPR prior to addition of 6-MP, a much greater decrease in both ATP concentration and total adenine nucleotides occurred. The experiments performed in Charts 5 and 6 were carried out at different times with different batches of cells which accounts for the variations seen in the adenine nucleotide concentrations at zero time.

It may be concluded from the experiments of Charts 5 and 6 that the decreases in the ATP concentrations induced by incubation of Sarcoma 180 cells with a combination of 6-MP and MMPR are the result of at least 2 factors: an effect on the energy metabolism of the cell which is reflected in a decrease in the energy charge and, more importantly, an overall decrease in the total adenine nucleotides of the cell. The decreases in the adenylate energy charge of the cell were observed during the 1st 30 min of incubation and were restored to a value approximating the zero-time adenylate charge at the end of 1 hr. The decrease in total adenine nucleotides is consistent with the known inhibitions of enzymes in the pathway of adenine nucleotide metabolism produced by the analog nucleotides 6-thioIMP and MMPR-5'.

DISCUSSION

The experiments reported here confirm and extend a number of observations made in other laboratories. For example, the synergistic effect of 6-MP and MMPR was reported by Schabel et al. (36) in mice bearing L1210 cells. We observed a similar effect with Sarcoma 180 cells, and also a synergistic effect was seen with the combination of 6-TG and MMPR. Not only were the nucleotides of 6-MP and MMPR synthesized without mutual interference but also stimulation of 6-thioIMP formation occurred when the cells were preincubated with MMPR in agreement with the findings of Wang et al. (39). In addition, for the first time a marked
Synergistic Effect of 6-MP and MMPR

Chart 6. Effect of preincubation with MMPR on adenine nucleotide levels. Two g of washed Sarcoma 180 cells were incubated in a final volume of 12 ml. In 1 case (○-○) MMPR was preincubated with the Sarcoma 180 cells for 30 min followed by addition of 6-MP as indicated. The cells were then incubated for 1 more hr. To an additional 2 g of Sarcoma 180 cells obtained from the same pool that the preincubation experiment was carried out on, 6-MP and MMPR were added at the same time (○-○) followed by a 1-hr incubation period. In both cases, 1-ml aliquots were removed at the indicated times. Neutralized perchloric acid extracts were prepared as described in "Materials and Methods," and 8-μl aliquots were analyzed for their nucleotide levels on the Varian Aerograph LCS-1000. Each point represents the average of 2 separate determinations. ●, AMP + ADP + ATP; △, ATP; ■, ADP; □, AMP. Numbers in parentheses are the adenylate energy charges.

decrease has been demonstrated in the steady-state concentrations of the adenine nucleotides of tumor cells incubated for a short time with a combination of 6-MP and MMPR.

A lag in the initiation of 6-thioIMP synthesis was seen which was shortened by concurrent administration of MMPR (Chart 2). In addition, the rate of 6-thioIMP formation was greater in the presence of MMPR. These observations are in accord with the concept that MMPR-5'-P inhibits the de novo synthesis of purines and thus makes available more PRPP for the salvage reactions (18, 29, 32). The formation of MMPR-5'-P from 6-MP, first described by Allan et al., was confirmed (1). However, it was also shown that this conversion was markedly inhibited in cells incubated with MMPR, suggesting that product inhibition of the enzyme system is responsible for this 5'-methylation. The remarkably high concentration of MMPR-5'-P achieved in these cells, e.g., in this range of 1 to 2 mM, could result in a number of other metabolic effects not explored in the present study. For example, it is possible that this high concentration of MMPR-5'-P might saturate enzymes such as 5'-nucleotidase and through competitive substrate effects decrease the rate of degradation in the cell of other analog nucleotides such as 6-thioIMP or 6-thioGMP.

Many investigators who have studied the effects of analog nucleotides on enzymes of purine metabolism have speculated that purine analogs might interfere with the metabolism of normal purine nucleotides. The present studies demonstrate for the first time that tumor cells treated with a combination of 6-MP and MMPR undergo a drastic decrease during 1 hr of incubation in the steady-state concentrations of the adenine nucleotides. Sartorelli and Booth (35) found that treatment of mice bearing Sarcoma 180 ascites cells with azaserine caused a significant decrease in the steady-state concentrations of adenine nucleotides after 9 hr.

Although incubation of Sarcoma 180 cells with a combination of 6-MP and MMPR results in a marked decrease in the ATP levels and a depletion of the steady-state adenine nucleotide concentrations, it may not be assumed that this is the cause of cellular death. It seems likely that under resting conditions tumor cells might tolerate prolonged periods of diminished adenine nucleotide levels without serious damage. However, at certain crucial stages of the cell cycle such an effect might be fatal. Since little is known about the adenine nucleotide flux, i.e., the rates of synthesis, utilization, and degradation, and since the cells used in these experiments were not synchronized but were randomly distributed throughout the cell cycle, it is possible that some of the cells had little decrease in the adenine nucleotides, whereas other cells were almost completely depleted, which might have resulted in an average depletion of about 50%. Attempts should be made to determine the nucleotide flux in cells and to perform experiments similar to those described in this paper in synchronized cells at various stages of the cell cycle. For example, one might expect that cells in the S phase that are rapidly synthesizing nucleic acids would be more profoundly affected than cells in other stages of the cell cycle and, therefore, one might observe a marked decrease in the adenine nucleotides of S-phase cells but little effect on other cells. Some of the experiments described above, in which relatively lesser effects on the adenine nucleotide levels were observed,
may have been performed with cells approaching the plateau of tumor growth rather than in the stage of exponential growth. If so, these cell populations might have contained a large percentage of drug-insensitive cells.

The response of the intracellular adenine nucleotides to treatment with 6-MP or MMPR, or both drugs in combination, suggests that at least 2 different effects take place. First of all, when tumor cells were incubated with both drugs in combination, there was a progressive decrease in the sum of the adenine nucleotides, whereas with either drug alone no striking changes in the total adenine nucleotide concentrations were seen. Decreases in the adenylyl energy charge of the cell were observed during the first 30 min of incubation with either drug alone or with both drugs in combination. The energy charge was restored to a value approximating the zero-time adenylyl energy charge by the end of 1 hr. This effect on the adenylyl energy charge could be due to a greater rate of consumption than of synthesis of high-energy phosphate bonds during the early stages of analog nucleotide formation. On the other hand, the lowered adenylyl energy charge might have been the result of interference with high-energy phosphate generation. The fact that, in each case, at the end of 1 hr of incubation the adenylyl energy charge was restored to about the zero-time value suggests that the former explanation is more likely. However, there does not appear to be strict correlation between the rapid synthesis of MMPR-5'-P and the decrease in the adenylyl energy charge. The metabolic consequences of decreases in the concentrations of adenine nucleotides might be reflected in decreases in a number of key enzymatic reactions for which these nucleotides serve as substrates. In addition, since Atkinson (2) and his colleagues have shown that a number of important enzymes in energy metabolism appear to be regulated by the adenylate energy charge of the cell, it is conceivable that certain key enzymatic reactions may be either stimulated or inhibited as a result of the decrease in adenylyl energy charge during the 1st 0.5 hr of analog nucleotide formation. The possible role of these phenomena in the mechanism of action of these analogs is a suitable subject for future study.

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Synergistic Effect of 6-Mercaptopurine and 6-Methylmercaptopurine Ribonucleoside on the Levels of Adenine Nucleotides of Sarcoma 180 Cells

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