The Mechanism of Action of 6-Mercaptopurine

I. Biochemical Effects

JOSEPHINE SEE SALSER AND M. EARL BALIS

(Division of Nucleoprotein Chemistry, Sloan-Kettering Institute for Cancer Research, Sloan-Kettering Division of Cornell University Medical College, New York, New York)

SUMMARY

The tumor Sarcoma 180 (S-180) was compared to host liver in an effort to evaluate the inhibitory action of 6-MP. The concentration of endogenous adenine nucleotides in S-180 was about 40% of that found in liver, that of guanine nucleotides was about the same in both tissues, while that of inosinate was much lower in tumor. The liver of normal mice and S-180-bearing mice exhibited the same capacity to convert IMP to AMP. A slightly greater conversion of 6-MP to 6-thio IMP in S-180 was noted but it was not by itself of sufficient magnitude to explain the anti-tumor specificity of the compound. An S-180 sensitive to 6-MP and one resistant to it each showed about one and a half times as much capacity to synthesize purines in vitro as host liver.

The conversion of IMP to AMP by either S-180 or liver was inhibited by 6-thio IMP; the inhibition, though low, was greater in the tumor. The actions of 6-thio IMP, reported by others in mammalian systems, are analogous to those previously found in bacterial and avian systems; i.e., the conversion of IMP to AMP and XMP is inhibited. It is suggested that the basis for the anti-tumor specificity may lie in differences in the enzymatic capacity of tumors and host tissues to carry out the conversions blocked by 6-thio IMP.

MATERIALS AND METHODS

IMP-8-14C was obtained from Schwarz BioResearch, Inc.; 6-MP-8-14C and 4-amino-5-imidazole-carboxamide-4-14C (AICA), from Southern Research Institute; glycine-1-14C, from Tracerlab; ATP (disodium salt); IMP (sodium salt); NAD (DPN); fructose-1,6-diphosphate (sodium salt); α-ketoglutarate; L-glutamine; ribose-5-phosphate (sodium salt); and tetrahydrofolic acid, were obtained from Sigma Chemical Company. 6-Thioinosinate (6-thio IMP) was kindly supplied by Dr. A. Hampton.

Growth and isotope incorporation studies with bacterial systems (3–5, 12, 13, 17, 18) have led to the hypothesis that the active form of 6-MP is its ribonucleotide (thioinosinate, 6-thio IMP), which inhibits the further conversion by inosinate to other purine ribonucleotides (4, 5). This inhibition by thioinosinate of the formation of adenylate and guanylate has been demonstrated in cell-free preparations from Streptococcus faecalis and pigeon liver, respectively (26). In L1210, studies on the incorporation of labeled hypoxanthine into nucleic acid purines are consistent with the inhibition at the level of hypoxanthine, but only the conversion to adenylate seems to be seriously affected by 6-MP (10). More recent studies with partially purified inosine-5'-phosphate-NAD-oxidoreductase (IMP dehydrogenase) from bacteria (15) and Ehrlich ascites tumor cells (1) have confirmed the observation that 6-thio IMP is actually an effective competitive inhibitor of the conversion to xanthylate, the intermediate in the formation of guanylate.

Since the conversion of inosinate to other purine ribonucleotides occurs in both normal and tumor tissues, the selective susceptibility of tumors such as Sarcoma 180 (S-180) to the action of 6-MP must be considered in terms of the total purine synthesis and interconversion mechanisms. This paper presents some aspects of purine metabolism in S-180 and host liver pertinent to nucleotide synthesis and conversion. The synthesis in vivo of 6-thio IMP, the synthesis of purines de novo in in vitro systems, and the inhibition of adenylate formation by 6-thio IMP in S-180 and liver have been studied.
HA/ICR Swiss mice (approximately 20 gm each) with a 5- or a 7-day-old subcutaneous implant of S-180 were kindly supplied by Dr. H. Schwartz and Dr. C. Reilly of Sloan-Kettering Institute. Mice with 11-day-old subcutaneous implants of a resistant variant of S-180 were supplied by Dr. D. Clarke. Necrotic tumors were discarded in all instances.

Acetone powders of the tissues were prepared by homogenizing the liver or tumor with 10 volumes of cold acetone (−25°C) in a Waring Blendor, followed by filtering and rehomogenizing the resuspended cake in fresh cold acetone. The last 2 steps were repeated twice. The yield of acetone powder from different batches of liver was about 25% of the wet weight while that from S-180 was 12-15%.

6-MP incorporation in vivo.—Mice with 5-day-old implants of S-180 were given i.v. (caudal vein) injections of 6-MP-8-14C (50 mg/kg body weight, specific activity = 2.3 × 106 cpm/μmole). The animals were sacrificed at 2, 6, and 24 hr after a single injection and at 48 hr after 3 successive daily injections of the labeled compound. The liver and tumor were removed and chilled in chopped ice. All subsequent steps, unless otherwise specified, were carried out at 4°C. The chilled tissues were homogenized briefly in a Potter-Elvehjem homogenizer with 1 volume of cold distilled water. Sufficient cold 60% TCA was then added to give a final concentration of 10% TCA and homogenization was continued for another 3 min. The precipitated protein was removed by centrifugation, resuspended and rehomogenized 3 more times with cold 5% TCA. The combined supernatant solutions were adjusted to 0.01 N with respect to HBr and extracted 8–10 times with ether to remove the TCA.

The components of this acid-soluble fraction (adjusted to pH 9 with concentrated NH4OH) were separated on Dowex-1 (bromide form, X10, 200–400 mesh) columns into 3 fractions: (a) feed and wash water, containing nucleosides, nicotinamide, amino acids, and flavins, (b) 0.006 N HBr, containing free purines, kNIP, and IMP, and (c) 0.18 N HBr, containing 6-thio IMP with traces of GMP and ATP. These fractions were concentrated to dryness and taken up in a minimal measured volume of water. The radioactivities of aliquots of the fractions were determined on infinitely thin films on aluminum planchets in a Geiger-Müller internal flow counter. Paper chromatography of the 3rd fraction in various solvent systems (16) showed that all the radioactivity was associated with 6-thio IMP. The identity was further confirmed spectrally.

Purine synthesis de novo in cell-free preparations.—Frozen 7-day-old implants of S-180 (sensitive) and 11-day-old implants of S-180 (resistant) as well as their controls and nontumor-bearing animals, the concentration of IMP was 1.25 μmoles/10 ml incubation. After a 4-hr incubation at 20°C in a Dubnoff metabolic shaker, the reaction was stopped and the incubation mixture deproteinized after

* The presence of glutamine stabilizes the purine-synthesizing enzymes. (J. M. Buchanan, personal communication.)

Each 10 ml of incubation mixture contained 2 gm wet weight or the equivalent amount of acetone powder; 2.5 mmoles ATP; 2.5 mmoles L-aspartate; 5 mmoles fructose-1,6-diphosphate; 0.5 mmoles MgCl2; 5 mmoles sodium phosphate buffer, pH 7.4; 2.5 mmoles α-ketoglutarate; 1 mmole NAD; and “x” μmoles IMP-8-14C (specific activity = 6.31 × 106 cpm/μmole).

The comparison of these fractions will be part of another communication.

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In the comparative studies of acetone powder and frozen tissue preparations of livers of tumor-bearing and nontumor-bearing animals, the concentration of IMP was 1.25 μmoles/10 ml incubation. After a 4-hr incubation at 20°C in a Dubnoff metabolic shaker, the reaction was stopped and the incubation mixture deproteinized after

Incubations at this temperature have been shown to promote accumulation of succinoadenylate (21), a compound of interest in studies of nucleotide interconversions.
ether extraction. The clear solution was neutralized and, if necessary, stored at -20°C. The components of such solutions were separated on Dowex-1 (chloride form, X10, 200-400 mesh) columns by a modification of the method of Deutsch and Nilsson (11). The identities of the various fractions were checked spectrally and by paper chromatography and the radioactivities were determined. This method of fractionation was used to determine the endogenous nucleotide concentration of the 2 tissues.

In the inhibition studies, the reaction mixtures contained 5.16 μmoles IMP/10 ml. The levels of 6-thio IMP studied were 1.94 and 4.85 μmoles/10 ml. After a 2-hr incubation at 37.5°C, the reaction was stopped and deproteinization carried out as described in the foregoing section. The solutions were lyophilized to dryness, taken up in freshly prepared 1 N HCl and hydrolyzed for 2½ hr at 100°C. The purines were isolated by chromatography on Dowex-50 (hydrogen form, AGW, X8, 200-400 mesh) columns by elution with 0.15 N HCl for xanthine, 6-MP and hypoxanthine and 1.5 N HCl for guanine and adenine.

RESULTS AND DISCUSSION

The enzymatic conversion of 6-MP to its active form, 6-thio IMP, by S-180 and host liver was studied. Table 1 summarizes the data obtained from a typical experiment in vivo. The 6-thio IMP levels of S-180 were consistently about 10-15% higher than that of host liver, with the highest levels being found in animals sacrificed 2 hr after injection. Catabolism of 6-thio IMP proceeded at a steady rate, but a detectable amount was still present after 24 and 48 hr.

The synthesis de novo of purines by whole homogenates of the 2 tissues under consideration is given in Table 2. A 6-MP-resistant variant of S-180, and the corresponding host liver was studied. Preliminary studies with unlabeled IMP have shown that the net formation of AMP, GMP, or their intermediates (based on spectral data), was too low in cell-free extracts of both liver and tumor to be determined accurately. To facilitate the determination of small changes of this nature, all subsequent studies were carried out with 14C-labeled IMP.

In all these comparative studies, the liver of the tumor-bearing animals was used as the normal tissue since there is an ambiguity as to the exact tissue or cell of origin of S-180. Furthermore, the conversion to adenylate is the same in the liver of nontumor and S180-bearing animals, i.e., about 40 μmoles/gm wet weight of tissue/hr. Table 3 summarizes the data obtained with frozen tissues and acetone-powder preparations of these 2 tissues. The latter growing tumor. Consequently, it is not surprising that the concentration of free ribonucleotides is much lower in the tumor than in the liver (see Table 4). Such differences, though small, may well be reflected in the relative sensitivity of the 2 tissues to purine antagonists, such as 6-MP.

Preliminary studies with unlabeled IMP have shown that the net formation of AMP, GMP, or their intermediates (based on spectral data), was too low in cell-free extracts of both liver and tumor to be determined accurately.
Whole homogenate concentrations used are in excess of those occurring in the tissues, and because of errors inherent in using wet weight data presented here show that the capacity for this conversion in S-180 is not appreciably greater (only 10–15%) than that in host liver. In vitro experiments\(^7\) show essentially the same thing. These experimental values are in the same range as those observed in mice bearing a resistant variant of S-180 (22). The higher values at 2 hr, the shortest experimental time period, followed by a steady decrease with time suggest that most of the synthesis probably occurred within 2 hr. The 6-thio IMP persists for a considerable period of time in both liver and S-180. This rather small difference in synthesis of 6-thio IMP can hardly explain by itself the great difference observed in the biologic response of the 2 tissues to 6-MP (9).

An inhibition in the pathway de novo, probably at the phosphoribosylamidotransferase step, has recently been suggested as the primary site of action of 6-MP in experimental tumors in vivo (6). However, in biochemical studies of this enzyme system, adenylate has been reported to be 5% as effective as 6-thio IMP and the inhibitions by the 2 compounds are additive (20). The endogenous adenylate concentration of S-180 is over 1000 times greater than the 6-thio IMP level at 2 hr after injection of an LD50 dose of 6-MP. Thus, in this tumor, the contribution by the 6-thio IMP formed would be negligible and could hardly by itself explain the observed anti-tumor specificity found only with 6-MP. Although 6-thio IMP may exert feed-back inhibition, it does not appear likely that the bio-

\* See "Materials and Methods."
\* Samples had been hydrolyzed to free purines prior to column chromatography.

values were adjusted to a wet weight basis (see "Materials and Methods"). On the basis of these results, either preparation can be used for studies of this nature.

Table 4 summarizes the endogenous purine nucleotide concentration of liver and S-180. These values were obtained from acid-soluble extracts of whole homogenates as well as the blanks for some of the incubations with undialyzed preparations.\(^7\) The concentration of adenine nucleotides\(^9\) in S-180 is about 40% of that in the liver, while that of guanine nucleotides\(^9\) is but slightly less in S-180.

The inhibition by 6-thio IMP of the conversion of inosinate to adenylate was examined in 15,000 \(\times g\) supernatants of liver and S-180. The results are given in Table 5. The inhibition in the liver at the 2 levels studied was about half that observed in the tumor. Since the IMP concentrations used are in excess of those occurring in the tissues, and because of errors inherent in using wet weight

\* These were tissue blanks used in assaying studies of the conversion of inosinate to adenylate (to be published).
\* This represents the sum of the mono-, di-, and triphosphates.

### Table 4

**Concentration of Endogenous Nucleotides**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Purine Nucleotides (\text{mole/gm wet weight})</th>
<th>(\text{mole/gm wet weight})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenylate</td>
<td>Guanylate</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>7.63</td>
<td>0.90</td>
</tr>
<tr>
<td>Whole homogenate blank(^7)</td>
<td>8.25</td>
<td>1.02</td>
</tr>
<tr>
<td>Supernatant 15,000 (\times g) blank(^7)</td>
<td>7.82</td>
<td>0.85</td>
</tr>
<tr>
<td>S-180</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>2.90</td>
<td>0.75</td>
</tr>
<tr>
<td>Whole homogenate blank(^7)</td>
<td>3.05</td>
<td>0.80</td>
</tr>
<tr>
<td>Supernatant 15,000 (\times g) blank(^7)</td>
<td>2.74</td>
<td>0.68</td>
</tr>
</tbody>
</table>

\* See "Materials and Methods."
\* See footnote 8.
\* See footnote 7. Cell-free extracts from 4 gm wet weight of tissue made up to 20 ml with buffer and adjusted with cold 60% trichloroacetic acid to give a final concentration of 7%.

### Table 5

**Inhibition of Inosinate to Adenylate Conversion in Cell-free Preparation**

<table>
<thead>
<tr>
<th>Inosine, monophosphate</th>
<th>6-Thioinosinate</th>
<th>(\text{mole/gm wet weight})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.16</td>
<td>0</td>
<td>201</td>
</tr>
<tr>
<td>5.16</td>
<td>1.94</td>
<td>188</td>
</tr>
<tr>
<td>5.16</td>
<td>4.85</td>
<td>177</td>
</tr>
</tbody>
</table>

\* See "Materials and Methods."
\* Samples had been hydrolyzed to free purines prior to column chromatography.
logic result can be primarily attributed to that biochemical action alone. Furthermore, the effects of 6-MP on incorporation of formate and pre-formed purines (6) can be interpreted in terms of the "sparing" action of 6-MP on formate utilization (4) and the inhibition of xanthine oxidase by 6-MP (28, 29). The latter would result in less degradation of purines per se. Consequently, there would be less need for purine synthesis de novo and the utilization of tracer doses of purines would not be decreased. Studies with bacteria have indicated that 6-MP exerts its inhibitory action as the ribonucleotide, 6-thio IMP, which blocks the further conversion of IMP to AMP and GMP (4, 5). The inhibition of these nucleotide interconversions has subsequently been demonstrated to occur at reasonable 6-thio IMP concentrations in bacterial (15, 26) and avian (26) systems in vitro. The present study, as well as those of Atkinson et al. (1), indicate that these inhibitions do occur in mammalian systems with 6-thio IMP levels comparable to those which exist in tissues in vivo. The lower endogenous level of IMP and the slightly higher synthesis of 6-thio IMP in S-180 (compared to host liver) could indicate some specificity. However, it does not seem likely that these differences alone can explain the degree of specificity observed in vivo with S-180. The reported inhibitions in vitro by 6-thio IMP of the conversion of IMP to succino-AMP and XMP suggest that the basis of the selective susceptibility of tissues to 6-MP might be expected, in part, to lie in differences in the ability of tissues to carry out such interconversions. Attempts to verify this suggestion have been carried out and will be reported.

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