Resistance to Purine Antagonists in Experimental Leukemia Systems

R. W. BROCKMAN
Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama

SUMMARY

Resistance to purine analogs by loss of nucleotide-forming capacity was observed in a number of experimental tumor systems. Such resistance frequently was accompanied by loss of specific purine ribonucleotide pyrophosphorylase activities. However, it is evident that other mechanisms of resistance to purine analogs also exist both in mouse leukemia and in human leukemia.

Attention was focused on a locus of action of 6-mercaptopurine, as the ribonucleotide, at an early step on the purine biosynthetic pathway—namely, the site of end-product inhibition by purine ribonucleotides. In a human neoplasm grown in cell culture, 6-mercaptopurine was a potent inhibitor of this early step in purine biosynthesis, in agreement with work in mouse neoplasms.

The present discussion centers on cellular resistance to purine analogs in experimental murine leukemias. Much of the work under consideration was based on the premise that such resistance could be defined in biochemical terms and that such knowledge could be of value to a basic understanding of mechanisms of resistance. It is hoped that such knowledge might contribute to the control of leukemia. Skipper et al. (52, 53) have discussed means by which populations of leukemia cells may escape inhibition by therapeutic agents without undergoing a process of selection of resistant variants. The discussion to be presented here focuses on biochemical alterations in resistant cells that afford them a survival advantage in the presence of the therapeutic agent. Also to be considered is 6-mercaptopurine inhibition in leukemia cells of an early step in purine biosynthesis. This is thought to be the feedback site that is subject to regulation by purine ribonucleotides—end products of this biosynthetic pathway.

A comprehensive review of purine analogs and of resistance is beyond the scope of this presentation; several recent reviews are available (7, 8, 27, 32, 45).

RESISTANCE TO PURINE ANALOGS BY LOSS OF NUCLEOTIDE-FORMING CAPACITY

Law (36) developed resistant variants of the lymphocytic neoplasm L1210 by serial transfer of the leukemia cells in mice receiving treatment with purine analogs or with folic acid analogs. The sublines resistant to 8-aza-

guanine, 6-mercaptopurine, and 6-thioguanine, as well as a line resistant to amethopterin, appeared to develop in a stepwise fashion. Resistance to these analogs was shown to be stable, irreversible, and heritable in the continued absence of the analog used in their selection. Law (36) concluded that "mutation and selection appear to constitute the mechanism involved" in the origin of these resistant populations of neoplastic cells. In our laboratory a comparison was undertaken of the metabolism of purine bases and analogs in the sensitive and resistant L1210 lines of Law. Results of these studies have been reported in detail (6, 9, 13, 54); extensions of these studies in microorganisms (10), mouse leukemia cells grown in culture (12), and human carcinoma cells in culture (11) have also been reported.

These studies in our own and in other laboratories demonstrated that azaguanine-resistant L1210 cells incorporated much less 8-azaguanine-2-14C into nucleic acids than did sensitive L1210 cells (4). Further study confirmed this observation and extended it to show that L1210 cells metabolized azaguanine to azaguanine ribonucleotides, whereas the L1210/8-Aza cells failed to do so (9). Results of this study are graphically summarized in Chart 1. The lower half of this graph compares the extent of incorporation of 8-azaguanine-2-14C into azaguanic acid of the soluble fraction of sensitive and resistant tumors;
CHART 1.—Comparison of the incorporation of 8-azaguanine-2-¹⁴C into azaguanine ribonucleotides and into nucleic acids by sensitive and resistant neoplasms in vitro (9).

Treatment of animals bearing bilateral implants of L1210 and L1210/8-Aza with 4-amino-5-imidazolecarboxamide, which inhibits deamination of azaguanine to azaxanthine (41), resulted in increased incorporation of azaguanine into nucleic acids of sensitive L1210 but not into those of L1210/8-Aza (Chart 1A). Thus, inhibition of azaguanine degradation did not alter the pattern of incorporation of azaguanine-2-¹⁴C into nucleic acids of the resistant tumor.

Similar studies were made with sensitive and resistant ascites tumor cells, and the results are summarized in Charts 1C and 1D. From these data it is again evident that the sensitive cells metabolized azaguanine to nucleotides and incorporated the analog into nucleic acids, whereas resistant cells were deficient in these respects.

The striking contrast in the incorporation of the analog into the nucleotide and polynucleotide components of these cells led to a consideration of the metabolism of azaguanine (Chart 2). Way and Parks (58) showed that azaguanine and other purine analogs underwent an enzyme-catalyzed reaction with 5-phosphoribosyl-1-pyrophosphate to yield the ribonucleotide. It had previously been found in the laboratories of Kornberg (35) and of...
and exhibit a similar decrease in enzymatic capacity to form guanylic, azaguanylic, or inosinic acids. Formed only small amounts of the ribonucleotide rel which puriries are salvaged for utilization by cells. An enzymatic reaction of natural purines with 5-phosphoribosyl-1-pyrophosphate yielded the ribonucleotide pyrophosphorylase activities of 6-mercaptopurine in L1210 sublines that were resistant to such analogs in experimental systems was frequently accompanied by a decrease or loss of inosinic and guanylic acid pyrophosphorylase activities. Analysis of purine ribonucleotide pyrophosphorylase activities showed the pattern summarized in Table 1; loss of nucleotide-forming capacity, except for adenine, is evident. Comparable results were obtained in a study of azaguanine-resistant P388 murine lymphocytic leukemia in cell culture (12).

Concurrent with the work on resistance to purine analogs in neoplastic cells, studies were carried out on resistant microorganisms in collaboration with Dr. D. J. Hutchison (10). Support was obtained for the developing hypothesis that nucleotide formation was essential for activity of these hypoxanthine and guanine analogs and that resistance to such analogs in experimental systems was frequently accompanied by a decrease or loss of inosinic and guanylic acid pyrophosphorylase activities. Results of such investigations were next extended to human epidermoid carcinoma cells (H.Ep. 2) in culture (11). A mercaptopurine-resistant subline was developed by exposing a population of cells to low concentrations of 6-mercaptopurine (0.5–1 μg/ml) and isolating surviving clones (33). The resistant population of cells selected in this manner was highly resistant to 6-mercaptopurine. A study of the metabolism of purine bases and analogs showed that the resistant cells (H.Ep. 2/MP) grown in suspension culture did not convert guanine, azaguanine, hypoxanthine, or mercaptopurine to nucleotides; adenine was extensively metabolized to nucleotides. Analysis of purine ribonucleotide pyrophosphorylase activities showed the pattern summarized in Table 1; loss of nucleotide-forming capacity, except for adenine, is evident. Comparable results were obtained in a study of azaguanine-resistant P388 murine lymphocytic leukemia in cell culture (12).

Buchanan (34) that enzymatic reaction of natural purines with 5-phosphoribosyl-1-pyrophosphate yielded the ribonucleotide (Chart 3). This is the major pathway by which purines are salvaged for utilization by cells. An examination was made of enzyme preparations from sensitive and azaguanine-resistant cells for their capacity to form ribonucleotides by the reaction depicted in Chart 3. The results revealed that the resistant cells were deficient in ribonucleotide-forming capacity. Resistant cells did not form guanylic, azaguanylic, and inosinic acids; however, formation of adenylic acid from adenine was unimpaired in the resistant cells. It appeared, in the mouse neoplasms studied, that sensitivity to azaguanine might be correlated with formation of the nucleotide and, conversely, that failure to form the nucleotide was accompanied by insensitivity to the analog.

Pursuant to this idea, a study was made of the metabolism of 6-mercaptopurine in L1210 sublines that were resistant to 8-azaguanine, to 6-mercaptopurine, and to 6-thioguanine. Each of these cell lines was resistant to inhibition by each of these purine analogs. Chart 4 summarizes the results of a study of the metabolism of 6-mercaptopurine to 6-mercaptopurine ribonucleotide in L1210 ascites tumor cells in vivo (6). These data show that the mercaptopurine-resistant L1210 cells formed only small amounts of the ribonucleotide relative to the sensitive cell line. Also, an examination of the formation of nucleotides by enzyme preparations from these cell lines showed that the resistant cells (L1210/MP, L1210/TG, and L1210/8-Aza) were deficient in purine ribonucleotide pyrophosphorylase activity for formation of inosinic acid and 6-mercaptopurine ribonucleotide, as well as for formation of guanylic and azaguanylic acids (6). Again, full enzymatic capacity for conversion of adenine to adenylic acid was retained by these resistant cells.

Such investigations were next extended to human epidermoid carcinoma cells (H.Ep. 2) in culture (11). A mercaptopurine-resistant subline was developed by exposing a population of cells to low concentrations of 6-mercaptopurine (0.5–1 μg/ml) and isolating surviving clones (33). The resistant population of cells selected in this manner was highly resistant to 6-mercaptopurine. A study of the metabolism of purine bases and analogs showed that the resistant cells (H.Ep. 2/MP) grown in suspension culture did not convert guanine, azaguanine, hypoxanthine, or mercaptopurine to nucleotides; adenine was extensively metabolized to nucleotides. Analysis of purine ribonucleotide pyrophosphorylase activities showed the pattern summarized in Table 1; loss of nucleotide-forming capacity, except for adenine, is evident. Comparable results were obtained in a study of azaguanine-resistant P388 murine lymphocytic leukemia in cell culture (12).
enzyme, although this is not yet certain (8). The enzyme (or enzymes) converts hypoxanthine, mercaptopurine, guanine, thioguanine, and azaguanine to the corresponding nucleotides. Xanthylie acid pyrophosphorylase activity is present in microbial cells but is weak or lacking in mammalian cells. It can be readily understood from this summary that, if purine analogs, such as mercaptopurine, thioguanine, or azaguanine, become biochemically active only after conversion to the nucleotide, then a decrease or a loss of inosinic-guanyl acid pyrophosphorylase activity would decrease or eliminate the inhibitory effect of these analogs.

Other workers have also observed resistance to purine analogs accompanied by decreased capacity for formation of the analog ribonucleotide. Remy and Smith (48) observed that resistance to 2,6-diaminopurine in Escherichia coli was accompanied by decreased formation of diaminopurine ribonucleotide. Kallé and Gots (30), working with Salmonella typhimurium, and Lieberman and Ove (39), working with fibroblasts in cell culture, observed loss of enzymatic capacity for conversion of diaminopurine to the ribonucleotide. These workers (31, 39) also observed loss of enzymatic capacity for formation of nucleotides of mercaptopurine and azaguanine in resistant cells. Salser et al. (49) observed decreased IMP pyrophosphorylase activities in mercaptopurine-resistant Streptococcus faecalis. Like ourselves (12), Davidson et al. (16) found that resistance to 8-azaguanine in P388 murine lymphocytic leukemia in cell culture was accompanied by a marked decrease in IMP-GMP pyrophosphorylase activity. Littlefield (40) isolated lines of mouse fibroblasts with a small degree of resistance to 8-azaguanine as well as more highly resistant lines. The IMP pyrophosphorylase content of cells with a low degree of resistance was intermediate between that of the sensitive cells and of the highly resistant cells, which showed loss of IMP pyrophosphorylase activity. Thus, the partially resistant cells showed a quantitative decrease in the enzyme activity rather than a loss of activity, as was seen in other cell lines (11, 12, 16). Results such as these were also observed in D98 bone marrow cells resistant to purine analogs (56). It is also of interest that IMP pyrophosphorylase in the partially resistant cells appeared to be the same as that in the sensitive cells with respect to heat stability and Michaelis constants (40).

More extensive reviews of resistance to purine analogs have been presented elsewhere (7, 8). From the above discussion it is evident that the loss of specific purine ribonucleotide pyrophosphorylase activity accompanying resistance to purine analogs has been observed in a number of laboratories.

ON MODEL SYSTEMS

The mechanism of resistance considered thus far has been explored in model systems, such as microorganisms, mouse leukemia cells, or neoplasms in cell culture. Linderstrøm-Lang (19) co-authored a monograph that contained the following gem concerning model systems: “One well established and generally accepted method of treating systems which are complicated beyond comprehension is to construct simple models and see whether they fit the systems in question. If they do, you will immediately become suspicious, and so will your colleagues, most certainly, with the result that a blooming literature springs up (or breaks out) dealing with the problem of how you have managed to make all your errors cancel one another. If they do not fit, the beauty of the models themselves may shine for years untainted by the squallid awkwardness of reality.”

We have presented a résumé of our studies of resistance to purine analogs in model systems and considered some of the work of others who have obtained similar results in model systems; now the following pertinent question comes to the fore: How do results of these studies with model systems relate to the problem at hand—awkward reality in the form of human leukemia?

RESISTANCE TO MERCAPTOPURINE IN HUMAN LEUKEMIA

Davidson and Winter (17) undertook an investigation of purine ribonucleotide pyrophosphorylase activity in human leukemia cells that were considered clinically resistant to 6-mercaptopurine and compared the enzyme activities with those in sensitive human leukemia cells. Of 6 leukemias that were classified as rigidly resistant to mercaptopurine, 1 showed a low inosinic acid pyrophosphorylase activity and a low capacity to form mercaptopurine ribonucleotide. The other resistant human leukemia cells did not appear to differ significantly from sensitive cells in purine ribonucleotide pyrophosphorylase activities. Even though this sample was necessarily small, it does appear that decreased IMP pyrophosphorylase activity may, perhaps, be 1 mechanism by which human leukemia cells may become resistant to mercaptopurine. Obviously, this mechanism does not account for resistance in the other cases.
A biochemical basis for resistance to thioguanine in these cell lines is not yet available, although several leads appear promising. These findings also suggest that resistance mechanisms may vary from one neoplasm to another.

Paterson (45) recently reviewed his studies of resistance to mercaptopurine. His findings indicated an intact cell effect in resistance to 6-mercaptopurine in Ehrlich ascites tumor cells. The intact resistant cells were unable to synthesize mercaptopurine ribonucleotide, even though undiminished pyrophosphorylase activity was demonstrated in cell-free enzyme preparations from the resistant cells. Paterson (46, 47) considered the possibility that cell permeability might be a factor in mercaptopurine resistance in Ehrlich ascites carcinoma cells. However, Davidson (14) obtained no evidence for altered permeability as a mechanism for resistance to mercaptopurine in L1210 cells, and Jacquez (28, 29) found that purine and pyrimidine bases gained entry into cells by diffusion rather than by an active transport mechanism. It is evident from the studies of Paterson, LePage, and others that there are mechanisms of resistance other than decreased capacity for formation of the analog nucleotide; but an understanding of the biochemical basis for such resistance, at least in the case of 6-mercaptopurine, has not yet been achieved.

ON THE MECHANISM OF ACTION OF 6-MERCAPTOPURINE: INHIBITION AT THE FEEDBACK CONTROL SITE

Reconsideration of mechanisms of mercaptopurine inhibition of nucleic acid synthesis may be of value in further exploration of mechanisms of resistance to this analog. Salsor et al. (49), working with microorganisms, observed that mercaptopurine ribonucleotide inhibited the enzymatic conversion of inosinic acid to adenylosuccinic acid. Hampton (23) observed inhibition of cleavage of adenylosuccinic acid to adenylic acid. Bridger and Cohen (5) showed that mercaptopurine ribonucleotide, or a copper complex of the nucleotide, inhibited the conversion of adenylosuccinate to adenylic acid. Evidence that mercaptopurine inhibits susceptible tumors as a consequence of its incorporation into DNA. They observed that incorporation of thioguanine into DNA of susceptible tumors was significantly higher than was incorporation of the analog into thioguanine-insensitive tumors. Decreased conversion of thioguanaylic acid to thiodoxyguanylic acid was considered as a possible resistance mechanism.

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TABLE 2

<table>
<thead>
<tr>
<th>PURINE RIBONUCLEOTIDE PYROPHOSPHORYLASES</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Adenylic acid pyrophosphorylase</td>
</tr>
<tr>
<td>Adenine + PRPP → AMP</td>
</tr>
</tbody>
</table>
| Active also for aminoimidazolecarboxamide, 2,6-
  diaminopurine, 2-fluoroadenine, 4-aminopyra-
  zolo(3,4-d)pyrimidine |
| II. Inosinic-guanic acid pyrophosphorylase |
| A. Hypoxanthine + PRPP → IMP |
| Active also for 6-mercaptopurine, 4-hydroxy-
  pyrazolo(3,4-d)pyrimidine |
| B. Guanine + PRPP → GMP |
| Active also for 6-thioguanine, 8-azaguanylic |
| III. Xanthic acid pyrophosphorylase |
| Xanthine + PRPP → XMP |
| Active also for 8-Azaxanthine |

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* The abbreviations used are: PRPP, 5-phosphoribosyl-1-pyrophosphate; AMP, adenosine mono-
  phosphate; IMP, inosine monophosphate; GMP, guanosine monophosphate; and XMP, xanthosine 
  monophosphate.

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OTHER MECHANISMS OF RESISTANCE TO PURINE ANALOGS

It is evident from work of LePage and of Paterson that multiple mechanisms are possible by which cells may become resistant to purine analogs. Sartorelli et al. (50) observed that thioguanine-resistant Ehrlich ascites carcinoma cells formed less thioguanic acid than the sensitive line. This result was attributed to thioguanine catabolism by the resistant neoplasm. Ellis and LePage (18) developed 2 lines of thioguanine-resistant Ehrlich ascites carcinoma. One was developed with a low level of 6-thioguanine (2 mg/kg) and the other was obtained with a higher level of 6-thioguanine (10 mg/kg). In studies with intact cells, both lines showed decreased formation of thioguanic acid and decreased incorporation of thioguanine into nucleic acids. The subline developed in the presence of a low level of thioguanine did not exhibit loss of enzymatic capacity for formation of thioguanic acid, whereas the more highly resistant subline showed a greatly decreased pyrophosphorylase activity for thioguanic acid synthesis. This interesting study clearly demonstrated that resistance mechanisms vary and are dependent to some extent on the selection pressure (drug concentration) used, an observation likely to be pertinent, also, for human leukemia. Similar observations were made with D98 cells partially resistant to 8-

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captopurine inhibited adenylic acid synthesis in intact L1210 cells in vitro was obtained by Davidson (15). Enzymatic synthesis of xanthyllic acid from inosinic acid by enzyme preparations from pigeon liver (49), from Aerobacter aerogenes (22), and from Ehrlich ascites tumor cells (1) also was inhibited by mercaptopurine ribonucleotide.

Baker and Bennett (2), working with enzyme preparations from Adenocarcinoma 755 cells, also observed inhibition of the conversion of inosinic acid to adenylic acid, but only by relatively high concentrations of mercaptopurine ribonucleotide. These findings led to reconsideration of other sites of action of 6-mercaptopurine.

It has been known for some time that mercaptopurine inhibited the incorporation of glycine and formate into nucleic acid purines (51). LePage (37) and Heidelberger

<table>
<thead>
<tr>
<th>Purine</th>
<th>Concentration (µg/ml)</th>
<th>FGAR-¹⁴C accumulation in the soluble fraction (% of control) without purines*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Adenine</td>
<td>5.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>50.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>50.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>100</td>
</tr>
</tbody>
</table>

* Azaserine (10 µg/ml) was added to 100-ml suspension cultures; 30 min later the purine base was added. One hr after addition of azaserine, sodium formate-¹⁴C (25 µC/flask) was added; 2 hr after the tracer was added cells were harvested. The soluble fraction was analyzed by 2-dimensional chromatography-radioautography, and quantitative determinations of radioactivity were made.

** H.Ep. ²/8 designates mercaptopurine-sensitive cells; H.Ep. ²/MP designates mercaptopurine-resistant cells.

(24) observed mercaptopurine inhibition of glycine and formate labeling of nucleic acid purines. Gots and Gollub (21) found that mercaptopurine, as well as natural purines, inhibited the accumulation of 4-amino-5-imidazolecarboxamide in a purine-requiring E. coli mutant and considered this to be a feedback effect. LePage and Jones (38) showed that mercaptopurine inhibited azaserine-induced formylglycinamide ribonucleotide accumulation in ascites tumor cells in vivo. However, even though these studies pointed to inhibition by mercapto-
purine of an early step in purine biosynthesis, emphasis seems to have remained on the observed inhibitions of further metabolism of inosinic acid.

Bennett et al. (3) undertook an extensive study of the effect of mercaptopurine on the incorporation of radioactive formate, glycine, 4-amino-5-imidazolecarboxamide, and purine bases into the nucleic acids of mouse neoplasms in vivo. Chart 5 summarizes the tracers used and the sites of inhibition that could be studied in this way. Nucleotide formation from adenine or 2,6-diaminopurine is beyond the known loci of mercaptopurine inhibition. Radioactive formate and glycine enter the pathway early and are therefore subject to inhibition at a site early on the pathway (see 1 on Chart 5). Aminomidazolecarboxamide enters the purine biosynthetic pathway at a later point but before formation of inosinic acid and, therefore, provides a measure of effects of the analog on nucleotide interconversions. Table 3 presents, in abbreviated form, representative data from the study with L1210 mouse leukemia in vivo (3). The data shown give the specific activities of nucleic acid adenine and guanine as a percentage of the controls not exposed to mercaptopurine. Had mercaptopurine shown no inhibition of the incorporation of the precursor into nucleic acids, the values would be 100. From the data of Table 3 it can be seen that formate and glycine incorporation was significantly inhibited; the values ranged from 34 to 58% of controls. Incorporation of aminomidazolecarboxamide was considerably less inhibited; the effect observed is presumably due to inhibition of further metabolism of inosinic acid (see 2 and 4 on Chart 5). Adenine incorporation was actually enhanced by mercaptopurine treatment; this increased utilization of a salvage pathway is readily understandable in terms of mercaptopurine inhibition of inosinic acid synthesis de novo. Similar data were obtained by Bennett and associates (3) in studies with Sarcoma 180 and with Ehrlich ascites tumor cells in vivo. The findings strongly support the view that a locus on the purine pathway before aminomidazolecarboxamide ribonucleotide synthesis is the primary site of mercaptopurine inhibition.

Additional evidence supporting this view was obtained in a study of the effect of mercaptopurine on azaserine-induced formylglycinamide ribonucleotide synthesis. The initial steps of purine biosynthesis are shown in Chart 6. It is well known that azaserine, a glutamine analog, strongly inhibits the glutamine-mediated amidation of formylglycinamide ribonucleotide (FGAR); in the presence of sodium formate-14C radioactive FGAR accumulates. This then serves as a tool for an analysis of inhibition of a step prior to FGAR formation and thereby permits exploration of possible feedback inhibition. LePage and Jones (38) showed that such azaserine-induced FGAR accumulation in neoplastic cells was inhibited to varying degrees by natural purines and also by mercaptopurine or thioguanine. Similarly, Henderson (25) showed that natural purines inhibited FGAR synthesis in Ehrlich ascites tumor cells.6 Such inhibition was presumed to take place by inhibition of the 1st step on the pathway—the synthesis of phosphoribosylamine. An examination was made with the LePage technic of the effect of purines and analogs on the accumulation of formylglycinamide ribonucleotide in sensitive and resistant neoplasms. Adenine, hypoxanthine, and mercaptopurine, even at concentrations of 0.5 μg/ml, were potent inhibitors of formylglycinamide ribonucleotide synthesis in sensitive E.H.Ep. and sarcoma 180 cells grown in culture (Table 4). In H.Ep. and sarcoma 180 cells, which have lost inosinic and guanylic acid pyrophosphorylase activities, adenine was still effective at low concentrations, but hypoxanthine and mercaptopurine were inactive even at 50 μg/ml—100 times the effective concentration in the sensitive cells. This study was extended to sensitive and mercaptopurine-resistant L1210 cells and with similar results. Data summarized in Table 5 show that in L1210-sensitive cells adenine, hypoxanthine, and mercaptopurine inhibited formylglycinamide ribonucleotide synthesis to about 35% of control after a single dosage of 20 mg/kg of the given purine; of these 3 bases only adenine was an effective inhibitor of FGAR synthesis in L1210/MP cells, which are known to have lost capacity for formation of nucleotides of hypoxanthine and mercaptopurine. These findings, in agreement with previous work (38), show that, in neoplastic cells, mercaptopurine, probably as the ribonucleotide, was an effective inhibitor of a step in purine biosynthesis prior to the formation of formylglycinamide ribonucleotide. In addition, these results show that mercaptopurine was effective only in cells capable of forming the nucleotide. The levels of mercaptopurine that were inhibitory to FGAR synthesis are in good agreement with the concentration required to inhibit growth in cell culture of H.Ep. and proliferation in vivo of L1210 cells.

6 However, in a recent study, Henderson (25) did not observe mercaptopurine inhibition of FGAR synthesis in Ehrlich ascites tumor cells exposed to the analog in vitro.
**TABLE 6**

<table>
<thead>
<tr>
<th>Purinethiol</th>
<th>Concentration (amoles/ml)</th>
<th>FGAR-¹³C Accumulation in the Soluble Fraction (% of Control without Purinethiols)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H.Ep. #2/MP</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>0.3</td>
<td>92</td>
</tr>
<tr>
<td>6-Mercaptopurine ribonucleoside</td>
<td>0.3</td>
<td>55</td>
</tr>
<tr>
<td>6-Mercaptopurine ribonucleotide</td>
<td>0.15</td>
<td>99</td>
</tr>
<tr>
<td>Bis((thioinosine)-5',5''-phosphate</td>
<td>0.3</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.015</td>
</tr>
</tbody>
</table>

*a* See 1st footnote to Table 4 for the procedure used.

*b* H.Ep. #2/MP designates mercaptopurine-resistant cells; H.Ep. #2/TG designates thioguanine-resistant cells.

phosphoribosylamine synthesis in enzyme preparations from pigeon liver; the free bases and nucleosides were inactive. This has led to the happy situation for both this group and ourselves that the studies in growing cells proved to be in good agreement with the findings with the cell-free enzyme.

Evidence has been presented in this section supporting the view that the most sensitive site of inhibition of purine biosynthesis by 6-mercaptopurine is an early step in the synthesis of purines, probably the feedback-controlled synthesis of phosphoribosylamine. Many examples are known in which the end product of a series of biochemical reactions inhibits an early step on the pathway. Pardee and Wilson (44) recently considered the operation of such controls in higher animals. In the specific example under consideration it is known that natural purine ribonucleotides inhibit phosphoribosylamine synthesis. The observation that 6-mercaptopurine ribonucleotide also inhibits this reaction in leukemia cells is of considerable interest. More precise definition of the mechanism of feedback inhibition by purine nucleotides and analogs will no doubt contribute to further understanding of inhibition mechanisms and of resistance mechanisms.

**CIRCUMVENTION OF RESISTANCE**

The therapist, when confronted with resistance to a therapeutic agent, of necessity turns to another agent with a different mode of action. As an experimental approach, effort in our laboratories has been directed toward introducing into the resistant cell what was deemed to be the active form of the inhibitor. Thus, if resistance to mercaptopurine arises as a consequence of failure to form the nucleotide, then mercaptopurine ribonucleotide itself might be an active inhibitor. Apparent impermeability of the cells to the nucleotide blocked so direct an approach. The idea that mercaptopurine ribonucleotide might be inhibitory to resistant cells gained support from a study of the effect of mercaptopurine ribonucleotide on the conversion of IMP to AMP by enzyme preparations from cells that were resistant to mercaptopurine by virtue of inability to form the analog ribonucleotide. Mercaptopurine ribonucleotide inhibited the conversion of IMP to AMP in mercaptopurine-resistant Adenocarcinoma 755 cells (2). Furthermore, it was equally inhibitory whether the enzyme was obtained from sensitive cells or from resistant cells. Thus, in this case an enzyme from resistant cells was found to retain sensitivity to mercaptopurine ribonucleotide.

Since McCollister et al. (42) found that mercaptopurine ribonucleotide inhibited the first step of purine biosynthesis, it became of interest to explore also the sensitivity to mercaptopurine ribonucleotide of this enzyme from resistant cells. We have obtained indirect evidence that mercaptopurine ribonucleotide, or a derivative, may inhibit formylglycinamide ribonucleotide synthesis in mercaptopurine-resistant cells. Thomas and Montgomery (57) synthesized bis((thioinosine)-5',5''-phosphate (Chart 7). It appears that this compound may be enzymatically cleaved to yield mercaptopurine ribonucleotide. Therefore, we examined its effect on formylglycinamide ribonucleotide synthesis in mercaptopurine-resistant H.Ep. #2 cells. The results, summarized in Table 6, showed that mercaptopurine and mercaptopurine ribonucleotide did not inhibit FGAR synthesis but that lower concentrations of bis((thioinosine)-5',5''-phosphate did inhibit this synthesis.
This nucleotide ester also was found to inhibit the growth of mercaptopurine-resistant H.Ep. #2 cells in culture (43). These preliminary results suggest that it might be possible to circumvent resistance that develops by loss of nucleotide-forming capacity.

Another interesting aspect of these experiments is the observed inhibition of FGAR synthesis in resistant cells by relatively high concentrations of 6-mercaptopurine ribonucleoside (Table 6). This inhibition, although not strong, was found to be reproducible. This result indicates that some phosphorylation of mercaptopurine ribonucleoside directly to the nucleotide may have occurred. The activity of 6-mercaptopurine ribonucleotide that was sometimes observed (Table 6) may be a consequence of breakdown to the ribonucleoside, entry into the cell, and subsequent phosphorylation. We have recently obtained some evidence for weak inosine and guanosine kinase activities in resistant H.Ep. #2 cells that lack IMP-GMP pyrophosphorylase activities. The kinase pathway appears to be a minor one relative to the pyrophosphorylase pathway in the tumor cells that we have examined. Low activity for phosphorylation of inosine and of mercaptopurine ribonucleoside would account for the observed cross-resistance to mercaptopurine ribonucleoside in mercaptopurine-resistant cells.

ACKNOWLEDGMENTS

The author is indebted to Dr. L. L. Bennett, Jr., for helpful discussions of aspects of this work and to Miss Sue Chumley and Mrs. Frank Hays for valuable laboratory assistance.

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