Deereased Feedback Inhibition in a 6-(Methylmercaptoropurine Ribonucleoside-resistant Tumor

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SUMMARY

Some biochemical effects of 6-(methylmercapto)purine ribonucleoside (Me6MPR) have been compared in cells of the Ehrlich ascites carcinoma and of a Me6MPR-resistant subline. The sole metabolite of Me6MPR in both cell types was Me6-MPR-5'-phosphate, and both formed the nucleotide at the same rate. This metabolite persisted for longer periods in the sensitive cells than in the resistant cells.

Me6MPR and guanine were less effective as feedback inhibitors of pyrimidine biosynthesis de novo in drug-resistant cells than in cells of the sensitive parent tumor, whereas adenine was equally effective in either. The phosphoribosylpyrophosphate amidotransferase activity of resistant cells was inhibited less than that of sensitive tumor cells. These results are consistent with the conclusion that the Me6MPR-resistant cells possess an altered amidotransferase which binds Me6MPR-5'-phosphate less well, or for which such binding has less inhibitory effect than for the enzyme in sensitive cells.

INTRODUCTION

The selection from the Ehrlich ascites carcinoma of a subline (here designated EAC-R1) which is resistant to 6-MP has been described by Paterson (15). Cells of this subline do not convert 6-MP to 6-MP ribonucleoside 5'-phosphate, which is either the pharmacologically active form of this drug, or an intermediate in the formation of the active metabolite. However, the enzyme which catalyzes 6-MP nucleotide formation, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8), is as active in extracts of these resistant cells as in extracts of the 6-MP-sensitive parent line of tumor cells (16). This is in contrast to many other 6-MP-resistant tumor lines (see Ref. 5), in which this enzyme is lacking.

The EAC-R1 tumor is also resistant to Me6MPR, which strongly inhibits growth of the Ehrlich ascites carcinoma (17). Although the mechanisms of action of 6-MP and Me6MPR are not known, the results of combination chemotherapy experiments (M. C. Wang, A. I. Simpson, and A. R. P. Paterson, manuscript in preparation) suggest that they are probably different. The principal metabolite of Me6MPR found in Ehrlich ascites carcinoma cells is the 5'-monophosphate, which is formed by a purine nucleoside kinase, probably adenosine kinase (EC 2.7.1.20) (1, 6). Me6MPR-resistant sublines have been isolated from the Ehrlich ascites carcinoma (7) and from H.Ep. No. 2 cells (2), a line of cultured cells derived from a human epidermoid carcinoma. In both instances, the Me6MPR-selected sublines are deficient in purine nucleoside kinase and therefore are unable to convert Me6MPR to its nucleotide derivative. The work reported here shows that such is not the case with the EAC-R1 subline.

In this study the metabolism and some biochemical effects of Me6MPR have been examined in cells of the resistant EAC-R1 tumor and the sensitive Ehrlich ascites carcinoma parent line in an attempt to understand the biochemical basis of resistance of EAC-R1 cells to the nucleoside analog.

MATERIALS AND METHODS

Me6MPR-methyl-14C was synthesized in this laboratory (6). Adenine-8-14C, 30.7 mc/mmole; guanine-8-14C, 10 mc/mmole; hypoxanthine-8-14C, 7.7 mc/mmole; and glycine-2-14C, 1 mc/mmole, were obtained from New England Nuclear Corp. Aza-serine and Me6MPR were provided by the Cancer Chemotherapy National Service Center.

Feedback inhibition of purine biosynthesis de novo was measured, as previously described (9), in cells pretreated with aza-serine to block phosphoribosylformylglycinamidine synthetase (EC.6.3.5.3). The amount of FGAR which accumulates as a result of this block is a measure of the rate of the early steps of this pathway. In the presence of exogenously supplied purines and purine analogs, the amount of FGAR which accumulates is reduced, and this reduction is taken as a measure of feedback inhibition of the pathway.

Nucleotide synthesis from radioactive purine bases and Me6MPR was measured by paper chromatography of measured volumes of incubation mixtures (18) in 1-butanol:glacial acetic acid:water (5:3:2, by volume) together with carrier nucleotide, nucleoside, and base. The total radioactivity in each class of compound was measured as previously described (18).

The apparent initial rate of glycine-14C influx into tumor cells was measured after an incubation period of 2 min as described by Sterling and Henderson (20). For the determination of specific activities of intracellular glycine and of protein in tumor cells exposed to glycine-14C, cells were first separated from medium and extracted with boiling water (20). The insoluble residue was then extracted with hot 0.4 M perchloric acid and the specific radioactivity of the protein determined (20). Glycine was isolated...
RESULTS

Me6MPR Metabolism

The metabolism of Me6MPR both in vivo and in vitro was compared in cells of the EAC-R1 line, which is resistant to both 6-MP and Me6MPR, and of the parent Ehrlich ascites carcinoma line, which is chemotherapeutically sensitive to these two analogs. Acid extracts of Ehrlich ascites carcinoma and EAC-R1 cells were prepared 6 hr after injection of Me-14C-6MPR into the peritoneal cavity of mice bearing these two ascites tumors, and the extracts were fractionated by ion-exchange chromatography on diethylaminoethyl-Sephadex (I. C. Caldwell, manuscript in preparation). Typical chromatographic profiles are shown in Chart 1. The major peak of radioactivity, eluted immediately after adenylate, is Me6MPR-5'-phosphate; the minor peak of radioactivity is unchanged Me6MPR.

Extracts of EAC-R1 and Ehrlich ascites carcinoma cells were also prepared at intervals up to 24 hr after a single intraperitoneal injection of Me-14C-6MPR into mice bearing these tumors. Radioactivity in the extracts was measured and extracts obtained after each interval were examined by ion-exchange chromatography. In each instance, Me6MPR-5'-phosphate was the only radioactive metabolite detected, and it was concluded that the metabolism of Me6MPR is qualitatively the same in both Ehrlich ascites carcinoma and EAC-R1 cells. The data in Chart 2 show, however, that although the amount of the analog nucleotide made initially was only slightly lower in EAC-R1 cells than in Ehrlich ascites carcinoma cells, this metabolite persisted longer in the sensitive Ehrlich ascites carcinoma cells. This difference in drug persistence may provide at least a partial explanation for the difference in drug sensitivity between the 2 tumor lines.

The initial rate of Me6MPR-5'-phosphate synthesis in vitro was virtually the same in both Ehrlich ascites carcinoma and EAC-R1 cells, as is shown in Chart 3. Both types of cells were incubated with Me-14C-6MPR for up to 3 hr and neutralized perchloric acid extracts of the cells were then examined by paper and ion-exchange chromatography, the analog nucleotide was the only detectable radioactive metabolite.

Purine Biosynthesis de Novo

Although the difference in drug persistence may account in part for the different chemotherapeutic sensitivities of Ehrlich ascites carcinoma and EAC-R1 cells to Me6MPR treatment, the possibility that Me6MPR-5'-phosphate might have different biochemical effects in these two lines of cells was also considered.

Me6MPR is known to be a potent inhibitor of PRPP amidotransferase (EC 2.4.2.14), the first enzyme in the pathway of purine biosynthesis de novo (11), and this inhibition is believed to be of a "feedback" or "allosteric" nature. For this reason, a comparison was made of the ability of Me6MPR to inhibit the early steps of this pathway in Ehrlich ascites carcinoma and EAC-R1 cells. Chart 4 (top) shows the inhibitory effects of a wide range of Me6MPR concentrations on FGAR synthesis in both tumors. It is apparent that this drug had less effect in the EAC-R1 cells than in the parent line of cells at all
Persistence of MeGMPH-5'-phosphate in sensitive and resistant tumor cells in vitro. Me-14C-MPR (1.0 μmole, 3 × 10⁶ cpm) was injected into the ascitic fluid of tumor-bearing mice. Tumor cells were collected as noted, and MeGMPH-5'-phosphate was measured in acid extracts of the cells. Ehrlich ascites carcinoma (■); MeGMPR-resistant subline of Ehrlich ascites carcinoma (▲); MeGMPR, 6-(methylmercapto)purine ribonucleoside; Me-14C-MPR, methyl-labeled MeGMPR.

In order to examine further the question of the relative sensitivities to feedback inhibition of purine biosynthesis de novo in the two cell lines, the effects of adenine and guanine on FGAR accumulation were tested in a similar manner. The data shown in Chart 4 (bottom) indicate that although adenine was equally effective as an inhibitor of FGAR synthesis in both Ehrlich ascites carcinoma and EAC-R1 cells, guanine, like MeGMPR, was able to exert more inhibition in Ehrlich ascites carcinoma cells than in the resistant subline. Again, these results are consistent with the existence in the EAC-R1 cells of an altered PRPP-amidotransferase which is less sensitive to MeGMPR-5'-phosphate than is this enzyme in Ehrlich ascites carcinoma cells.

This hypothesis was tested directly by a comparison of PRPP-amidotransferase activities in intact Ehrlich ascites carcinoma and EAC-R1 cells in the presence and absence of MeGMPR by measurements of the glutamine-dependent disappearance of PRPP. Table 1 shows the inhibition of this enzyme by two concentrations of MeGMPR in both cell lines and demonstrates that the lesser effect was exerted in the EAC-R1 cells. These data, therefore, more directly support the conclusion that there is a quantitative difference in the PRPP-amidotransferases in the two tumors. Because this enzyme assay cannot be carried out in the presence of feedback inhibitors which themselves react with PRPP by alternative pathways, adenine and guanine could not be used in similar experiments.

Alternative explanations for the different effects of MeGMPR and guanine in the sensitive and resistant cells were also considered. One possibility was that Ehrlich ascites carcinoma and EAC-R1 cells, under the conditions used in these experiments (which are slightly different from those used in the experiments of Chart 3), converted purine bases and MeGMPR to nucleotides at different rates. Table 2 shows the results of an experiment in which this possibility was tested. Cells were incubated with purine bases and MeGMPR at concentrations which would give moderate to weak feedback inhibition, and the amount of nucleotide formed after 1 hr was measured. Hypoxanthine was not included in the experiments of Chart 4 because it is believed to cause feedback inhibition primarily after conversion to adenine nucleotides (11). It can be seen that in each instance EAC-R1 cells synthesized at least as much nucleotide as did Ehrlich ascites carcinoma cells. Such an experiment does not, however, rule out the possibility that nucleotides are distributed differently among intracellular compartments in the sensitive and resistant cells.

One other difference between sensitive and resistant tumor cells with respect to purine biosynthesis de novo was found. The total amount of radioactivity from glycine incorporated into FGAR in the absence of inhibitors was about twice as great in EAC-R1 cells as that in Ehrlich ascites carcinoma cells. This
apparent increase in the rate of the purine biosynthetic pathway may reflect altered intrinsic characteristics of PRPP-aminotransferase, the rate-limiting enzymes, e.g., increased catalytic activity or decreased feedback inhibition by endogenous purine nucleotides. Alternatively, it could reflect a difference in one of several aspects of glycine metabolism in these cells. Table 3 shows that the apparent initial rate of glycine influx, the specific activity of total intracellular soluble glycine, and the specific activity of protein synthesized from radioactive glycine were all similar in the Ehrlich ascites carcinoma and EAC-R1 cells. Changes in glycine metabolism may, therefore, be excluded as an explanation for the different apparent rates of purine biosynthesis de novo in the sensitive and resistant tumor cells.

**DISCUSSION**

The tumor subline EAC-R1 which is resistant to treatment with Me6MPR forms the presumptive pharmacologically active metabolite, Me6MPR-5'-phosphate, at the same rate as does the sensitive Ehrlich ascites carcinoma parent line both in vivo and in vitro. Me6MPR-5'-phosphate, however, persists at high concentrations much longer in the sensitive cells, and this difference may contribute to the difference in drug sensitivity between these two tumor lines. Bieber and Sartorelli (3) reported that thioguanine nucleotide disappeared from cells of a thioguanine-resistant subline of Sarcoma 180 more rapidly than from cells of the thioguanine-sensitive parent line, and drug resistance has been attributed in specific cases to increased rates of destruction of purine analogs before conversion to their nucleotide derivatives (12, 19). The exact relationship of this difference between Ehrlich ascites carcinoma and EAC-R1 cells and the observed chemotherapeutic resistance of the latter remains uncertain, however, and must be studied further.

The difference in sensitivity of purine biosynthesis de novo to feedback inhibition by Me6MPR and guanine in Ehrlich ascites carcinoma and EAC-R1 cells, in contrast to the identical effects of adenine on this process in each tumor, supports the conclusions of other workers (8, 14) that PRPP-aminotransferase has two types of binding sites for feedback inhibitors, 1 for 6-aminopurine nucleotides and 1 for 6-hydroxypurine nucleotides. Evidence has previously been adduced (11) that Me6MPR acts as a 6-hydroxypurine nucleotide in this system. These results indicate that although the PRPP-aminotransferases from Ehrlich ascites carcinoma and EAC-R1 cells have similar sensitivities to feedback inhibition by adenine nucleotides, either the EAC-R1 enzyme either binds 6-hydroxypurine nucleotides and their analogs less well, or such binding has less effect on the enzyme. Studies of
Decreased Feedback Inhibition

**CHART 4.** Inhibition of $\alpha$-N-formylglycineamidibis ationate formation by Me6MPR and purine bases in sensitive and resistant tumor cells. Ehrlich ascites carcinoma and an Me6MPR-resistant subline of Ehrlich ascites carcinoma tumor cells, 6 mg wet weight, were incubated in calcium-free Krebs-Ringer phosphate medium, pH 7.4, in an air atmosphere for 1 hr at 37°C with 5.5 mM glucose, 2 mM glutamine, 1 mM glycine-$^{14}$C, 6.4 $\mu$M azaserine, and varying concentrations of 6-(methylmercapto)purine ribonucleoside (MeGMPR), adenine, or guanine. Ehrlich ascites carcinoma (■■■■); Me6MPR-resistant subline of Ehrlich ascites carcinoma (▲▲▲▲).

### TABLE 1

*Effect of Me6MPR on PRPP Concentration in the Presence of Glutamine in Sensitive and Resistant Tumor Cells*

EAC and EAC-R1 tumor cells were incubated as described in Chart 4 with 5.5 mM glucose, 2 mM glutamine, and 0.1 mM or 0.01 mM Me6MPR for 60 min. Control PRPP: 2.1 mmoles per gm.

<table>
<thead>
<tr>
<th>PRPP (% of glucose control)</th>
<th>EAC</th>
<th>EAC-R1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Me6MPR, 0.1 mM</td>
<td>92.3</td>
<td>93.1</td>
</tr>
<tr>
<td>Me6MPR, 0.01 mM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glutamine</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Glutamine + 0.1 mM Me6MPR</td>
<td>57.8</td>
<td>34.4</td>
</tr>
<tr>
<td>Glutamine + 0.01 mM Me6MPR</td>
<td>28.7</td>
<td>19.6</td>
</tr>
</tbody>
</table>

* Me6MPR, 6-(methylmercapto)purine ribonucleoside; PRPP, 5-phosphoribosyl-1-pyrophosphate; EAC, Ehrlich ascites carcinoma; EAC-R1, Me6MPR-resistant subline of EAC.

### TABLE 2

*Nucleotide Synthesis from Purine Bases and Me6MPR in Sensitive and Resistant Tumor Cells*

EAC and EAC-R1 tumor cells, 6 mg wet weight, were incubated as described in Chart 4 for 1 hr at 37°C with 5.5 mM glucose and adenine-$^{14}$C, hypoxanthine-$^{14}$C, guanine-$^{14}$C, or Me6MPR-$^{14}$C.

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Concentration (mM)</th>
<th>Total acid-soluble nucleotide (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>0.1</td>
<td>180,800</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.1</td>
<td>8,830</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>1.0</td>
<td>8,008</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.01</td>
<td>740</td>
</tr>
<tr>
<td>Me6MPR</td>
<td>0.1</td>
<td>214</td>
</tr>
<tr>
<td>Me6MPR</td>
<td>0.01</td>
<td>222</td>
</tr>
</tbody>
</table>

* Me6MPR, 6-(methylmercapto)purine ribonucleoside; EAC, Ehrlich ascites carcinoma; EAC-R1, Me6MPR-resistant subline of EAC.

### TABLE 3

*Glycine Metabolism in Sensitive and Resistant Cells*

EAC* and EAC-R1 tumor cells were incubated as described in Chart 4 with 5.5 mM glucose and 1 mM glycine-$^{14}$C. In separate experiments the total radioactivity in FGAR, the specific activity of cellular protein, and the specific activity of total intracellular glycine were determined after 60 min incubation. The apparent initial rate of glycine entry was measured after 2 min of incubation.

<table>
<thead>
<tr>
<th>Determination</th>
<th>EAC cells</th>
<th>EAC-R1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGAR (cpm)</td>
<td>28,800</td>
<td>54,800</td>
</tr>
<tr>
<td>Protein (cpm/mg)</td>
<td>3,056</td>
<td>2,770</td>
</tr>
<tr>
<td>Glycine pool (cpm/μmole)</td>
<td>134.500</td>
<td>135.900</td>
</tr>
<tr>
<td>Rate of glycine entry (μmole/ml H₂O/2 min)</td>
<td>0.313</td>
<td>0.315</td>
</tr>
</tbody>
</table>

* EAC, Ehrlich ascites carcinoma; EAC-R1, 6-(methylmercapto)purine ribonucleoside-resistant subline of EAC; FGAR, $\alpha$-N-formylglycineamidibis ationate.

PRPP-amidotransferase in cell-free systems are of course necessary to confirm this conclusion, and these will be the subject of a separate study. Caskey et al. (8) have shown that not only can nonspecific treatments in vitro cause the desensitization of this enzyme to feedback inhibition, but that loss of sensitivity to adenylate and to guanylate may occur at different rates.

The observed increased rate of purine biosynthesis de novo in EAC-R1 cells cannot be explained on the basis of increased substrate availability. Glutamine is present at saturating concentrations, and both these and the parent Ehrlich ascites carcinoma cells synthesize PRPP at the same rate. In addition, there appears to be no major difference in glycine metabolism between these cell lines, and in any case, none of these factors would account for the identical response to adenine and dissimilar responses to the 6-hydroxypurines and their analogs observed in these cell lines. This problem remains to be studied further.

In the absence of further information concerning the biochemical effects and mechanism of action of Me6MPR, these results prove neither that this drug inhibits cell growth through in-

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hibition of PRPP-amidotransferase nor that the observed decreased sensitivity of this enzyme in EAC-R1 cells to inhibition by Me6MPR contributes to their chemotherapeutic resistance. The results are consistent with both possibilities, however.

The results reported here illustrate the complexities of resistance and cross resistance studies, as the mechanisms of resistance of EAC-R1 cells to 6-MP and to Me6MPR appear to be quite different, and several different factors may contribute to resistance to Me6MPR.

REFERENCES


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