Biochemical Effects of Formycin, an Adenosine Analog

J. FRANK HENDERSON, A. R. P. PATerson, IAN C. CALDWELL, AND MAKOTO HORI

University of Alberta Cancer Research Unit (McEachern Laboratory), and Department of Biochemistry, Edmonton, Alberta, Canada

SUMMARY

The effects of formycin (7-amino-3-(β-D-ribofuranosyl)pyrazolo-(4,3-d)-pyrimidine), an adenosine analog, on purine metabolism in Ehrlich ascites tumor cells were studied. Purine biosynthesis de novo was blocked by formycin through inhibition of phosphoribosylpyrophosphate synthesis, and this did not occur in cells which did not phosphorylate formycin. This analog had no significant effect on adenine conversion to adenosine and guanine nucleotides, but did inhibit the synthesis of these nucleotides from hypoxanthine and guanine. The incorporation of purines into nucleic acids was inhibited slightly. Formycin did not inhibit the phosphorylation of guanosine or inosine, but did inhibit the incorporation of lysine and methionine into protein. Formycin 5'-phosphate inhibited adenine phosphoribosyltransferase but did not inhibit purine nucleoside kinase.

INTRODUCTION

The discovery of formycin in culture filtrates of Nocardiopsis interforma was reported in 1964 by Hori et al. (12), and this compound was shown to inhibit the growth of Ehrlich ascites carcinoma in vivo, Yoshida sarcoma and HeLa cells in tissue culture, Xanthomonas oryzae, and Mycobacterium 607 (12, 13). The structure of formycin has recently been determined by Koyama et al. (14), and by Robins et al. (21) to be 7-amino-3-(β-D-ribofuranosyl)pyrazolo(4,3-d)-pyrimidine (Chart 1), and it appears to be an adenosine analog possessing an unusual C-glycoside bond. N. interforma also produces the deamination product of formycin, 7-hydroxy-3-(β-D-ribosyl)pyrazolo(4,3-d)-pyrimidine (formycin B), which is an analog of inosine (22). Formycin B did not inhibit the growth of any of the previously mentioned test systems except X. oryzae, against which it was as active as was formycin itself (22).

The effects of formycin on purine metabolism in Ehrlich ascites tumor cells in vivo, and on several enzymes of purine metabolism, are reported in this paper. A preliminary report of studies of the metabolism of formycin has been presented (4).

MATERIALS AND METHODS

Formycin, formycin B, and the 5'-monophosphate and mixed 2'- and 3'-monophosphate derivatives of each were gifts of Prof. H. Umezawa, Institute of Microbial Chemistry, Tokyo. 6-Methylmercaptopurine ribonucleoside-(methyl)-14C was synthesized in this laboratory. Azaserine was obtained from the Cancer Chemotherapy National Service Center. Other chemicals were of commercial origin.

Details of the maintenance and preparation of the Ehrlich ascites tumor cells and incubation conditions for studies in vitro have been described (7). The derivation and biochemical characteristics of the 6-methylmercaptopurine ribonucleoside-resistant subline of this tumor will be presented elsewhere.

Inhibition of tumor growth by formycin was assessed by measurement of packed ascites cell volume 7 days after tumor implantation (19). Formycin treatment (0, 4, or 8 mg/kg) was begun 24 hr after tumor implantation and 4 additional doses were given, each at successive 24-hr intervals.

Nucleotide synthesis from radioactive purine bases was measured by paper chromatography of a measured aliquot of cell extracts (7) in n-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 (20).

The methods employed for the determination of PRPP accumulation (7), FGAR synthesis (6), and inosine synthesis (20) have been described. The isolation of nucleic acid purines for specific activity measurement was performed as previously described (15, 17), except that the final step was paper chromatography in isopropanol:2 N HCl (65:35) (24). For facilitation of the isolation of acid-soluble guanine from small amounts of tumor cells, 0.2 μmole of guanylate was added to each cell extract as carrier, and specific activities are expressed in terms of the guanine isolated. Purine bases and their nucleosides were removed from cell extracts prior to isolation of nucleotide purines by adsorption on 10- x 20-mm columns of Dowex 50-H+. To complete the recovery of nucleotides, such columns were eluted with 5 ml of 0.5 N HCl, which was combined with the initial effluent and heated to release the purine base.

The protein residue remaining after hydrolysis of nucleic acids with hot perchloric acid was washed twice with cold 0.4 N perchloric acid, twice with acetone, and allowed to air-dry. This residue was then dissolved in 1.0 ml of 0.1 N NaOH and 0.5 ml taken for radioactivity measurement in a liquid scintillation counter, using the scintillation phosphor solution of Bray (1). Another portion of this solution was analyzed for protein by the method of Lowry et al. (16).

The adenine phosphoribosyltransferase preparation used was that partially purified from Ehrlich ascites tumor cells by Hori and Henderson and was assayed as described (10). Purine

1 Supported by the Medical Research Council and National Cancer Institute of Canada.

2 Present address: Institute of Microbial Chemistry, Kamiosaki, Shinagawa-Ku, Tokyo, Japan.

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nucleoside kinase activity was measured with 6-methylmercapto-
urine ribonucleoside-\(^{14}\)C as substrate, as described by Caldwell et al. (3).

RESULTS

Growth Inhibition

Prior to studies of the biochemical effects of formycin, an
appraisal was made of its growth inhibitory effects on our line of
the Ehrlich ascites tumor. Tumor cell volume seven days after
implantation in control animals was 2.0 ± 0.32 ml (average of
results from ten animals); five daily treatments with 4 mg/kg
reduced this to 0.25 ± 0.10 ml, and similar treatment with 8
mg/kg resulted in a packed cell volume of 0.06 ± 0.03 ml. These
doses of formycin did not cause loss of weight of the animals,
and did demonstrate the sensitivity of these tumor cells to
formycin.

Purine Biosynthesis de Novo

The rate of the initial reactions of purine biosynthesis de novo
was determined by measurement of the incorporation of glycine-
\(^{14}\)C into FGAR in the presence of azaserine. The experiment
the results of which are shown in Chart 2 measured the effect of
various concentrations of formycin on this process, and demon-
strated that formycin was a potent inhibitor, although no more
than 86% inhibition was achieved at the highest concentration
used, 1 mM. Other experiments showed that 1 mM formycin B
causd no inhibition of FGAR synthesis under the same condi-
tions. Although inhibition of FGAR synthesis is generally
accepted as presumptive evidence of feedback inhibition of
PRPP-amidotransferase, the same result might be achieved if
PRPP synthesis were inhibited, and this possibility was there-
fore tested.

Tumor cells were incubated with glucose and various con-
centrations of formycin for 60 min and then analyzed for PRPP
content. Chart 3 shows that 0.1 mM formycin caused almost
complete inhibition of PRPP accumulation under these condi-
tions, and that this effect declined with decreasing drug concen-
tration. Comparison of the data in Charts 2 and 3 shows that
at high concentrations of formycin, PRPP accumulation was
inhibited more than was the PRPP-dependent synthesis of
FGAR. At lower formycin concentrations, however, the two
curves were similar. This apparent discrepancy between measured
PRPP concentration and PRPP availability has been studied
previously (8), and will be discussed again below.

Even though inhibition of FGAR synthesis by formycin in the
experiments just described could reasonably be ascribed to
inhibition of PRPP synthesis, the possibility remained that
formycin might also cause true feedback inhibition of PRPP-
amidotransferase. In order to test directly the effects of formycin
on this reaction, cells were incubated with glucose to allow
PRPP concentrations to rise, after which glutamine was added
to react with the PRPP by means of the amidotransferase
reaction. It has previously been demonstrated that feedback
inhibitors will block this glutamine-stimulated utilization of
PRPP (9). It is seen in Table 1 that formycin did not inhibit this
reaction, but rather, appeared to cause an increase in its rate.

In order to examine the question of the active form of
formycin, the inhibition of FGAR synthesis was compared in the
parent line of Ehrlich ascites tumor cells and in a subline which
does not phosphorylate formycin and the growth of which is not
inhibited by this compound (4). Formycin (0.1 mM) inhibited
FGAR synthesis by 78.8% in the parent line of cells, whereas
this figure was only 7.8% for the resistant subline. This is
presumptive evidence that formycin nucleotides are the active

\[ \text{Chart 1. Formycin: } 7\text{-amino-3-} (\beta\text{-D-ribofuranosyl})\text{pyrazolo-}
(4,3-d)\text{-pyrimidine.} \]

\[ \text{Chart 2. Effect of formycin on } \alpha\text{-N-formylglycinamide ribo-}
nucleotide (FGAR) synthesis. Ehrlich ascites tumor cells, 20 mg
\text{wet weight, were incubated in calcium-free Krebs-Ringer phos-
phate medium, pH 7.4, in an air atmosphere for 1 hr at 37°C with
2 mM glycine-}\(^{14}\)C, 2 mM glutamine, 5.5 mM glucose, 6.4 \mu M azaserine,
and several concentrations of formycin. Control FGAR: 44,600
cpm. Each point is a mean of values from separate analyses of
4-6 flasks in 2-3 experiments.} \]
Effects of Formycin

On the basis of the results described above, a concentration of 0.1 mM formycin, which inhibited FGAR synthesis by 80%, was chosen for most of the subsequent experiments.

Metabolism of Purine Bases

The effects of formycin on the formation of nucleotides from tracer concentrations of adenine, guanine, and hypoxanthine, and on the specific activities of nucleic acid and acid-soluble nucleotide adenine and guanine are shown in Table 2. These data show that there was no significant effect of formycin on either nucleotide synthesis or on the specific activity of nucleotide adenine and guanine synthesized from adenine. Nucleotide synthesis from both hypoxanthine and guanine was inhibited approximately 58%, and the specific activity of nucleotide adenine derived both from guanine and hypoxanthine was decreased about 66%. The specific activity of nucleotide guanine derived from hypoxanthine was depressed by 54%, while that derived from guanine was inhibited 43%.

Formycin inhibited the incorporation of adenine into nucleic acid purines and of hypoxanthine into nucleic acid guanine about 23%. Guanine incorporation into both nucleic acid purines and hypoxanthine incorporation into polynucleotide adenine were inhibited approximately 48%.

In parallel experiments it was found that almost all of the radioactivity in the nucleic acid fraction was in RNA and that the combined soluble plus messenger RNA fraction was most extensively labeled, followed by ribosomal and DNA-associated RNA. Formycin did not alter the distribution of radioactivity among these fractions, nor did it inhibit incorporation of radioactivity into any fraction preferentially. (These experiments will be described in more detail elsewhere.)

Purine Nucleoside Metabolism

Paterson has shown that Ehrlich ascites tumor cells and human erythrocytes convert hypoxanthine to inosine if another ribonucleoside which can donate ribosyl groups for this reaction is present (20). Certain nucleoside analogs inhibit this process, perhaps by affecting membrane transport of nucleosides. The ability of formycin to serve as a ribosyl donor for inosine synthetase was investigated.

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**Table 2**

<table>
<thead>
<tr>
<th>Purine</th>
<th>Formycin</th>
<th>Total acid-soluble nucleotide (cpm)</th>
<th>Acid-soluble</th>
<th>Nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adenine (cpm/µg) &amp; Guanine (cpm/µg)</td>
<td>Adenine (cpm/µg) &amp; Guanine (cpm/µg)</td>
</tr>
<tr>
<td>Adenine</td>
<td>-</td>
<td>10,720</td>
<td>16,690 &amp; 1090</td>
<td>1470 &amp; 131</td>
</tr>
<tr>
<td>Adenine</td>
<td>+</td>
<td>9,620</td>
<td>15,340 &amp; 945</td>
<td>1140 &amp; 101</td>
</tr>
<tr>
<td>Guanine</td>
<td>-</td>
<td>7,190</td>
<td>84.9 &amp; 447</td>
<td>7.54 &amp; 83.4</td>
</tr>
<tr>
<td>Guanine</td>
<td>+</td>
<td>3,080</td>
<td>29.6 &amp; 205</td>
<td>4.41 &amp; 49.8</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>-</td>
<td>12,070</td>
<td>10,830 &amp; 1140</td>
<td>110 &amp; 28</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>+</td>
<td>4,930</td>
<td>3,530 &amp; 657</td>
<td>61 &amp; 21</td>
</tr>
</tbody>
</table>

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**Chart 3.** Effect of formycin on 5-phosphoribosyl-1-pyrophosphate (PRPP) accumulation. Tumor cells were incubated as described in Chart 2 with 5.5 mM glucose and several concentrations of formycin. Each point is a mean of values from separate analyses of 4 flasks in 2 experiments. Control PRPP: 2.1 µmoles per gm of cells.

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Amino Acid Incorporation into Protein

The effect of formycin on the incorporation of radioactive glycine, DL-lysine, and L-methionine into total cellular protein is shown in Table 3. Incorporation of both methionine and lysine was inhibited about 38%, but this compound, even at 1 mM concentration, had no effect on glycine incorporation. The basis of this selectivity is not known.

Adenine Phosphoribosyltransferase and Purine Nucleoside Kinase

Table 4 shows that formycin 5'-phosphate was a potent inhibitor of adenylate synthesis by adenine phosphoribosyltransferase, and was as active as deoxyadenylate, the most potent inhibitor of this reaction previously known. Formycin B 5'-phosphate was about as active as was inosinate in this system (11).

The effect of formycin on another route of purine nucleotide synthesis, the direct phosphorylation of nucleosides, was also tested. Under conditions in which inhibitor concentration was 10-fold that of the substrate, 6-methylmercaptopurine ribonucleoside, neither formycin nor formycin B, nor their 5'- or mixed 2'- and 3'-phosphates inhibited the purine nucleoside kinase activity of Ehrlich ascites tumor cell extracts.

DISCUSSION

It is apparent from these studies that formycin, an adenosine analog, inhibits many aspects of purine metabolism in chemotherapeutically sensitive Ehrlich ascites tumor cells. It is not possible at the present time, however, to say which of these, or which combination of effects, is responsible for growth inhibition. In order of decreasing sensitivity to formycin, PRPP synthesis, nucleotide synthesis from guanine and hypoxanthine, conversion of these bases to adenine nucleotides, and nucleic acid synthesis, all seem to be affected.

PRPP synthesis is probably inhibited by formycin at the level of phosphoribose pyrophosphokinase, and the inhibition may be due to competition of formycin triphosphate, the major metabolite of this compound (4), with ATP. This type of inhibition has been reported in various systems to be effected by 3'-deoxyadenosine triphosphate (18), xylofuranosyladenine triphosphate (5), tubecidin (9), psicofuranine (2), decoyinine (2), and perhaps by 6-benzylthiopurine (9). Supplies of formycin triphosphate were not sufficient to study its effect directly on this or other enzymes of interest to this study, but this will be done when possible.

Inhibition of PRPP synthesis probably accounts for the observed decline in FGAR synthesis, which is a PRPP-dependent process. The lack of inhibition of FGAR formation in cells which cannot phosphorylate formycin and which are therapeutically resistant to it supports the assumption that nucleotides of formycin, and not formycin itself, effected the observed inhibition.

The inhibition of nucleotide synthesis from guanine and hypoxanthine, but not that from adenine, may be due to PRPP deficiency or to direct inhibition of the phosphoribosyltransferase. It has previously been shown that PRPP accumulation in tumor cells is not an accurate index of the actual availability of this compound for purine nucleotide synthesis (8). In addition, adenine phosphoribosyltransferase has a higher affinity for the available PRPP than does the guanine-hypoxanthine phosphoribosyltransferase (8). The relative insensitivity to formycin of adenine nucleotide synthesis also suggests that formycin 5'-monophosphate is not a major metabolite, and this conclusion is supported by the work of Caldwell et al. (4). Guanine-hypoxanthine phosphoribosyltransferase is known to be inhibited by ATP (23) and may also be sensitive to formycin triphosphate.

Apparent inhibition of other aspects of guanine and hypoxanthine metabolism is probably due in a large part to this inhibition of nucleotide synthesis, although there also appears to be a direct effect on the conversion of inosinate to adenylate. Some inhibition of RNA synthesis is also evident, but the mechanism is not known.

Caldwell et al. (4) have found that ATP concentrations are the

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Specific activity of protein (cpm/mg)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10,100</td>
<td></td>
</tr>
<tr>
<td>Formycin</td>
<td>10,100</td>
<td>103</td>
</tr>
<tr>
<td>Glycine</td>
<td>5,120</td>
<td>0</td>
</tr>
<tr>
<td>Formycin</td>
<td>5,230</td>
<td></td>
</tr>
<tr>
<td>DL-Lysine</td>
<td>6,150</td>
<td>37.5</td>
</tr>
<tr>
<td>Formycin</td>
<td>3,770</td>
<td></td>
</tr>
<tr>
<td>L-Methionine</td>
<td>16,300</td>
<td>38.9</td>
</tr>
<tr>
<td>Formycin</td>
<td>10,100</td>
<td></td>
</tr>
</tbody>
</table>

One µg of enzyme protein was incubated for 12 min at 30°C with 1 mM MgSO4, 1 µM adenine-14C, 0.01 mM PRPP, * 0.12 M Tris buffer (pH 7.4), and formycin derivatives in a total volume of 0.50 ml. Figures are means of results of 2 experiments.

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formycin</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Formycin</td>
<td>0.1</td>
<td>5</td>
</tr>
<tr>
<td>Formycin</td>
<td>1</td>
<td>93</td>
</tr>
<tr>
<td>Formycin</td>
<td>0.1</td>
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<tr>
<td>Formycin</td>
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<td>Formycin</td>
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<tr>
<td>Formycin</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>Formycin</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>Formycin</td>
<td>0.1</td>
<td>17</td>
</tr>
</tbody>
</table>

* PRPP, 5-phosphoribosyl-1-pyrophosphate.
same in control and formycin-treated cells, and Hori et al. (12) showed that formycin did not inhibit glycolysis in Yoshida ascites sarcoma cells. The effects of this drug found in this study can therefore probably not be ascribed to changes in energy metabolism.

ACKNOWLEDGMENTS

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