Further Studies on the Activity of Hadacidin*

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SUMMARY

Hadacidin, which was previously found to inhibit the biosynthesis de novo of adenylic acid, was also found to inhibit the formation of uridylic acid. However, the concentration of hadacidin required to partially suppress pyrimidine synthesis was much greater than that necessary to inhibit purine formation. Hadacidin did not directly inhibit the incorporation of glycine, L-leucine, and formate into proteins.

The effect of hadacidin when used with other known antitumor agents on the growth of Escherichia coli B was also investigated. The results indicated that hadacidin potentiated the growth-inhibitory activity of 2,6-diaminopurine and acted additively with 5-fluorouracil, aminopterin, amethopterin, psicofuranine, puromycin, and azaserine. Hadacidin antagonized the growth-inhibitory properties of 6-mercaptopurine and 6-azauracil.

The antibiotic hadacidin (N-formyl hydroxyaminoacetate) has been shown by Gitterman, Kaczka et al. (3, 7) to exhibit carcinostatic activity against human tumors growing in the embryonated egg. Previous work from this laboratory (18, 19) demonstrated that hadacidin suppressed the biosynthesis de novo of adenylic acid by virtue of its inhibitory action against adenylosuccinate synthetase (IMP : L-aspartate ligase [11]) which catalyzes the conversion of inosinate to adenylosuccinate. It was further shown that the inhibition was competitively reversed by L-aspartate.

To delineate further, by biochemical studies, the areas of potential chemotherapeutic value of hadacidin, its effect on the utilization of bicarbonate in the biosynthesis de novo of pyrimidine nucleotides has been investigated, and a study of the effects of the compound in combination with other antitumor agents that influence nucleic acid metabolism has also been made. The effect on pyrimidine synthesis was studied in Ehrlich ascites cells, and the results of combinations of hadacidin with other drugs were observed in growing Escherichia coli B.

MATERIALS AND METHODS

Materials.—NaHCO₃ and orotic acid-6-C¹⁴ were purchased from New England Nuclear Corporation. Glycine-U-C¹⁴, formate-C¹⁴, and L-leucine-U-C¹⁴ were obtained from Nuclear Chicago. The antitumor agents used in this investigation were obtained from the Cancer Chemotherapy National Service Center.

Ehrlich ascites tumor cell.—Tumor-bearing Swiss albino mice were used 7 days after transplantation. The ascitic fluid obtained from five to six mice was pooled by mixing with about 25 ml. of Robinson’s medium (13) in a chilled flask. Unless otherwise specified, Robinson’s medium contained 0.1 per cent glucose and 0.02 M KHCO₃ (6). The ascites cells were washed in the same medium, and experiments were performed with cell suspensions containing 6 to 9 X 10⁶ cells/ml.

Incubation of ascites cells.—Experiments with isotopic compounds were performed in 10-ml. Erlenmeyer flasks placed in a Dubnoff incubator and shaken constantly under air for a specified time at 37° C. The reactions were terminated by the addition of cold perchloric acid to a final concentration of 0.4 M.

Isolation of nucleotides and determination of radioactivity.—The acid-insoluble residues obtained above were washed with cold 0.4 M perchloric acid, and total nucleic acids were extracted with hot 10 per cent NaCl by the procedure of Schneider and Potter (15). The methods used for the hydrolysis of ribonucleic acid, the isolation of the ribonucleotides, and the determination of radioactivity were the same as described previously (18).

Purification and radioactivity measurements of protein.—After incubation of the ascites cells with...
radioactive amino acids and precipitation in cold 5 per cent trichloroacetic acid the total protein fractions were purified, and radioactivities were measured as described earlier (17).

**Cultivation of Escherichia coli B.—**Escherichia coli B was cultivated at 37° C. in a medium consisting of the following components per liter: 1.5 gm. KH₂PO₄, 13.5 gm. Na₂HPO₄, 0.2 gm. MgSO₄·7H₂O, 2.0 gm. NH₄Cl, 10 mg. CaCl₂, 0.5 mg. FeSO₄·7H₂O, and 4 gm. glucose (9). Fifty ml. of exponentially growing cells were centrifuged in the cold. The pellet was suspended in about 5 ml. of fresh medium and used as described below.

**Test of combined effects of hadacidin with antitumor agents.—**The method described by Elion et al. (2) was used to examine the combined effects of hadacidin and other antitumor drugs on the growth of *E. coli* B. The procedure consisted essentially of the determinations of dose-response curves of any two drugs tested separately and concurrent determinations with varying concentrations of one drug at three or four different concentrations of the second drug.

A suspension of *E. coli* B in 0.1 ml. was added to 4.5 ml. of glucose-salts medium containing various test components as specified in the legends to the tables and charts. The cultures were incubated at 37° C. for 3–3½ hours after which growth was estimated by measurements of turbidity (100 less per cent transmittance) at 600 μμ with a Beckman B spectrophotometer. The turbidity at zero time was 11 (89 per cent transmittance), and the turbidity value of 25 (75 per cent transmittance) was arbitrarily selected for measurements of the inhibitory capacities of the compounds. The “fractional inhibitory concentrations” for each test compound in the combination can be calculated, as described by Elion et al., by dividing the concentration of the first inhibitor (A) present in combination with the second inhibitor (B) by the concentration of A which would be necessary to give the same degree of inhibition (75 per cent transmittance) by itself. To determine the combined effects of the compounds, the fractional inhibitory concentrations of hadacidin were plotted against those of the test compound. An additive effect was indicated when the sum of the fractional inhibitory concentration was equal to unity. Values less than one or greater than one indicated synergism and antagonism, respectively.

**Incubation of Escherichia coli B with orotic acid-6-¹⁴C.**—Logarithmically growing cells of *E. coli* B were incubated with orotic acid-6-¹⁴C and azauracil in the presence and absence of hadacidin. After 3½ hours, the reactions were stopped by the addition of cold TCA. Nucleic acids were extracted by the method of Revel and Magasanik (12), and total radioactivity was determined.

**RESULTS AND DISCUSSION**

**Effect of Hadacidin on the Biosynthesis of Uridylic Acid**

To study the effect of hadacidin on pyrimidine biosynthesis *de novo*, suspensions of Ehrlich ascites cells were incubated with radioactive bicarbonate or orotic acid in the presence and absence of the antibiotic. The specific activities of the isolated nucleotides indicated that the incorporation of NaHC⁴O₃ into acid-insoluble uridylic acid was only slightly depressed (about 15–20 per cent) in the presence of 4.4 X 10⁻⁴ M hadacidin (Table 1).

<table>
<thead>
<tr>
<th>NUCLEOTIDES</th>
<th>EXPERIMENT 1</th>
<th>EXPERIMENT 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
</tr>
<tr>
<td>Adenylic acid</td>
<td>24*</td>
<td>4</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>Uridylic acid</td>
<td>95</td>
<td>75</td>
</tr>
</tbody>
</table>

* Counts/min/µmoles nucleotide.

The incorporation mixtures in a total volume of 4.78 ml. contained 0.05 M NH₄Cl, 0.005 M KHCO₃, 4.4 X 10⁻⁴ M hadadin, and 50 µc. NaHC⁴O₃ (two µmoles). The mixtures were incubated at 37° C. for 45 minutes.

It is of significance to note that, in the case of purine formation, under the same conditions a much lower concentration of hadacidin was sufficient to suppress the synthesis of adenylic acid by about 80 per cent (18). Such a differential sensitivity to hadacidin of reactions involving aspartate was also shown by the previous observation that the amidation of 5-amino-4-imidazole carboxylase with aspartate was not inhibited by hadacidin.

In agreement with the previous experiments (18), the incorporation of bicarbonate into adenylic acid was also markedly inhibited by hadacidin, whereas that into guanylic acid was not affected.

When orotic acid-6-¹⁴C was used as the precursor, the incorporation into uridylic acid and cytidylic acid was not affected (Table 2). The results indicated that the site of inhibition in the formation of uridylic acid observed above occurred prior to the formation of orotic acid. Since it had been shown previously (19) that hadacidin is a metabolic and apparent structural analog of aspartic acid, the inhibition of pyrimidine biosynthesis by hadacidin may be ascribed to the suppression of the formation of orotic acid.
of the reaction involving aspartic acid—namely, in the synthesis of carbamyl aspartate.

**Lack of Effect of Hadacidin on Amino Acid Incorporation into Total Cell Proteins**

The extreme specificity of the biological action of hadacidin was further demonstrated by the absence of any direct inhibitory effect on the incorporation of glycine, L-leucine, and formate into total cell proteins (Table 3). The slight increases in the specific activities of proteins in the hadacidin-treated cells may be due to the inhibition of incorporation of the labeled compounds into nucleic acids. It is conceivable that compounds that are prevented from entering a certain synthetic pathway will accumulate and be utilized, if possible, for other biosynthetic processes.

**Table 2**

**Incorporation of Orotic Acid-6-C14 into Acid-insoluble Pyrimidine Nucleotides of Ehrlich Ascites Cells**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
</tr>
<tr>
<td>Uridylic acid</td>
<td>655</td>
<td>640</td>
</tr>
<tr>
<td>Cytidylic acid</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

* Counts/min/µmole nucleotide.

**Experiment 1.**—Incubation mixture in a total volume of 4.95 ml. contained 0.5 × 10⁻⁴ M KHCO₃, 4 × 10⁻⁴ M hadacidin, and orotic acid-6-C¹⁴ (2.25 µC/0.75 µmole).

**Experiment 2.**—Incubation mixture in a total volume of 4.85 ml. contained 0.5 × 10⁻³ M KHCO₃, 4 × 10⁻³ M hadacidin, and orotic acid-6-C¹⁴ (2.25 µC/0.75 µmole).

Experiments were allowed to proceed for 45 minutes at 37° C.

**Effects of Combinations of Hadacidin with Other Antitumor Agents on the Growth of E. Coli B**

**Additive effects produced with hadacidin.**—The dose response curves obtained with azaserine alone and those in combination with various concentrations of hadacidin are shown in Chart 1A. Hadacidin at concentrations of 1, 2, 3, and 4 mg/ml gave turbidity readings of 40, 34, 26, and 24, respectively. The sum of the fractional inhibitory concentrations at four different levels of the inhibitors was found to be approximately equal to one (Chart 1B), indicating that the two compounds acted additively in depressing the growth of microorganism.

Similar additive effects were observed when hadacidin was used together with 5-fluorouracil, aminopterin, amethopterin, psicofuranine, and puromycin. The concentrations of these antitumor agents required to give a turbidity reading of 25 in the presence and absence of hadacidin are listed in Table 4.

These experiments show that the combination of hadacidin, which is essentially an antipurine compound, with either an antipurine, an antipyrimidine, an antipurine-antipyrimidine, or an inhibitor of protein synthesis may all produce a similar type of additive inhibition. The difficulties encountered in predicting the type of inhibition that may arise in combination treatments are apparent from these experiments.

**Synergistic effects produced with hadacidin and 2,6-diaminopurine.**—As shown in Chart 2, the growth-inhibitory activity of 2,6-diaminopurine was considerably potentiated by the addition of hadacidin. The sum of the fractional inhibitory

**Table 3**

**Effect of Hadacidin on Amino Acid Incorporation into Protein of Ehrlich Ascites Cells**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Radioactive precursor</th>
<th>Amount of radioactivity added</th>
<th>Concentration of hadacidin × 10⁻⁴ M</th>
<th>Specific activities of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Hadacidin-treated</td>
</tr>
<tr>
<td>1</td>
<td>Glycine-U-C¹⁴</td>
<td>0.5 µC/1.6 µmole</td>
<td>4.6</td>
<td>670*</td>
</tr>
<tr>
<td>2</td>
<td>Glycine-U-C¹⁴</td>
<td>0.5 µC/1.6 µmole</td>
<td>11.0</td>
<td>705</td>
</tr>
<tr>
<td>3</td>
<td>L-Leucine-U-C¹⁴</td>
<td>1.0 µC/0.16 µmole</td>
<td>9.3</td>
<td>355</td>
</tr>
<tr>
<td>4</td>
<td>Formate-C¹⁴</td>
<td>20.0 µC/1.45 µmole</td>
<td>4.8</td>
<td>370</td>
</tr>
</tbody>
</table>

* Counts/min/mg protein.

Incubation medium contained 0.1 per cent glucose, 0.02 M KHCO₃, and radioactive precursors and hadacidin in the amounts indicated. The mixtures were incubated at 37° C. for 45 minutes.
concentrations of the two components at the most effective level was estimated to be 0.46.

Although the over-all effect of 2,6-diaminopurine in cellular metabolism is diverse and not fully understood, the inhibition exerted by this compound has been in some cases reversed by adenine (10), suggesting an inhibition of the formation of adenine derivatives. It has also been shown that the conversion of guanylic acid to adenylic acid in human leukocytes is inhibited by 2,6-diaminopurine (16). The possibility is suggested by these observations that the employment of two drugs primarily suppressing the formation of a single metabolite (adenylic acid, in this case) and its interconversion to another (guanylic acid) may result in synergistic inhibition. Elion et al. (2) have demonstrated synergistic effects with similarly related compounds.

Antagonistic action toward 6-mercaptopurine and 6-azauracil.—As shown in Chart 3, the sum of the fractional inhibitory concentrations of 6-MP and hadacidin was greater than one, indicating that the growth-inhibitory property of 6-MP was somewhat antagonized by hadacidin. It has been shown recently that 6-MP inhibited the conversion of inosinate to adenylate and guanylate in ascites cells of lymphoid leukemia L1210 (1) and in enzyme systems obtained from Streptococcus faecalis and pigeon liver acetone powder (14). In view of the fact that hadacidin and 6-MP inhibit, among other reactions, a common site in the purine synthetic pathway, the unexpected antagonistic effects obtained with these two compounds may reflect the complex nature of the action of 6-MP. Evidence that two purine antimetabolites may be functioning differently in the presence of each other has also been obtained with tumor cells (5).

The possible antagonistic action of drugs was further exemplified by the effect produced by hadacidin and 6-azauracil. The dose response curves of azauracil alone and those in combination with various concentrations in hadacidin, as shown in Chart 4, indicate that, with increasing concentrations of hadacidin, increasing amounts of 6-azauracil were required to give a turbidity reading of 25. The results show that the potent inhibitory

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Plus 1 mg/ml of hadacidin (µg/ml)</th>
<th>No hadacidin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorouracil</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Puromycin</td>
<td>30.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Psicofuranine</td>
<td>160.0</td>
<td>175.0</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>55.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Amethopterin</td>
<td>180.0</td>
<td>220.0</td>
</tr>
</tbody>
</table>

The experimental conditions are described in the text under "Tests of Combined Effects of Hadacidin with Anti-Tumor Agents."
activity of 6-azauracil is competitively reversed by hadacidin.

The reversal of the inhibitory effect of azauracil by hadacidin was also shown directly in experiments with isotopic orotic acid (Table 5). In the presence of a constant concentration of 6-azauracil, addition of increasing amounts of hadacidin resulted in a parallel increase in growth of cells and in the incorporation of orotic acid-6-C\textsuperscript{14} into nucleic acids.

The suppression of growth by 6-azauracil has been shown to be due to the block in the conversion of orotidylic acid to uridylic acid exerted by 6-azauridine-5'-phosphate (4). The observation reported here that the incorporation of orotic acid into nucleic acids in the presence of an inhibitory concentration of 6-azauracil is enhanced by hadacidin indicated that the conversion of orotidylic acid to uridylic acid was in some manner, perhaps indirectly, affected by hadacidin. Antagonism between certain purine analogs has also been demonstrated with Salmonella typhimurium (8).

The unusual growth-promoting effects produced by two drugs which are in themselves growth-inhibitory demonstrate the necessity of cautious examinations of drugs which are to be used in combination for therapeutic purposes. Further information with regard to the mode of action of various compounds in the presence of...
each other will facilitate a more rational approach to combination chemotherapy.

REFERENCES
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