Effects of Acivicin and Dipyridamole on Hepatoma 3924A Cells

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ABSTRACT

Dipyridamole inhibited the incorporation of cytidine, thymidine, uridine, and guanosine in rat hepatoma 3924A cells with 50% inhibitory concentrations of 0.2 to 0.5 μM. For deoxycytidine, the 50% inhibitory concentration was about 100 times higher (23.8 μM). Addition of a combination of cytidine, deoxycytidine, and guanosine, at an optimal concentration of 80 μM each, protected the hepatoma cells from the growth-inhibitory action of the antiguammine drug, acivicin. The protection provided by the nucleosides was blocked by dipyridamole (6 μM), but not by nitrobenzylthioinosine (30 μM). The effect on cell survival of graded concentrations of 0.25 to 1.75 μM acivicin plus dipyridamole (5 μM) and 80 μM concentrations each of cytidine, deoxycytidine, and guanosine was investigated. At an acivicin concentration of 1.75 μM, survivals in the different groups were: (a) acivicin alone, 1%; (b) acivicin plus dipyridamole, 1%; (c) acivicin plus nucleosides, 78%; and (d) acivicin plus nucleosides plus dipyridamole, 3%. Acivicin and dipyridamole were cytotoxic for hepatoma 3924A cells with 50% inhibitory concentrations of 0.5 and 20.3 μM, respectively, as measured by clonogenic assay.

INTRODUCTION

The activities of pyrimidine salvage enzymes, thymidine kinase (EC 2.7.1.21), deoxycytidine kinase (EC 2.7.1.74), and uridine-cytidine kinase (EC 2.7.1.48) were increased in rat hepatomas. The former 2 enzymes are transformation and progression linked; the latter is transformation linked (4, 22, 23). These enzymatic activities were elevated in colon tumors in the mouse, rat (24), and human (2). In the transition from quiescence to a proliferative state, the earliest parameters to rise were the incorporation of cytidine and deoxycytidine, and, subsequently, of uridine and thymidine (26). These marked increases preceded the elevation in activities of enzymes of de novo pyrimidine synthesis. The activities of enzymes for salvage utilization of cytidine, deoxycytidine, thymidine, and uridine were high even in resting hepatoma cells (25). Inhibitors of de novo pyrimidine synthesis failed to cure human neoplasms (1, 9), probably because of increased activities of the salvage enzymes (2). These results emphasize the importance of the salvage pathways in cancer chemotherapy.

Acivicin is a glutamine antagonist with activity against L1210 leukemia (3), and human breast and lung tumor xenografts in athymic mice (6). Acivicin inhibited and inactivated several enzymes, including carbamyl phosphate synthetase II, CTP synthetase (27), amidophosphoribosyltransferase (27), GMP synthetase (8, 14, 15, 27), and selectively decreased CTP and GTP pools (11, 15, 27). The cytostatic effects of acivicin on L1210 cells in culture were antagonized by addition of nucleosides to the medium (14, 15). In utilization of exogenous nucleosides by the cell, transport is the initiating step. Dipyridamole and nitrobenzylthioinosine are 2 of the more intensively evaluated inhibitors of nucleoside transport (17, 19). This study reports the effect of combination of acivicin with dipyridamole on hepatoma 3924A cells in the presence and absence of nucleosides.

MATERIALS AND METHODS

Cell Culture. Rat hepatoma 3924A cells were grown in monolayers in McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% dialyzed fetal bovine serum; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. The medium contains 1.5 mM glutamine but no nucleoside or nucleobase. Cells were incubated at 37° in a 5% CO2-95% O2 humidified atmosphere. The hepatoma cells had a doubling time of 15 hr. Cells in logographically growing phase were used in all experiments.

Inhibition of Incorporation of Labeled Nucleosides. The 14C-labeled nucleosides were purchased from Amersham Corp., Arlington Heights, Ill. They were made up to 2 μCi/ml (50 mCi/mmol) before use. For measuring the incorporation of thymidine, uridine, and guanosine, cells were harvested after trypsinization (0.25%); for cytidine and deoxycytidine, cells were obtained by scraping. Aliquots of 5 × 10⁶ cells were seeded into 25-sq cm flasks containing 5 ml of medium. Six hr after seeding, medium was removed, filters were rinsed with phosphate-buffered saline containing, in g/liter, KCl, 0.2; KH2PO4, 0.2; NaCl, 8.0; and NaH2PO4·7H2O, 2.16, and 2 ml of serum-free medium were pipeted into the flasks. Different concentrations of dipyridamole (Sigma Chemical Co., St. Louis, Mo.) or nitrobenzylthioinosine (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were tested with duplicate samples for each concentration; the latter inhibitor was dissolved in dimethyl sulfoxide and appropriate aliquots were added to the aqueous solution. Controls received similar dimethyl sulfoxide aliquots. Immediately thereafter, 20 μl of 14C-labeled nucleosides were added and flasks were incubated for 20 min. After incubation, filters were analyzed for the incorporation of 14C-labeled nucleoside into TCA-insoluble material. Medium was discarded, and the filters were rinsed with ice-cold phosphate-buffered saline. Then 0.5 ml of 0.2 N NaOH was added to dissolve the cells, and 10 ml of ice-cold 10% TCA were added. After 30 min on ice, the contents of the flasks were filtered onto a glass fiber filter (Grade 934AH; size 2.4 cm, Whatman Inc., Clifton, N. J.) by a Millipore filter system. Filters were washed 3 times with 5 ml of 5% TCA and then with 0.5 ml of 95% ethanol. The filters were dried and counted in 10 ml of organic counting scintillator solution (“OCS.” Amersham Corp.).

Growth Inhibition. Logarithmically growing cultures were harvested and seeded into the 25-sq cm flasks (3 × 10⁶ cells/flask). Six hr after seeding, acivicin, nucleosides, and dipyridamole or nitrobenzylthioinosine were added to the medium. Triplicate samples were used for each concentration. After 72 hr of incubation, the medium was discarded, and the trypsinized cells were counted in a Coulter Counter.

Clonogenic Assay. Logarithmically growing cells were seeded at 500

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4. The abbreviations used are: TCA, trichloroacetic acid; I50, 50% inhibitory concentration.

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cells/25-sq cm flask. Six hr after seeding, acivicin, nucleosides, and dipyridamole in various combinations were added to the medium and maintained throughout the experiment without renewing the medium. Triplicate samples were used for each concentration. After 7 days of incubation, medium was discarded, and cells were stained with a saturated solution of crystal violet in 0.85% sodium chloride solution. Colonies consisting of more than 50 cells were counted, and the surviving fraction of treated cells was calculated as the percentage of the colonies formed from untreated cells.

**RESULTS**

**Inhibition of Nucleoside Incorporation by Dipyridamole and Nitrobenzylthioinosine.** For dipyridamole, IC_{50} values for inhibition of incorporation into hepatoma cells of cytidine, thymidine, uridine, and guanosine were 0.2 to 0.5 μM, and for deoxycytidine it was 23.8 μM. Dipyridamole was 9- to 27-fold more effective than nitrobenzylthioinosine (Table 1).

**Protection from Acivicin by Nucleosides.** Cytidine, deoxyctydine, thymidine, uridine, inosine, or adenosine did not protect, but guanosine or deoxyguanosine yielded partial protection from inhibition by 2 μM acivicin (Table 2). The best protection was observed with a combination of guanosine with cytidine and deoxycytidine, but adenosine with cytidine and deoxycytidine gave no protection.

**Protection from Acivicin by Graded Concentrations of Nucleoside Combinations.** Graded concentrations of equimolar guanosine, cytidine, and deoxycytidine were examined for protective action. Acivicin alone (2 μM) reduced the cell count to 10% of the untreated control (Chart 1). Cell counts increased in parallel with the added nucleoside concentration until 80 μM, when cell count was 62% of control. Increasing the nucleoside concentration to 160 μM was not more effective. Thus, the optimal concentration of the 3-nucleoside combination was 80 μM of each nucleoside.

**Effect of Nucleoside Transport Inhibitors on Protection.** The IC_{50} value for inhibition of proliferation of hepatoma cells was 22 μM. Addition to the medium of dipyridamole (6 μM) blocked the protection from acivicin provided by the nucleosides. At 80 μM concentrations of each nucleoside, the cell counts were 62%, but in presence of dipyridamole the cell count was significantly decreased to 17%. Similarly, at 40 μM concentrations of nucleosides, the cell counts decreased from 42 to 11% (Chart 1). In contrast, nitrobenzylthioinosine (30 μM) failed to overcome the protection that the nucleosides provided from acivicin (not shown).

The blocking action of dipyridamole was tested at concentrations of acivicin of 0.25 to 2 μM (Chart 2). At an acivicin concentration of 1 μM or lower, hepatoma cells were completely protected by addition of the 3 nucleosides (80 μM each). Dipyridamole (5 μM) partially blocked this protection. In absence of nucleosides, cell counts in the acivicin plus dipyridamole group, at the concentrations tested, did not differ from those of acivicin alone.

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**Table 1**

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Dipyridamole (μM)</th>
<th>Nitrobenzylthioinosine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50}</td>
<td>IC_{50}</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>23.8</td>
<td>69.0</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.4</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* IC_{50}, 90% inhibitory concentration.

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell count (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None, control</td>
<td>100</td>
</tr>
<tr>
<td>Acivicin</td>
<td>10</td>
</tr>
<tr>
<td>Acivicin + cytidine, 200 μM</td>
<td>11</td>
</tr>
<tr>
<td>Acivicin + deoxycytidine, 200 μM</td>
<td>12</td>
</tr>
<tr>
<td>Acivicin + thymidine, 200 μM</td>
<td>9</td>
</tr>
<tr>
<td>Acivicin + uridine, 200 μM</td>
<td>9</td>
</tr>
<tr>
<td>Acivicin + adenosine, 200 μM</td>
<td>12</td>
</tr>
<tr>
<td>Acivicin + guanosine, 200 μM</td>
<td>39*</td>
</tr>
<tr>
<td>Acivicin + deoxyguanosine, 200 μM</td>
<td>39*</td>
</tr>
<tr>
<td>Acivicin + inosine, 200 μM</td>
<td>8</td>
</tr>
<tr>
<td>Acivicin + adenosine, 200 μM + cytidine, 100 μM</td>
<td>12</td>
</tr>
<tr>
<td>Acivicin + guanosine, 100 μM + deoxycytidine, 100 μM</td>
<td>11</td>
</tr>
<tr>
<td>Acivicin + adenosine, 100 μM + cytidine, 50 μM + deoxycytidine, 50 μM</td>
<td>87*</td>
</tr>
<tr>
<td>Acivicin + guanosine, 100 μM + cytidine, 50 μM + deoxycytidine, 50 μM</td>
<td>73*</td>
</tr>
</tbody>
</table>

* Significantly different from acivicin-treated cells (p < 0.01).
Effect of Acivicin and Dipyridamole on Survival of Hepatoma Cells. Determination of the cytotoxicity by clonogenic assay indicated that for acivicin IC₅₀ was 0.5 μM (not shown), and for dipyridamole it was 20.3 μM (Chart 3). This is the first report that dipyridamole killed cancer cells. The effects of acivicin and dipyridamole in the presence of 3 nucleosides were examined (Chart 4). At 1.75 μM acivicin, the survivals of hepatoma cells were: (a) acivicin alone, 1%; (b) acivicin plus dipyridamole, 5 μM, 1%; (c) acivicin plus nucleosides, 80 μM each, 78%; and (d) acivicin plus dipyridamole plus nucleosides, 3%. Dipyridamole (5 μM) did not affect the killing action of acivicin, but it blocked the protection provided by the nucleosides. The results of clonogenic assays were similar to those obtained from growth inhibition.

DISCUSSION

Acivicin inhibited and inactivated glutamine-utilizing enzymes, CTP synthetase, GMP synthetase, amidophosphoribosyltransferase, and carbamyl phosphate synthetase II (8, 10, 14, 15, 27). The inhibitory effect of acivicin on L1210 cells in culture was partially reversed by guanosine plus cytidine (14). In this study we showed that rat hepatoma cells in culture can be protected from acivicin by a combination of guanosine, cytidine, and deoxycytidine. These in vitro experiments illuminate the importance of salvage pathways, which are relevant to the potential impact of circulating nucleosides on the outcome of cancer chemotherapy. Nucleoside concentration in rat plasma ranged from 1 to 32 μM (5, 7, 12, 20). Thus, the concentrations of nucleosides in plasma might protect tumor cells from inhibitors of de novo pathways. The circulating levels of cytidine and uridine were not remarkably altered in animals treated with inhibitors of de novo pyrimidine synthesis, such as N-(phosphonacetyl)-L-aspartic acid and pyrazofurin (12). Our studies in rats also showed that nearly complete inhibition by acivicin of the activity of carbamyl phosphate synthetase II in the transplanted hepatoma failed to decrease the concentration of UTP (27). Since this is attributed to the operation of the uridine salvage pathway, in anticancer treatment with acivicin it seems desirable to block this salvage pathway.

In the utilization of exogenous nucleosides, transport is the initiating step. Dipyridamole and nitrobenzylthioinosine are nucleoside transport inhibitors that have been studied to some extent (17–19). In this investigation, dipyridamole and nitrobenzylthioinosine inhibited the incorporation of nucleosides in hepatoma 3924A cells. Dipyridamole, but not nitrobenzylthioinosine, blocked the nucleoside-provided protection from acivicin. Dipyridamole is a vasodilator and an antithrombotic agent which has been used for the prophylaxis of both angina pectoris and thromboemboli in patients with prosthetic heart valves (13, 16). Antiviral activity of dipyridamole was observed (21). This appears
to be the first report that dipyridamole could act as a cytotoxic agent against hepatoma cells in tissue culture. This study suggests that dipyridamole might be used to block the salvage pathways, and should be useful in combination chemotherapy with antimetabolites of the de novo pathways.

**REFERENCES**


**Acivicin and Dipyridamole Effects on Hepatoma**

![Graph](attachment:image.png)

Chart 4. Protection from acivicin by nucleosides and blocking of protection by dipyridamole in hepatoma 3924A cells (clonogenic assay). Dipyridamole, 5 μM; nucleosides, guanosine plus cytidine plus deoxycytidine, 80 μM each. Points, means of triplicate assays; bars, S.E.
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