Enhancement of the Sensitivity of Human Colon Cancer Cells to Growth Inhibition by Acivicin Achieved through Inhibition of Nucleic Acid Precursor Salvage by Dipyridamole

Paul H. Fischer,2 Rifat Pamukcu, Gerard Bittner, and James K. V. Willson2,3

Department of Human Oncology, University of Wisconsin School of Medicine, Madison, Wisconsin 53792 [P. H. F., R. P., J. K. V. W.], and William S. Middleton Memorial Veterans Medical Center, Madison, Wisconsin 53705 [G. B., J. K. V. W.]

ABSTRACT

This study was undertaken to determine if salvage of nucleic acid precursors might constitute a mechanism of resistance to acivicin in human colon cancer cells and, if so, to establish whether dipyridamole, an inhibitor of nucleoside and nucleobase transport, can block the salvage process and restore sensitivity to acivicin. Acivicin inhibited the replication of human colon cancer cells (VACO 5) in vitro in a dose- and time-dependent fashion. In addition, marked cell lysis was evident after a 24-hr exposure to acivicin at concentrations greater than 1 µg/ml. The primary metabolic effect of acivicin was depletion of the cytidine triphosphate and guanosine triphosphate pools. Adenosine triphosphate levels were also reduced, but apparently as a consequence of the guanosine triphosphate depletion. VACO 5 cells exposed to acivicin (3 µg/ml) efficiently salvaged low levels (1 µm) of cytidine, guanosine, and guanine and could, therefore, restore the depleted nucleotide pools. The combination of cytidine and guanosine, but not either nucleoside alone, provided significant protection against the growth-inhibitory properties of acivicin. Dipyridamole, at a noncytotoxic concentration (5 µM), blocked repletion of the cytidine triphosphate and guanosine triphosphate pools in cells exposed to acivicin and the nucleic acid precursors. As a result, the growth-inhibitory effects of acivicin were maintained. The salvage of cytidine was particularly sensitive to inhibition by dipyridamole, and no restoration of cytidine triphosphate pools was evident. The cellular uptake of a variety of nucleic acid precursors was differentially sensitive to inhibition by dipyridamole. The 50% inhibitory dose values ranged from 0.01 to 2.5 µM for cytidine and uridine, respectively. The results of this study indicate that, although the replication of VACO 5 cells was inhibited by acivicin, low levels of nucleosides and nucleobases can circumvent the cytotoxicity. Dipyridamole effectively blocked the salvage pathways and restored the sensitivity of the cancer cells to the antiproliferative actions of acivicin.

INTRODUCTION

Acivicin,4 a structural analogue of L-glutamine, is an antitumor agent isolated from Streptomyces sviceus (6). The compound has activity against several murine tumors, the L1210 and P388 leukemias (6) and M5076 ovarian carcinoma (8), and human mammary and lung xenografts (8). Although acivicin inhibits numerous enzyme reactions involving the transfer of nitrogen from the γ-carboxamide of L-glutamine (10), including L-asparagine synthetase (3), carbamoyl phosphate synthetase II (1, 10), phosphoribosylformylglycinamidine synthetase (10), and amido-phosphoribosyl transferase (4, 10), inhibition of 2 other L-glutamine-dependent enzymes, CTP synthetase (4, 10, 18) and XMP aminase (10), appears to be most closely associated with the cytotoxicity of acivicin. Depletion of the CTP and GTP pools and expansion of the UTP pool have typically been seen (10, 13, 14, 30).

In rodent cancer cells, perturbation of both purine and pyrimidine metabolism is evidently critical, since cytosine and guanine nucleosides antagonize acivicin cytotoxicity much more effectively when they are used in combination than when they are used alone (18, 30). These data suggest the potentially important role which salvage of nucleic acid precursors might play in modifying the actions of acivicin. In particular, studies on rat hepatoma 3924A cells have documented the ability of nucleosides to circumvent the growth-inhibitory effects of acivicin (14, 24, 25, 30). Furthermore, dipyridamole, an inhibitor of nucleoside transport (11, 19–21), antagonized the protection afforded by salvage mechanisms and substantially enhanced the cytotoxicity of acivicin (24, 25, 30). Since the antitumor activity of acivicin in humans is now being evaluated in Phase II trials, including colon cancer, we felt that several important questions regarding the actions of this drug in human cells should be addressed. Does acivicin inhibit the growth of a human colon cancer cell line, such as VACO 5? Are the cytotoxic effects seen at clinically achievable concentrations and times of exposure to acivicin? Does salvage of nucleic acid precursors appear to be a likely mechanism of resistance to acivicin? Does the use of dipyridamole block repletion of the nucleotide pools and provide a reasonable therapeutic approach for maintaining the growth-inhibiting properties of acivicin?

MATERIALS AND METHODS

Materials. Acivicin (NSC 163501) was obtained from the National Cancer Institute, Investigational Drugs division. Dipyridamole, nucleotides, nucleosides, and nucleobases were purchased from either Sigma Chemical Co. (St. Louis, MO) or P-L Biochemicals (Milwaukee, WI). Aldrich Chemical Co. (Milwaukee, WI) supplied the Alamine (tri-n-octylamine) and Freon (1,1,2-trichlorotrifluoromethane). [5-3H]Guanosine (5 Ci/mmol), [5-3H]cytidine (30 Ci/mmol), and [8-14C]guanine sulfate (51 µCi/mmol) were purchased from Amersham/Searle Corp. (Arlington Heights, IL). [5-3H]Uridine (20 Ci/mmol) and [5-3H]deoxyctydine (25 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (Brea, CA).

Cell Culture. VACO 5 cells, a human colorectal cell line, were grown in Eagle's minimal essential medium supplemented with 2 mM L-gluta-
mine, 0.1 mM nonessential amino acids (Grand Island Biological Co., Grand Island, NY), insulin (2 μg/ml), transferrin (2 μg/ml), and 7.5 mM sodium selenite (Sigma), gentamicin (50 μg/ml) (Schering Co., Kenilworth, NJ), and 8% heat-inactivated fetal bovine serum, Lot 100374 (HyClone, Logan, UT). The contributions from the serum yielded final medium concentrations of less than 0.1 μM guanosine and cytidine and 2.1 μM guanine. Dissociation medium was identical to growth medium, except that a Ca²⁺- and Mg²⁺-free minimal essential medium was used and fetal bovine serum was excluded. VACO 5 cells were established from a poorly differentiated adenocarcinoma of the cecum and characterized as described previously (16). Stock cultures are passed weekly and have maintained morphological, karyotypic, and drug sensitivity characteristics during the 12-month course of this study. VACO 5 cells were determined to be negative for Mycoplasma contamination, as demonstrated by culture under aerobic and anaerobic conditions (Wisconsin State Laboratory of Hygiene, Madison, WI).

**Cytotoxicity.** Exponentially growing cells, initially plated at a density of 2 x 10⁴ cells/ml in 25-sq cm tissue culture flasks, were maintained at 37° in a humidified 5% CO₂ atmosphere. Medium containing the various additives was added at 12 hr later. In the experiment described in Chart 1, the exposure to acivicin was continued for an additional 2 to 96 hr. The cells were then washed twice, fresh medium was added, and the incubation was continued such that the entire growth period was 96 hr. For the experiment shown in Chart 3, the cells were exposed to acivicin, the nucleosides, or dipyridamole for 24 hr. The cells were then washed twice and incubated for 72 hr in fresh medium containing the nucleosides and dipyridamole as shown. Cells were harvested after a 1- to 2-hr exposure to a Ca²⁺ and Mg²⁺ free dispersion medium. Cells excluding trypan blue were counted using a hemocytometer.

**Measurement of Nucleoside Triphosphate Pools.** Nucleoside triphosphate pool extractions were carried out on duplicate flasks in parallel with the cell growth studies. VACO 5 cells (2.5 to 12 x 10⁴) were collected by centrifugation (10 min, 200 x g), the medium was removed, and the cells were resuspended in 1 ml of cold phosphate-buffered saline. The suspension was transferred to a 1.5-ml microfuge tube and centrifuged (30 sec, 11,500 rpm). The supernatant was removed, and the cell pellet was extracted in 0.1 ml of 0.5 M HClO₄ for 10 min at 4°. The extract was neutralized with an equal volume of 0.5 M Alamine in Freon (12). The neutralized samples were stored at -20° until analyzed by high-pressure liquid chromatography. Chromatography of the samples was performed on a system consisting of a Spectra-Physics 8700 solvent delivery system, a Whatman Partisil 10/25 SAX anion-exchange column equipped with a guard column, a Kratos Spectroflow 773 UV spectrophotometer monitoring 254 nm, and a Hewlett-Packard 3380A integrator. Peaks were identified by retention times. Peak areas were linearly related to the amount injected over the range of 0.08 to 12 nmol, and extracted standards were run on each day of analysis. Recovery of nucleotides by extraction averaged 93%. The buffer system used an isocratic elution of the nucleotides with 0.3 M sodium phosphate, pH 5.0, at a flow of 1 to 2 ml/min as determined by the age of the column. Typical retention times were: UTP, 6.6 min.; CTP, 9.0 min.; ATP, 11.3 min.; and GTP, 17.2 min.

**RESULTS AND DISCUSSION**

**Effects on Cell Growth.** The inhibition of VACO 5 cell replication by acivicin was influenced strongly by both the drug concentration and the duration of exposure (Chart 1). For example, following a 72-hr exposure, increasing concentrations of acivicin (0.1, 1.0, and 10 μg/ml) inhibited cell growth by 30, 82, and 94.5%, respectively. Similarly, acivicin (3.0 μg/ml) reduced cell replication by 34, 72, and 92% after exposures of 6, 24, and 72 hr. These concentrations are in the same range as the peak plasma levels of 4.5 and 0.8 μg/ml achieved in the phase I trials in which acivicin was administered as a 24-hr (27) or 72-hr (5) continuous infusion. Cell lysis, as well as inhibition of replication, was produced by acivicin. There were 20, 44, and 74% fewer cells 24 hr after exposure to acivicin at 1.0, 3.0, and 10 μg/ml than were present at the time of drug addition. Such cell loss is an important parameter and must be considered when analyzing pool size data. In addition, acivicin produced marked changes in the cellular morphology, including a flattened cuboidal appearance and avid attachment to the plastic flasks (data not shown).

**Effects of Acivicin on Ribonucleoside Triphosphate Pools.** Exponentially growing VACO 5 cells were exposed to increasing concentrations of acivicin for either 6 hr (data not shown) or 24 hr prior to extraction with 0.5 M perchloric acid. The data are expressed as the percentage of control base on either nmol/10⁶ cells (B) or nmol/flask (F). The control values (nmol/10⁶ cells) were: CTP, 0.30; GTP, 0.71; ATP, 3.8; and UTP, 1.55. These data are from a single experiment; however, in other experiments in which the time of exposure and concentration of acivicin were varied, similar results were obtained.
Inhibition of Nucleic Acid Precursor Salvage

with a 24-hr exposure to acivicin, the results are presented both as nmol/flask, which reflect overall changes in the cell population, and as nmol/10^6 cells, which depict pool sizes in those cells surviving the exposure to acivicin. The data, expressed as nmol/flask (solid bars), illustrate a dose-dependent reduction in the ribonucleoside triphosphate pools. Acivicin depleted the CTP pools most effectively, followed by the GTP, ATP, and UTP pools. The effects on CTP and GTP implicate CTP synthetase and XMP aminase as the most sensitive targets. These results are consistent with the relatively low K_i values reported for these reactions (13, 26). In addition, studies of the action of acivicin in vivo had shown rapid inactivation of CTP synthetase and depletion of CTP and GTP pools in rat hepatomas (4, 26). The data, expressed as nmol/10^6 cells. Acivicin (3 μg/ml) markedly lowered the CTP and GTP pools most effectively, followed by the GTP, ATP, and UTP pools. Cytidine (10 μM) did not deplete the CTP, GTP, or ATP pools more than 1 μg/ml. Thus, in the cells surviving a 24-hr exposure to acivicin, the nucleotide pool depletions were much less than in the population as a whole. It should be noted that, when plated in drug-free medium, the cells surviving exposure to acivicin regrew with a normal population doubling time of 24 hr. The mechanisms underlying the relative insensitivity of the surviving cells have not been established.

Repletion of Ribonucleoside Triphosphate Pools and Modulation of Cytotoxicity. The capacity of VACO 5 cells to replete nucleotide pools by salvaging nucleic acid precursors present at low concentrations (1 to 10 μM) was assessed (Table 1). Since utilization of either a nucleoside (guanosine) or a nucleobase (guanine) could potentially replete GTP pools, both were evaluated. In these experiments, the cells were exposed to acivicin for 6 hr prior to extraction. Significant cell lysis did not occur over this time period, and the data are expressed as nmol/10^6 cells. Acivicin (3 μg/ml) markedly lowered the CTP and GTP pools, slightly reduced the ATP pools, and expanded the UTP pools. Cytidine (1 μM) entirely repleted the CTP pool without altering the other pools. Both GTP and ATP were restored by guanosine (1 μM) and guanine (1 μM). In the presence of 10 μM of these precursors, the corresponding nucleotide pools were further expanded to 414% for CTP and 188% for GTP. Similar increases were produced in cells not exposed to acivicin (data not shown). Dipyridamole (5 μM) antagonized these salvage mechanisms and entirely blocked the repletion of CTP pools by cytidine (10 μM). A substantial but somewhat lesser degree of inhibition of GTP pool repletion was seen.

The data presented in Table 1 indicate that VACO 5 cells can readily restore depleted ribonucleoside triphosphate pools through salvage mechanisms. As expected, however, cytidine did not replete GTP pools, and CTP pools were not expanded by the purines. These results, as well as the findings with mouse (18) and rat (14, 30) cells, suggested that a pyrimidine and a purine source would be necessary to replete both pools and to effectively antagonize cytotoxicity. As shown in Chart 3, the growth-inhibitory effects of a 24-hr exposure to acivicin (3 μg/ml) were strongly reduced by the simultaneous presence of cytidine and guanosine, whereas 10 μM guanosine alone was ineffective. Similarly, cytidine alone reduced the inhibition produced by acivicin only marginally, from 75 to 67 and 53%, respectively, when present at concentrations of 1 and 10 μM (data not shown). The antagonism of acivicin toxicity produced by a combination of cytidine (10 μM) and guanosine (10 μM) was, however, fully blocked by dipyridamole (Chart 3). This effect was achieved at a concentration of dipyridamole, 5 μM, which did not alter VACO 5 cell growth. The important therapeutic implication is that blockade of either CTP or GTP repletion would maintain

Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>CTP</th>
<th>GTP</th>
<th>ATP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.28 ± 0.007 a (3)</td>
<td>1.2 ± 0.04 (3)</td>
<td>5.7 ± 0.13 (3)</td>
<td>3.0 ± 0.27 (3)</td>
</tr>
<tr>
<td>ACV</td>
<td>0.07 ± 0.002 a (2)</td>
<td>0.28 ± 0.06 (2)</td>
<td>4.0 ± 0.6 (2)</td>
<td>5.9 ± 1.2 (2)</td>
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<tr>
<td>ACV + 1 μM cytidine</td>
<td>0.33 ± 0.03 (3)</td>
<td>0.22 ± 0.05 (3)</td>
<td>3.8 ± 0.38 (3)</td>
<td>5.9 ± 0.6 (3)</td>
</tr>
<tr>
<td>ACV + 10 μM cytidine</td>
<td>1.2</td>
<td>0.2</td>
<td>2.8</td>
<td>4.6</td>
</tr>
<tr>
<td>ACV + 1 μM guanosine</td>
<td>0.036 ± 0.007 (3)</td>
<td>0.36 ± 0.05 (3)</td>
<td>2.6 ± 0.62 (3)</td>
<td>2.3 ± 0.68 (3)</td>
</tr>
<tr>
<td>ACV + 1 μM guanosine + 5 μM DP</td>
<td>0.07</td>
<td>0.3</td>
<td>3.2</td>
<td>4.6</td>
</tr>
<tr>
<td>ACV + 10 μM guanosine + 5 μM DP</td>
<td>0.046 ± 0.017 (3)</td>
<td>0.87 ± 0.18 (3)</td>
<td>4.4 ± 0.9 (3)</td>
<td>2.75 ± 0.85 (3)</td>
</tr>
<tr>
<td>ACV + 10 μM guanosine</td>
<td>0.03</td>
<td>2.2</td>
<td>5.3</td>
<td>3.3</td>
</tr>
<tr>
<td>ACV + 1 μM guanosine + 5 μM DP</td>
<td>0.046 ± 0.002 (3)</td>
<td>0.41 ± 0.07 (3)</td>
<td>2.94 ± 0.62 (3)</td>
<td>2.5 ± 0.81 (3)</td>
</tr>
<tr>
<td>ACV + 1 μM guanosine</td>
<td>0.15 ± 0.001 (2)</td>
<td>1.45 ± 0.16 (2)</td>
<td>7.1 ± 0.76 (2)</td>
<td>4.7 ± 0.04 (2)</td>
</tr>
<tr>
<td>ACV + 10 μM guanosine</td>
<td>0.023 ± 0.003 (3)</td>
<td>2.16 ± 0.23 (3)</td>
<td>7.05 ± 0.8 (3)</td>
<td>4.5 ± 0.52 (3)</td>
</tr>
<tr>
<td>ACV + 1 μM guanosine + 5 μM DP</td>
<td>0.05 ± 0.006 (3)</td>
<td>0.83 ± 0.21 (3)</td>
<td>5.7 ± 1.2 (3)</td>
<td>5.23 ± 0.81 (3)</td>
</tr>
<tr>
<td>ACV + 10 μM guanosine + 5 μM DP</td>
<td>0.025 ± 0.001 (2)</td>
<td>0.88 ± 0.08 (2)</td>
<td>4.1 ± 1.1 (2)</td>
<td>3.0 ± 1.5 (2)</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* Numbers in parentheses, number of experiments.
* ACV, acivicin; DP, dipyridamole.
* VACO 5 cells were exposed to acivicin (3 μg/ml) for 6 hr.
* Mean ± range.

Table 1

Perturbation of nucleotide pools

Inhibition of GTP pool repletion was seen.

The data presented in Table 1 indicate that VACO 5 cells can readily restore depleted ribonucleoside triphosphate pools through salvage mechanisms. As expected, however, cytidine did not replete GTP pools, and CTP pools were not expanded by the purines. These results, as well as the findings with mouse (18) and rat (14, 30) cells, suggested that a pyrimidine and a purine source would be necessary to replete both pools and to effectively antagonize cytotoxicity. As shown in Chart 3, the growth-inhibitory effects of a 24-hr exposure to acivicin (3 μg/ml) were strongly reduced by the simultaneous presence of cytidine and guanosine, whereas 10 μM guanosine alone was ineffective. Similarly, cytidine alone reduced the inhibition produced by acivicin only marginally, from 75 to 67 and 53%, respectively, when present at concentrations of 1 and 10 μM (data not shown). The antagonism of acivicin toxicity produced by a combination of cytidine (10 μM) and guanosine (10 μM) was, however, fully blocked by dipyridamole (Chart 3). This effect was achieved at a concentration of dipyridamole, 5 μM, which did not alter VACO 5 cell growth. The important therapeutic implication is that blockade of either CTP or GTP repletion would maintain
the cytotoxicity of acivicin. The changes in ribonucleoside triphosphate pools associated with the modulation of acivicin cytotoxicity were also determined. Pool sizes were measured in cells exposed for 24 hr to the experimental conditions described in Chart 3. Since these treatments were associated with large changes in cell number, the data were normalized on a per-cell and per-flask basis (Table 2). The effects of acivicin on GTP and ATP pools were reversed by the addition of guanosine (10 μM). In fact, on a per-cell basis, these pools were expanded 2.5 to 3 times over control values. Although 1 μM cytidine fully repleted the CTP pool following a 6-hr exposure (Table 1), only partial reversal was evident after a 24-hr treatment (Table 2). However, 10 μM cytidine was fully restorative, and CTP pools were approximately 2-fold larger than were controls. Although CTP and GTP pools were reversed by the addition of cytidine (10 μM) and guanosine (10 μM), cell growth was still somewhat less than the control rate (Chart 3). This may have resulted from the depressed ATP pools associated with these conditions (Table 2). Dipyridamole entirely blocked the salvage of cytidine (10 μM) and restored the cytotoxicity of acivicin. All of the ribonucleoside triphosphate pools were reduced on a per-flask basis, whereas only minimal changes were evident on a per-cell basis.

Although a wide range of values have been reported, the levels of most nucleosides in human plasma appear to be in the low micromolar (0.1 to 1) range (7, 9, 17, 22, 23). In these experiments, cytidine, 1 μM of guanosine, or guanine fully counteracted the effect of acivicin on CTP or GTP pools during a 6-hr exposure (Table 1). Thus, it would appear that physiological levels of nucleic acid precursors are, in fact, potentially sufficient to facilitate the restoration of pool sizes by human cancer cells.

### Effect of Dipyridamole on the Uptake of Nucleic Acid Precursors

The uptake process involves both the transport and metabolism of nucleosides and nucleobases (29). Since dipyridamole inhibits the first step, transport, the uptake of both nucleosides and nucleobases can be altered as well. This action would, presumably, account for the ability of dipyridamole to prevent the repletion of ribonucleotide pool sizes. Therefore, the ability of dipyridamole to inhibit the uptake of a variety of these compounds into the 60% methanol-soluble and insoluble portions of VACO 5 cells was assessed. Since the effects on incorporation of the precursors into macromolecules and into the nucleotides were essentially identical, only data on the methanol-soluble fraction are presented (Chart 4). Considerable variation in the degree to which dipyridamole inhibited the uptake of different precursors was apparent. Dipyridamole reduced cytidine uptake by 50% at approximately 10 nM, whereas a similar effect on uridine uptake required approximately 2.5 μM of the inhibitor. Guanosine, guanine, and deoxycytidine were sensitive to an intermediate degree. The influence of acivicin pretreatment on the ability of dipyridamole to inhibit nucleoside uptake was also evaluated. The type of experiment described in Chart 4 was repeated for several nucleosides using VACO 5 cells which had been exposed to acivicin (3 μg/ml) for 6 hr, suggesting that pool size depletion does not alter the inhibitory effects of dipyridamole on the uptake of cytidine, guanosine, or deoxycytidine (data not shown).

Numerous studies have shown the feasibility of blocking nucleoside uptake both in vitro and in vivo with inhibitors of nucleoside transport (2, 15, 19–21). Dipyridamole, which inhibits the transport of both nucleosides and nucleobases, is also clinically used as a vasodilator and an antiplatelet drug. Because of our interest in conducting a clinical evaluation of acivicin in combination with a transport inhibitor, dipyridamole was chosen for use in this study. In the course of these experiments, several studies from the laboratory of G. Weber (24, 25, 30) reported on the interaction of acivicin and dipyridamole in rat hepatoma cells. Their data clearly showed that dipyridamole can block the ability of nucleosides to reverse the cytotoxicity of acivicin. In addition,
the replication of rat hepatoma cells was inhibited by dipyridamole, with a 50% inhibitory dose of approximately 20 µM (30).

The results of this study, taken together with the data reported by others (24, 25, 30), suggest that the salvage of nucleic acid precursors is likely to antagonize the cytotoxic effects of acicin in a clinical setting. Intervention with dipyridamole, as suggested by Weber et al. (25), would appear to be a realistic approach to this problem. A critical question is whether the anticancer effects of acicin can be enhanced by dipyridamole without an equivalent increase in toxicity to normal tissues. As a first step toward answering this question, we have initiated a phase I trial of acicin given in combination with dipyridamole.

ACKNOWLEDGMENTS

The authors wish to thank Karen Blomstrom for her assistance in preparing this manuscript.

REFERENCES


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