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BIOCHEMICAL PHARMACOLOGY OF ACIVICIN IN RAT HEPATOMA CELLS*

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Abstract—The antiglutamine agent acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, inhibited the growth of hepatoma 3924A cells in culture. After 7 days of incubation with the drug, an LC_{50} of 1.4 μM was observed by determination of colony forming ability. A combination of cytidine (1 mM), deoxycytidine (10 μM) and guanosine (10 μM) completely protected the hepatoma cells against the cytotoxic action of acivicin, but each nucleoside by itself had no effect. Acivicin (0.1 mM) inhibited the incorporation of uridine and thymidine into macromolecules, but not that of leucine. Acivicin depressed the pools of CTP, GTP, dCTP, dGTP and dTTP to 46, 62, 40, 64 and 53%, respectively, but it increased UTP level to 152% of the values of untreated cancer cells. The activity of a highly purified CTP synthetase (EC 6.3.4.2) from rat liver and hepatoma 3924A was inhibited by acivicin. The inhibition was competitive with respect to L-glutamine, and the K_i values with liver and hepatoma enzymes, determined by Dixon and reciprocal plots, were 1.1 and 3.6 μM respectively. The hydroxy analog of acivicin was also a competitive inhibitor, but it was less effective than acivicin, with a K_i value of 1.8 mM for the hepatoma enzyme. Our observations on the impact of acivicin on the behavior of pools of ribonucleotides and deoxyribonucleotides and the competitive inhibition of purified CTP synthetase from hepatoma cells suggest that a major mechanism of action for this drug is the inhibition of CTP synthetase and GMP synthetase (EC 6.3.5.2).

Previous work in this laboratory showed that CTP synthetase (EC 6.3.4.2) activity was markedly increased in all rat hepatomas examined and that it was elevated to 10-fold of the normal liver value in the rapidly-growing neoplasms [1]. CTP synthetase activity was also increased in chemically induced, transplantable rat kidney tumors, and in colon neoplasms of rat and mouse and in human primary colon carcinomas [1–3]. CTP synthetase which catalyzes the conversion of UTP and CTP in the liver requires glutamine for amidation. This is the final and rate-limiting enzyme in the biosynthesis of CTP [4]. Because of its transformation- and progression-linked increase in activity in neoplasia, CTP synthetase should be a promising target for cancer chemotherapy. Indeed, CTP synthetase had been utilized as a target in the design of enzyme-pattern-directed chemotherapy [5]. It became of interest to examine the possible action of the anti-glutamine agent, acivicin‡, against rapidly growing hepatoma 3924A cells in tissue culture and to test the action of this agent against purified extracts of CTP synthetase prepared from solid hepatoma 3924A.

Acivicin, isolated from *Streptomyces sviceus* [6, 7], had antitumor activity against L1210 and P388 leukemias in mice and human breast and lung tumor xenografts in athymic mice [8, 9, §].

In L1210 cell culture, acivicin blocked DNA synthesis and arrested cell cycle progression in G_1 and early S phase; glutamine protected the cells [10]. Acivicin also inhibited the activities of various bacterial and mammalian enzymes which catalyze the transfer of the amido group of L-glutamine [11]. Further investigation indicated that acivicin was an inhibitor of purified CTP synthetase from rat liver [12].

This paper compares the effects of acivicin and hydroxy acivicin on purified CTP synthetases from rapidly growing hepatoma 3924A and rat liver. The dose–response of acivicin in hepatoma 3924A cells in culture was investigated. The effects of acivicin on concentrations of ribonucleotides and deoxynucleotides were in line with a proposed primary mechanism of action as an inhibitor of CTP and GMP synthetases.

MATERIALS AND METHODS

Materials. Acivicin and hydroxy acivicin were provided by courtesy of the Upjohn Co., Kalamazoo, MI. Labeled isotopes were purchased from Amersham (Arlington Heights, IL). Other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). PEI cellulose plates were obtained from Brinkmann Instruments, Inc. (Westbury, NY).

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‡ Abbreviations: acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; and PALA, *N*-(phosphonacetyl)-L-aspartic acid.

§ G. L. Neil, A. E. Berger, L. J. Hanka and S. L. Kuentzel, Abstracts of the Eleventh International Cancer Congress, Florence, Italy, 4: panel 69 (1974).

Purified CTP synthetase from rat liver and hepatoma 3924A. CTP synthetases from rat liver and hepatoma 3924A were purified 1100-fold and 400-fold, respectively, as previously reported [13]. The biological and biochemical properties of this hepatoma have been reported elsewhere [14–16]. The protein concentration was determined by a standard method [17] using crystalline bovine albumin as standard.

Enzyme assay. CTP synthetase activity was determined by measuring the conversion of [^{14}C]UTP to [^{14}C]CTP, as previously reported [18] with slight modifications. The reaction mixture in 50 μl contained 35 mM Tris–HCl, 8 mM ATP, 1 mM GTP, 100 mM 2-mercaptoethanol, 18 mM $MgCl_2$, 2 mM UTP, 0.0625 μCi [4-^{14}C]UTP, 10 mM L-glutamine, different concentrations of the inhibitor, and enzyme sample. The final pH was 7.4. Reaction was carried out at 37° and terminated at 5-min intervals for 15 min by placing the tubes in boiling water for 3 min. The reaction tubes were centrifuged for 1 min in a Microfuge B (Beckman), and 10 μl of carrier solution containing the mono-, di- and triphosphates of uridine and cytidine was added, each at 2 mM. Chromatography and measurement of radioactivity were conducted as reported [18]. Enzyme activity was expressed as nmoles product formed per hr per mg protein.

The inhibition studies were carried out by the determination of the initial velocity of the enzyme reaction as a function of L-glutamine concentration in the presence and absence of acivicin or hydroxy acivicin, and reciprocal plots were made from the resulting data. The K_i value was also determined by Dixon plot.

Cell culture. Hepatoma 3924A cell line was grown in monolayer cultures in McCoy's 5A medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). The medium contained 1.5 mM glutamine. The hepatoma cells had a doubling time of 15 hr and were subcultured twice a week to keep them continually in logarithmic growth.

Survival studies. Five hundred exponentially growing cells were seeded into each 25 cm^2 flask. Different concentrations of acivicin were added after 24 hr and cultures were incubated for 7 days. Colonies were stained with a saturated solution of crystal violet in 0.85% sodium chloride, and the surviving fraction of treated cells was calculated as the percentage of colonies formed by untreated cells.

Protection by nucleosides from inhibition by acivicin. Cultures of 10 ml were established in 30 ml plastic flasks (Falcon Plastics, Oxford, CA) at an initial cell density of approximately 1×10^5 cells. Drugs and nucleosides were added to the medium after 4 hr and the cells were counted at 72 hr by electronic particle counting (Coulter Counter, model ZB1). The increase in the cell count of the treated cultures was expressed as a percentage of the rise in the control cell count.

Incorporation of precursors into macromolecules. The effect of acivicin on incorporation of radioactive precursors into TCA-insoluble macromolecules of hepatoma 3924A was studied as described previously [19]. Cultures were preincubated with different acivicin concentrations and then pulsed with radioactive precursors for 30 min.

Nucleotide and deoxynucleotide pools. Approximately 10^7 cells were incubated with 1 μM acivicin for 16 hr. Cells were harvested, ribonucleotides and deoxyribonucleotides were extracted, and their concentrations were measured as reported earlier [20, 21].

RESULTS

The validity of the enzyme assay method [18] and the properties of purified enzymes from liver and hepatoma [13] were discussed previously. The inhibitory potencies of acivicin and hydroxy acivicin at different concentrations were studied. Acivicin (1 mM) completely inhibited CTP synthetase from both hepatoma and liver, whereas the hydroxy analog inhibited only 10–20%. The CTP synthetases from liver and hepatoma showed Michaelis–Menten kinetics, and the K_m values for L-glutamine for liver

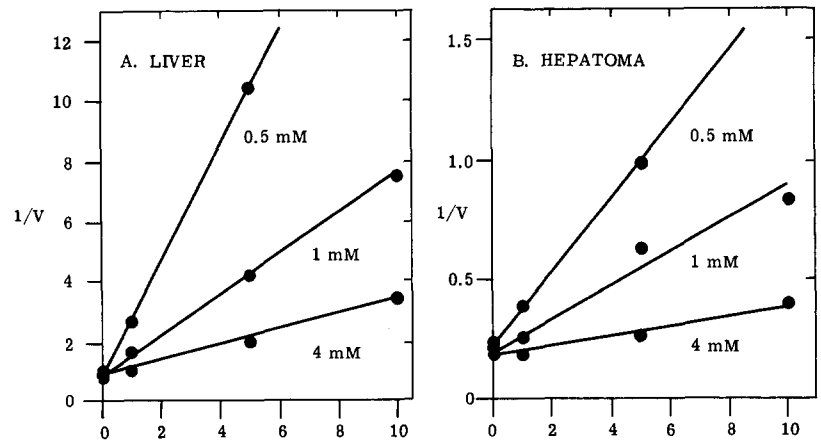

Fig. 1. Dixon plots of the velocity of CTP synthetase activity (ordinate) as a function of the acivicin concentration (on the abscissa in mM $\times 10^{-2}$) in liver (A) and hepatoma (B). Reaction was carried out as described in the text. The concentrations of L-glutamine are indicated in the figure.

Table 1. Protection from acivicin in hepatoma 3924A cells by nucleosides*

Drug	Cell count (% of control)
None, control	100
Acivicin (0.1 mM)	45†
Cytidine (1 mM)	115
Deoxycytidine (0.01 mM)	102
Guanosine (0.01 mM)	90
Acivicin (0.1 mM) plus cytidine (1 mM)	48†
Acivicin (0.1 mM) plus deoxycytidine (0.01 mM)	60†
Acivicin (0.1 mM) plus guanosine (0.01 mM)	52†
Acivicin (0.1 mM) plus cytidine (1 mM) plus deoxyctidine (0.01 mM)	79†
Acivicin (0.1 mM) plus cytidine (1 mM) plus deoxycytidine (0.01 mM) plus guanosine (0.01 mM)	103

* Cells were incubated with the given concentrations of acivicin and nucleosides for 72 hr. Percent of controls was calculated from means of three experiments.

† Significantly different from control ($P < 0.05$).

and hepatoma enzymes were 0.11 and 0.13 mM respectively.

The velocity of the CTP synthetase reaction in enzyme preparations from liver and hepatoma was examined at different substrate concentrations in the presence and absence of acivicin. For both enzymes, acivicin was a competitive inhibitor to the substrate, L-glutamine. The K_i values for liver and hepatoma enzymes obtained from the reciprocal plots were 1.2 and 3.0 μM respectively. The K_i values were also determined by plotting the initial velocity of the reaction as a function of the concentration of the inhibitor (Fig. 1) and calculated to be 1.0 and 4.2 μM respectively. The average K_i values were 1.1 μM for the liver and 3.6 μM for the hepatoma enzyme. Hydroxy acivicin was also a competitive inhibitor to glutamine for the hepatoma enzyme with a K_i of 1.8 mM.

Survival studies. Logarithmically growing cells were treated with different acivicin concentrations. Colonies were stained and counted after 7 days. For acivicin, LC_{50} was 1.4 μM and cell survival dropped sharply at higher concentrations of the drug.

Protection by nucleosides from inhibition by acivicin. An acivicin concentration of 0.1 mM gave 55% inhibition of growth when measured after 72 hr (Table 1). Cytidine (1 mM), deoxycytidine (10 μM), and guanosine (10 μM) had no significant effect on growth of hepatoma 3924A cells. Each of the nucleosides by itself yielded little protection against acivicin. Cytidine (1 mM) and deoxycytidine (10 μM) together provided partial protection with 79% growth. However, the three nucleosides together completely protected the cells against the action of acivicin. Even at a concentration of acivicin which inhibits 90% cell growth, the three nucleosides together were able to protect the cells yielding near normal growth.

Effect of acivicin on the incorporation of radioactive precursors into macromolecules. Hepatoma 3924A cells were incubated with acivicin for 2 or 4 hr and then pulsed with radioactive precursors for 30 min. The incorporation of radioactive precursors was compared with that of untreated cultures. At 1 μM acivicin for 2 hr, only uridine incorporation was inhibited by 32%; the drug had no effect on

Table 2. Effect of acivicin on ribonucleoside and deoxyribonucleoside triphosphate concentrations in hepatoma 3924A cells*

Nucleotides	Control (no drug added)	Acivicin-treated (1 μM)	% of control
ATP	3373 ± 98	2920 ± 195	87
GTP	741 ± 8	462 ± 33	62†
CTP	904 ± 64	413 ± 65	46†
UTP	1654 ± 107	2506 ± 116	152†
dATP	157 ± 4	194 ± 37	124
dGTP	104 ± 6	66 ± 8	64†
dCTP	147 ± 15	59 ± 5	40†
dTTP	250 ± 27	132 ± 17	53†

* Treatment was for 16 hr. Nucleotide concentrations are expressed in nmoles/10^9 cells. Means ± S.E. of three experiments are given.

† Significantly different from control ($P < 0.05$).

thymidine and leucine incorporation. When cells were treated with the same concentration of acivicin for 4 hr, uridine and thymidine incorporation dropped to 43 and 55% of control respectively. Increasing the concentration of acivicin to 0.1 mM further decreased uridine and thymidine incorporation to 18 and 21%. Even at this concentration of acivicin, incorporation of leucine was 82% of control.

Effect of acivicin on ribonucleoside and deoxyribonucleoside triphosphate pools. Table 2 shows the effect of 16-hr treatment with 1 μM acivicin on the ribonucleoside and deoxyribonucleoside triphosphate pools of hepatoma 3924A cells. The concentration of CTP was depressed to 46%, whereas that of UTP accumulated to 152% of controls. GTP level was depressed to 62% and ATP was at 87% of the control. Similar treatment with acivicin depressed the pools of dCTP to 40%, dGTP to 64%, dTTP to 53% and did not significantly change that of dATP.

DISCUSSION

Acivicin and hydroxy acivicin have been shown to inhibit several enzymes which catalyze the transfer of the amido group of L-glutamine in the crude supernatant enzymes of fetal rat liver and in purified enzymes of bacteria [8]. Our present study showed a complete inhibition of purified CTP synthetases from rat liver and hepatoma by 1 mM acivicin, but only 10–20% inhibition by hydroxy acivicin. Acivicin was also a more potent inhibitor for purified hepatoma CTP synthetase than hydroxy acivicin. This is in line with the report that the hydroxy compound was four to ten times less potent than acivicin against L1210 leukemia in mice [22].

For the study of the biochemical pharmacology of acivicin, hepatoma 3924A cells in culture are a suitable model because the biological, cytological and biochemical parameters have been well characterized in this laboratory [19]. The ribonucleotide and deoxyribonucleotide pools of the solid hepatoma 3924A, from which this cell line was derived, have been reported [23]. This cell line was also used in enzyme-pattern-directed chemotherapy with other antipyrimidines [20]. In 7 days of incubation with acivicin, the LC_{50} was 1.4 μM. This LC_{50} was similar to that reported for KB human epidermoid carcinoma cells in culture, but was slightly higher than that of L1210 and P388 mouse leukemia cells [24]. It is interesting that the LC_{50} in hepatoma 3924A was in the same range as the K_i of acivicin against CTP synthetase from solid hepatoma 3924A and in L1210 cells [12].

Cytidine, deoxycytidine and guanosine individually gave little protection to hepatoma 3924A cells from inhibition by acivicin. The protection from inhibition achieved only by the combination of cytidine, deoxycytidine and guanosine suggested that acivicin interfered with both cytidylate and guanylate synthesis. Analysis of the nucleotide pools confirmed that the activities of the glutamine utilizing enzymes CTP synthetase and GMP synthetase may be blocked as indicated by the depression of both CTP and GTP pools.

The lack of change in ATP and the rise in UTP indicated that *de novo* purine and pyrimidine syntheses were affected to a lesser extent, and/or that the salvage pathway could provide protection. The depression of dCTP and dGTP was in line with the postulate of a primary action of acivicin on CTP and GTP synthesis. The depression of dTTP content suggested that CTP may be the primary precursor of dTTP in these cancer cells. Our present study showing that acivicin decreased GTP and CTP pools in hepatoma cells agrees with those previously published by Neil *et al.* [12] in L1210 cells in culture.

The inhibition of incorporation of uridine, but not of thymidine or leucine, at 1 μM acivicin for 2 hr suggested that RNA synthesis was blocked first. DNA synthesis was inhibited by the same concentration of acivicin at 4 hr. Acivicin did not interfere with protein synthesis under our conditions.

The relevance of these investigations is emphasized by a recent report that the combination of acivicin and PALA yielded therapeutic synergism in the LL/PALA-2 variant of the Lewis lung carcinoma [25]. An additional dimension is added to our understanding of the possible mechanism of action of acivicin by indication that this drug inactivated anthranilate synthase and glutamate synthase in *Serratia marcesceus* cells through alkylation [26]. Recent investigations in this laboratory brought evidence that injection of acivicin in rats carrying transplantable hepatoma resulted, in the host liver and the tumors, in a rapid inactivation of amidophosphoribosyltransferase, carbamoyl-phosphate synthetase II, CTP synthetase [27–29] and GMP synthetase.* A profound decrease in the tissue concentrations of CTP and GTP followed these enzymic alterations [28, 29]. These current results provide direct evidence indicating that the biochemical pharmacology of acivicin may entail competitive and tight-binding inhibition or inactivation of key enzymes of glutamine utilization in mammalian tumors.

* M. S. Lui and G. Weber, results to be published.

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