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IN VIVO INACTIVATION OF FORMYLGLYCINAMIDINE RIBONUCLEOTIDE SYNTHETASE IN RAT HEPATOMA*

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Abstract—The antitumor drug acivicin, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, irreversibly inactivated *in vivo* formylglycinamidine ribonucleotide synthetase (FGAM synthetase, EC 6.3.5.3) in transplantable rat hepatoma 3924A while the activity in host liver remained unchanged. At acivicin doses of 1.0 and 5.0 mg/kg body weight, enzyme activity in the hepatoma decreased to 26 and 5%, respectively, after 2 hr. The activity of the *in vivo* inactivated hepatoma 3924A enzyme could not be restored by gel filtration or 40 hr of dialysis. In the absence of L-glutamine, acivicin *in vitro* inactivated both liver and hepatoma FGAM synthetase in a time-dependent fashion, with an inactivation constant $K_{inact} = 66\ \mu M$ and a minimum inactivation half-time $T = 1.0$ min. In the presence of L-glutamine, competitive inhibition was observed with a $K_i = 5\ \mu M$. Protection against *in vitro* inactivation was observed in the presence of 1 mM L-glutamine, suggesting that L-glutamine concentrations are important in the selective toxicity of acivicin on hepatoma cells *in vivo*. Irreversible inhibition of FGAM synthetase by acivicin is consistent with the view that this antibiotic is an active site-directed affinity analog of L-glutamine and indicates that this enzyme is a sensitive target of acivicin action.

Previous work in this laboratory showed that the activities of key enzymes involved in the *de novo* biosynthesis of purines and pyrimidines are increased in hepatomas and in other tumors [1], in particular five enzymes that utilize L-glutamine as an amino (amide nitrogen) group donor. In pyrimidine biosynthesis the enzymes are carbamoyl phosphate synthetase II (EC 6.3.5.5) [2] and CTP synthetase (EC 6.3.4.2) [3], in purine biosynthesis, amidophosphoribosyltransferase (EC 2.4.2.14) [4], GMP synthetase (EC 6.3.5.2) [5], and FGAM‡ synthetase (EC 6.3.5.3) [6]. Based on the postulate that the increase in enzyme activities confers a selective proliferative advantage on cancer cells, they are important potential targets in the design of chemotherapy. On this assumption, investigations were started using various L-glutamine antagonists. Acivicin, an antitumor antibiotic [7, 8], irreversibly inhibited the activities of L-glutamine amidotransferases from *Serratia marcescens*, L5178Y leukemia, and fetal rat liver [9–11]. Investigations in this laboratory showed that acivicin injection into rats bearing transplantable hepatoma 3924A decreased specific activities of amidophosphoribosyltransferase, GMP synthetase, CTP synthetase, and carbamoyl phosphate synthetase II in the tumor [12].

FGAM synthetase catalyzes the irreversible formation of FGAM from FGAR and the amide group of glutamine, the driving force of the reaction being the hydrolysis of ATP. The specific activity of FGAM synthetase was elevated significantly in a series of chemically-induced, transplantable hepatomas, and the increase correlated positively with the proliferation rate of the tumors [6]. In this report, evidence is provided that *in vivo* injection of acivicin irreversibly inactivates FGAM synthetase in hepatoma. *In vitro* incubation of desalted enzyme with acivicin also resulted in rapid, irreversible inactivation. FGAM synthetase proved to be one of the most sensitive targets of acivicin action among the L-glutamine-utilizing enzymes.

MATERIALS AND METHODS

Materials. Hepatoma 3924A was maintained as a bilateral, subcutaneous transplant in male inbred rats (ACI/N); it takes 14 days to reach a diameter of 1.5 cm. Maintenance and killing of animals and excising of livers and tumors were as described [2, 6]. Acivicin was a gift from the Upjohn Co., Kalamazoo, MI. For *in vivo* studies acivicin was dissolved in 0.9% NaCl solution and the drug (1 or 5 mg/kg body wt) or solvent (controls) was injected intraperitoneally into rats bearing the hepatoma. Rats were killed at the indicated times, and supernatant fluids were prepared from the host livers and hepatoma.

Enzyme assay. Activity of FGAM synthetase was measured by converting FGAM produced to AIR using chicken liver AIR synthetase. The reaction

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‡ Abbreviations: FGAM, formylglycinamidine ribonucleotide; acivicin, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; FGAR, formylglycinamide ribonucleotide; AIR, aminoimidazole ribonucleotide; and PRPP, 5-phosphoribosyl-1-pyrophosphate.

Table 1. Selective *in vivo* inactivation by acivicin of FGAM synthetase activity in hepatoma 3924A*

Tissues	Acivicin dose (mg/kg body wt)	FGAM synthetase activity	
		nmoles/hr/mg protein	% of Control
Host liver	0	6.8 ± 0.3	100
	1.0	6.5 ± 0.4	96
	5.0	6.6 ± 0.2	97
Hepatoma	0	28.1 ± 1.1	100
	1.0	7.3 ± 0.3	26†
	5.0	1.4 ± 0.2	5†

* Values are the mean ± S.E. of three or more samples in each group. Animals were killed 2 hr following injection.
† Significantly different from control ($P < 0.05$).

mixture contained 20 mM Tris buffer, 2 mM L-glutamine, 13 mM ATP; 13 mM $MgCl_2$, 100 mM KCl, 0.1 mM FGAR, AIR synthetase (0.20 mg/ml), and the enzyme sample in a 300-μl final volume. The final pH of the reaction mixture was 7.4. Reaction was initiated by addition of supernatant fluid followed by AIR synthetase addition. Tubes without supernatant fluid served as blanks. Incubation at 37° was terminated by the addition of 100 μl of 1.33 M potassium phosphate, pH 1.4, containing 20% trichloroacetic acid. Tubes were centrifuged for 1 min at 21° in a Microfuge B. The following reagents were then mixed with the supernatant solutions without disturbing the protein pellets: 50 μl of 0.1% sodium nitrite; after 3 min, 50 μl of 0.5% ammonium sulfamate; after 3 min, 50 μl of 0.1% *N*-1-(naphthyl)-ethylenediamine dihydrochloride. The mixture was allowed to stand for at least 10 min for complete color development. Following a 30-sec centrifugation, the absorbance at 500 nm was recorded. Under these conditions, the extinction coefficient of the salmon-colored derivative is 24,600 $M^{-1} cm^{-1}$. Further details of this assay were published [6]. Enzyme activity in the standard assay was proportionate with elapsed time and protein amount added. For host liver and hepatoma of control rats, the specific activities were in the range of 6–10 and 28–34 nmoles/hr per mg protein respectively.

Enzyme inactivation. For *in vitro* studies, supernatant fluids were first passed through a Sephadex G-25 column (1.5 × 5.0 cm) in order to remove L-glutamine. *In vitro* inactivation was carried out at 37° in a solution containing 0.25 M sucrose, pH 7.4, 10 mM $MgCl_2$, and 20 mM KCl, the enzyme and acivicin. Inactivation was initiated by the addition of enzyme, and at specified intervals an aliquot of the inactivation mixture (0.1 or 0.15 ml) was transferred to a second mixture (0.2 or 0.15 ml) to determine the remaining activity of FGAM synthetase. The combined mixture (0.3 ml) consisted of the standard reaction mixture described previously [6]. Appropriate control experiments were run to determine the net inactivation of the enzyme by acivicin. Additional control experiments showed that acivicin does not interfere with AIR synthetase or the diazotization of AIR.

RESULTS AND DISCUSSION

Selective in vivo *inactivation by acivicin of FGAM synthetase in hepatoma* 3924A. When rats bearing hepatoma 3924A were given injections of acivicin (1.0 or 5.0 mg/kg, i.p.), in 2 hr the FGAM synthetase activity in the hepatoma decreased to 26 and 5% of the controls. By contrast, in the host liver, the synthetase activity did not change significantly (Table 1).

To test whether the *in vivo* decrease in enzymic activities was reversible, the enzyme extracts were subjected to 40-hr dialysis or G-25 chromatography in an attempt to remove the drug from the enzyme and to restore activity. The liver enzyme which was not inhibited by *in vivo* treatment with acivicin was not affected by dialysis or chromatography. *In vivo* acivicin treatment (1 mg/kg) decreased FGAM synthetase activity to 24% in the hepatoma. When extracts from the hepatoma were subjected to dialysis or G-25 chromatography, the enzyme activities were not restored to the values observed in the uninjected rats (Table 2).

Time course of in vivo *selective inactivation of FGAM synthetase by acivicin.* When acivicin (1 mg/kg, i.p.) was injected in rats bearing hepatoma 3924A, FGAM synthetase activity in the tumor at 15, 30, 60 and 120 min after treatment decreased to 70, 58, 41 and 25% of control values. This experiment yielded an *in vivo* inactivation half-time of $t_{\frac{1}{2}} = 40$ min. In sharp contrast, in the host liver there was no inhibition of the synthetase activity (Fig. 1).

Competitive inhibition of FGAM synthetase by acivicin in vitro. To gain information on the mechanism of inhibition of synthetase, the effect of acivicin was studied at different concentrations of L-glutamine. As the double-reciprocal plots in Fig. 2 show, for the liver and hepatoma 3924A enzymes, the inhibition by acivicin was competitive with respect to L-glutamine, yielding a $K_i = 5$ μM. This K_i is comparable to that reported for carbamoyl phosphate synthetase II [13], CTP synthetase [14] and anthranilate synthetase [11].

In vitro, *irreversible inactivation of FGAM synthetase and protection by L-glutamine.* For these studies the liver and hepatoma supernatant fluids were passed through a Sephadex G-25 column to

Table 2. Irreversible inactivation of FGAM synthetase *in vivo**

Tissue	Treatment	FGAM synthetase activity (nmoles/hr/mg protein) − Acivicin (Control)	+ Acivicin (1 mg/kg)	% Control
Liver	None	9.4 ± 0.1	8.8 ± 0.1	94
	Dialysis	10.0 ± 0.1	9.4 ± 0.1	94
	G-25 chromatography	10.1 ± 0.1	9.5 ± 0.1	95
Hepatoma 3924A	None	32.2 ± 0.2	7.7 ± 0.2	24†
	Dialysis	36.3 ± 0.7	8.7 ± 0.5	24†
	G-25 chromatography	34.4 ± 0.4	8.6 ± 0.2	25†

* Two hours following acivicin injection, the enzyme–acivicin complex was either dialyzed for 40 hr against isotonic buffer, pH 7.4, (four changes, 1:100) containing 5 mM L-glutamine or desalted on Sephadex G-25 in an attempt to remove the drug from the inactivated complex and restore activity. Values are the mean ± S.E. of three or more determinations.

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

remove L-glutamine as described in Materials and Methods. In liver and hepatoma extracts, acivicin (10 μM) decreased synthetase activity to 42 and 15%, respectively, but addition of L-glutamine (1 mM) to the inactivation mixture was able to protect the enzyme from inactivation. Dialysis alone decreased both the liver and hepatoma enzymic activities, but G-25 chromatography had no effect. Neither procedure was able to reactivate the enzyme to reverse the action of acivicin (Table 3).

In vitro *inactivation of FGAM synthetase of host liver and hepatoma* 3924A. When 10 μM acivicin and 2 mM L-glutamine were present together in the assay mixture, approximately 100% of the liver or the hepatoma enzyme activity remained after the 20-min incubation in the standard assay (Fig. 2).

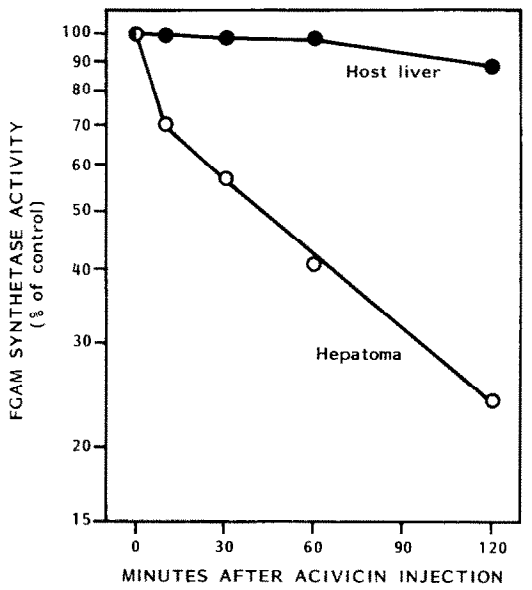

Fig. 1. Time course of *in vivo* inactivation of FGAM synthetase by acivicin. Rats were injected with acivicin (1 mg/kg, i.p.) and killed at the indicated times. Synthetase activity was measured in the supernatant fluids of host liver and hepatoma as outline in Materials and Methods.

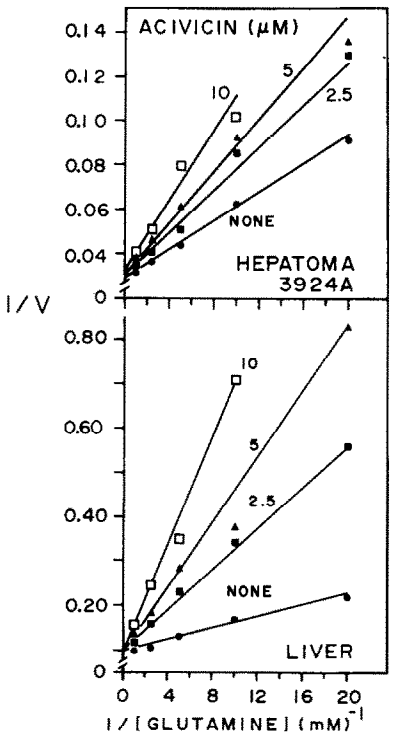

Fig. 2. Competitive inhibition of liver and hepatoma FGAM synthetase activity by acivicin. Synthetase activities were measured after a 20-min incubation in the standard assay system at acivicin concentrations of 0, 2.5, 5 and 10 μM and at varied concentrations of L-glutamine. Synthetase activity is given in nmoles/hr/mg protein. Assays were carried out in the 100,000 *g* supernatant fluids from which L-glutamine was removed as described in Materials and Methods. Linear regression by the method of least squares was used to determine the best fit of lines.

Table 3. Irreversible inactivation of FGAM synthetase *in vitro* and protection by L-glutamine

Tissue	Treatment*	FGAM synthetase activity: nmol/hr/mg protein		
		− Acivicin (Control)	+ Acivicin (10 μM)	% Control
Liver	No L-glutamine	7.5 ± 0.1	3.2 ± 0.1	42†
	Plus L-glutamine (1 mM)	8.9 ± 0.5	9.0 ± 0.7	101
	Dialysis	4.4 ± 0.4	1.0 ± 0.1	23†
	G-25 chromatography	8.1 ± 0.1	2.1 ± 0.1	26†
Hepatoma	No L-glutamine	28.1 ± 1.6	4.3 ± 2	15†
	Plus L-glutamine (1 mM)	31.6 ± 0.9	30.0 ± 1	95
	Dialysis	18.8 ± 0.4	1.5 ± 0.6	8†
	G-25 chromatography	38.0 ± 0.8	2.7 ± 0.7	7†

* Supernatant fractions were desalted on Sephadex G-25. Inactivation by 10 μM acivicin for 20 min was carried out in the presence or absence of 1 mM L-glutamine as described in Materials and Methods. Following inactivation in the absence of L-glutamine, the enzyme–acivicin complex was either dialyzed for 40 hr against isotonic buffer, pH 7.4, (four changes, 1:100) containing 5 mM L-glutamine or desalted on Sephadex G-25 in an attempt to remove the drug from the inactivated complex. Values are the mean ± S.E. of three or more determinations.

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

When L-glutamine was removed from samples of liver and hepatoma, incubation of the extracts with acivicin (10 μM) resulted in a progressive loss of activity at the same rate for both liver and hepatoma enzymes (Fig. 3). Under these circumstances, 60% of the activity was lost in 12 min. These data indicate that the different responsiveness of the liver and hepatoma enzymes in the crude extracts may be due, at least in part, to the differing L-glutamine concentrations which are high in liver and low in the hepatoma.

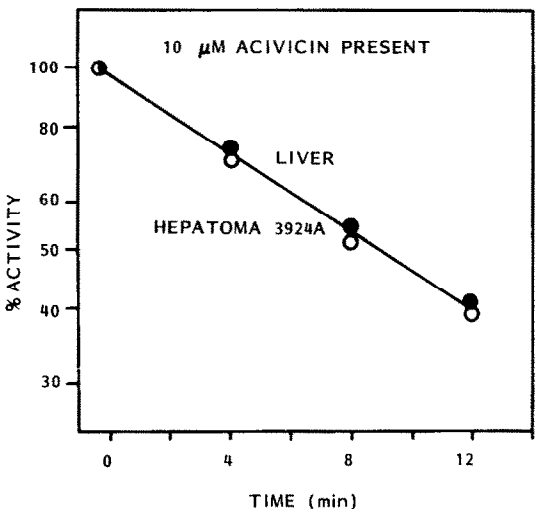

Fig. 3. Acivicin (10 μM) inactivation of liver and hepatoma FGAM synthetase. Activity was measured in the 100,000 *g* supernatant fluid from which L-glutamine was removed as described in Materials and Methods. The activity was measured by the standard enzyme assay. Enzyme activities in nmoles/hr: 6.5 for liver (0.47 mg protein) and 8 for hepatoma (0.26 mg protein).

The time course of inactivation of hepatoma FGAM synthetase by different concentrations of acivicin demonstrated first-order kinetics up to 80% inactivation (Fig. 4A). The plots of inactivation half-time vs 1/[acivicin concentration] exhibited a linear relationship (saturation kinetics) (Fig. 4B), consistent with the formation of a reversible intermediate preceding irreversible inactivation as suggested earlier [11, 15]. Kinetic parameters for acivicin inactivation of the enzyme were calculated from the linear plots, based on the equation [15]:

$$\text{inactivation half-time} = T\,K_{inact}/[I] + T,$$

where I is the inhibitor concentration, T, which can be calculated from the intercept of the ordinate, is the minimum inactivation half-time at infinite concentration of inactivator, and K_{inact} is the inactivation constant. For acivicin the inactivation constant was 66 μM and the minimum inactivation half-time was 1.0 min. These values are similar to the inactivation constant and minimum inactivation half-time of 90 μM and 0.7 min, respectively, reported for carbamoyl-phosphate synthetase II [13].

L-Glutamine protection against acivicin inactivation. L-Glutamine present at the start of incubations protected FGAM synthetase from inactivation so that saturation kinetics were observed (Fig. 2). However, without L-glutamine present, rapid and irreversible inactivation occurred (Fig. 3, Table 2). *In vitro* inactivation of hepatoma 3924A FGAM synthetase using crude supernatant fluid rather than desalted enzyme resulted in lower relative inactivation rates. These data again suggest that tissue L-glutamine concentrations may be a determinent for *in vivo* inactivation as the concentration of L-glutamine in rat liver (4.5 mM) is 9-fold higher than that in hepatoma 3924A (0.5 mM) [16]. The higher L-glutamine concentration in liver versus hepatoma 3924A may protect the liver against acivicin inactivation, while providing selective tox-

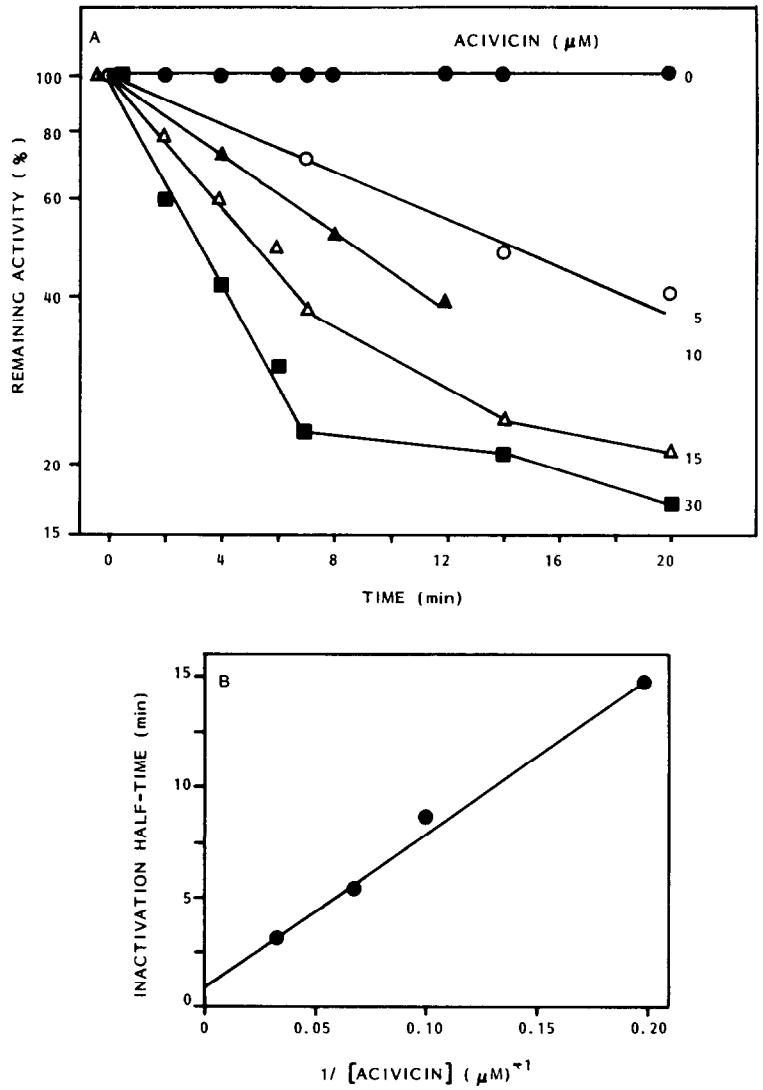

Fig. 4. Effect of acivicin concentration on inactivation of FGAM synthetase. Inactivation of hepatoma 3924A enzyme from which L-glutamine was removed was carried out as described in Materials and Methods, except for the addition of different concentrations of acivicin, as indicated. Key: (A) time-course of inactivation by different concentrations of acivicin; and (B) the plot of inactivation half-time vs 1/[acivicin]. Enzyme activity in nmoles/hr: 8.0 (0.29 mg protein).

icity against the hepatoma cells. The importance of tissue L-glutamine content is supported by the fact that the initial binding of acivicin to FGAM synthetase and other L-glutamine utilizing enzymes is competitive [13, 14]. Thus, the affinity for L-glutamine, the K_i for acivicin, and tissue L-glutamine concentration are all important determinants in the sensitivity of an enzyme to this agent.

FGAM synthetase from hepatoma 3924A with respect to the other L-glutamine utilizing enzymes of purine and pyrimidine *de novo* biosynthesis is as sensitive to *in vivo* inactivation by acivicin (5 mg/kg body wt) as CTP synthetase and GMP synthetase (both about 5% of control) and more sensitive than carbamoyl phosphate synthetase II (35% of control) and amidophosphoribosyltransferase (45% of control) [12]. The marked sensitivity of tumor FGAM synthetase to acivicin demonstrates the importance of this enzyme as a target for L-glutamine antagonists. *In vivo* inhibition of FGAM synthetase and amidophosphoribosyltransferase, the two L-glutamine utilizing enzymes of *de novo* IMP biosynthesis, may contribute, in part, to the 5-fold elevation in PRPP levels following acivicin injection [12]. Thus, with purine salvage capacity remaining constant following acivicin treatment [17], agents that block the salvage or transport of purine nucleosides and bases should heighten the effect of L-glutamine antagonists as shown *in vitro* with the combination of acivicin and dipyridamole [18].

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