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ENZYMIC PROGRAMS OF RAT BONE MARROW AND THE IMPACT OF ACIVICIN AND TIAZOFURIN*

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Abstract—The *in vivo* actions of two antimetabolites, acivicin (NSC-163501) and tiazofurin (NSC-286193), were examined on the enzymic programs of rat bone marrow. From the bone marrow of the femurs, 100,000 *g* supernatant fractions were prepared; enzymic activities were measured by isotopic assays, and cellularity was determined. In the normal bone marrow, the specific activities of pyrimidine *de novo* synthetic enzymes, CDP reductase, dTMP synthase, CTP synthase, carbamoyl-phosphate synthase II (synthase II), orotidine 5′-phosphate decarboxylase and aspartate carbamoyltransferase, were 1, 2.7, 5, 10, 63 and 601 nmol/hr/mg protein, respectively, whereas those of the salvage enzymes, deoxycytidine, thymidine, cytidine and uridine kinases were 3, 43, 149, and 367 nmol/hr/mg protein, respectively. In purine biosynthesis, the activities of the *de novo* synthetic enzymes, IMP dehydrogenase, formylglycinamidine ribonucleotide (FGAM) synthase, GMP synthase, amidophosphoribosyltransferase (AT) and adenylosuccinate synthase were 16, 8, 107, 78 and 124 nmol/hr/mg protein, respectively, and those of the salvage enzymes, adenine, hypoxanthine and guanine phosphoribosyltransferases, were 340, 407, and 1018 nmol/hr/mg protein, respectively. The sequence of events was elucidated after a single i.p. injection of acivicin (5 mg/kg) or tiazofurin (200 mg/kg). Within 2 hr after acivicin injection, CTP, GMP and FGAM synthases lost 85–90%, while AT and synthase II lost 50 and 80%, respectively, of their activities. The activities rose to near normal range by 72–96 hr. The bone marrow cellularity decreased, reaching a nadir at 24 and 48 hr, and returning to normal range by 72 and 92 hr; thymidine kinase activity followed a similar pattern. Tiazofurin injection depressed IMP dehydrogenase activity to 20% by 2 hr with a rebound to normal range by 48 and 72 hr. The cellularity decreased more slowly, reaching its lowest point at 24 hr and returning to normal range at 72 hr. For acivicin the marked depletion of the activities of the glutamine-utilizing enzymes and for tiazofurin that of IMP dehydrogenase might account, in part at least, for the bone marrow toxicity of these antimetabolites. Because of the presence in the bone marrow of high activities of purine and pyrimidine salvage enzymes, it should be possible to design methods utilizing nucleosides and nucleobases to protect the bone marrow from the action of antimetabolites.

Acivicin [L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, NSC-163501, AT-125], an antiglutamine agent, has been shown to competitively inhibit and inactivate *in vitro* and *in vivo* enzymes of glutamine utilization, to possess potent anti-tumor activity against murine tumors, and is now in phase II trials [1–12]. Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC-286193) exhibits potent anti-tumor action against murine tumors and is curative against Lewis lung tumor, a neoplasm refractory to most oncolytic agents [13, 14]. It has been established that tiazofurin is metabolized to an analog of NAD, and the active metabolite, TAD‡, is a strong inhibitor of IMP dehydrogenase, resulting in a depression of GTP and dGTP concentrations which leads to inhibition of tumor cell proliferation [15–17]. Tiazofurin as a single agent exhibits potent cytotoxicity against rat hepatoma 3924A in culture and *in vivo* [17–19], and this drug is in phase I and II trials [20, 21]. Because of these ongoing studies, it was of interest to elucidate the impact of these drugs on the bone marrow. Results indicated a marked depression in bone marrow cellularity in the rat and in the specific activities of enzymes sensitive to acivicin or tiazofurin. The elucidation of the enzymic programs of the bone marrow should have relevance in the protection against the impact of these side effects.

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‡ Abbreviations: AT, amidophosphoribosyltransferase; FGAM, formylglycinamidine ribonucleotide; synthase II, carbamoyl-phosphate synthase II; and TAD, thiazole-4-carboxamide adenine dinucleotide.

MATERIALS AND METHODS

Materials. Acivicin was obtained from the Upjohn Co., Kalamazoo, MI, and tiazofurin was provided by Dr. Ven Narayanan, National Cancer Institute,

NIH, Bethesda, MD. For the enzyme assays, the isotopes were purchased from Amersham Radiochemicals, Arlington Heights, IL, and from New England Nuclear, Boston, MA. Other chemicals were also of the highest quality available, purchased from Sigma, St. Louis, MO.

Biochemical studies. ACI/N inbred male rats of 180–220 g of weight were kept in individual cages. Rapidly growing hepatoma 3924A was transplanted subcutaneously as described [4, 5]. Drugs were injected intraperitoneally. Rats were killed by stunning and decapitation. Blood was obtained from severed neck vessels and centrifuged, and the serum was isolated for determination of acivicin content. Femurs were cut and bone marrow was suctioned out within 90 sec of decapitation. The liver and hepatoma were excised rapidly. From the tissues, 100,000 *g* supernatant fractions were prepared after centrifugation of the homogenates for 30 min as described [4, 5]. The protein concentration was determined by the biuret method, using crystalline serum albumin as a standard. Assays for activities of enzymes of biosynthesis of pyrimidines [3–5, 9] and purines [4, 10, 22, 23] and concentrations of L-glutamine [6, 8] were carried out as cited. Cell counts were determined in a Coulter counter and were verified by determinations in a hemocytometer.

Expression of biochemical results and statistical evaluation. Enzymic activities were calculated in nmol substrate metabolized per hr per mg protein, as specific activity; data were also expressed as percentages. Results were subjected to statistical evaluation by the *t*-test for small samples. Differences between means yielding a probability of less than 5% were considered as statistically significant.

RESULTS AND DISCUSSION

Pyrimidine and purine enzymic program of the bone marrow. A comparison of the specific activities of the enzymes of *de novo* and salvage pyrimidine and purine biosynthesis of rat liver and bone marrow is provided in Table 1. Results are also expressed as percentages of the rat liver values and a further

Table 1. Enzymc activitics of *de novo* and salvage pyrimidine and purine biosynthesis in rat bone marrow

Synthetic enzymes		Activity (nmol/hr/mg protein)		% of Liver	
Pyrimidine	EC No.	Liver	Bone marrow	Bone marrow	Rapid hepatomas*
De novo					
CDP reductase	1.17.4.1	0.03	1	3,000†	18,348†
Thymidylate synthase	2.1.1.45	0.2	2.7	1,350†	12,536†
CTP synthase	6.3.4.2	4	5	125	1,122†
Carbamoyl-phosphate synthase II	6.3.5.5	9	10	111	950†
OMP decarboxylase	4.1.1.23	54	63	116	889†
Aspartate carbamoyl transferase	2.1.3.2	483	601	124	706†
Salvage					
Deoxycytidine kinase	2.7.1.74	1	3	300†	1,400†
Thymidine kinase	2.7.1.21	2	43	2,150†	7,000†
Cytidine kinase	2.7.1.48	60	149	248†	694†
Uridine kinase	2.7.1.48	123	367	298†	694†
Purine					
De novo					
IMP dehydrogenase	1.1.1.205	4	16	400†	1,350†
FGAM synthase	6.3.5.3	7	8	114	378†
GMP synthase	6.3.5.2	35	107	305†	548†
Amidophosphoribosyl-transferase	2.4.2.14	57	78	137†	280†
Adenylosuccinate synthase	6.3.4.4	148	124	84†	308†
Salvage					
Adenine phosphoribosyltransferase	2.4.2.7	379	340	89	170†
Hypoxanthine phosphoribosyltransferase	2.4.2.8	405	407	100	70†
Guanine phosphoribosyltransferase	2.4.2.8	1,206	1,018	83	70†

Data are from three or more samples. Standard errors were $<\pm 5\%$ of the means.
* Values for rapidly-growing hepatomas were from Ref. 4.
† Significantly different from liver ($P < 0.05$).

comparison is given with the relative values of rapidly-growing hepatomas 3924A and 3683F reported elsewhere [4]. In the bone marrow, the rate-limiting enzyme of overall pyrimidine synthesis is CDP reductase, which had the lowest activity of all enzymes in purine and pyrimidine metabolism. In bone marrow, CDP reductase activity was 30-fold and dTMP synthase activity 13.5-fold higher than in the liver, but the other four pyrimidine synthetic enzymes had activities similar to that of liver. By contrast, the activities of the pyrimidine salvage kinases were 2.5- to 21-fold higher in the bone marrow than in liver. This indicates a heightened capacity of the bone marrow to salvage particularly thymidine but also uridine, cytidine and deoxycytidine.

In *de novo* purine biosynthesis in bone marrow the enzymic activities that channel IMP to guanylate biosynthesis, GMP synthase and IMP dehydrogenase, were 3- to 4-fold higher than those in liver. The other enzymes measured were near the range of the liver. The salvage enzymic activities were similar in bone marrow and liver. However, because of the high specific activities of the purine salvage enzymes, the bone marrow is well suited for the salvage processing of adenine, hypoxanthine and guanine; the activities were much higher than those of the enzymes of *de novo* purine biosynthesis.

Since the bone marrow is a tissue of high cellular renewal, the enzymic programs were also contrasted with those of rapidly growing rat hepatomas 3924A and 3683F. The comparison indicates that the enzymic capacity for pyrimidine and purine biosynthesis in the rapidly growing hepatomas was much higher than in the bone marrow. In the hepatoma, all pyrimidine enzymic activities increased 6.9- to 183-fold and in purine *de novo* biosynthesis, 2.8- to 13.5-fold. The only activities that were comparable in bone marrow and hepatoma were those of the purine salvage enzymes, however, these specific activities were orders of magnitude higher than those of the rate-limiting enzymes of *de novo* synthesis, such as CDP reductase, dTMP synthase or IMP dehydrogenase. The heightened capacity for cell renewal in bone marrow is reflected in the high activities, compared to those of liver, of the key enzymes of dTMP biosynthesis and salvage.

The enzymic programs indicate that the bone marrow, as compared to liver, has an amplified capability for purine and pyrimidine nucleotide biosynthesis which is chiefly achieved by the high activities of pyrimidine and purine salvage enzymes and the elevated activities of CDP reductase, dTMP synthase, IMP dehydrogenase and GMP synthase. This enzymic program is bone marrow specific and significantly different from that of the reference low cell renewal tissue, the adult resting liver, and the high cell renewal neoplastic tissues, the rapidly growing hepatomas.

Dose–response studies with acivicin. Acivicin dose–response studies have been carried out in the mouse [24]. In the rat, different doses were employed in the study of the mechanism of action of acivicin in liver [3, 4, 10], hepatoma [3, 4 10] and sarcoma [12] and recently in brain [25]. Since no biochemical pharmacology investigations appear to have been conducted on the action of acivicin on rat bone marrow, dose–response studies were carried out.

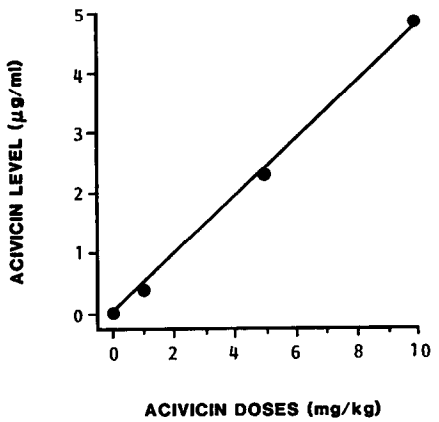

Fig. 1. Effect of different i.p. doses of acivicin on serum drug level. Acivicin was injected (mg/kg, i.p.), animals were killed 2 hr after treatment, and blood was obtained as outlined in Materials and Methods.

Previous work revealed that, in the rat after a single i.p. injection of acivicin, the drug concentration peaks in the plasma at 2 hr after treatment [12]. In the present studies, single i.p. injections of acivicin (1, 5 and 10 mg/kg) were given to groups of rats. The rats were killed and blood was obtained as outlined in Materials and Methods. The results indicated that the serum acivicin concentrations were proportionate with the amount of drug injected (Fig. 1). These results are in line with those obtained in patients where plasma concentrations measured at the end of the infusion correlated with the acivicin dose [26].

Effect of different acivicin doses on enzymic activities of bone marrow. Acivicin (1, 5 and 10 mg/kg, i.p.) was injected in single doses; the rats were killed 2 hr after treatment, bone marrows were removed, and the activities of the glutamine-utilizing enzymes were determined. The activities of synthase II and of CTP and GMP synthases decreased most markedly, whereas that of amidophosphoribosyltransferase was inhibited the least (not shown). These results are in line with our earlier studies on the action of acivicin on activities of enzymes of glutamine utilization in hepatoma 3924A [3, 4]. On the basis of such studies, a dose of 5 mg/kg was selected for the present investigation.

Impact of acivicin on bone marrow cellularity, protein concentration, and activities of pyrimidine and purine enzymes of glutamine utilization. Preliminary studies showed that *in vitro* addition of acivicin to 100,000 *g* supernatant fractions from bone marrow rapidly decreased the activity of the glutamine-utilizing enzymes in an incubation time and dose–response dependent fashion; thymidine kinase activity was not affected (not shown). This agrees with earlier observations on the *in vitro* inactivation of glutamine-utilizing enzymes in hepatoma and in human colon carcinoma [3–8].

After a single injection of acivicin the protein concentration in the bone marrow did not change.

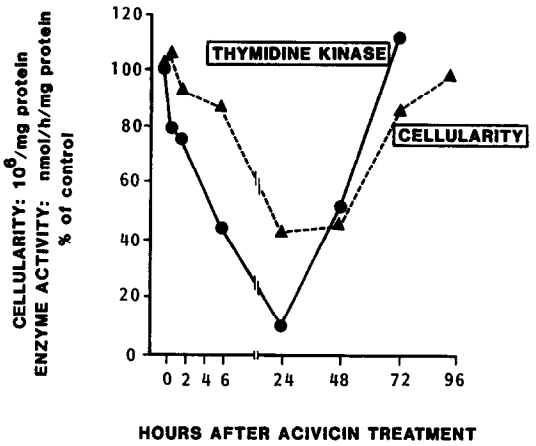

Fig. 2. Sequence of events in cell counts and thymidine kinase activity after a single injection of acivicin (5 mg/kg, i.p.). Animals were killed at various intervals after treatment, and bone marrow was removed. Each point represents four or more determinations, and standard errors were <±5%.

The cell counts were depressed with a nadir of 40% of controls at 24 hr; cellularity returned to control range at 72 hr after treatment (Fig. 2). Thymidine kinase activity was also determined because it was not inhibited by acivicin and thus it was expected to reflect the behavior of cellularity in the bone marrow. The results support this idea because the thymidine kinase activity decreased and returned to control level roughly parallel with the cellularity of the bone marrow (Fig. 2).

A single injection of acivicin sharply reduced the bone marrow CTP synthase activity, which reached 10% of control in 30 min, with activity remaining barely measurable for 48 hr and then returning to control range by 168 hr. The activity of carbamoylphosphate synthase II decreased steeply to 21% at 2 hr, remaining at this level for 48 hr, and then returning to control range at 72 hr (Fig. 3). Among the purine enzymes the depression of the activity of GMP synthase was the most marked, with FGAM synthase less affected and amidophosphoribosyltransferase inhibited the least. All three enzymic activities returned to control range by 72–168 hr.

Selective impact of acivicin. To compare the biochemical impact of acivicin in bone marrow, hepatoma and host liver, a single dose of the drug (5 mg/kg, i.p.) was given to rats bearing hepatoma 3924A. The sequence of events was determined by killing rats at different time intervals after injection and measuring the activities of the glutamine-utilizing enzymes. The acivicin-induced *in vivo* half-lives of the enzyme are shown in Table 2. In the host liver, the apparent $T_{\frac{1}{2}}$ values varied between 1.5 and 3.3 hr. By contrast, in the bone marrow, the $T_{\frac{1}{2}}$ values were between 0.2 and 1.3 hr. These values were 2.5- to 6-fold shorter than those in the liver, and they were similar to those in the rapidly growing hepatoma 3924A where the $T_{\frac{1}{2}}$ values varied between 0.1 and 0.9 hr. The different susceptibilities to acivicin of the enzymes in the tissues may reflect the glutamine contents which were for liver, bone marrow and hepatoma 3924A 5.0, 0.5 and 0.5 mM respectively. The prompt decrease in the activities for a number of glutamine-utilizing enzymes may also reflect the rapidity of transport and binding of acivicin in competition with glutamine to the active centers of the various enzymes. The acivicin inhibitory action was shown to be irreversible since the decreased enzymic activities could not be reactivated *in vitro* by incu-

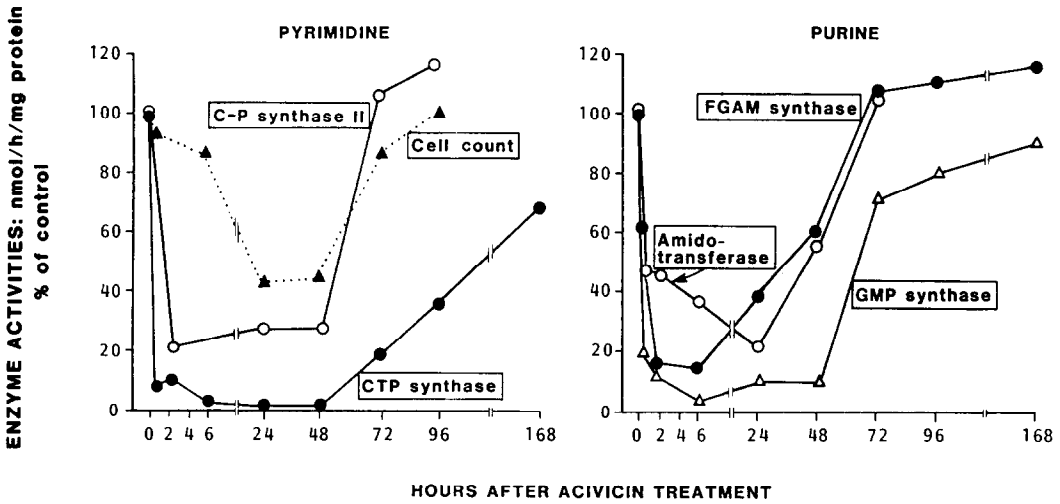

Fig. 3. Sequence of events in bone marrow cellularity and glutamine-utilizing enzyme activities after a single injection of acivicin (5 mg/kg, i.p.). Animals were killed at different periods after drug treatment, and cellularity and enzyme activities were measured as cited in Materials and Methods. Enzymic activities were calculated in nmol substrate metabolized per hr per mg protein and expressed as percentages of the values of control, untreated rats. Values are means of four or more determinations for each point; the standard errors were <±5%.

Table 2. Acivicin-induced *in vivo* apparent half-lives ($T_{\frac{1}{2}}$) of glutamine-utilizing enzymes in different rat tissues

Enzymes	$T_{\frac{1}{2}}$ (hr)		
	Liver	Bone marrow	Hepatoma 3924A
CTP synthase	2.3	0.2	0.5
GMP synthase	2.0	0.3	0.1
Amidophosphoribosyltransferase	1.5	0.5	0.7
FGAM synthase	3.3	0.8	0.9
Carbamoyl-phosphate synthase II	2.1	1.3	0.5

Four or more time points were taken to determine $T_{\frac{1}{2}}$.

bation of the enzymes with high concentrations of L-glutamine or by dialysis [3–8]. The results indicated to us that an inactivation of the enzymes by acivicin occurred by affinity labeling of the glutamine-binding site as was shown for bacterial enzymes [27]. The selectivity of acivicin action to the glutamine site is shown by the fact that the activity of aspartate carbamoyltransferase, which is part of the same protein complex in which synthase II activity was inactivated, was retained; furthermore, the ammonia-utilizing activity is also unaffected [3–7].

Impact of tiazofurin injection on bone marrow. A single injection of tiazofurin (200 mg/kg) did not alter the bone marrow protein content, but the cellularity declined to 46% of the control at 24 hr and returned to control range at 72 hr after treatment (Fig. 4). The thymidine kinase activity followed roughly the same pattern as the cellularity but with a more profound decrease to 10%, rebounding to above the control range at 48 hr.

The primary target of tiazofurin, IMP dehydrogenase activity, rapidly decreased to 52% in 30 min, reaching a nadir of 22% of control at 2 hr. The IMP dehydrogenase activity was decreased markedly for 48 hr, returning to control range at 72 hr. The speed of decline, extent of depletion, and rate of rebound resembled the response of IMP dehydrogenase activity in rapidly growing hepatoma 3924A in the rat [17]. These observations suggest that the clinically observed granulocyte depression in bone marrow in tiazofurin treatment [21] may be due, in part at least, to the decrease in the activity of IMP dehydrogenase.

Whereas the drug concentrations were not measured in the bone marrow, the prompt impact

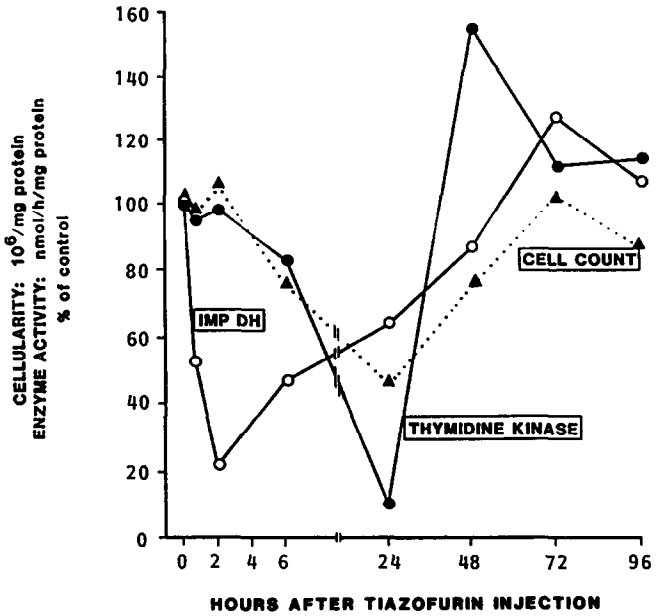

Fig. 4. Sequence of events in bone marrow cellularity and enzymic activities after a single tiazofurin injection (200 mg/kg, i.p.). Rats were killed at different intervals after treatment. Enzymic activities were measured in nmol substrate metabolized per hr per mg protein and expressed as percent of values of normal control rats. Each point is the mean of four or more determinations with a standard error of $<\pm 5\%$.

on the target enzymes of acivicin or tiazofurin in the dose–response and sequence studies indicated that these drugs rapidly permeated the bone marrow. Because of the presence of powerful activities of pyrimidine and purine salvage enzymes, it should be possible to design treatment with nucleosides and nucleobases for protection of the bone marrow in the anticancer treatment with these drugs.

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