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Enhancement by Tetrahydrouridine (NSC-112907) of the Oral Activity of 5-Azacytidine (NSC-102816) in L1210 Leukemic Mice^{1,2,3}

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SUMMARY

5-Azacytidine is more active when administered parenterally than orally in the treatment of L1210 leukemic mice. Oral coadministration of tetrahydrouridine, a pyrimidine nucleoside deaminase inhibitor with no intrinsic antitumor activity, greatly increases the oral activity of 5-azacytidine. 5-Azacytidine (or cytotoxic equivalent) blood levels in BDF₁ mice are much higher after oral administration of the 5-azacytidine-tetrahydrouridine combination than when 5-azacytidine is administered alone by the same route. The therapeutic results (L1210 leukemia) achieved with the oral combination are similar to those observed with parenteral 5-azacytidine alone.

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5-Azacytidine has shown antitumor activity against transplanted tumors (T-4 lymphoma [1] and L1210 leukemia [1,2]) and spontaneous tumors (AK lymphoma [3]) in mice. It is also an immunosuppressant (4) and an abortifacient (5) in this species. The biochemical

mode of action of 5-azacytidine has been actively studied (4,6-9) and clinical trials of its effect in the treatment of human neoplasia are in progress (10-17). 5-Azacytidine has been shown to be an effective treatment of acute leukemia in children (12-14) including tumors resistant to another cytosine nucleoside antineoplastic, cytosine arabinoside (12,13).⁷

We have reported the effects of tetrahydrouridine, a potent inhibitor of pyrimidine nucleoside deaminase, on the oral activity of cytosine arabinoside (18). Although it possesses no antitumor activity itself, tetrahydrouridine markedly increases the oral activity (in L1210 leukemic mice) of cytosine arabinoside by modifying its metabolism and effective absorption. The effects of tetrahydrouridine on the antitumor activity of 5-azacytidine are reported here.

METHODS

The effects of 5-azacytidine and combinations of 5-azacytidine and tetrahydrouridine on the survival of L1210 leukemic mice were studied

⁷ Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1- β -D-arabinofuranosyl-, monohydrochloride; cytarabine; Cytosar; ara-C.

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² This study was supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

³ Tetrahydrouridine: CAS reg. No. 5627-05-4; 2(1H)-pyrimidinone, tetrahydro-4-hydroxy-1- β -D-ribofuranosyl-; THU. Provided by Dr. Arthur R. Hanze (deceased) or Dr. Clifford Y. Peery of The Upjohn Company, Kalamazoo, Mich.

5-Azacytidine: CAS reg. No. 320-67-2; 8-triazin-2(1H)-one, 4-amino-1- β -D-ribofuranosyl-; 5-azaCR. Obtained from the Alfred Bader Chemical Company or through the courtesy of Dr. David G. Martin of The Upjohn Company.

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using previously described methods (18) following protocols described by Drug Research and Development, DCT, NCI (19). In all experiments, female BDF₁ (C57BL × DBA/2) mice weighing 18–20 g were used (eight animals/group). Agents were dissolved in sterile 0.9% saline and administered (intraperitoneal [ip] injection or oral intubation) in a constant total volume of 0.2 ml/mouse. Fresh solutions were prepared each day. Animals were inoculated ip on Day 0 with 1×10^6 L1210 cells/mouse and treatment was initiated the following day.

Previous studies (18) have shown that tetrahydrouridine has no intrinsic antitumor effect. Thus, control animals received only oral doses of saline. Where combinations were employed, half of the tetrahydrouridine dose (0.1-ml volume) was administered one-half hour before 5-azacytidine administration and the other half immediately prior to 5-azacytidine administration. Mean survivals, with standard deviations, were calculated for treated and control groups. Percent increases in lifespan (% ILS) were then calculated from these data. Each group of animals was weighed on Days 0 and 5 and the average weight change (g/mouse) was calculated.

Levels of 5-azacytidine (or cytotoxic equivalent) in BDF₁ mouse blood were determined after administration of 5-azacytidine in a dose of 50 mg/kg, ip (experiment 1) or orally (experiment 2) alone or in combination with tetrahydrouridine in a dose of 10 mg/kg by the same route. A divided tetrahydrouridine dose (see above) was used. Blood was obtained from the vena cava of anesthetized (Metofane, Pittman Moore Division, Dow Chemical Co., Southgate, Mich) mice (three/time) at various times after 5-azacytidine administration and was stored on ice for less than 3 hours until assay. Blood or a 1:5 dilution in 0.9% saline from each mouse was assayed separately.

The assay employed was a modification of the microbiologic disc-plate assay reported by Haňka et al (1). The assay organism was *Escherichia coli* (ATCC-26) growing in a fully synthetic medium. The synthetic medium consisted of a broth containing KH₂PO₄, 3.0 g; K₂HPO₄, 7.0 g; MgSO₄, 0.1 g; (NH₄)₂SO₄, 1.0 g; sodium citrate (Na₃C₆H₅O₇ · 2H₂O), 1.0 g; glu-

cose, 2 g; tetrahydrouridine, 50 mg; and distilled water to make 1 liter, supplemented with 1.5% agar (high-grade granulated agar, BBL, Cockeysville, Md). Subsequent to autoclaving, the glucose and tetrahydrouridine^{*} were added. The seed consisted of 1.0 ml of culture (16–18 hours)/500 ml of agar. Aliquots of 100 ml each were then dispensed into plastic trays (20 cm × 50 cm) (20).

Standard solutions were prepared by dissolving 5-azacytidine (0.5–16 µg/ml) in whole mouse blood (heparinized) or mouse blood diluted (1:5) with 0.9% saline. Standard solutions, in triplicate, and test samples, full-strength blood and 1:5 dilutions in saline in duplicate, were applied to 12.7-mm diameter paper discs (Carl Schleicher and Schuell Co., Keene, NH) on the seeded agar trays. After preincubation at 4° C for 2 hours (which was found to improve sensitivity) trays were incubated overnight at 32° C. Diameters of zones of inhibition were then measured.

Typical standard curves are shown in figure 1. Standard curves were constructed using linear regression analysis on individual assay values; however, only mean values are shown. The sensitivity limits in experiments 1 and 2 were 1.0 and 0.5 µg of 5-azacytidine (or equivalent)/ml of mouse blood, respectively. The concentrations of 5-azacytidine in the test samples were determined using the appropriate standard curve.

The sensitivity of the assay organism to a number of available metabolites or degradation products of 5-azacytidine was also determined. 5-Azacytosine^{*} (VI, fig 2), ribose (VIII, fig 2), guanidine (V, fig 2), and biuret (XIII, fig 2)

^{*} Previous experiments not described here showed that the addition of tetrahydrouridine (50 µg/ml) to the assay medium increased sensitivity from two- to four-fold, depending on the diluent employed. Similar results have been observed with the sensitivity of *Streptococcus faecalis* (actinobolin-resistant) to cytosine arabinoside (20). No additional increase in sensitivity was observed with concentrations of 100 or 200 µg/ml of tetrahydrouridine. In addition, levels of tetrahydrouridine in blood samples themselves (up to 50 µg/ml) had no effect on the 5-azacytidine values observed.

^{*} 5-Azacytosine and 5-azauracil were purchased from Calbiochem, Los Angeles, Calif, and Nutritional Biochemicals Corporation, Cleveland, Ohio, respectively.

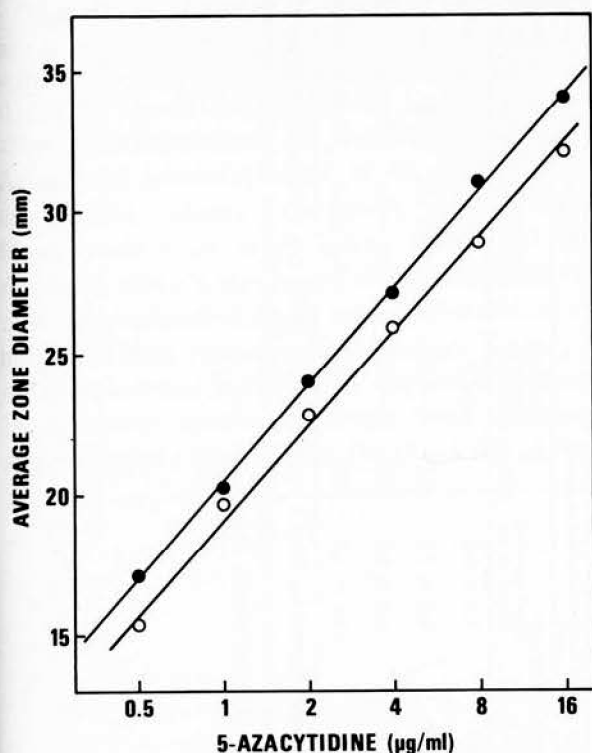


FIGURE 1.—Standard curve for inhibition of *E. coli* by 5-azacytidine. ○ = solutions in mouse blood; ● = solutions in mouse blood diluted (1:5) with 0.9% saline. Pearson's correlation coefficients (*r*) were 0.9995 and 0.9980 for the mouse blood curve and the diluted blood curve, respectively.

were completely inactive at concentrations of 1 mg/ml. 5-Azauracil^o (VII, fig 2) had approximately one seventh the activity of 5-azacytidine. Thus, all data are expressed as "5-azacytidine (or cytotoxic equivalent)."

RESULTS

The effects of tetrahydrouridine on 5-azacytidine therapy of L1210 leukemic mice (single-dose and daily dose schedules) are shown in tables 1 and 2, respectively. 5-Azacytidine used alone was much more effective (especially on a daily dose schedule) when administered ip than when administered orally. For example, daily treatment with 2.5 mg/kg of 5-azacytidine ip was considerably more effective (150% ILS) than treatment with an oral dose of 10 mg/kg/day (83% ILS) (table 2). Using both sched-

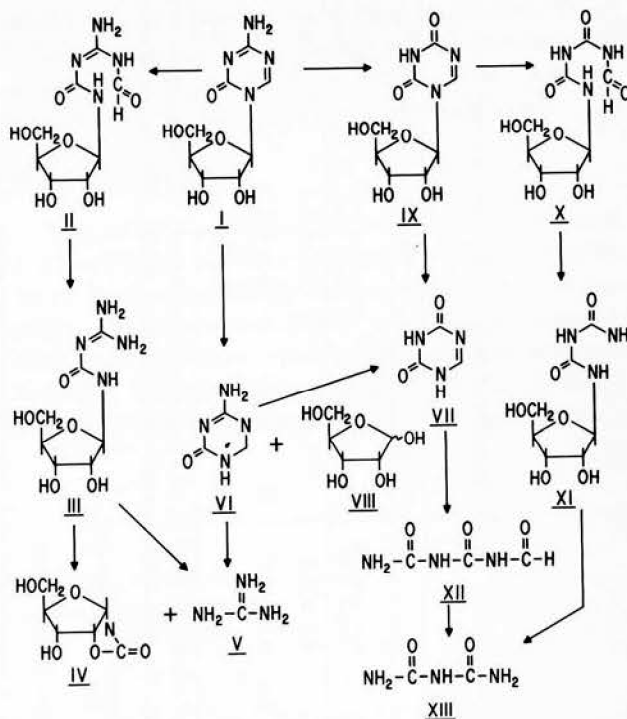


FIGURE 2.—Possible pathways for the degradative metabolism of 5-azacytidine. I = 5-Azacytidine; II = 1-ribofuranosyl-3-(formylguanyl)urea (unstable); III = 1-ribofuranosyl-3-guanylurea; IV = α -D-ribofuro[1',2':4,5]-2-oxazolidone; V = guanidine; VI = 5-azacytosine; VII = 5-azauracil; VIII = ribose; IX = 5-azauridine (unstable); X = ribofuranosyl-formyl biuret; XI = ribofuranosylbiuret; XII = 1-formylbiuret; XIII = biuret.

ules, ip tetrahydrouridine had little effect, if any, on ip therapy with 5-azacytidine. However, tetrahydrouridine significantly *increased* the effectiveness of 5-azacytidine when both agents were administered orally. Oral therapy (both schedules) with this combination was approximately as effective as therapy with ip 5-azacytidine used alone. Toxicity, as measured by early mortality and weight loss, was also increased when tetrahydrouridine was employed in an oral combination with 5-azacytidine. In figure 3, mean survival is shown as a function of weight change. The weight loss observed at a particular ILS appeared to be independent of the use of tetrahydrouridine. Thus, the therapeutic ratio with the combination does not appear to differ significantly from that observed with 5-azacytidine used alone.

TABLE 1.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—single-dose therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—				Oral administration of—			
	5-Azacytidine		5-Azacytidine + tetrahydrouridine		5-Azacytidine		5-Azacytidine + tetrahydrouridine	
	Mean survival (days) $\pm$ SD	% ILS	Weight change† (g/mouse)	Mean survival (days) $\pm$ SD	% ILS	Weight change† (g/mouse)	Mean survival (days) $\pm$ SD	Weight change† (g/mouse)
0 (control)	6.9 $\pm$ 0.4		+2.3	6.9 $\pm$ 0.4		+2.3	6.9 $\pm$ 0.4	+2.3
12.5	9.8 $\pm$ 0.6	42	-0.4	11.3 $\pm$ 1.1	64	-1.7	8.0 $\pm$ 0.7	+1.4
50.0	12.7 $\pm$ 0.4	84	-2.3	12.6 $\pm$ 0.4	83	-2.8	9.9 $\pm$ 0.9	-1.0
							13.9 $\pm$ 1.4	100
								-3.4

*Mice were inoculated on Day 0 with 1×10^6 L1210 ascites cells; therapy was started on Day 1; tetrahydrouridine (10 mg/kg) was administered in a divided dose (see Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times ($\pm$ SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

TABLE 2.—Therapy of L1210 leukemic mice with combinations of 5-azacytidine and tetrahydrouridine—daily therapy*

5-Azacytidine dose (mg/kg)	Ip administration of—				Oral administration of—			
	5-Azacytidine		5-Azacytidine + tetrahydrouridine		5-Azacytidine		5-Azacytidine + tetrahydrouridine	
	Mean survival (days) $\pm$ SD	% ILS	Weight change† (g/mouse)	Mean survival (days) $\pm$ SD	% ILS	Weight change† (g/mouse)	Mean survival (days) $\pm$ SD	Weight change† (g/mouse)
0 (control)	7.1 $\pm$ 0.6		+2.2	7.1 $\pm$ 0.6		+2.2	7.1 $\pm$ 0.6	+2.2
2.5	17.8 $\pm$ 1.6	150	-3.5	20.3 $\pm$ 3.1	186	-4.7	8.6 $\pm$ 1.2	-0.2
5.0	19.3 $\pm$ 2.0	172	-4.1	19.4 $\pm$ 1.6	173†	-4.9	9.6 $\pm$ 0.8	-2.3
10.0	19.0 $\pm$ 0.8	168‡	-4.7	5.1 $\pm$ 1.3	Toxic	-4.7	13.0 $\pm$ 1.7	-3.6
							8.3 $\pm$ 2.4	Toxic
								-4.9

*Mice were inoculated on Day 0 with 1×10^6 L1210 ascites cells; therapy was started on Day 1 and continued daily for 4 days; tetrahydrouridine (10 mg/kg/day) was administered in a divided dose (see Methods) by the same route as employed for 5-azacytidine. Control animals received only saline orally. Results are expressed as mean survival times ($\pm$ SD) and % ILS calculated therefrom.

†Animals were weighed on Days 0 and 5.

‡Two toxic deaths not included in calculation of mean survival times.

§1/8 Long-term (45-day) survivor.

The effects of tetrahydrouridine on 5-azacytidine mouse blood levels after ip or oral administration of the combination are shown in figure 4. Levels are compared to those found after administration of 5-azacytidine alone. After oral administration of 50 mg/kg of 5-azacytidine alone, maximum 5-azacytidine blood levels were much lower (about 2.1 $\mu\text{g}/\text{ml}$) than after a similar ip dose (about 43 $\mu\text{g}/\text{ml}$). Coadministration of tetrahydrouridine resulted in little increase of maximum 5-azacytidine levels when both agents were administered ip. However, maximum levels were increased approximately three times (to about 6.2 $\mu\text{g}/\text{ml}$)

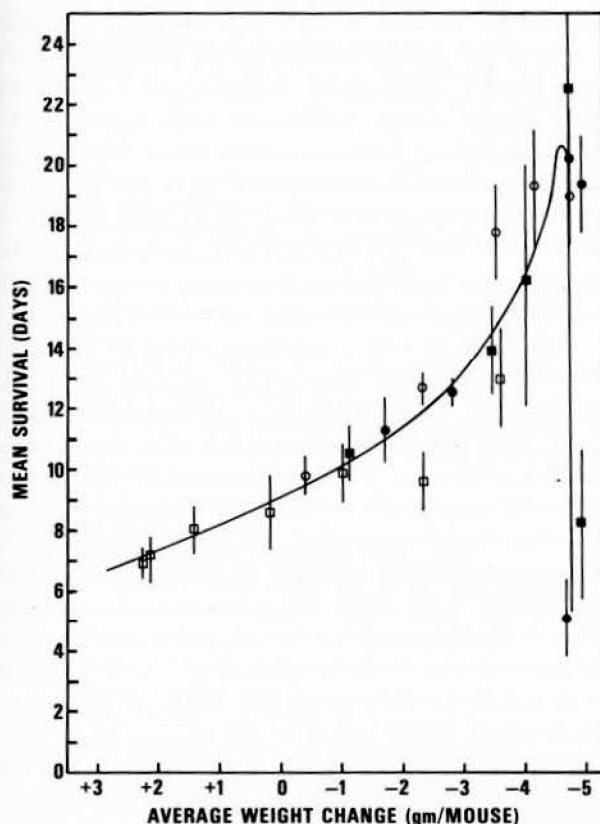


FIGURE 3.—Therapeutic ratio with 5-azacytidine and 5-azacytidine + tetrahydrouridine combinations in the treatment of L1210 leukemic mice. Survival of L1210 leukemic mice (mean $\pm$ SD) is shown as a function of weight change (average g/mouse, Days 0–5) observed with various treatments (routes and schedules) with 5-azacytidine or 5-azacytidine + tetrahydrouridine combinations. Data are composite and were obtained from tables 1 and 2. $\bullet$ = Ip 5-azacytidine + tetrahydrouridine; $\circ$ = ip 5-azacytidine; $\blacksquare$ = oral 5-azacytidine + tetrahydrouridine; $\square$ = oral 5-azacytidine.

when an oral combination was employed. With both routes, the time during which levels of 5-azacytidine could be detected in blood was increased when tetrahydrouridine was used.

DISCUSSION

The effects of tetrahydrouridine on the oral antileukemic activity of cytosine arabinoside have been extensively discussed (18). The results of the present study with tetrahydrouridine and another cytosine nucleoside antimetabolite, 5-azacytidine, are very similar to those of the previous study with cytosine arabinoside.

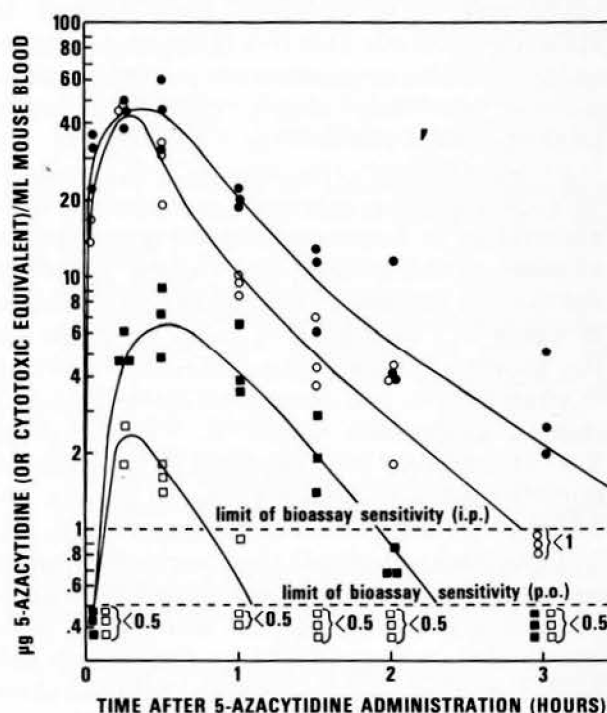


FIGURE 4.—Blood levels of mice receiving 5-azacytidine (or cytotoxic equivalent) after a single administration of 5-azacytidine or a 5-azacytidine + tetrahydrouridine combination. 5-Azacytidine (50 mg/kg) was administered (ip or orally) alone or in combination with tetrahydrouridine (10 mg/kg by the same route). Blood from three mice/time was obtained 5, 15, 30, 60, 90, 120, and 180 min after 5-azacytidine administration. Blood levels were determined with a microbiologic disc-plate assay employing *E. coli* growing in a fully synthetic medium supplemented with tetrahydrouridine (50 $\mu\text{g}/\text{ml}$). $\bullet$ = Ip 5-azacytidine + tetrahydrouridine; $\circ$ = ip 5-azacytidine; $\blacksquare$ = oral 5-azacytidine + tetrahydrouridine; $\square$ = oral 5-azacytidine.

In both cases, the oral activity of the antitumor agent, administered alone, is much less than that observed parenterally. With both agents, this oral activity is markedly increased by concomitant oral administration of the deaminase inhibitor. Also, in both cases, coadministration of tetrahydrouridine resulted in increased blood levels of the antitumor agent (or cytotoxic equivalent) after oral administration.

In the case of cytosine arabinoside, enzymatic deamination is certainly implicated as a major reason for the low oral activity observed, and the inhibition of this deamination by tetrahydrouridine is apparently the reason for the observed increase in oral activity (18). With 5-azacytidine, it is somewhat more difficult to state unequivocally that this is the case. Whereas the catabolic metabolism of cytosine arabinoside is exquisitely simple (the deamination product, uracil arabinoside [NSC-68928] being the only metabolite observed), the metabolism, including nonenzymatic degradation, of 5-azacytidine is very complex. A representation of some of the possible degradative pathways involved in 5-azacytidine metabolism is shown in figure 2. This scheme is based primarily on the work of Sorm's group (4,21-24) involving *in vitro*, *in vivo*, and nonenzymatic (hydrolysis) studies. Compounds I, III, V, VII, XII, and XIII (fig 2) have been identified in the urine of mice treated with 5-azacytidine (4,21). In addition to these metabolites, compounds IV, VI, VIII, and, transiently, II (fig 2) arise in aqueous hydrolysis studies under relatively mild conditions (23). 5-Azacytidine has been shown to be deaminated to 5-azauridine by *Escherichia coli* (24). Recent results of Chabner et al (25) show that 5-azacytidine is deaminated by a human leukemic cell deaminase (the same enzyme which deaminates cytosine arabinoside) and that tetrahydrouridine inhibits this deamination.¹⁰ 5-Azauridine (IX, fig 2) appears (13,14) to be even less stable to hydrolysis than 5-azacytidine and thus it may not be too surprising that it was not observed in mouse urine. Further degradation products of the 5-azauracil

moiety (XII and XIII, fig 2) were found however (21). Although these products could arise via pathways (eg, I $\rightarrow$ VI $\rightarrow$ VIII, fig 2) other than those involving deamination of 5-azacytidine to 5-azauridine (I $\rightarrow$ IX, fig 2), this latter route is certainly probable. Indeed the postulated conversion of 5-azacytidine (I, fig 2) to 5-azacytosine (VI, fig 2) is rather unlikely since it might be expected that, like cytidine, it would not be a substrate for any of the known mammalian phosphorylases (26).

Although the deamination product of cytosine arabinoside (uracil arabinoside) has shown no biologic activity and, in particular, does not increase the lifespan of L1210 leukemic mice (unpublished results, these laboratories), the biologic effects of the 5-azacytidine metabolites are less clearly understood. 5-Azauracil (VI, fig 2) has shown some antitumor activity (27) although it has been reported to be inactive in L1210 leukemia. Although no data are available concerning their antitumor activities, 5-azauridine itself and *N*-formylbiuret (compound XII, fig 2) have been shown to possess some, but relatively low, antibacterial activity (28,29).

The lack of any significant effect of tetrahydrouridine on the therapeutic activity of ip administered 5-azacytidine is reasonably consistent with the rather marginal effects of tetrahydrouridine on 5-azacytidine blood levels (especially maximum levels) after administration by that route. Similarly, the enhancement of 5-azacytidine's oral activity by tetrahydrouridine correlates well with the enhanced blood levels observed. However, although the antitumor activity of the oral combination was roughly equivalent to that observed with ip 5-azacytidine alone, blood levels in the former case were clearly inferior to those observed with ip administration. This discrepancy is not easily explained. In our studies, blood was the only tissue assayed for 5-azacytidine activity. Perhaps studies of the kinetics of the disposition of 5-azacytidine in other tissues (particularly lymphoid tissues, ascites fluid, and the tumor itself) would yield more meaningful data for such correlations.

Further studies aimed at determining the importance of nucleoside deamination and its inhibition by tetrahydrouridine on the disposition,

¹⁰ Indeed, of the reactions illustrated in figure 2, the deamination of 5-azacytidine to 5-azauridine is the only one which has definitively been demonstrated to proceed enzymatically.

metabolism, and biologic activity of 5-azacytidine are in progress in our laboratories.

In conclusion, tetrahydrouridine has been shown to increase the effectiveness of another antitumor cytosine nucleoside antimetabolite, 5-azacytidine. The possible clinical use of this inhibitor in combination with agents such as cytosine arabinoside and 5-azacytidine would seem to warrant careful consideration.

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