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ANALOGS OF 1-β-D-ARABINOFURANOSYLCYTOSINE. STUDIES ON MECHANISMS OF ACTION IN BURKITT'S CELL CULTURE AND MOUSE LEUKEMIA, AND *IN VITRO* DEAMINATION STUDIES

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Abstract—Analogs of 1-β-D-arabinofuranosylcytosine (ara-C), 1-β-D-arabinofuranosyl-5-fluorocytosine (ara-FC), cytidine (CR), and 2′-deoxycytidine (CdR) were studied for deamination and as deaminase inhibitors (ara-C as substrate) in human liver and mouse kidney enzyme systems. N^4-Hydroxy, N^4-methyl, and 4-hydrazino derivatives of ara-C and ara-FC, and N^4-acetyl-ara-C were studied for deamination and as deaminase inhibitors and also for their effects on mouse leukemias L1210 and P815 and in Burkitt's tumor cells in cell culture. None of the N^4-substituted analogs of ara-C and ara-FC were deaminated by human liver and mouse kidney homogenates *in vitro*. N^4-Hydroxy analogs were active in mice but only slightly active in Burkitt's cell culture. The inhibitory effect against Burkitt's cell cultures *in vitro* was blocked by deoxycytidine but not by thymidine, and N^4-hydroxy-ara-C was active *in vivo* in a line of leukemia resistant to 5-fluorouracil but not in a line resistant to ara-C, suggesting this analog has a similar mechanism of action to ara-C. The N^4-methyl and 4-hydrazino derivatives of ara-C and ara-FC are inactive in mouse leukemia and Burkitt's cell culture. Only N^4-hydroxy-ara-C and N^4-methyl-ara-FC are deaminase inhibitors.

N^4-Hydroxy-cytidine, N^4-hydroxy-5-methyl-deoxycytidine, and N^4-hydroxy-5-fluoro-deoxycytidine are all deaminase inhibitors, but attempts at potentiating the effect of ara-C in mouse leukemia *in vivo* by administering the first two of these compounds in combination with ara-C resulted in partial blocking of the effect of ara-C, presumably due to reduction of the N^4-hydroxy derivative to deoxycytidine or to a derivative which acts like deoxycytidine.

N^4-Hydroxy-deoxycytidine has minimal activity in Burkitt's cell culture, but its inhibitory effect was blocked completely at thirty times the ID_{50} (50 per cent inhibitory dose) by thymidine at equimolar concentrations but only slightly by deoxycytidine. Although this analog is not deaminated, and is a strong deaminase inhibitor, it blocked rather than potentiated the effect of ara-C in L1210 leukemia, suggesting that in cell culture it acts as a thymine antimetabolite, whereas *in vivo* it is reduced to deoxycytidine.

N^4-Hydroxy-5-fluoro-deoxycytidine is active in Burkitt's cell culture, and its effect could be blocked by thymidine, and to a lesser degree, by deoxycytidine, suggesting that it too acts in this system as a thymine antimetabolite.

1-β-D-Arabinofuranosylcytosine (cytosine arabinoside, ara-C) has shown useful activity in the clinical treatment of acute leukemia and lymphomas,[1–6] but pharmacologic studies in man and mouse have shown that it is rapidly deaminated to 1-β-D-arabinofuranosyluracil (ara-U), an inactive metabolite.[3, 7] A synthetic program has

been undertaken to find derivatives of ara-C which are less easily deaminated and therefore possibly more effective, or which might serve as deaminase inhibitors if administered in conjunction with ara-C. Several N^4-substituted analogs of ara-C, 1-β-D-arabinofuranosyl-5-fluorocytosine (ara-FC), 2′-deoxycytidine (CdR), and cytidine (CR) have been studied for antileukemic activity in L1210 and P815 mouse leukemias, for their inhibitory effects in two lines of Burkitt's tumor cells in cell culture, and for deamination and as deaminase inhibitors in the *in vitro* system of Camiener and Smith,[7a] using ara-C as substrate (Figs. 1 and 2).

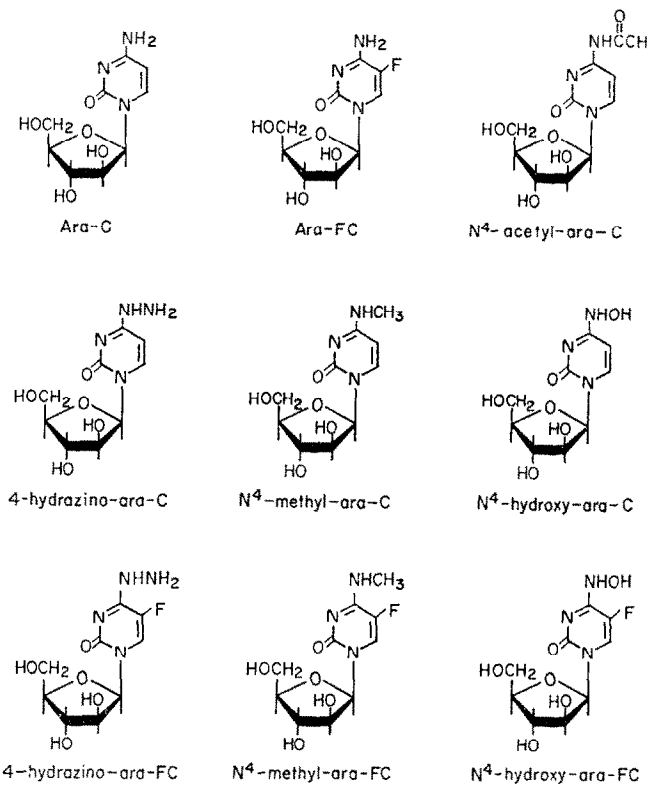

FIG. 1. Analogs of ara-C and ara-FC.

MATERIALS AND METHODS

Compounds

Analogs of ara-C, ara-FC,[8] cytidine[9, 10] and deoxycytidine[10] were synthesized in our laboratories and will be reported elsewhere. (J. J. Fox, N. C. Miller, I. Wempen, E. A. Falco, manuscript in preparation). Analogs were dissolved in normal saline for injection into mice or in distilled water for studies with Burkitt's tumor cells. Samples of ara-C-3′-phosphate (ara-C-3′-P) and ara-C-5′-phosphate (ara-C-5′-P) were kindly supplied by Dr. W. Wechter, Upjohn Laboratories, Kalamazoo, Michigan. Ara-C for animal and cell culture experiments was supplied by Upjohn Laboratories.

Burkitt's tumor cells in cell culture

The technique for assay of chemotherapeutic agents in this system has been described.[11, 12] Two cell lines of Burkitt's tumor, EBl[13] and Kudi,[14] were grown at 37°

in Eagle's minimal essential medium plus added non-essential amino-acids, glutamine, 15% fetal calf serum, and 200 units/ml of penicillin and 200 μg/ml of streptomycin. An initial cell concentration of 30,000 cells/ml was used and the cells were grown in 16-ml screw-capped tubes by the Fischer technique.[15] Each tube contained cells, 5 ml of medium, and 0·1 ml of a 50-fold concentration of the drug. Drugs were tested at

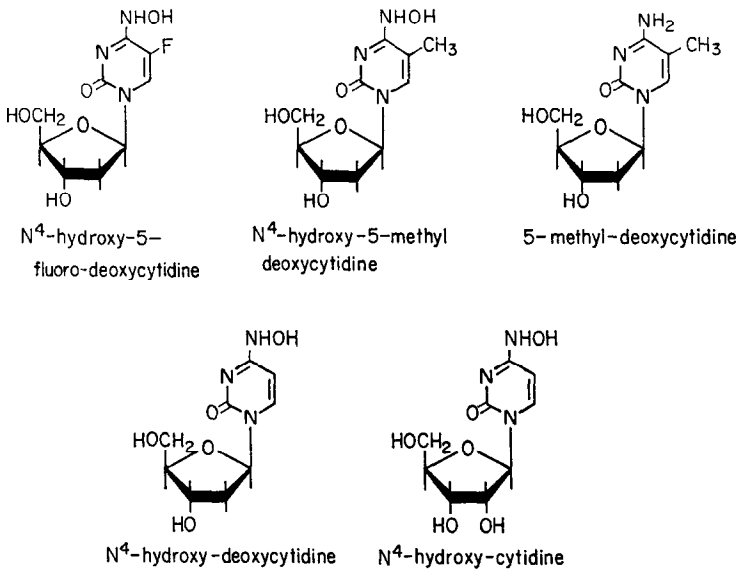

FIG. 2. Analogs of cytidine and deoxycytidine.

3-fold dilutions (e.g. 3, 1, 0·3, 0·1 μg/ml, etc.). Tubes were incubated without agitation for 168 hr at 37°, and were then agitated gently to break up clumps of cells, diluted 1:20, and counted in a Coulter counter, Model A. Control counts usually were between 500,000 cells/ml (four generations) and 1 million cells/ml (five generations). Following counting of an aliquot, some tubes were also studied for deamination of the experimental drug, as described below.

Mouse leukemia

The technique for evaluating the chemotherapeutic activity of a drug to prolong the survival time of mice with transplanted leukemia has been reported previously.[16] The experiments described here were done with leukemias P815[17] and L1210[18] and their sublines resistant to ara-C and 5-fluorouracil.[19] The various lines of mouse leukemia were carried in F_1 hybrids of the C57Bl/6 × DBA/2 cross (BDF1). One million leukemic cells in a saline suspension were inoculated intraperitoneally (IP) into each animal, producing an ascitic leukemia which later became generalized. Control and treatment groups contained ten mice each (in a few cases only five animals were used, due to the small amount of compound available). Treatment was initiated 24 hr after the inoculation of leukemia and continued once daily IP to a total of ten doses. The mice were weighed twice weekly and autopsied at death for gross evidence of leukemia.

Results are expressed in percent increased life span (ILS%), calculated as 100 × increased survival over controls (days)/control survival (days). Values over 50 percent ILS are considered significant, and values over 100 percent, highly significant.

Studies of deamination and inhibition of deamination

Deamination studies were carried out by a method similar to that of Camiener and Smith.[7a] The various analogs were incubated with homogenates of human liver or mouse kidney, which have been shown to have large amounts of deoxycytidine deaminase,[7a] to determine if deamination occurs. Spectrophotometric assay of the reaction products is based on the fact that, under acidic conditions, ara-C has an absorption maximum at 280 mμ, whereas its deamination product, ara-U, has an absorption maximum at 261 mμ. Similarly, ara-FC and its deamination product, ara-FU, can be determined spectrophotometrically, allowance being made for the bathochromic shift in the absorption maximum due to the 5-fluorine atom. The identity of the reaction products was confirmed by paper chromatography in two systems, as described below.

Preparation of tissue homogenates

Normal liver tissue was obtained at autopsy shortly after death from patients without evident liver pathology. Mouse kidneys and other tissues were obtained from BDF1 mice. Tissue was stored in the frozen state. Tissue homogenates (20%) were freshly prepared by adding Krebs–Ringer buffer at pH 7·4, modified to be low in bicarbonate, and supplemented with 100 μg each of penicillin and streptomycin per ml,[20] and homogenizing at 0° in a Potter–Elvehjem style homogenizer. The homogenate–buffer mixture was then centrifuged for 15 min at 1000 rpm at 5°, and the supernatant was used for subsequent determinations. The term "tissue homogenate" subsequently used refers to this supernatant. The crude enzyme preparation is reasonably stable, retaining considerable activity after incubation for 2 hr at 37°.[7a] Activity is retained in the frozen state for at least 6 months.

Assay for deamination and deamination inhibition

In a typical determination the reaction mixture (0·5 ml) in a 15-ml centrifuge tube contains 156 or 625 mμM of substrate (e.g. ara-C or analog), 0·2 ml tissue homogenate (human liver or mouse kidney), and Krebs–Ringer buffer at pH 7·4. After incubation for 1 hr at 37° the reaction is stopped by addition of 1·5 ml 5% TCA and cooling to 5°, and protein is removed by centrifugation. The supernatant is diluted 1:1, 1:2, or 1:4 with 0·01 N HCl, and absorption spectra are determined in a Cary recording spectrophotometer. The presence of deaminase activity in other tissues was determined by incubating ara-C with tissue homogenate for 1 hr, and determining the absorption maximum, as described above. Inhibition of deamination was studied by preincubating various analogs with tissue homogenate for 20 min, then adding substrate (ara-C) in various ratios, and incubating for 1 or 2 hr. The reaction mixtures were then processed as described previously. All experiments were performed, in duplicate, on two separate occasions, using different crude enzyme preparations.

The relative degree of deamination was estimated by comparing the observed absorption maximum with a standard curve (Fig. 3) relating absorption maximum with percent of ara-C in a mixture of ara-C and ara-U. This curve was constructed

by preparing twenty-seven random mixtures of ara-C and ara-U in various proportions in 0·01 N HCl containing 5% TCA and determining the absorption maximum of each mixture. In every case, single absorption peaks intermediate between ara-C (280 mμ) and ara-U (261 mμ) were obtained with mixtures of the two

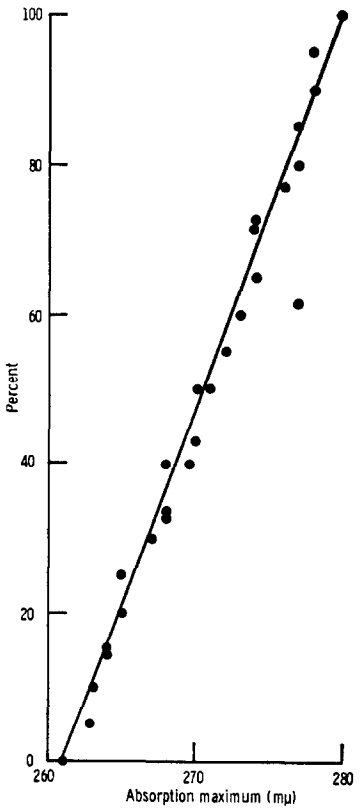

FIG. 3. Standard curve for determination of percent of ara-C in an ara-C/ara-U mixture. Absorption maxima of twenty-seven random mixtures of ara-C and ara-U were determined in 0·01 N HCl. The solid line represents the theoretically expected relationship between absorption maximum and per cent ara-C in an ara-C/ara-U mixture.

substances, confirming theoretical intermediate absorption maxima obtained mathematically. We have not been able to identify more than these two major components to the deamination reaction. Comparison of the values obtained by use of the standard curve (Fig. 3) with the known percent of ara-C indicated an average error of 5 percent in this method of estimation. Results of deamination inhibition experiments obtained by this method, expressed in Table 4, are indicated as "no deamination inhibition" (0), representing less than 25 percent ara-C in the incubation mixture at the end of incubation, "complete deamination inhibition" (+), representing more than 75 percent ara-C, and "partial deamination inhibition" (±), representing 25 percent to 75 percent ara-C remaining at the end of incubation.

Chromatographic studies

Identity of reaction products was confirmed by paper chromatography, using two systems, "A", isopropanol–HCl–water (68:17:14·4), and "B", *n*-propanol–ethyl acetate–water (1:4:2) (Table 1). Deproteinized reaction mixtures were spotted on

TABLE 1. PAPER CHROMATOGRAPHY OF ANALOGS OF ARA-C, ARA-FC, DEOXYCYTIDINE AND CYTIDINE

Compound	R_f values System A*	System B†
Ara-C	0·50	0·03
N^4-Hydroxy-ara-C	0·51	0·11
N^4-Methyl-ara-C	0·61	0·05
4-Hydrazino-ara-C	0·59	0·01
N^4-Acetyl-ara-C	0·50	0·15
Ara-U	0·72	0·16
Ara-C-3′-P	0·60	0·03
Ara-C-5′-P	0·54	0·03
Ara-FC	0·51	0·08
N^4-Hydroxy-ara-FC	0·51	0·19
N^4-Methyl-ara-FC	0·57	0·13
4-Hydrazino-ara-FC	0·44	0·14
Ara-FU	0·82	0·37
Cytidine	0·39	0·03
Deoxycytidine	0·52	0·05
Uridine	0·71	0·14
Uracil	0·67	0·36
Deoxyuridine	0·80	0·26
Thymidine	0·82	0·53
5-Methyl-deoxycytidine	0·58	0·05
N^4-Hydroxy-cytidine	0·40	0·08
N^4-Hydroxy-5-methyl-deoxycytidine	0·54	0·26
N^4-Hydroxy-deoxycytidine	0·65	0·23
N^4-Hydroxy-5-fluoro-deoxycytidine	0·62	0·39

* System A: isopropanol–HCl–water 68:17:14·4.
† System B: *n*-propanol–ethyl acetate–water 1:4:2.
Substances dissolved in Krebs–Ringer buffer at pH 7·4, spotted on Whatman #3MM paper and developed by descending technique.

Whatman #3MM paper, developed by the descending technique, dried, sprayed with 0·01 N HCl, redried, and the location of all ultraviolet-absorbing zones determined by inspection under ultraviolet light (Sensitivity: 3 μg arabinofuranosyluracil).

Deamination studies with Burkitt's tumor cells

After counting the cells at the end of the 168-hr incubation period, 0·5–1·0 ml of 25% sodium lauryl sulfate was added to lyse the cells, and protein was precipitated by the addition of 5 ml 10% TCA. The mixture was centrifuged for 30 min at 1500 rpm at 5°, and absorption spectra were obtained by reading the supernatant directly in a Cary recording spectrophotometer. In some studies, homogenized rather than intact Burkitt's cells were used. Substrate (ara-C, CdR or CR) was incubated for 1 hr with homogenized cells, and subsequent analysis was similar to that described under "assay for deamination".

Studies on deamination of ara-C

Tritiated ara-C of specific activity 1·0 mc/μM, uniformly labelled, over 97 percent pure by chromatographic analysis, was purchased from Schwarz Bioresearch Inc.,

Orangeburg, New York. Solutions of ara-C containing 8·3 μc/0·1 ml (530 mμM) were incubated for 1 hr with freshly prepared 20 percent human liver homogenate, under identical conditions as the deamination studies described above. The reaction was terminated by addition of 1·5 ml of 5% TCA and cooling to 5°. The supernatant was spotted directly on Whatman #3MM paper, 500 μl being applied to each spot, and developed by the descending technique in chromatographic systems "A" and "B" described previously. Controls were run without addition of crude enzyme, and also without addition of enzyme and without incubation. An aliquot of labelled ara-C was spotted on paper but not chromatographed. Activity of the crude enzyme was verified by incubating unlabelled ara-C (625 mμM/0·1 ml) with liver homogenate for 1 hr; shift in absorption maximum from 280 mμ to 262 mμ was observed, indicating conversion of ara-C to ara-U.

Following drying of the developed chromatograms, each lane, including both types of controls, was cut into thirty-eight or forty-four consecutive 1-cm segments. Each segment was placed in a scintillation vial, 0·8 ml distilled water added, and after 2 hr at 37°, 20 ml Diatol added and the radioactivity determined by counting in a Packard Tri-Carb liquid scintillation spectrometer, Model 314E. The radiochromatogram was reconstructed to determine the locations of all radioactive zones. The final amount of labelled ara-C in the incubation mixtures in this experiment was adjusted to detect minor components or products of the deamination reaction present in concentrations of 0·01 percent of the total amount of ara-C.

The validity of the determination of the degree of deamination from the observed absorption maximum of an ara-C/ara-U mixture (Fig. 3) depends on there being only two substances measured. The radiochromatographic studies described above demonstrated that ara-U is the only reaction product (see Results). All radioactivity, except the 3 percent impurity noted previously, present in the initial aliquot of ara-C, could be accounted for in the ara-U spot.

RESULTS AND DISCUSSION

N^4-hydroxy analogs of ara-C and ara-FC

N^4-Hydroxy-ara-C and N^4-hydroxy-ara-FC are active against L1210 mouse leukemia at about the same dose and at about twice the dose, respectively, as the parent compounds (Table 2) but are about 1000-fold less inhibitory in cultures of P815 mouse leukemic cells (Fig. 4) and Burkitt's tumor (Fig. 5). This suggests the possibility that they are reduced to ara-C and ara-FC respectively in the intact mouse but not in the cell cultures. The inhibitory effects of these compounds in Burkitt's cell cultures are blocked by deoxycytidine but not by thymidine (Fig. 6), as is the case with the parent compounds.[12] Both N^4-hydroxy derivatives are active in a line of P815 leukemia resistant to 5-fluorouracil, but not in a line resistant to ara-C (Table 2). Blocking of the inhibitory effects in Burkitt's cell culture by deoxycytidine but not by thymidine and the behavior against the 5-fluorouracil and ara-C resistant lines of P815 leukemia again suggest that these compounds are acting in the same manner as the parent compounds.[11]

N^4-Hydroxy-ara-C and N^4-hydroxy-ara-FC are not deaminated by human liver or mouse kidney enzyme systems, although both systems have a high content of deoxycytidine deaminase. Since ara-C and ara-FC are deaminated, to an equal extent

under these conditions, by both enzyme systems it is concluded that hydroxyl substitution on the N^4 position (4-amino group) blocks deamination.

Since the N^4-methyl and N^4-amino (alternatively called 4-hydrazino) analogs of ara-C and ara-FC are also not deaminated, these data indicate that the deaminase possesses a high degree of specificity with respect to alterations in the exocyclic amino-function.

TABLE 2. EFFECT OF N^4-HYDROXY ARA-C AND N^4-HYDROXY-ARA-FC ON SURVIVAL TIME OF MICE WITH LEUKEMIAS L1210 AND P815

Compound	Dose (mg/kg)*	7-day weight change (g)	Survival time (days)	ILS, %†
Control (L1210)		+1·2	10·1	
N^4-Hydroxy-ara-C	40	−1·5	19·6	94
	20	−0·2	17·1	69
Ara-C	20	−0·6	18·8	86
	10	+0·8	18·1	79
N^4-Hydroxy-ara-FC	40	−0·6	22·5	123
	20	0·0	19·8	96
Control		+3·0	9·1	
Ara-FC	10	+0·6	19·6	115
Control (P815/ara-C)‡		+4·9	11·1	
N^4-Hydroxy-ara-C	20	+2·7	9·8	−12
Ara-C	20	+3·7	9·7	−13
5-Fluorouracil	13	−0·5	20·0	80
Control (P815/ara-C)		+5·6	10·8	
N^4-Hydroxy-ara-FC	20	+5·1	9·9	−8
Ara-C	20	+5·1	10·8	0
5-Fluorouracil	13	+0·6	20·1	86
Control (P815/5FU)§		+3·0¶	9·9	
N^4-Hydroxy-ara-C	20	+0·3¶	15·7	58
5-Fluorouracil	13	+2·6¶	10·2	3
Ara-C	25	−0·9¶	34·3+‖	246+‖
Control (P815/5FU)		+4·5**	9·4	
N^4-Hydroxy-ara-FC	20	+0·7**	18·2	94
5-Fluorouracil	13	+4·3**	10·3	10
Ara-C	20	−0·6**	33·6+††	257+††

* Treatment begun 24 hr after injection of leukemia.
† Increase in life span in percent.
‡ Resistant to 1-β-D-arabinofuranosylcytosine(ara-C).
§ Resistant to 5-fluorouracil (5-FU).
¶ Six-day weight change.
‖ Three of nine animals surviving at 50 days.
** Eight-day weight change.
†† Two of ten animals surviving at 50 days.

When N^4-hydroxy-ara-C is given IP to mice, arabinofuranosyluracil (ara-U), but no uracil, appears in the urine, supporting the theory that reduction to ara-C occurs in the intact mouse in some tissue other than the kidney (ara-U is not formed from N^4-hydroxy-ara-C by mouse kidney *in vitro*), and is then followed by deamination. Ara-U is also not formed by incubation of N^4-hydroxy-ara-C in the human liver enzyme system.

Although N^4-hydroxy-ara-C is a deaminase inhibitor (Table 4), N^4-hydroxy-ara-FC is not. N^4-Hydroxy-ara-C does not potentiate the action of ara-C in Burkitt's cell

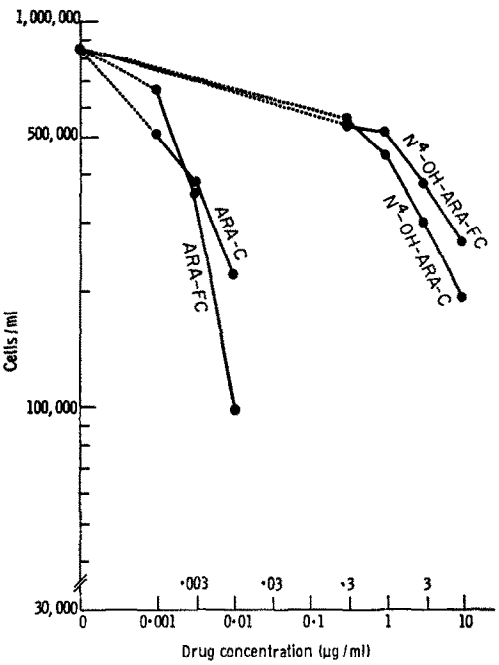

FIG. 4. Comparison of inhibitory effect of N^4-hydroxy-ara-C with ara-C, and of N^4-hydroxy-ara-FC with ara-FC in P815 leukemic cell culture (cell counts at 96 hr).

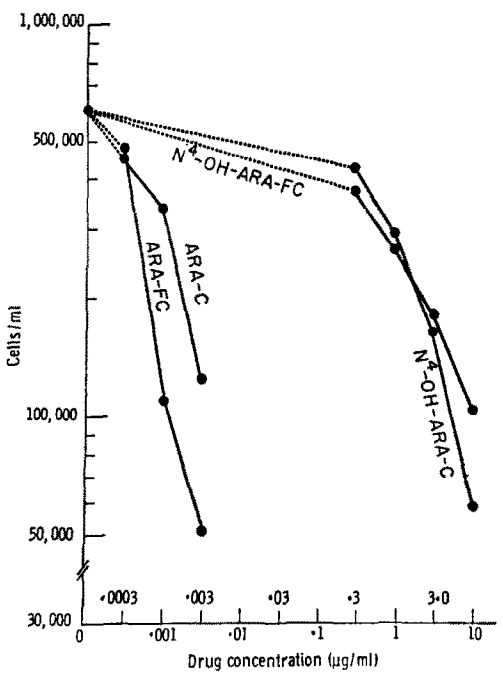

FIG. 5. Comparison of inhibitory effect of N^4-hydroxy-ara-C with ara-C, and of N^4-hydroxy-ara-FC with ara-FC in EBl Burkitt's cell culture (cell counts at 168 hr).

culture, nor does N^4-methyl-ara-FC, a weak deaminase inhibitor. This is not surprising since both intact and homogenized Burkitt's tumor cells are unable to deaminate ara-C, deoxycytidine, and cytidine *in vitro*, indicating that deaminase activity is absent in these cells and that deamination is probably not essential for cytotoxic action. None of the N^4-substituted analogs of ara-C and ara-FC were deaminated by intact Burkitt's tumor cells in culture.

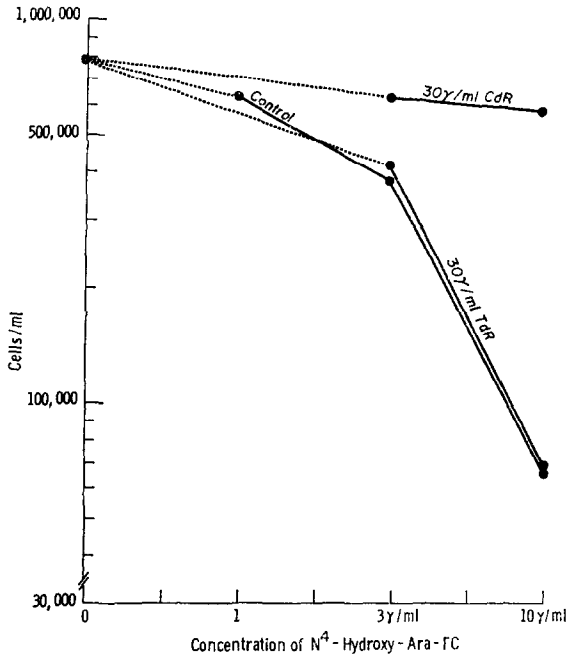

FIG. 6. Effect of deoxycytidine (CdR) and thymidine (TdR) on inhibitory activity of N^4-hydroxy-ara-FC on Kudi Burkitt's cell culture (cell counts at 168 hr).

N^4-Methyl and 4-hydrazino derivatives of ara-C and ara-FC

The N^4-methyl and 4-hydrazino analogs of ara-C and ara-FC are not deaminated by human liver or mouse kidney homogenates. All four compounds are inactive and non-toxic in mice with L1210 leukemia at doses four to eight times the active doses of ara-C or ara-FC. Unfortunately, higher doses could not be given due to limited supply of these analogs. Of these four analogs, only the 4-hydrazino-ara-FC has weak activity against Burkitt's tumor cells in culture, being 1/100 as active as ara-C. The lack of effect of the 4-hydrazino and N^4-methyl derivatives of ara-C and ara-FC in mice with L1210 leukemia at doses four to eight times greater than would be necessary for the parent compounds (Table 5) suggests that they are not degraded to the parent compounds to any significant degree, and their activity in cell culture suggests that if this activity is due to the parent compounds, there is only 1/100th of the amount being produced. Thus, any significant activity of the ara-C and ara-FC analogs in Burkitt's cell culture seems to depend on the 4-amino group being unsubstituted. Neither of the 4-hydrazino derivatives nor N^4-methyl-ara-C inhibits

deamination of ara-C *in vitro* by human liver or mouse kidney enzyme systems; N^4-methyl-ara-FC is a weak deaminase inhibitor (Table 4).

N^4-Acetyl-ara-C

N^4-Acetyl-ara-C has moderate activity against Burkitt's tumor cells in culture (Table 3), with about 1/10th the activity of ara-C. This activity is blocked by deoxycytidine but not by thymidine. N^4-Acetyl-ara-C is relatively inactive against L1210

TABLE 3. 50 PERCENT INHIBITORY DOSE (ID_{50}) OF ANALOGS OF ARA-C, ARA-FC, CYTIDINE, AND DEOXYCYTIDINE AGAINST EB1 AND KUDI LINES OF BURKITT'S TUMOR CELLS IN CELL CULTURE, AND BLOCKING BY DEOXYCYTIDINE (CdR) OR THYMIDINE (TdR)

Compound	ID_{50} (μg/ml)*		Blocking		
	KUDI	EB1	By CdR†	By TdR†	Of ara-C or ara-FC
Ara-C‡	0·003	0·003	+(1:100)	0	
Ara-FC‡	0·007	0·003	+(1:100)	0	
N^4-Hydroxy-ara-C	3 (1–10)	3 (1·5–3)	+(1:10)	0	0
N^4-Hydroxy-ara-FC	3	3	+(1:10)	0	
N^4-Methyl-ara-C	None at 30	None at 30			
N^4-Methyl-ara-FC	None at 30	20% at 30			0
4-Hydrazino-ara-C	1·4	3–10			
4-Hydrazino-ara-FC	0·3	0·8–1·6			
N^4-Acetyl-ara-C	0·017	32% at 0·1			
N^4-Hydroxy-5-methyl-deoxycytidine	70% at 3	3 (3–30)			0
5-Methyl-deoxycytidine	25% at 30	15% at 30			0 at 1:300 + at 1:1000
N^4-Hydroxy-cytidine	None at 1	74% at 3			0
N^4-Hydroxy-deoxycytidine	0·42–0·9	1	±(1:10)	+(10:1)	
N^4-Hydroxy-5-fluoro-deoxycytidine	0·007	61% at 0·01	+(1:30)	+(1:30)	
Deoxycytidine	None at 30	None at 30			+(1:100)
Cytidine	None at 30	None at 30			0
Thymidine	None at 30	None at 30			0
Ara-U‡		None at 1			

* ID_{50} shown; compounds indicated as "none" had no growth inhibition at 30 μg/ml, the highest concentration tested. Where range of concentrations tested was above or below the ID_{50} level, the growth inhibition and dose nearest the ID_{50} is shown.

‡ Ara-C: 1-β-D-arabinofuranosylcytosine.
Ara-FC: 1-β-D-arabinofuranosyl-5-fluorocytosine.
Ara-U: 1-β-D-arabinofuranosyluracil.

† Ratios given are the lowest ratio of analog/CdR or TDR at which blocking is observed.

mouse leukemia at doses up to four to eight times those effective for ara-C, supporting the assumption that this compound is not de-acetylated significantly to ara-C *in vivo* in mice (Table 6).

N^4-Substituted analogs of cytidine and deoxycytidine

N^4-Hydroxy-cytidine, N^4-hydroxy-5-methyl-deoxycytidine, and N^4-hydroxy-5-fluoro-deoxycytidine (Fig. 2) are all deaminase inhibitors, the first two compounds being the strongest inhibitors in this group (Table 4).

Camiener[7d] (Presented by Dr. C. Smith at meeting of the Acute Leukemia Task Force, Bethesda, Maryland, June 23, 1966) has previously reported that N^4-hydroxy-5-methyl-deoxycytidine and 5-methyl-deoxycytidine are deaminase inhibitors. We have confirmed also that the latter compound is such an inhibitor. None of these

TABLE 4. INHIBITION OF DEAMINATION OE ARA-C† BY ANALOGS OF ARA-C, ARA-FC‡, CYTIDINE AND DEOXYCYTINE*

Compound	Ratio of substrate/inhibitor							
	100/1	10/1	5/1	2/1	1/1	1/5	1/10	1/20
N^4-Hydroxy-ara-C		0/0	0/±	±/±	±/+	±/+	+/+	+/+
N^4-Hydroxy-ara-FC					0/0	0/0	0/0	0/0
N^4-Hydroxycytidine	0/0	±/±	+/±	+/+	+/+			
N^4-Hydroxy-5-methyl-deoxycytidine	0/0	±/+	+/+	+/+	+/+			
N^4-Hydroxy-5-F-deoxy-cytidine	0/0	0/±	0/+	0/+	0/+	+/+	+/+	+/+
N^4-Hydroxy-deoxycytidine	0/	0/0	0/±	±/±	±/±	±/+	±/+	±/+
N^4-Acetyl-ara-C					0/0	0/0	0/0	0/0
4-Hydrazino-ara-C					0/0	0/0	0/0	0/0
4-Hydrazino-ara-FC					0/0	0/0	0/0	0/0
N^4-Methyl-ara-C					0/0	0/0	0/0	0/0
N^4-Methyl-ara-FC					0/0	0/0	+/+	+/+
5-Methyl-deoxycytidine	0/0	0/±	±/±	±/±	±/±	+/+		

* Reaction conditions: organ homogenates incubated with ara-C and inhibitor for 1 hr at 37° (results expressed as: human liver/mouse kidney). No inhibition of deamination = 0; complete inhibition = +; partial inhibition = ±. Each symbol represents two independent experiments.
† ara-C: 1-β-D-arabinofuranosylcytosine.
‡ ara-FC: 1-β-D-arabinofuranosyl-5-fluorocytosine.

TABLE 5. EFFECT OF N^4-METHYL AND 4-HYDRAZINO DERIVATIVES OF ARA-C AND ARA-FC ON SURVIVAL TIME OF MICE WITH L1210 LEUKEMIA

Compound	Dose (mg/kg)* qd × 10	6-day weight change (g)	Survival time (days)	ILS, %†
Control		+2·6‡	10·4	
N^4-Methyl-ara-C	80	+1·2‡	11·0	6
Control		+3·0	8·9	
N^4-Methyl-ara-FC	40	+3·5	9·3	4
Control		+2·6	10·4	
4-Hydrazino-ara-C	80	+3·2	11·0	6
Control		+3·0	8·9	
4-Hydrazino-ara-C	40	+2·8	9·2	3
4-Hydrazino-ara-FC	40	+3·0	8·8	−1

* Treatment begun 24 hr after injection of leukemia.
† Increase in life span in percent.
‡ Five-day weight change in this experiment.

N^4-hydroxy derivatives of cytidine and deoxycytidine undergo deamination by human liver or mouse kidney enzyme systems, again confirming the blocking of enzymatic deamination by N^4-substitution.

N^4-Hydroxy-cytidine, although a deaminase inhibitor, did not potentiate the effect of ara-C in L1210 mouse leukemia (Table 7). This might be due to *in vivo* reduction of N^4-hydroxy-cytidine to cytidine. Both N^4-hydroxy-cytidine and cytidine have no activity in L1210 mouse leukemia. N^4-Hydroxy-cytidine did not show activity in Burkitt's cells in culture and did not block or potentiate the effects of ara-C when given in combination.

TABLE 6. EFFECT OF N^4-HYDROXY-DEOXYCYTIDINE, N^4-HYDROXY-5-FLUORO-DEOXYCYTIDINE, AND N^4-ACETYL-ARA-C ON SURVIVAL TIME OF MICE WITH L1210 LEUKEMIA

Compound	Dose (mg/kg)*	6-day weight change (g)	Survival time (days)	ILS, %†
Control		+4·0	8·9	
N^4-Hydroxy-deoxycytidine	40	+3·5	9·0	1
N^4-Acetyl-ara-C	40	+2·0	12·2	37
Control		+2·0‡	8·4	
N^4-Hydroxy-deoxycytidine	20	+4·2‡	8·6	2
N^4-Acetyl-ara-C	20	+3·6‡	8·8	5
Control		+4·0	8·9	
N^4-Hydroxy-5-fluoro-deoxycytidine	20	−1·6	10·9	22
	10	+0·7	11·1	25
	5	+2·4	9·7	9
Control		+2·5§	10·4	
Ara-C	20	+0·8§	21·7	109
	10	+0·3§	18·0	73
	5	+0·9§	14·6	40

* Treatment begun 24 hr after injection of leukemia.
† Increase in life span in percent.
‡ Eight-day weight change in these experiments.
§ Seven-day weight change in these experiments.

5-Methyl-deoxycytidine had a minimal inhibitory activity in Burkitt's cell culture, and blocked the action of ara-C when given in ratios of 1:1000 (Table 3), indicating it probably inhibits ara-C in the same manner as deoxycytidine, although much less efficiently.

N^4-Hydroxy-5-methyl-deoxycytidine, a strong deaminase inhibitor, blocked rather than potentiated the effect of ara-C in L1210 mouse leukemia (Table 7), but did not potentiate or block the inhibitory effect of ara-C in Burkitt's cells (Table 3). This might be due to reduction *in vivo* in mice to 5-methyl-deoxycytidine, which then might act in the same way as deoxycytidine, as described previously. Aside from consideration of possible conversion to the nucleotide, its lack of effect *in vitro* in Burkitt's cells might be due to lack of the reductase necessary to convert the compound to 5-methyl-deoxycytidine.

The therapeutic hope of potentiation of action of ara-C *in vivo* by inhibition of deamination has apparently not been realized with these analogs, presumably due to conversion into other substances which probably act in a manner similar to deoxycytidine. Since one mechanism of action of ara-C on the nucleotide level

involves an inhibition of conversion of cytidylate to deoxycytidylate, as demonstrated by Chu and Fischer[21, 22] in mouse leukemia 5178Y cells, and the action of ara-C can be blocked by deoxycytidine in several systems, including Ehrlich carcinoma,[23] T4 lymphoma,[23] mouse fibroblasts,[24] and L1210 leukemia *in vivo* (Table 8) and Burkitt's tumor cells *in vitro*,[12] any potentially useful analogue intended to block deamination must not also act like deoxycytidine or be converted to substances which act like deoxycytidine.

TABLE 7. EFFECT OF N^4-HYDROXY-5-METHYL-DEOXYCYTIDINE AND N^4-HYDROXYCYTIDINE, ALONE AND IN COMBINATION WITH ARA-C, ON SURVIVAL TIME OF MICE WITH LEUKEMIA L1210

Compound	Dose (mg/kg)*	6-day weight change (g)	Survival time (days)	ILS, %†
Control		+3·1	8·8	
Ara-C	20	−0·2	18·5	110
	10	−0·7	17·1	94
	5	+0·3	13·5	53
N^4-Hydroxy-5-methyl-deoxycytidine (OH-Me-CdR)	100	+3·2	9·2	4
N^4-Hydroxycytidine (OH-CR)	20	+3·2	8·8	0
Ara-C	10			
+OH-Me-CdR	100‡	+0·4	13·2	50
Ara-C	5			
+OH-Me-CdR	100‡	+2·6	11·2	27
Ara-C	10			
+OH-CR	20‡	−0·8	17·8	102
Ara-C	5			
+OH-CR	20‡	+0·5	15·1	72

* Treatment begun 24 hr after injection of leukemia.

† Increase in life span in percent.

‡ OH-Me-CdR or OH-CR injected 1 hr before ara-C.

The history, methods of synthesis, bacterial metabolism, and effects on mammalian cells of D-arabinosyl nucleosides have been ably reviewed by Cohen.[25]

N^4-Hydroxy-deoxycytidine is a deoxycytidylate deaminase inhibitor as shown by Maley and Maley[26] for the nucleotide in chick embryos, and by us in human liver and mouse kidney enzyme systems (Table 4). It is inactive in L1210 mouse leukemia (Table 6). Although it has only minimal activity in Burkitt's cell culture (Table 3), these inhibitory effects of N^4-hydroxy-deoxycytidine in Burkitt's cells were blocked completely at thirty times the ID_{50} (50 percent inhibitory dose) by thymidine at 1/10th equimolar concentrations but only slightly by deoxycytidine (Table 9).

Although N^4-hydroxy-deoxycytidine is not deaminated by and is indeed an inhibitor of deamination of ara-C in human liver or mouse kidney enzyme systems, it did not potentiate or block the inhibitory effects of ara-C in Burkitt's cells, and it blocked rather than potentiated the effect of ara-C in leukemia L1210 *in vivo* (Table 9). This suggests that in cell culture it was acting as an antimetabolite of thymine, whereas it was reduced in the intact mouse to deoxycytidine, which in turn blocked the effect of ara-C.

TABLE 8. EFFECT OF N^4-HYDROXY-DEOXYCYTIDINE, ALONE AND IN COMBINATION WITH ARA-C, ON SURVIVAL TIME OF MICE WITH LEUKEMIA L1210

Compound	Dose (mg/kg)*	6-day weight change (g)	Survival time (days)	ILS, %†
Control		+3·5	8·6	
Ara-C	20	0·0	20·2	135
	10	+0·1	20·4	137
	5	+0·1	15·0	74
N^4-Hydroxy-deoxy-cytidine (OH-CdR)	20	+3·0	9·2	7
Ara-C	10			
+OH-CdR	40‡	+2·5	10·9	27
Ara-C	10			
+OH-CdR	20‡	+1·8	12·8	49
Ara-C	5			
+OH-CdR	20‡	+2·5	11·3	31
Ara-C	10			
+CdR	20‡	+2·8	11·1	29
Ara-C	5			
+CdR	20‡	+3·3	11·6	35

* Treatment begun 24 hr after injection of leukemia.
† Increase in life span in percent.
‡ N^4-Hydroxy-deoxycytidine (OH-CdR) or deoxycytidine (CdR) injected 10 min before ara-C.

TABLE 9. EFFECTS OF N^4-HYDROXY-ARA-C (N^4-OH-ARA-C) AND N^4-HYDROXY-DEOXYCYTIDINE (N^4-OH-CdR) ON GROWTH OF BURKITT'S CELL CULTURE, AND BLOCKING BY DEOXYCYTIDINE (CdR) OR THYMIDINE (TdR)

Compound	Dose/ml (μg)	Growth inhibition (%)	Blocking
Thymidine	30	5	
Deoxycytidine	30	5	
N^4-OH-Ara-C	30	93	
N^4-OH-Ara-C/TdR*	30/30*	92	
N^4-OH-Ara-C/TdR	30/10	91	
N^4-OH-Ara-C/CdR	30/30	9	+
N^4-OH-Ara-C/CdR	30/10	16	+
N^4-OH-Ara-C/CdR	30/3	30	+
N^4-OH-Ara-C	10	79	
N^4-OH-Ara-C/TdR	10/30	86	
N^4-OH-Ara-C	3	47	
N^4-OH-Ara-C/TdR	3/30	39	
N^4-OH-CdR	30	82	
N^4-OH-CdR/TdR	30/30	14	+
N^4-OH-CdR/TdR	30/10	11	+
N^4-OH-CdR/TdR	30/3	3	+
N^4-OH-CdR/CdR	30/30	53	±
N^4-OH-CdR/CdR	30/10	64	
N^4-OH-CdR/CdR	30/3	75	
N^4-OH-CdR	10	81	
N^4-OH-CdR/CdR	10/30	50	±
N^4-OH-CdR	3	70	
N^4-OH-CdR/CdR	3/30	43	

* Ratio given is growth inhibitor/potential blocking agent.

These results in general confirm those of Nelson and Carter,[27] who showed that in L5178Y leukemic cells the mechanism of N^4-hydroxy-deoxycytidine inhibition was interference with thymidylate biosynthesis. This was shown by the specific protection afforded by thymidine and deoxyuridine, by the decrease in incorporation of labeled deoxycytidine and deoxyuridine into DNA thymine, by the competitive inhibition by deoxyuridine and thymidine of the phosphorylation of N^4-hydroxy-deoxycytidine, and the demonstration that N^4-hydroxy-deoxycytidylate was a competitive inhibitor of thymidylate synthetase in cell free preparations of *E. Coli* and leukemia 5178Y cells.

N^4-Hydroxy-5-fluoro-deoxycytidine, which bears close structural analogy to ara-C and to 5-fluoro-deoxycytidine was studied, since it was thought that it might act in the same manner as ara-C and/or as a thymidylate synthetase inhibitor. This compound shows considerable activity in Burkitt's cell culture, requiring about twice the concentration as ara-C to achieve the same degree of inhibition (Table 3). Its effect was blocked by thymidine, and to a much lesser degree by deoxycytidine. It was much more potent in Burkitt's cell culture than N^4-hydroxy-deoxycytidine, but was still not as active as the parent compound, 5-fluoro-deoxycytidine. N^4-Hydroxy-5-fluoro-deoxycytidine has no significant therapeutic effect on L1210 mouse leukemia (Table 6). Although it is a strong deaminase inhibitor, the difference in molar ratios of substrate to inhibitor necessary to achieve complete inhibition of deamination in human liver as compared to mouse kidney enzyme systems suggests that there may be a species difference in the deaminase.

Studies on deamination

Radiochromatograms of the commercially purchased ara-C showed radioactivity corresponding to ara-C (97%), and two minor impurities ($< 1\%$ each). Radiochromatograms obtained by incubation of labeled ara-C with human liver homogenate and subsequent development in chromatographic systems "A" and "B" showed spots corresponding to ara-U, as well as spots corresponding to the known impurities in the tritiated ara-C. No other spots were evident, confirming that deamination of ara-C is complete in 1 hr under these conditions, and that there are no other end products of deamination except for ara-U. Similar results have been reported by Camiener and Smith,[7a, c] Although the presence of phosphorylated derivatives of ara-C (ara-C-3′-P and ara-C-5′-P) as end products of deamination has been ruled out, we have not excluded the possibility that these derivatives might exist early in the deamination reaction, perhaps as part of the reaction mechanism.

Deaminase activity in other tissues

Camiener and Smith have reported deaminase activity in mouse kidney but not in mouse liver, heart or muscle.[7a] Deaminase activity was not found by us in liver, brain, or blood of BDFl mice, nor in L1210 leukemic cells *in vitro*. Several normal human subjects did not have deaminase activity in their serum. A patient being treated with ara-C for chronic granulocytic leukemia had a white blood cell count of 200,000. He tolerated high doses of ara-C without hematologic effect, and had high levels of deaminase in his plasma, as demonstrated by complete conversion of 156 mμM of ara-C to ara-U in 5 min, using 0·2 ml of his plasma in the test system described previously.

CONCLUSIONS

These studies indicate that Burkitt's cells and cells of mouse leukemia L1210 lack the ability to deaminate ara-C and have very little, if any, ability to reduce the N^4-hydroxy derivatives, whereas both reduction of N^4-hydroxy-ara-C and subsequent deamination occur readily in the leukemic mouse. Both ara-C and ara-FC are rapidly deaminated by both human liver and mouse kidney enzyme systems, but hydroxy-, methyl-, or amino-substitutions on the N^4-amino group give compounds which cannot be deaminated by these systems. Some of these derivatives, particularly N^4-hydroxy-cytidine, N^4-hydroxy-5-fluoro-deoxycytidine, and N^4-hydroxy-5-methyl-deoxycytidine, inhibit the deamination of ara-C by these systems. This inhibition of deamination unfortunately does not potentiate the action of ara-C either in cell culture, where there is normally no deamination of ara-C, or in the mouse, where N^4-hydroxy-ara-C and probably also the other N^4-hydroxy derivatives are reduced to the amino analogs.

Some of these derivatives of ara-C, ara-FC, cytidine, and deoxycytidine show differences in mechanisms of action in cell culture and in leukemic mice, but no more effective derivatives nor any that potentiate ara-C have so far been found.

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