

# Effect of Uracil Arabinoside on Metabolism and Cytotoxicity of Cytosine Arabinoside in L5178Y Murine Leukemia

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## Abstract

Pretreatment of L5178Y murine leukemia cells with uracil arabinoside (ara-U) enhances the cytotoxicity of cytosine arabinoside (ara-C). This effect is mediated by the cytostatic effect of ara-U, which causes a delay of cell progression through S-phase. Consequently, the specific activity of enzymes that peak during S-phase increases, and deoxycytidine kinase increases 3.6-fold over untreated controls. This allows enhanced anabolism of ara-C to nucleotides, as well as increased incorporation into DNA with ultimate synergistic cytotoxicity. It is postulated that the systemic metabolism of high-dose ara-C to sustained high levels of ara-U in patients with acute leukemia may enhance the activity of subsequent doses of ara-C, and thus contribute to a means for pharmacologic self-potential, contributing to the unique therapeutic activity of high-dose ara-C.

## Introduction

Cytosine arabinoside (ara-C)<sup>1</sup> is one of the most effective drugs for the treatment of acute non-lymphocytic leukemia (ANLL). When ara-C is used as a single agent in conventional doses and schedules, a complete remission is achieved in ~25% of patients, most of whom will relapse with ara-C refractory disease (1). The active form of ara-C is the nucleotide triphosphate, ara-CTP, which competes with deoxycytidine triphosphate (dCTP) as an alternate substrate for DNA polymerase (2). The extent to which ara-C is incorporated into DNA seems to correlate with its cytotoxicity (3).

Intravenous infusions of high dose ara-C (HiDAC) are effective in reinducing remission in patients who fail induction or who relapse while being treated with regimens that include

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1. *Abbreviations used in this paper:* ACS, aqueous counting scintillant; ANLL, acute non-lymphocytic leukemia; ara-C, cytosine arabinoside; ara-U, uracil arabinoside; CDP, cytidine diphosphate; CMP, cytidine monophosphate; CTP, cytidine triphosphate; dCyd, deoxycytidine; Hiara-U, high levels of uracil arabinoside; HiDAC, high dose cytosine arabinoside.

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conventional doses of ara-C (4). This observation has been confirmed by many investigators (5-10). In studies of the pharmacokinetics and metabolism of HiDAC in patients with ANLL, rapid metabolism to high levels of 1- $\beta$ -D-arabinofuranosyl uracil (ara-U), or Hiara-U, were discovered. These high levels persist in the plasma with a relatively long half-life ( $t_{1/2}$  3.75 h) (11). Since these levels of ara-U would persist throughout multiple-dose treatment with HiDAC, they would represent pretreatment of the second and all subsequent doses of ara-C with Hiara-U. Consequently, the interaction of Hiara-U and ara-C was investigated.

## Methods

*Materials.* Ara-C was provided by the Upjohn Co., Kalamazoo, MI. Ara-U, CDP-choline, deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP), and dCTP were obtained from Sigma Chemical Co., St. Louis, MO. Tetrahydrouridine was obtained from the National Cancer Institute, National Institutes of Health. [<sup>3</sup>H]ara-C 10.2 Ci/mmol was purchased from Amersham Corp., Arlington Heights, IL. [<sup>14</sup>C]cytidine (29.7 mCi/mmol), [2-<sup>14</sup>C]thymidine (58 mCi/mmol), and <sup>32</sup>P (carrier-free) were purchased from New England Nuclear, Boston, MA.

*Cell culture conditions and clonogenic assay.* L5178Y cells were cultured in Fischer's medium and supplemented with 10% horse serum. The cells were routinely passaged twice weekly. All cultures were grown at 37°C in an atmosphere of 5% CO<sub>2</sub>:95% air. Exponentially growing cells were used for all experiments. Clonogenic assay was performed by the method previously described (12).

*Ara-C accumulation by L5178Y cells.* To determine the cellular accumulation of ara-C in cells with or without pretreatment with ara-U (10<sup>-3</sup> M for 24 h), 10<sup>6</sup> cells/ml were incubated in a shaking water bath at 37°C. [<sup>3</sup>H]ara-C and ara-C were added to make a final concentration of 10<sup>-6</sup> M (10  $\mu$ Ci/ml) or 10<sup>-4</sup> M (100  $\mu$ Ci/ml). 1 ml of the cell suspension was sampled at designated time intervals after exposure to ara-C alone, or ara-U and ara-C concurrently, after ara-U pretreatment for 24 h. The cells were centrifuged rapidly in an Eppendorf microfuge for 30 s, and then washed three times with 1.5 ml of ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 1 ml PBS. 50  $\mu$ l was transferred to a vial containing 10 ml of aqueous counting scintillant (ACS; Amersham Corp.) for liquid scintillation counting. The remaining cell suspension was centrifuged, and the cell pellets were extracted with 65  $\mu$ l of 1.5 N perchloric acid. The acid-soluble fraction was neutralized with 100  $\mu$ l of 1 N KOH and 40  $\mu$ l of KH<sub>2</sub>PO<sub>4</sub> buffer. 50  $\mu$ l was counted in 10 ml of ACS. The remainder of the extract was used for separation of ara-C metabolites. The acid-insoluble precipitate was washed once with ice-cold perchloric acid and dissolved in 100  $\mu$ l of dimethylsulfoxide, 10 ml ACS were added, and radioactivity was counted in a liquid scintillation counter.

*Separation of ara-C metabolites.* 25  $\mu$ l of the acid soluble fraction and 5-10  $\mu$ l of a marker solution containing known amounts of ara-C, ara-U, dCMP, dCDP, and dCTP were spotted on polyethyleneimine impregnated thin-layer chromatography plates (layer, 0.1 mm cellulose MN 300 polyethyleneimine impregnated) for nucleotide separation. The plates were developed in a tank containing 2 M glacial acetic acid with 0.5 M lithium chloride until the solvent front was ~15 cm above

the origin. The plates were then dried, the ultraviolet-absorbing spots were marked in the dark, and the polyethyleneimine-cellulose with nucleotides was removed using magic transparent tape, and cut out in equal lengths according to the position of the marker metabolites. 10 ml of ACS was added to each vial for liquid scintillation counting. Since this method did not separate ara-CMP from ara-CDP-choline, a DEAE Sephadex A 25 anion exchange column chromatography system, developed by A. Townsend, University of North Carolina, was used to distinguish ara-CDP-choline from ara-CMP. The acid-soluble fraction of the cells was isolated and then loaded onto DEAE Sephadex-25 anion exchange columns. The columns were eluted with stepwise gradient of 0–1.0 M triethylammonium formate. 1.5 ml of each fraction was collected. Ara-CDP-choline and ara-CMP were eluted from the columns using 0.1 M and 0.2 M, respectively.

**Incorporation of [ $^3\text{H}$ ]-ara-C into nucleic acid.** 10  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-ara-C (Sp. Act. 15.5 Ci/mM) and 7.5  $\mu\text{Ci}$  of  $\text{H}_3^{32}\text{PO}_4$  were added to cells pretreated with or without ara-U for 24 h. The cells were incubated at 37°C in a shaking water bath. After further incubation with ara-C alone or ara-U and ara-C for 2 and 4 h,  $10^7$  cells were sampled and centrifuged. The cell pellets were washed twice with PBS and resuspended in 1 ml of PBS. A mixture containing 0.5 ml of 10% sodium lauryl sulfate, 0.01 M EDTA, 0.01 M Tris, and 100  $\mu\text{g}$  proteinase K was added, and the cell suspension was incubated at 37°C for 24 h. Nucleic acids were extracted twice with 5 ml water-saturated phenol and precipitated by the addition of 0.1 vol of 4 M NaCl and 2 vol of absolute ethanol. The samples were kept at –20°C overnight. After centrifugation, the pellets were dissolved by 0.5 ml of 5 mM EDTA and 0.5 ml of formamide at 80°C in a hot water bath for 5 min. Samples of 0.8 ml were loaded onto a solution of cesium sulfate (Sigma Chemical Co.), which had a final density of 1.548 g/ml and a refractive index of 1.3776. The sample was centrifuged at 25,000 rpm at 20°C in an Ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) with a SW 50.1 rotor for 60 h. Fractions of five drops each were collected from the top to the bottom of the tube with a fraction collector (Auto Densi-Flow II C Fraction Collector; Buchler Instruments, Inc., Fort Lee, NJ). The DNA had a density of  $\sim 1.42$  (native) and 1.44 (denatured). RNA had a density of 1.65.

**Deoxycytidine (dCyd) kinase, thymidine kinase, dCMP deaminase, and dCyd deaminase assay.** All cellular enzymatic activities were measured using methods previously described (13–15).

**DNA histogram and cell cycle analysis.** A nuclear suspension of the L5178Y cell line, treated under the conditions described, was obtained via a modification of the Thornwaite (16) procedure. In brief, 1 ml of the cell suspension ( $1\text{--}5 \times 10^6$  cell/ml) was treated with 0.1 ml of Dulbecco's phosphate medium containing 6% NP-40, 500  $\mu\text{g}$ /ml of propidium iodide, and 280 U RNAase. These solutions were mixed and allowed to incubate for at least 5 min at 0°C. Analysis by fluorescent microscopy gave a single suspension of red nuclei when excited with blue light. If any measurable clumping occurred, the suspension was gently syringed using a 26.5 gauge needle. These cells were, however, very sensitive to this procedure. DNA histogram analysis was performed (17) using a flow cytometer (TPS-2; Coulter Electronics, Inc., Hialeah, FL) with a 5-W laser (Spectra-Physics, Inc., Mountain View, CA). The cells were excited at 488 nm and the red fluorescence related to DNA content per nuclei was determined using a 510-nm barrier filter and a 530-nm long pass absorbance filter. Analysis was ended when 4,000 cells were collected in the peak channel of the histogram. The cell cycle kinetic determinations were performed using the Dean (18) program. The determination of significant differences between the defined groups was calculated by the standard *t* test.

## Results

The effect of ara-U on cell cycle progression of L5178Y cells is shown in Fig. 1 and Table I. DNA histogram analysis of the cells by flow cytometry after 24-h pretreatment with  $10^{-3}$  M

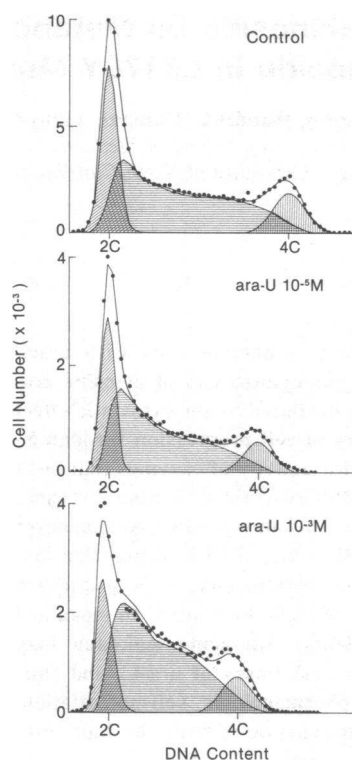


Figure 1. DNA histogram analysis of L5178Y cells after 24-h treatment with ara-U. Cells were prepared and analyzed as described in Methods.

ara-U reveals a significant accumulation of cells in S-phase. A similar cytostatic effect is observed with lower doses of ara-U ( $10^{-5}$  M and  $10^{-4}$  M). However, this requires a 48–72-h period of drug exposure (data not shown). Assessment of enzyme activity in treated vs. control cells corresponds with the DNA histogram data in that the accumulation of the cells in S-phase is associated with an increase in the specific activity of cell-cycle-dependent enzymes, thymidine kinase, and deoxycytidine kinase by 179 and 360% of control, respectively (Table II). The activity of dCMP deaminase varies little with cell cycle distribution, and dCyd deaminase activity is not detectable in this cell line.

Accumulation of ara-C in L5178Y cells is largely a reflection of “trapping” due to metabolism of the nucleoside to the nucleotide. Figs. 2 A and B depict ara-U enhancement of ara-C accumulation at two clinically relevant concentrations:  $10^{-4}$  and  $10^{-6}$  M (11). These concentrations are achievable as steady-state during infusion of 3 g/M<sup>2</sup> of ara-C, and during the post-infusion  $\gamma$  phase, respectively. Fractionation of intracellular metabolites of ara-C shows that the largest fraction of

Table I. DNA Histogram Distribution of L5178Y Cells in Culture After Treatment with Ara-U for 24 h

	No. of determinations	G <sub>1</sub>	S	G <sub>2</sub> M
Control	4	27.9 $\pm$ 1.8	60.8 $\pm$ 2.7	11.4 $\pm$ 3.4
$10^{-5}$ M	3	26.4 $\pm$ 2.7	62.3 $\pm$ 3.7	11.3 $\pm$ 1.2
$10^{-3}$ M	4	20.1 $\pm$ 2.2	70.0 $\pm$ 3.9	10.0 $\pm$ 2.9
		( <i>P</i> < 0.005)	( <i>P</i> < 0.005)	( <i>P</i> > 0.25)

Table II. Effect of Ara-U Treatment on Enzymatic Activities in L5178Y Cells

ara-U concentration	Thymidine kinase		dCyd kinase		dCMP deaminase	
	U/10 <sup>7</sup> cells	Percent increase over control	U/10 <sup>7</sup> cells	Percent increase over control	U/10 <sup>9</sup> cells	Percent increase over control
(M)						
—	0.75±0.13	—	0.05±0.02	—	0.32±0.08	—
10 <sup>-5</sup>	0.85±0.19	113	0.06±0.02	122	0.37±0.13	116
10 <sup>-4</sup>	1.16±0.29	155	0.1±0.02*	202	0.41±0.1	128
10 <sup>-3</sup>	1.34±0.32*	179	0.18±0.03‡	360	0.47±0.2	149

10<sup>5</sup> L5178Y cells were incubated with or without ara-U at 37°C for 24 h. These data are results of 3 experiments. There was no detectable dCyd deaminase activity. \*  $P < 0.05$ , ‡  $P < 0.001$  vs. control.

intracellular drug is in the form of acid-soluble nucleotides, the main constituent of which is ara-CTP (Table III). While the total cellular concentrations of metabolites are substantially increased as a result of ara-U pretreatment (Figs. 2 A and B), the qualitative distribution of metabolites is relatively similar to the control. The effect of ara-U pretreatment on formation of ara-CDP choline is shown in Fig. 3. Ara-U results in a fourfold enhancement of ara-CDP choline formation. In accord with other observers (3), cesium sulfate density gradient separation of the acid insoluble precipitate indicates that all of the radioactivity is incorporated into the DNA fraction (Fig. 4). Consistent with the expanded ara-CTP pool size, ara-U pretreatment resulted in enhanced ara-C incorporation into DNA.

Recent studies have correlated the extent of ara-C incorporation into DNA with its cytotoxic effect (3). Given the observed ara-U enhancement of ara-C anabolism and incorporation into DNA, the clonogenic fraction after drug treat-

ment(s) was used as an index of cytotoxicity. As seen in Fig. 5, exposure of cells to 10<sup>-6</sup> M ara-C for up to 4 h was not cytotoxic. At 10<sup>-4</sup> M ara-C, there is increasing toxicity associated with longer duration of exposure. In both instances, ara-U pretreatment resulted in enhancement of the cytotoxic effect of ara-C, especially at 4 h of drug exposure, compared with that expected if the cytotoxic effect from both drugs was additive.

## Discussion

Ara-C is included in virtually all clinical protocols dealing with the treatment of patients with ANLL. As such, the nucleoside ara-C is inactive and must be phosphorylated to the nucleotide triphosphate, ara-CTP, to exert its cytotoxic effect. The rate-limiting process in this regard is the specific activity of dCyd kinase, which metabolizes ara-C to ara-CMP.

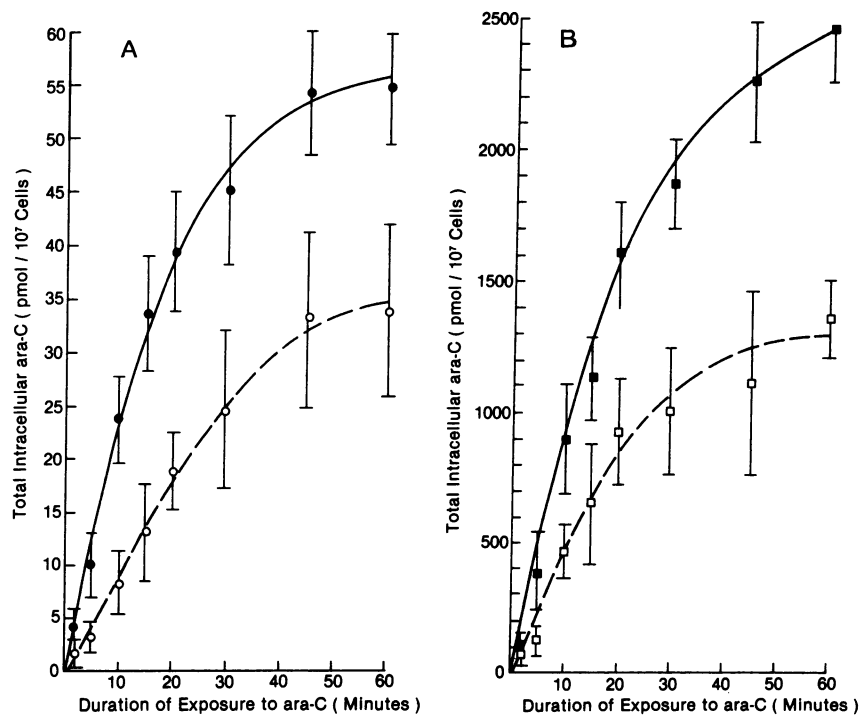


Figure 2. Effect of pretreatment of L5178Y cells (with 10<sup>-3</sup> M ara-U) on the cellular accumulation of ara-C. Cells in the exponential phase of growth were pretreated with 10<sup>-3</sup> M ara-U for 24 h, after which [<sup>3</sup>H]ara-C was added. Serial samples were collected over a period of 60 min. The total cellular concentration of ara-C was determined as described in Methods. (A) ---○---, ara-C 10<sup>-6</sup> M; ---●---, ara-U pretreatment. (B) ---□---, ara-C 10<sup>-4</sup> M; ---■---, ara-U pretreatment.

Table III. Distribution of Ara-C Metabolites in Acid-Soluble Fraction of Cells After Pretreatment with Ara-U

Pretreatment	Hours of exposure to ara-C	ara-CMP + ara-CDP-choline		ara-CDP		ara-CTP	
		%	pmol/10 <sup>7</sup> cells	%	pmol/10 <sup>7</sup> cells	%	pmol/10 <sup>7</sup> cells
None	1	14.1	3.37	2.3	0.55	81.8	19.55
	2	13.3	3.58	2.7	0.73	83.5	22.46
	3	17.1	3.59	2.6	0.55	80.3	16.86
	4	20.0	4.40	6.1	1.34	73.8	16.24
ara-U (10 <sup>-3</sup> M)	1	13.7	5.78	4.9	2.07	80.1	33.80
	2	15.2	6.22	5.3	2.17	79.2	32.39
	3	11.9	4.87	7.2	2.93	80.9	32.93
	4	17.5	7.96	6.3	2.87	75.9	34.53

Cells were pretreated with or without 10<sup>-3</sup> M ara-U for 24 h, at which time [<sup>3</sup>H]ara-C was added to the cultures. After further incubation with both drugs for 1, 2, 3, and 4 h, the intracellular acid-soluble fractions were extracted and the nucleotides were analyzed by thin layer chromatography. (Thickness of the plastic sheet was 0.1 mm cellulose MN 300 polyethyleneimine impregnated. Solvent system contained 2 M acetic acid and 0.5 M lithium chloride. Data are expressed as pmol/10<sup>7</sup> cells and percent of total radioactivity).

There are several means for increasing the cellular activity of dCyd kinase. Since the specific activity of this enzyme increases in cells as they enter S-phase (19), any means for retarding cellular progression in this phase of the cell cycle would enhance ara-C anabolism.

Ara-C cytotoxicity may be enhanced by decreasing the pool size of the normal metabolite, dCTP, by so-called biochemical modulation. Higher pool size of dCTP has been shown to be a mechanism of resistance to ara-C in both murine (20) and human neoplasms (21). The higher pool of dCTP could interfere with ara-C action by inhibiting anabolism to ara-CMP by acting as an allosteric inhibitor of dCyd kinase, and also by competing with ara-CTP for incorporation into DNA. A decrease in dCTP pool by pretreatment with hydroxy-

urea (22), deazauridine (23), or high doses of thymidine (24) has potentiated the cytotoxicity of ara-C. However, simple escalation of the extracellular concentration of ara-C has resulted in an amount of ara-C incorporated in DNA equivalent to that after thymidine pretreatment (25).

Earlier screening of ara-U against neoplastic cells in culture showed virtually no cytotoxic activity (26). However, isobologram studies by Mueller et al. (27) indicate a synergistic cytotoxic effect from protracted (72 h) exposure to ~10<sup>-5</sup> M ara-U and ara-C. This concentration of ara-U is clinically relevant in that plasma concentration in patients after a 3-h infusion of 3 g/M<sup>2</sup> ara-C results in sustained ara-U concentrations in the range of 1–3 × 10<sup>-4</sup> M (11). Given that all of the reported protocols with HiDAC in man use intermittent short-term (1–3 h) infusion, the high plasma levels of ara-U resulting from the metabolism of HiDAC are, in essence, pretreatment for subsequent infusion of HiDAC. Thus, in addition to the dose-dependent increase in ara-CTP formation and incorporation into DNA, the unique efficacy of HiDAC in the treatment of acute leukemia may be contributed by ara-U effect on ara-C metabolism. In *in vitro* studies, high levels of ara-U may retard ara-C catabolism by dCyd deaminase via product competitive inhibition (11). This effect may contribute to the long terminal (γ) half-life of ara-C noted after HiDAC infusion (11). Second, the cytostatic effect of high concentrations of ara-U causes a hold-up of cells in the S-phase of the cell cycle. This accumulation of cells in S-phase allows a relative increase in S-phase enzymes such as dCyd kinase, which in turn enhances the anabolic rate of ara-C to ara-CMP, and consequently, ara-CTP and ara-C-DNA.

Ara-CTP also serves as a substrate for the synthesis of ara-CDP-choline (28, 29), the synthesis of which is also enhanced by ara-U pretreatment (Fig. 3). Since this reaction is reversible, ara-CDP-choline may serve as a reservoir for ara-CTP, or may serve as a substrate for membrane phospholipid synthesis. The rapid lysis of leukemic blasts noted in clinical trials with HiDAC (30) may suggest a mechanism of action in addition to inhibition of DNA synthesis. Preliminary studies in our laboratory (unpublished) suggest that membrane phospholipid

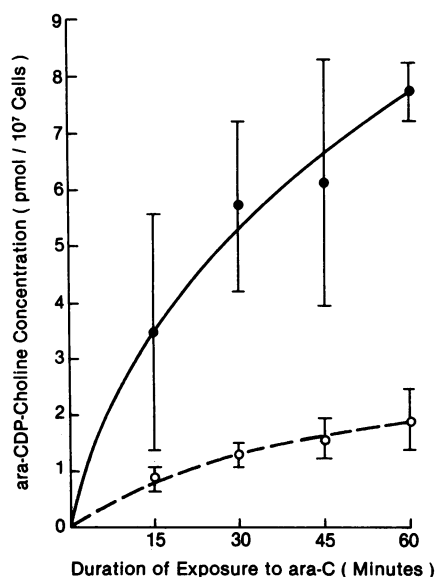


Figure 3. Effect of ara-U (10<sup>-3</sup> M) pretreatment on formation of ara-CDP-choline. Similar conditions were followed as described for Fig. 2. Ara-C exposure was 10<sup>-6</sup> M.

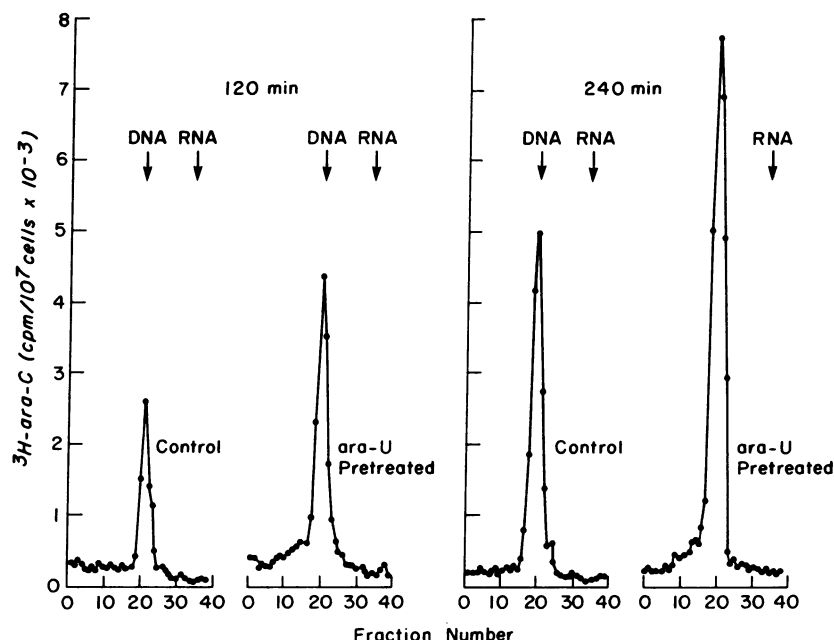


Figure 4. Effect of ara-U pretreatment on incorporation of ara-C into nucleic acid of L5178Y cells. Cells were pretreated with  $10^{-3}$  M ara-U for 24 h, at which time [ $^3\text{H}$ ]ara-C was added to the cultures. The extraction and cesium sulfate separation of nucleic acid is as described in Methods.

synthesis is substantially enhanced after ara-U pretreatment followed by HiDAC.

Thus, the efficacy of HiDAC in the treatment of acute leukemia may, in part, be related to direct dose-dependent effects in addition to indirect effects from ara-C metabolism. The HiDAC, in essence, serves as a pro-drug for high levels of ara-U that may retard ara-C systemic catabolism (11), as well as enhance the cellular anabolism of ara-C, thus providing a dual means for pharmacologic self-potential.

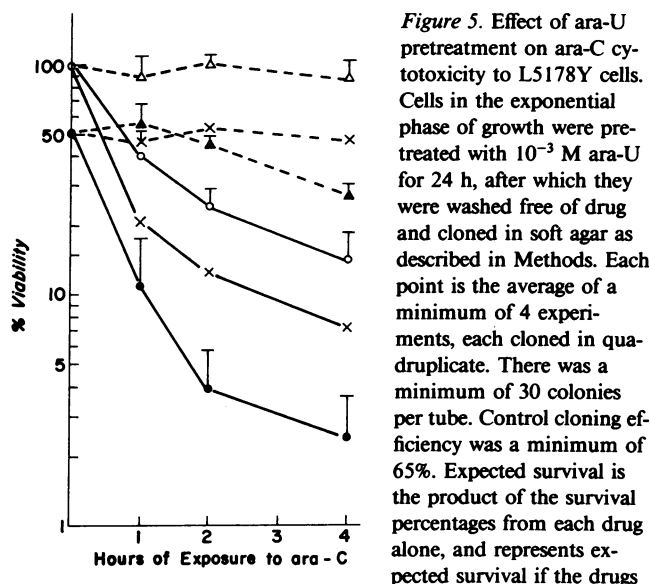


Figure 5. Effect of ara-U pretreatment on ara-C cytotoxicity to L5178Y cells. Cells in the exponential phase of growth were pretreated with  $10^{-3}$  M ara-U for 24 h, after which they were washed free of drug and cloned in soft agar as described in Methods. Each point is the average of a minimum of 4 experiments, each cloned in quadruplicate. There was a minimum of 30 colonies per tube. Control cloning efficiency was a minimum of 65%. Expected survival is the product of the survival percentages from each drug alone, and represents expected survival if the drugs

were to exert an additive effect. Ara-C alone: ---  $\Delta$  ---,  $10^{-6}$  M; ---  $\times$  ---,  $10^{-4}$  M. Expected survival after  $10^{-3}$  M ara-U pretreated for 24 h followed by ara-C: ---  $\times$  ---,  $10^{-6}$  M; ---  $\Delta$  ---,  $10^{-4}$  M. Observed survival after  $10^{-3}$  M ara-U pretreatment for 24 h followed by ara-C: ---  $\Delta$  ---,  $10^{-6}$  M ara-C; ---  $\bullet$  ---,  $10^{-4}$  M ara-C.

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