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Deoxypyrimidine-induced inhibition of the cytokinetic effects of 1-β-D-arabinofuranosyluracil*

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Summary. Ara-U-induced S-phase accumulation and the interaction between high concentrations of ara-U (HiCAU) and ara-C were investigated in L1210 leukemia cells in vitro. Treatment of exponentially growing L1210 murine leukemia cells with ara-U (200–1000 μM) for 48 h caused a dose-dependent accumulation of cells in the S-phase. The extent of this ara-U-induced S-phase accumulation correlated with ara-U incorporation into DNA and with increases of up to 172% and 464% in the specific activities of deoxycytidine kinase and thymidine kinase, respectively, over control values. Metabolism of 1 μM ara-C following the exposure of cells to ara-U (1 mM) resulted in 4.5 pmol ara-C DNA/mg protein vs 2.1 pmol/mg protein in control cells. Although 48-h exposure of cells to 200 and 400 μM ara-U is not cytotoxic, it enhances the cytotoxicity of ara-C (10–100 μM) 4- to 10-fold. Ara-U-induced S-phase accumulation is inhibited by deoxypyrimidine nucleosides but not by pyrimidine or deoxypurine nucleosides. Some of the ara-U and ara-C concentrations used in this study are achievable in clinical practice, and ara-U/ara-C interactions may explain in part the unique therapeutic utility of high-dose ara-C.

Introduction

The cytotoxic effects of ara-C are mediated in the S-phase of the cell cycle [14, 16, 25, 33]. Because the specific activity of dCyd kinase, the rate-limiting enzyme for drug anabolism, peaks during the S-phase [11, 32] laboratory and clinical therapeutic experiments have attempted to modulate the cell cycle so as to increase the cytotoxic effects of ara-C [21].

Our previous studies of the clinical pharmacokinetics of high-dose ara-C (HiDAC) in leukemic patients revealed high plasma levels (100–500 μM) of ara-U, the only circulating catabolite of ara-C [3]. As compared with ara-C, ara-U has a long half-life (3.8 h); thus, patients treated with repetitive short-term infusions (1–3 h) of HiDAC at 12-h intervals over 2–6 days [2, 4] exhibit a relatively sustained high plasma level of ara-U [3]. This clinical observation raises a question of practical therapeutic importance related to a possible effect of ara-U on ara-C metabolism and cytotoxicity in leukemia cells. Our previous studies using murine leukemia cells in vitro and in vivo indicate that sustained high extracellular levels of ara-U enhance ara-C anabolism and cytotoxicity by causing an accumulation of leukemia cells in the S-phase [8, 32] as well as altering systemic ara-C pharmacokinetics by diminishing its catabolism by cytidine-deoxycytidine deaminase [8]. These combined cytokinetic and pharmacokinetic effects of ara-U result in pharmacologic synergy with ara-C [8, 32].

The present study explores the cytokinetic effects of ara-U on murine L1210 leukemia cells in the presence and absence of normal nucleosides in an attempt to discern the mechanism of action of ara-U as a guide for further laboratory-clinical investigation.

Materials and methods

Chemicals. Ara-C was provided by the Upjohn Co. (Kalamazoo, Mich.). Ara-U, creatine phosphate, creatine phosphokinase, deoxycytidine, thymidine, bromodeoxyuridine (BrdUrd), and adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). [2-^{14}C]-Thymidine was supplied by New England Nuclear (Boston,

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Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-U, 1-β-D-arabinofuranosyluracil; ara-CTP, 1-β-D-arabinofuranosylcytidine triphosphate; HiDAC, high-dose ara-C; HiCAU, high concentrations of ara-U; dCTP, deoxycytidine triphosphate; HiDAU, high-dose ara-U; FiTC, Fluoroisothiocyanate; dUDP, deoxyuridine diphosphate; dUTP, deoxyuridine triphosphate; dTTP, thymidine triphosphate; BrdUrd, bromodeoxyuridine; dCyd kinase, deoxycytidine kinase

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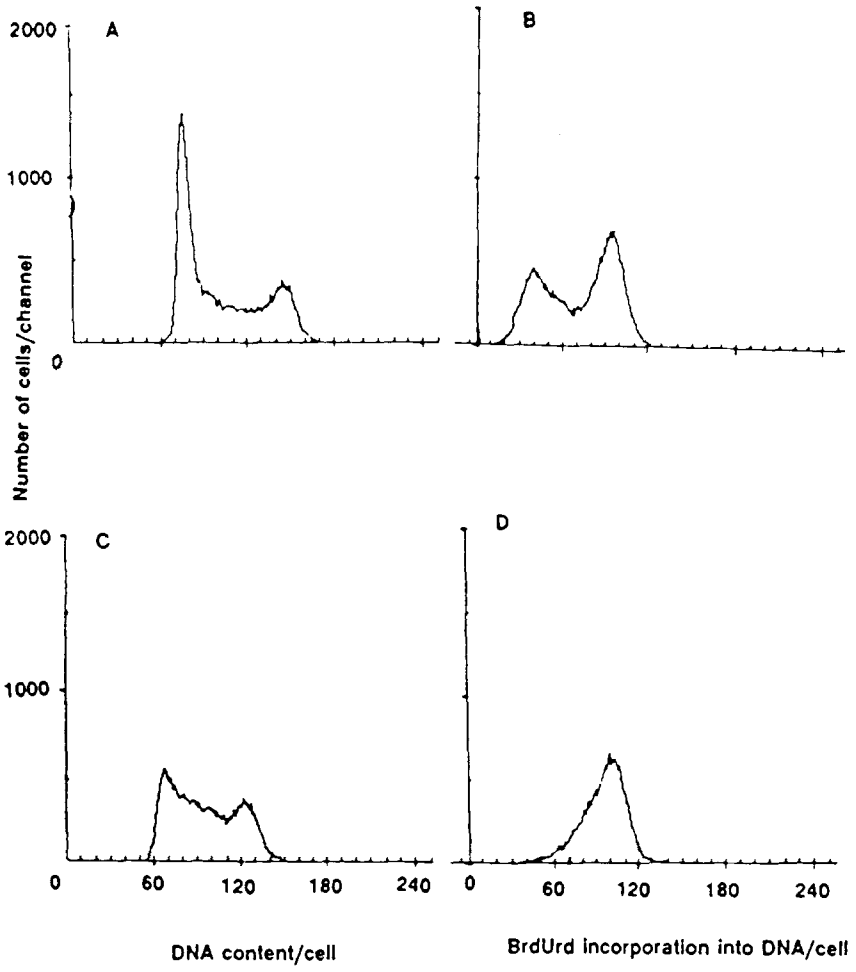

Fig. 1A–D. Dual-label analysis for DNA content and BrdUrd incorporation in L1210 cells labeled as described in Materials and methods. **A, C** DNA histograms of propidium iodide-stained cells for control and ara-U-treated (1 mM for 48 h) L1210 cells, respectively. **B, D** Histograms of the BrdUrd incorporation by control and ara-U-treated cells, respectively

Mass.). [^{3}H]-Ara-C and [2-^{14}C]-deoxycytidine were obtained from Amersham (Arlington Heights, Ill.). DEAE-Sephadex A-25 resin was purchased from Pharmacia Inc. (Piscataway, N.J.). Tetrahydrouridine was obtained from the National Cancer Institute, National Institutes of Health. [5, 6-^{3}H]-Ara-U was purchased from Moravek Biochemicals, Inc. (Brea, Calif.).

Cell culture. L1210 murine leukemia cells were cultured in Fischer's medium supplemented with 10% horse serum. The cells were kept in exponential growth by passage twice weekly. All cultures were grown at 37° C in an atmosphere comprising 5% CO_2 and 95% air. Exponentially growing cells were used for all experiments.

Drugs. Stock solutions of 100 mM ara-C and ara-U were prepared in sterile water and further diluted according to the needs of the experiment.

Determination of [5,6-^{3}H]-ara-U incorporation into DNA. Leukemic cells ($2-4\times10^4$/ml; 50 ml) were incubated with [^{3}H]-ara-U (18 Ci/mmol; 20 μCi; 1 mM) for 48 h. The cells were spun at 2,000 *g* for 1 min (Sorvall RT 6000 refrigerated centrifuge; DuPont Co., New Town, Conn.), washed with medium containing 10 μM dipyridamole to inhibit nucleoside influx or efflux, processed, and quantitated as described elsewhere [31]. Essentially, the cells were suspended in 0.5 ml 0.9% NaCl at 0° C; protein and nucleic acids were precipitated by the addition of 0.5 ml 0.4 *N* $HClO_4$ at 0° C. After 10 min, the precipitate was washed five times with 0.2 *N* $HClO_4$ at 0° C and then dissolved in 0.5 ml 0.3 *N* NaOH, and RNA was digested by incubation at 37° C for 1 h. Following chilling to 0° C, DNA and protein were reprecipitated by the addition of 50 μl 5.2 *N* $HClO_4$ and then separated by centrifugation. The precipitate was washed five times with 0.2 *N* $HClO_4$ at 0° C, suspended in 0.5 ml 0.5 *N* $HClO_4$, and incubated at 70° C for 25 min to digest the DNA. After cooling, the precipitate was separated by centrifugation, the supernatant was sampled, and the level of radioactivity in the DNA digest was determined.

Cell-cycle analysis using a monoclonal antibody to BRdUrd. The cells to be analyzed for BrdUrd incorporation were first treated with 30 μM BrdUrd for 30 min at 37° C, then washed and fixed in 70% cold ethanol. These cells were subsequently treated with RNAse, acid-denatured, and treated with a mouse monoclonal antibody to BrdUrd [12]. The antibody was a gift from Dr. J. Gray (Lawrence Livermore Laboratories, Livermore, Calif.). A mouse IgG was added to controls to correct for nonspecific binding. The cells were then washed and treated with FITC-labeled sheep antimouse IgG (Sigma Chemical Co). The DNA was stained with 10 μg propidium iodide/ml, and the dual-labeled cells were processed on a Becton Dickinson FACS 440 flow cytometer (Becton Dickinson FACS Systems, Sunnyvale, Calif.) and analyzed using the Consort 40 program. Determination of the distribution of cells in the G_1, S, and G_2+ M phases of the cell cycle was based on a cell-size-gated population using defined markers for these parameters.

Enzyme assays. The specific activities of deoxycytidine and thymidine kinase in leukemic cells were measured using methods described elsewhere [9, 10]. The total volume of the enzyme-assay mixtures was 0.1 ml and included 0.1 M TRIS-HCl (pH 7.5) and 2 mM dithiothreitol. The reaction mixture for thymidine kinase activity contained 2 mM ATP, 2 mM $MgCl_2$, 0.1 mg bovine serum albumin, 1 unit creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 0.1 mM thymidine, and 0.1 μCi [2-^{14}C]-thymidine (46 Ci/mol). The deoxycytidine reaction mixture contained 6 mM ATP, 6 mM $MgCl_2$, 0.2 mM tetrahydrouridine, 0.2% (w/v) bovine serum albumin, 1 unit creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 0.3 mM deoxycytidine, and 0.1 μCi [2-^{14}C]-deoxycytidine (52 Ci/mol). Enzyme activity was expressed in international units, which corresponds to nanomoles per minute per 10^7 cells.

Leukemia-cell metabolism of cytosine arabinoside. The procedure described by Yang et al. [32] was modified as previously reported [8] for the determination of ara-C and its metabolites in L1210 cells.

Clonogenic assay. The method reported earlier [32] was used with modifications. L1210 cells were cloned in sterile petri dishes (35 × 10 mm; Becton Dickinson and Company, Oxnard, Calif.) instead of test tubes, and the concentration of bacto agar used was 0.3%.

Nucleoside-inhibition studies. Exponentially growing L1210 cells ($2-4\times10^4$ cells/ml) were treated with ara-U (1 mM) and/or individual nucleosides (10 μM of either thymidine, deoxycytidine, deoxyuridine, deoxyadenosine, or deoxyguanosine; 100 μM of either cytidine or uridine). The two control conditions consisted of medium or ara-U (1 mM). Cells were sampled after 0, 24, and 48 h treatment, and the cell count, size, and volume and cell-cycle analysis were determined at each point as described above. At the end of the 48-h ara-U ± normal nucleoside treatment, aliquots of cells from each condition were exposed to ara-C (10–100 μM) for 3 h, washed free of drug, and cloned for cytotoxicity assessment [32].

Results

Figure 1 displays the flow cytometric results obtained for control cells (Fig. 1A, B) and for cells exposed to 1 mM ara-U for 48 h (Fig. 1C, D). Figures 1A and 1C show the cell-cycle distribution as determined by DNA staining with propidium iodide, and Figs. 1B and 1D illustrate the S-phase accumulation as determined by BrdUrd incorporation into DNA. Both methods demonstrated ara-U-induced accumulation of cells in the S-phase as compared with controls.

Table 1 displays the ara-U dose-response effect on cell-cycle traverse. Exposure of L1210 cells to ara-U for 24 and 48 h resulted in a significant ($P = 0.01$) concentration-dependent increase in the number of cells in the S-phase of the cell cycle as compared with controls. At all time points, >95% of the cells excluded trypan blue dye. Over the first 24 h of treatment with ara-U, we found a progressive accumulation of cells in the S-phase along with a corresponding decrease in the proportion of cells in the G_1 phase. Over the ensuing 24 h, cell division occurred (Table 2) along with the exit of cells from G_2M and their reentry into G_1 (Table 1). The extent to which cells proceeded from S to G_2M from 24 to 48 h was inversely related to the ara-U concentration (Table 1). This effect on cell-cycle traverse correlated with the amount of ara-U incorporated into DNA (Table 2).

Consistent with this cytokinetic effect was an increase in the specific activities of the S-phase enzymes deoxycytidine and thymidine kinase (Table 3). This, in turn, correlated with an increase in ara-C incorporation into DNA when the ara-C was added after 48 h pretreatment with 1 mM ara-U (Table 3). Fractionation of the intracellular metabolites of ara-C (1 μM) revealed that most of the drug occurred in the form of acid-soluble nucleotides, the main constituent of which was ara-CTP (85%–91%; data not shown). Prior studies have related the amount of ara-CTP formed [17, 29] to ara-C incorporation into DNA [20] and its subsequent cytotoxic effect.

Given the observed ara-U-induced enhancement of ara-C anabolism and incorporation into DNA, the viability of the leukemic cells after single and combined drug treatment was assessed by clonogenic assay. As noted in Fig. 2, treatment with 10, 50, and 100 μM ara-C for 3 h resulted in 49%, 41% and 36% viability, respectively, as compared with controls. Treatment of cells with 200 or 400 μM ara-U, the concentrations achieved in plasma during treatment of leukemia patients with high-dose ara-C [3], was not cytotoxic. Pretreatment of cells with these noncytotoxic concentrations of ara-U followed by ara-C resulted in a 4- to 10-fold enhancement of cytotoxicity over the value that would be expected if the combined cytotoxic effects of both drugs were additive, additivity being defined as the product of the observed fractional viability for each drug alone × 100. The greatest degree of potentiation occurred at the highest doses of both drugs: 1 mM ara-U and 100 μM

Table 1. Concentration-dependent effect of ara-U on the cell-cycle distribution of L1210 cells

Ara-U (μM)	24 h			48 h		
	G_1	S	G_2+M	G_1	S	G_2+M
0	37±1	55±3	8±1	44±4	54±4	5±0.4
200	27±3	62±3	9±1	43±3	52±2	7±0.3
400	22±2	68±3	9±2	33±4	58±4	7±0.2
600	17±3	73±3	9±3	25±2	67±2	7±1
800	15±1	75±3	10±2	23±1	70±1	6±0.4
1000	13±4	76±7	10±2	22±1	71±2	6±1

Data represent mean values ± SD. Exponentially growing L1210 cells were exposed to increasing concentrations of ara-U for 24 and 48 h, then exposed to 30 μM BrdUrd for 30 min. The cells were fixed, processed, and analyzed as described in Materials and methods

Table 2. Effect of ara-U on the growth of murine L1210 leukemia cells as related to its incorporation into DNA

Ara-U (μM)	Cell number (× 10^4)			[^{3}H]-Ara-U in DNA at 48 h (pmol/10^6 cells)
	0 h	24 h	48 h	
0	2.1	8.1	34	0
200	2.1	5.2	15	29±9
400	2.1	3.2	10	38±11
600	2.1	3	6.4	52±14
800	2.1	2.6	6	61±17
1000	2.1	2.2	5.7	69±19

Data represent mean values ± SD for 3 determinations. Exponentially growing L1210 leukemia cells were exposed to increasing concentrations of [^{3}H]-ara-U for up to 48 h. The cells were sampled at 0, 24, and 48 h, then counted, and the extent of ara-U incorporation into DNA was determined as described in Materials and methods

Table 3. Effect of pretreatment of L1210 cells with 1 mM ara-U for 48 h on the specific activity of enzymes and ara-C incorporation into DNA

	Specific activity (pmol min^{-1} $mg\ protein^{-1}$)		[^{3}H]-Ara-C incorporation into DNA[a] (pmol/mg protein)
	Deoxycytidine kinase	Thymidine kinase	
Control	135±7	73±4	2.1±0.1
Ara-U	367±12	412±24	4.5±0.3
% Increase over control	172	464	114

Data represent mean values ± SD

[a] [^{3}H]-Ara-C incorporation into DNA was measured after 4 h incubation with 1 μM ara-C

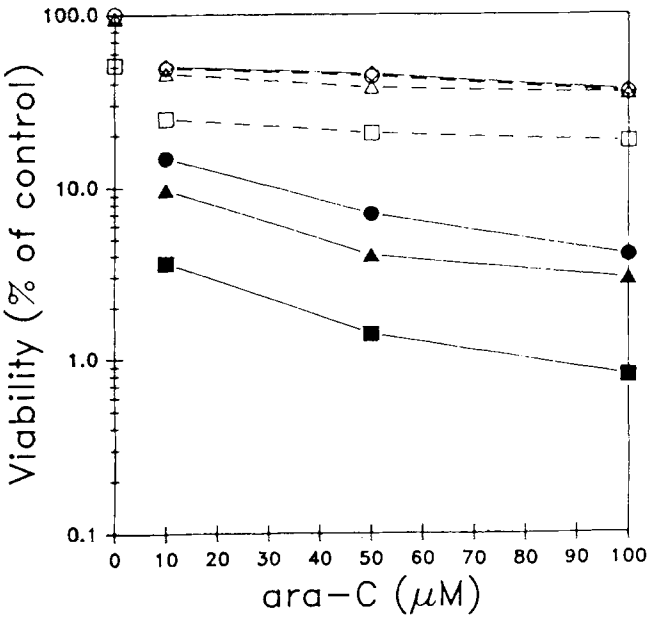

Fig. 2. Effect of ara-U pretreatment for 48 h on the cytotoxicity of ara-C. L1210 cells (4×10^4 cells/ml) were incubated in the presence or absence of ara-U (200–1000 μM) for 48 h (○, 200 μM ara-U; △, 400 μM ara-U; □, 1 mM ara-U; ara-C was added at 10–100 μM an additional 3-h incubation. The cells were then centrifuged, processed, and cloned as described in Materials and methods. Each point represents the mean of ≥2 determinations. *Dashed lines with open symbols* represent the expected viability following treatment with ara-U and ara-C (○----○, 200 μM ara-U + ara-C; △----△, 400 μM ara-U + ara-C; □----□, 1 mM ara-U + ara-C). *Solid lines with closed symbols* represent the observed viability following treatment with ara U and ara-C (●——●, 200 μM ara-U + ara-C; ▲——▲, 400 μM ara-U + ara-C; ■——■, 1 mM ara-U + ara-C). Estimates of expected viability were derived as described in the footnote to Table 6.

ara-C. As single agents, these concentrations resulted in 50% and 36% viability, respectively. If the effect of both drugs were additive, the expected viability would be 18% ($0.5 \times 0.36 \times 100$). In contrast, the cytotoxicity following sequential exposure to 1 mM ara-U and 100 μM ara-C increased 23-fold (0.8% viability) over that expected.

Arabinosyl nucleosides mimic the cellular biochemistry of deoxyribonucleosides. In an attempt to derive insight into the potential mechanism of ara-U-induced accumulation of cells in the S-phase, the effect of concurrent exposure of cells to ara-U and normal nucleosides was explored. As shown in Table 4, exposure for 48 h to 10 μM deoxynucleosides and 100 μM ribonucleosides alone did not alter cell-cycle progression as compared with controls. As noted previously (Table 1, Fig. 1), 48 h treatment with 1 mM ara-U resulted in a significant decrease in the G_1 population ($P = <0.001$) and a significant increase in the S-phase population ($P = <0.001$) as compared with controls. This effect was abrogated by concurrent exposure of the cells to both ara-U and any of the three deoxypyrimidine nucleosides. In contrast, deoxypurine nucleosides and ribonucleosides had no effect on ara-U-induced S-phase accumulation. As shown in Table 5, the same deoxypyrimidines could reverse the established effect of ara-U. Pretreatment of the leukemic cells with 1 mM ara-U for 48 h followed by exposure to ara-U and deoxypyrimidine nucleosides for an additional 24 h reversed the ara-U-induced S-phase accumulation ($P = <0.001$). The removal of ara-U from the medium (washout) and subsequent incubation in ara-U-free medium for an additional 24 h had a similar effect. In contrast, the deoxypurine and pyrimidine ribonucleosides had no effect.

Consistent with the above data, the determination of viability by clonogenic assay of leukemic cells revealed

Table 4. Effect of concurrent exposure of L1210 cells to ara-U and/or normal nucleosides on cell-cycle distribution

	G_1	S	G_2+M
Control	31±2	58±1	11±2
Thymidine	32±1	54±2	12±1
Deoxycytidine	32±2	56±5	13±3
Deoxyuridine	32±2	59±4	11±1
Deoxyadenosine	28±1	62±4	12±1
Deoxyguanosine	28±1	62±4	12±1
Uridine	30±3	57±5	13±3
Cytidine	31±3	57±2	12±2
Ara-U	15±5	73±3	11±1
Ara-U + thymidine	27±3	61±1	11±1
Ara-U + deoxycytidine	27±3	63±3	11±1
Ara-U + deoxyuridine	34±1	58±2	9±1
Ara-U + deoxyadenosine	18±4	73±6	9±1
Ara-U + deoxyguanosine	19±1	73±2	8±1
Ara-U + uridine	14±2	75±4	9±5
Ara-U + cytidine	14±4	76±5	9±4

Data represent mean values ± SD. Exponentially growing L1210 cells were exposed to ara-U (1 mM) and/or normal nucleosides for 48 h (concentrations: deoxyribonucleosides, 10 μM; ribonucleosides, 100 μM). At 48 h, the cells were treated with 30 μM BrdUrd for 30 min, spun to remove the BrdUrd, and washed with the appropriate medium; they were then fixed with 70% ethanol, processed, and analyzed as described in Materials and methods

Table 5. Effect of ara-U removal or sequential exposure of L1210 to ara-U followed by normal nucleosides on ara-U-induced S-phase accumulation

	G_1	S	G_2+M
Control	31±2	58±2	10±1
Ara-U	14±1	82±1	4±0.4
Ara-U, then washed out	30±1	62±2	10±1
Ara-U → thymidine	31±1	61±1	8±1
Ara-U → deoxycytidine	28±1	63±3	7±1
Ara-U → deoxyuridine	29±1	62±2	9±1
Ara-U → cytidine	16±1	80±2	6±1
Ara-U → uridine	16±1	80±1	6±1
Ara-U → deoxyadenosine	15±1	80±3	4±1
Ara-U → deoxyadenosine	14±1	83±1	3±0.4

Data represent mean values ± SD. Exponentially growing L1210 cells were exposed to ara-U (1 mM) for 48 h, at which time the cells were spun and the supernatant was discarded. All samples except the controls and the ara-U "washout" group were supplemented with fresh medium containing ara-U and the designated nucleoside and allowed to grow for an additional 24 h (concentrations: deoxyribonucleosides, 10 μM; ribonucleosides, 100 μM). At the end of this 72-h incubation, all samples were exposed to BrdUrd and cell-cycle distribution was analyzed as described in Materials and methods

Table 6. Effect of pretreatment of L1210 cells with ara-U and/or nucleosides on the cytotoxic effect of ara-C

	Viability (% of control)	
	Observed	Expected
Ara-U	50	–
Ara-C (10^{-5} M)	50	–
Ara-U → ara-C	4	25
Deoxycytidine → ara-C	60	50
Thymidine → ara-C	58	50
Deoxyuridine → ara-C	75	50
Ara-U + deoxycytidine → ara-C	68	2.7
Ara-U + thymidine → ara-C	58	2.3
Ara-U + deoxyuridine → ara-C	84	3
Ara-C (10^{-4} M)	36	–
Ara-U → ara-C	0.8	18
Deoxycytidine → ara-C	50	36
Thymidine → ara-C	60	36
Deoxyuridine → ara-C	46	36
Ara-U + deoxycytidine → ara-C	37	0.4
Ara-U + thymidine → ara-C	47	0.5
Ara-U + deoxyuridine → ara-C	48	0.4

L1210 cells (4×10^4/ml) were exposed to ara-U (1 mM) and/or nucleoside [thymidine, deoxycytidine, or deoxyuridine (10 μM)] for 48 h followed by ara-C (10 or 100 μM) for 3 h. The cells were washed with Fischer's medium, and a clonogenic assay for viability estimation was performed as described in Materials and methods. Estimates of the expected viability were derived from the product of the observed viability from single-drug exposure in the case of the two-drug combination, e. g., ara-U→ara-C, or from the product of the observed viabilities from dual agent exposures, e. g., the expected viability following treatment with ara-U + deoxycytidine → ara-C was derived as follows: observed viability for (ara-U → 10 μM ara-C = 0.04) × (deoxycytidine → 10 μM ara-C = 0.68) = 0.024 × 100 = 2.7%. Each number represents the mean of ⩾3 determinations

that concurrent exposure of the cells to ara-U and any one of the deoxypyrimidine nucleosides abrogated the ara-U-induced synergistic interaction with ara-C (Table 6). The observed percentage of viable cells produced by ara-U plus deoxypyrimidine nucleoside followed by ara-C was similar to that of the respective controls (deoxypyrimidine nucleoside followed by ara-C, Table 6).

Discussion

The clearance of ara-C from the plasma is primarily the result of systemic deamination of the drug to ara-U [1]. The clinical use of conventional doses of ara-C (100–200 mg/m^2) results in low plasma concentrations of ara-U (<1 μM) [15, 30]. However, the use of HiDAC (3 g/m^2) [24] almost immediately produces plasma concentrations of ara-U ranging from 0.1 to 0.5 mM [3]. In this setting, the half-life of ara-U is sufficiently long (3.75 h) that repetitive doses of HiDAC given at 12-h intervals result in sustained HiCAU in the plasma [3].

Sequential exposure of murine leukemia L5178Y cells to ara-U and ara-C in vitro results in synergistic cytotoxicity [23]. This synergy has been related to ara-U-induced slowing of cell-cycle traverse with a resultant accumulation of cells in the S-phase [8, 32]. This cytokinetic effect allows enhanced anabolism of ara-C to ara-CTP and incorporation into DNA [8, 32]. Similar effects have been noted following sequential use of these drugs in the treatment of mice bearing L5178Y leukemia [8].

The present study indicates that the cytokinetic effects of ara-U are not limited to L5178Y leukemia but also occur in L1210 leukemia cells in culture and relates this observation to the incorporation of ara-U into DNA. This effect of ara-U was blocked by concurrent or sequential exposure to thymidine, deoxycytidine, or deoxyuridine but not by deoxyadenosine, deoxyguanosine, cytidine, or uridine (Tables 4–6).

Ara-U-induced accumulation of cells in the S-phase was concentration-dependent Table 1), an effect that correlated with ara-U incorporation into DNA (Table 2). After the drug-exposed cells had been washed and resuspended in fresh ara-U-free medium, they quickly reverted to normal growth. This resumption of growth was associated with the loss of detectable ara-U from DNA. In contrast, cells that had been resuspended in ara-U-containing medium showed 74 ± 24 pmol/10^6 cells in DNA. This suggests that ara-U-induced S-phase accumulation is probably due to the incorporation of ara-U into DNA. The rapid removal of ara-U from DNA is likely to result from the activity of uracil-DNA glycosylase, an enzyme found in murine and human leukemia cells [6, 13, 19, 22, 28]. Likewise, after the leukemic cells had been preexposed to ara-U for 48 h and then concurrently exposed to both ara-U and deoxypyrimidine nucleosides for an additional 24 h, the cytokinetic effects of ara-U were reversed, an effect that was not achieved by deoxypurine or pyrimidine ribonucleosides (Table 5). The reversal of these ara-U effects by the three deoxypyrimidines may possibly be explained by a final common pathway such as competition between dTTP and ara-UTP for incorporation into DNA; this implies the serial metabolic conversion of deoxycytidine and deoxyuridine to dTMP followed by further phosphorylation.

These observations are consistent with the previously described role of uracil-DNA glycosylase in preserving the fidelity of DNA synthesis by removing misincorporated uracil residues [18]. Ara-U at high concentrations is metabolized to ara-UTP [7]. The concentration of this metabolite may be regulated by dUTPase [5]. However as long as high concentrations of ara-U are sustained in the extracellular medium, there is sufficient anabolism of ara-U to nucleotides and, hence, incorporation into DNA. Uracil-DNA glycosylase may readily remove these residues from DNA, resulting in repair of the apyrimidinic site, and ara-U may be reincorporated in the repair site. The removal of ara-U from the medium or the provision of deoxypyrimidine nucleosides in slight excess allows this repair process to proceed with fidelity [26], resulting in release of the S-phase block and resumption of cell growth.

The precise mechanism(s) of S-phase accumulation are under investigation. Possibilities include (1) the slowing of DNA synthesis by inhibition of DNA-primase or DNA-polymerase activity, (2) the termination of DNA-chain elongation by the insertion of two or more arabinosyl residues at the chain terminus [27], and (3) the creation of apyrimidinic sites by the action of uracil-DNA glycosylase [26]. The effects of this repair process may also facilitate

the subsequent incorporation of ara-C residues as noted in Table 3 and as previously reported [8, 32]. In addition or alternatively, arabinosyl uracil nucleotides may have an effect on pyrimidine-metabolizing enzymes. These possibilities are under investigation since their elucidation may provide new leads for cancer chemotherapy.

As noted above, our laboratory studies of the interaction between ara-U and ara-C were stimulated by the clinical observation of HiCAU following the clinical use of HiDAC. The demonstration of a synergistic interaction between ara-U and ara-C on L5178Y [32] and L1210 cells in vitro as well as in vivo in mice bearing L5178Y leukemia [8] raises the question as to whether or not ara-U contributes to the clinical efficacy of HiDAC. Clinical trials have shown that HiDAC can induce remission in 30% of patients who have either relapsed from or been refractory to conventional doses of ara-C [2]. This dose-related secondary response to the same drug is unusual in clinical oncology. The response to HiDAC observed in failed leukemic patients equals that associated with the use of newly introduced drugs [2]. Although a straightforward dose-response relationship is possible, other features such as the interaction between ara-U and ara-C may help to explain the renewed therapeutic effects associated with the use of HiDAC.

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References

1. Caminier GW, Smith CG (1965) Studies of the enzymatic deamination of cytosine arabinoside. Biochem Pharmacol 14: 1405
2. Capizzi RL, Powell BL (1987) Sequential high dose ara-C and asparaginase versus high dose ara-C alone in the treatment of patients with relapsed and refractory acute leukemias. Semin Oncol 14 [Suppl 1]: 40
3. Capizzi RL, Yang J-L, Cheng E, Bjornson D, Sahasrabudhe D, Tan R-S, Cheng Y-C (1983) Alteration of the pharmacokinetics of high dose ara-C by its metabolite high ara-U in patients with acute leukemia. J Clin Oncol 1: 763
4. Capizzi RL, Yang J-L, Rathmell JP, White JC, Cheng E, Cheng Y-C, Kute T (1985) Dose-related pharmacologic effects of high dose ara-C and its self-potentiation. Semin Oncol 12 [Suppl 3]: 65
5. Caradonna SJ, Cheng Y-C (1980) The role of deoxyuridine triphosphate nucleotidohydrolase, uracil-DNA glycosylase, and DNA polymerase α in the metabolism of FUdR in human tumor cells. Mol Pharmacol 18: 513
6. Caradonna SJ, Cheng YC (1980) Uracil DNA-glycosylase. Purification and properties of this enzyme isolated from blast cells of acute myelocytic leukemia patients. J Biol Chem 255: 2293
7. Chandrasekaran B, Capizzi RL, Thomas MJ (1986) Ara-U metabolism in murine and human leukemias (abstract). Proc Am Assoc Cancer Res 27: 304
8. Chandrasekaran B, Capizzi RL, Kute TE, Morgan T, Dimling J (1989) Modulation of the metabolism and pharmacokinetics of 1-β-D-arabinofuranosylcytosine by 1-β-D-arabinofuranosyluracil in leukemic mice. Cancer Res 49: 3259
9. Cheng Y-C, Prusoff WH (1974) A new rapid assay for measuring deoxycytidylate- and deoxythymidylate-kinase activities. Anal Biochem 60: 545
10. Cheng Y-C, Domin B, Lee L-S (1977) Human deoxycytidine kinase: purification and characterization of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia patients. Biochim Biophys Acta 481: 481
11. Chiba P, Tihan T, Eher R, Koller U, Wallner C, Gobl R, Linkesch W (1989) Effect of cell growth and cell differentiation on 1-β-D-arabinofuranosylcytosine metabolism in myeloid cells. Br J Haematol 71: 451
12. Dolbeare F, Beisker W, Pallavicini MG, Vanderlaan M, Gray JW (1985) Cytochemistry for bromodeoxyuridine/DNA analysis. Cytometry 6: 521
13. Domena JD, Mosbaugh DW (1985) Purification of nuclear and mitochondrial uracil-DNA glycosylase from rat liver. Identification of two subcellular forms. Biochemistry 24: 7320
14. Fietkau R, Friede H, Maurer-Schultze B (1984) Cell kinetic studies of the cytostatic cytocidal effect of 1-β-D-arabinofuranosylcytosine on the L1210 ascites tumor. Cancer Res 44: 1105
15. Ho DHW, Frei E (1971) Clinical pharmacology of 1-β-D-arabinofuranosylcytosine. Clin Pharmacol Ther 12: 944
16. Karon M, Shirakawa S (1969) The locus of action of 1-β-D-arabinofuranosylcytosine in the cell cycle. Cancer Res 29: 687
17. Kinahan JJ, Kowal EP, Grindey GB (1981) Biochemical and antitumor effects of the combination of thymidine and 1-β-D-arabinofuranosylcytosine against leukemia L1210. Cancer Res 41: 445
18. Koistinen P, Ferola E, Vilpo JA (1986) Uracil-DNA glycosylase activity in chronic lymphoproliferative disorders. Leuk Res 10: 1307
19. Koistinen P, Ferola E, Vilpo JA (1987) Uracil-DNA glycosylase activity in human acute leukemia. Leuk Res 11: 557
20. Kufe DW, Major PP, Egan ER, Beardsley P (1980) Incorporation of ara-C into L1210 DNA as a correlate of cytotoxicity. J Biol Chem 2355: 8997
21. Lampkin BC, Nagao T, Mauer AM (1971) Synchronization and recruitment in acute leukemia. J Clin Invest 50: 2204
22. Makino F, Munakata N (1979) Excision of uracil from bromodeoxyuridine-substituted and UV-irradiated DNA in cultured mouse lymphoma cells. Int J Radiat Biol 36: 349
23. Muller WEG, Zahn RK (1979) Metabolism of 1-β-D-arabinofuranosyluracil in mouse L5178Y cells. Cancer Res 39: 1102
24. Rudnick SA, Cadman EC, Capizzi RL, Skeel RT, Bertino JR, McIntosh S (1979) High dose cytosine arabinoside (HDARAC) in refractory acute leukemia. Cancer 44: 1189
25. Skipper HE, Schabel FM Jr, Wilcox WS (1967) Experimental evaluation of potential anticancer agents: XXI. Scheduling of arabinosylcytosine to take advantage of its S-phase specificity against leukemia cells. Cancer Chemother Rep 51: 125
26. Strauss BS (1985) Cellular aspects of DNA repair. Adv Cancer Res 45: 45
27. Townsend AJ, Cheng Y-C (1987) Sequence-specific effects of ara-5-aza-CTP and ara-CTP on DNA synthesis by purified human DNA polymerases in vitro: visualization of chain elongation on a defined template. Mol Pharmacol 32: 330
28. Vilpo JA (1988) The DNA-repair enzyme uracil-DNA glycosylase in the human hematopoietic system. Mutat Res 193: 207
29. Walsh CT, Craig RT, Agarwal RP (1980) Increased activation of 1-β-D-arabinofuranosylcytosine by hydroxyurea in L1210 cells. Cancer Res 40: 3286
30. Wan SH, Hoffman DH, Azarnoff DL (1974) Pharmacokinetics of 1-β-D-arabinofuranosylcytosine in humans. Cancer Res 34: 392
31. White JC, Rathmell JP, Capizzi RL (1987) Membrane transport influences the rate of accumulation of cytosine arabinoside in human leukemia cells. J Clin Invest 79: 380
32. Yang J-L, Cheng EH, Capizzi RL, Cheng Y-C, Kute T (1985) Effect of uracil arabinoside on metabolism and cytotoxicity of cytosine arabinoside in L5178Y murine leukemia. J Clin Invest 75: 141
33. Young RSK, Fischer GA (1968) The action of arabinosylcytosine on synchronously growing populations of mammalian cells. Biochem Biophys Res Commun 32: 23