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Cell cycle-specific metabolism of arabinosyl nucleosides in K562 human leukemia cells*

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Summary. Exponentially growing K562 cells incubated with 1-β-D-arabinofuranosylcytosine (ara-C) accumulate ara-C triphosphate (ara-CTP) at a higher rate and to a greater concentration after pretreatment with 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) than do cells treated with ara-C alone. Potentiation of ara-C metabolism is due in part to an indirect effect of F-ara-A triphosphate (F-ara-ATP)-mediated reduction in deoxynucleotide pools and consequent activation of deoxycytidine kinase. Because the levels of deoxynucleotide pools and the activity of deoxycytidine kinase are cell cycle-specific, we investigated the effect of cell cycle phases on the accumulation of ara-CTP and the influence of F-ara-A pretreatment on such accumulation. Exponentially growing K562 cells were fractionated into G_1, S, and G_2+M phase-enriched subpopulations (each enriched by >60%) by centrifugal elutriation. The rate of ara-CTP accumulation was 22, 25, and 14 μM/h and the rate of F-ara-ATP accumulation was 38, 47, and 33 μM/h in the G_1, S, and G_2+M subpopulations, respectively. The rate of elimination of arabinosyl triphosphates was similar among the different phases of the cell cycle. After pretreatment with F-ara-A, the rate of ara-CTP accumulation in the G_1, S, and G_2+M phase-enriched subpopulations was 43, 37, and 26 μM/h, indicating a 1.7-, 1.5-, and 1.9-fold increase, respectively. These results suggest that a combination of F-ara-A and ara-C may effectively potentiate ara-CTP accumulation in all phases of the cell cycle. This observation is consistent with the results of studies on the modulation of ara-C metabolism by F-ara-A in lymphocytes and leukemia blasts obtained from patients with chronic lymphocytic leukemia and acute myelogenous leukemia, respectively.

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Introduction

1-β-D-arabinofuranosylcytosine (ara-C) and 9-β-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) are the most effective agents for the treatment of acute myelogenous leukemia and chronic lymphocytic leukemia, respectively [13, 22–24]. To act as cytotoxic drugs, these analogues have to be phosphorylated to their respective 5′-triphosphates, ara-CTP and F-ara-ATP [9, 33, 38]. The rate-limiting step in the synthesis of these triphosphates is their initial phosphorylation to monophosphates, which is catalyzed by deoxycytidine (dCyd) kinase [8, 11, 12, 29]. The activity of dCyd kinase is influenced by the cell cycle phase and by the physiological concentration of deoxynucleotides [25]. Although to a substantially lesser extent than that of thymidine kinase [7, 39], the activity of dCyd kinase assayed in cell-free extracts has been found to increase as cells pass from the G_1 to the S phase [6, 21, 42, 43]. Additionally, endogenous deoxynucleotide pools (dNTPs) are elevated in S phase cells as compared with G_1 phase cells [1, 5, 10, 25, 40]. In intact cells, these two factors combine to regulate the activity of dCyd kinase, which can be expressed as the phosphorylation of ara-C to ara-CTP [25].

Our previous studies have demonstrated that exponentially growing K562 cells pretreated with F-ara-A accumulate ara-CTP at a faster rate and to a greater concentration than do cells treated with ara-C alone [14, 15]. Potentiation of ara-C metabolism is due to a direct effect of F-ara-ATP on dCyd kinase and to an indirect effect of F-ara-ATP on this enzyme that is mediated by the inhibition of ribonucleotide reductase and a consequent decrease in dNTPs [14]. Because of the reported cell-cycle specificity of dCyd kinase and the levels of dNTP pools in other cell lines, it is important to know whether K562 cells exhibit similar cell-cycle fluctuations in the activity of dCyd kinase, in dNTP levels, in the intracellular accumulation and retention of ara-CTP and F-ara-ATP, and in the stimulation of ara-CTP accumulation by preincubation with F-ara-A. This is important because if there is no interphasic difference in the potentiation of ara-CTP accumulation by F-ara-A, this

sequential combination could be used both in actively dividing cells such as acute myelogenous leukemia and in indolent chronic lymphocytic leukemia cells.

Materials and methods

Chemicals and cell line. Ara-C and ara-CTP were purchased from Sigma Chemical Co. (St. Louis, Mo.). F-ara-A and tetrahydrouridine were provided by Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md.). F-ara-ATP was synthesized as previously described [31]. All other chemicals were of the highest grade available. The K562 cell line [26] was obtained from the American Type Culture Collection (Rockville, Md.). The maintenance of the cultures, mycoplasma analysis, and growth characteristics of this cell line have been described elsewhere [14].

Enrichment for G_1, S, and G_2+M cells. Exponentially growing K562 cells ($2-4\times10^8$) were suspended in 20–30 ml RPMI medium supplemented with fetal calf serum containing 20 μg DNAse I/ml [28]. After they had been filtered through a nylon mesh, the cells were loaded into a Beckman J6-M/E centrifuge (Beckman Instruments, Palo Alto, Calif.) equipped with a standard horizontal chamber. Elutriation was done at a rotor speed of 1800–2000 rpm and at flow rates of between 10 and 60 ml/min at 4° C. From each elutriation run, 12–15 fractions were collected and both the number of cells in each fraction and the cell volume were determined with a Coulter counter equipped with a Coulter channelizer 256 (Coulter Electronics, Inc., Hialeah, Fla.). Based on the cell volume, 2–3 fractions were pooled to make the required G_1, S, and G_2+M subpopulations. Cells were fixed from each fraction and from pooled G_1, S, and G_2+M subpopulations to determine the percentage of cells in each phase.

Cytofluorometry. The cells were fixed in ethanol and stored at –20° C until staining. Propidium iodide was used to stain DNA after incubation with RNAse. The percentage of cells in each phase was determined with a FACSCAN flow cytometer (Becton/Dickinson, Calif.) equipped with an argon laser delivering 15 mW at 488 nm. Red fluorescence was measured through a long-pass filter (620 nm).

Metabolism of ara-C and combination studies. G_1, S, and G_2+M phase-enriched populations were incubated with 10 μM ara-C or 300 μM F-ara-A for 4 h. Aliquots were analyzed hourly for intracellular ara-CTP or F-ara-ATP. For investigation of the influence of F-ara-ATP on the anabolism of ara-C, cells were loaded with F-ara-ATP by incubation with 300 μM F-ara-A for 2 h, washed into drug-free medium, and incubated with 10 μM ara-C. Aliquots were removed at hourly intervals for 4 h during this incubation period and then analyzed for triphosphates of F-ara-A and ara-C.

Nucleotide extraction and analysis. After their incubation with drugs, the cells were washed twice with ice-cold phosphate-buffered saline (8.1 g NaCl, 0.22 g KCl, 1.1 g Na_2HPO_4, and 0.27 g KH_2PO_4/l H_2O, pH 7.4). After centrifugation, the cell pellet was extracted with $HClO_4$ as previously described [32]. The nucleoside triphosphates in the neutralized acid-soluble extract were analyzed by high-performance liquid chromatography (HPLC; Waters Associates, Milford, Mass.) as described elsewhere [17]. The identity of nucleoside analogue triphosphates was confirmed by their coelution with authentic triphosphates, their resistance to periodate oxidation, and their UV absorbance. Quantitation of the nucleotides in the $HClO_4$ extracts was determined by electronic integration and reference to response factors calculated from external standards [17].

Determinations of dNTPs. Neutralized $HClO_4$ extracts of treated and untreated cells were evaporated to dryness in an Evapomix volume-reduction apparatus (Buchler Instruments, Fort Lee N.J.). Ribonucleotides in the extracts were degraded by treatment with $NaIO_4$ [30], and dNTPs were separated by anion-exchange column on a Waters HPLC system as previously described [14].

Effect of F-ara-A and ara-C on cell clonogenicity. The reproductive viability of the cells was determined by cloning. G_1, S, and G_2+M phase-enriched populations were incubated under the following conditions: in the absence of drug, in the presence of 10 μM ara-C, and in the presence of 10 μM ara-C after a 2-h incubation with 300 μM F-ara-A. Aliquots were removed hourly for 3 h and diluted in drug-free medium. After being mixed with Iscove's medium (Gibco, Grand Island, N.Y.) supplemented with 30% fetal bovine serum and 5% glutamine, 500–2000 cells were plated in 35×10-mm tissue-culture plates with 0.3% agar. The plates were incubated for 10 days at 37° C in a humidified incubator containing 5% CO_2, and colonies of more than 50 cells were scored under a microscope. The cytotoxicity of the drugs was expressed as a percentage of survival relative to the untreated control value [16]. The expected additive cytotoxicity of the combined drugs was the product of survival fractions of cells treated with either F-ara-A (for 3 h) or ara-C (0–3 h). The cloning efficiency of the control cells was the same (29% ± 1%) for all phases of the cell cycle.

Deoxycytidine kinase assay. Cell extracts were prepared and dialyzed for 6 h to remove NTPs and dNTPs. The phosphorylation of ara-C was determined with DE-81 anion-exchange filter discs [14]. [^{3}H]-ara-C (100 μM, 1 Ci/mmol) was used as a substrate, and tetrahydrouridine was added to the reaction mixture to a final concentration of 100 μM to prevent ara-C deamination. Next, 30–40 μg protein was added to start the reaction, and 10 μl of each reaction mixture was spotted onto filter discs every 10 min for 40 min. The reaction was linear up to this time. After the discs had been washed, the radioactivity was counted as previously described [14]. Protein content was determined with the Bio-Rad Bradford assay (Bio-Rad Laboratories, Richmond, Calif.) using bovine plasma gamma globulin as a standard.

Statistical considerations. The results of all analyses (dNTPs, F-ara-ATP and ara-CTP levels, and clonogenicity) represent mean values for 2–4 separate experiments; the standard deviations were less than 15% of the mean values.

Results

Enrichment of the synchronous cell population

The exponentially growing K562 population constituted about 55% G_1, 35% S, and 10% G_2+M phase cells (Fig. 1A). The cells that were below channel number 150 and above 560 (most probably doublets and quadruplets) were not included in the calculations. We achieved enrichments of more than 70%, 60%, and 70% for G_1, S, and G_2+M phase cells, respectively (Fig. 1B–D). The mean cellular volume was 900, 1350, and 1950 fl for G_1, S, and G_2+M phase-enriched cells, respectively. The differences in the cell size represent a 1.5-fold increase in S phase cells and a 2.2-fold increase in G_2+M phase cells as compared with G_1 phase cells. To normalize for differences in cell size, we expressed the concentration of physiologic and analogue nucleotides in micromolar values; in this calculation, it is assumed that all the nucleotides are evenly distributed in the cell volume.

Accumulation of ara-CTP

Previous studies of exponentially growing K562 cells indicated that the accumulation of ara-CTP was saturated by 10 μM exogenous ara-C. The effect of different cell cycle phases on the accumulation of ara-CTP was studied using this concentration of ara-C in populations enriched for G_1, S, and G_2+M phases by centrifugal elutriation. The accu-

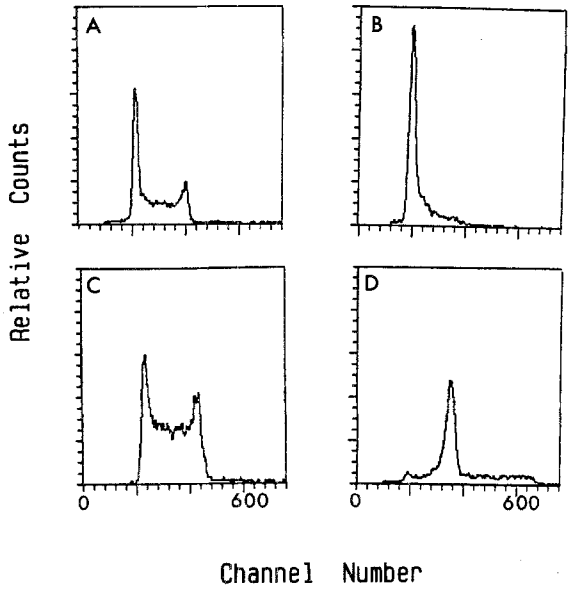

Fig. 1 A – D. Flow-cytometric analysis of exponentially growing or cell cycle-enriched populations of K562 cells. After centrifugal elutriation, cells were fixed, stained with propidium iodide, and analyzed using a FACS. **A** Exponentially growing cells (55% G_1, 35% S, and 10% G_2+M). **B – D** G_1 phase-enriched (81% G_1, 16% S, and 3% G_2+M), S phase-enriched (22% G_1, 60% S, and 18% G_2+M), and G_2+M phase-enriched (7% G_1, 12% S, and 81% G_2+M) subpopulations, respectively

mulation of ara-CTP was linear for up to 2 h in G_1 and S phase cells and for up to 4 h in G_2+M phase cells. The rate of ara-CTP accumulation during this linear phase was 25, 25, and 14 μM/h in the G_1, S, and G_2+M phases, respectively (Fig. 2), indicating that no apparent increase in the phosphorylation of ara-C occurred during the S phase. This observation contrasts with the results of previous studies, which described ara-CTP accumulation in picomoles of ara-CTP per given number of cells [42]. When calculated and expressed in this way, the rates of ara-CTP accumulation were linear for up to 4 h and amounted to 25, 40, and 30 pmol/10^6 cells in the G_1, S, and G_2+M phases, respectively. The maximal ara-CTP concentration was achieved at 4 h and was similar in all three phases of the cell cycle (Fig. 2).

Accumulation of F-ara-ATP

Because F-ara-A and ara-C are both phosphorylated by dCyd kinase, we also analyzed the accumulation of F-ara-ATP during different phases of the cell cycle. Cells were incubated with 300 μM F-ara-A, the concentration that saturates the rate of F-ara-ATP formation. F-ara-ATP synthesis was linear for up to 3 h in all three phases and the rates were 38, 47, and 33 μM/h in the G_1, S, and G_2+M phases, respectively (Fig. 3). At 3 h, the cells accumulated 100 – 140 μM F-ara-ATP.

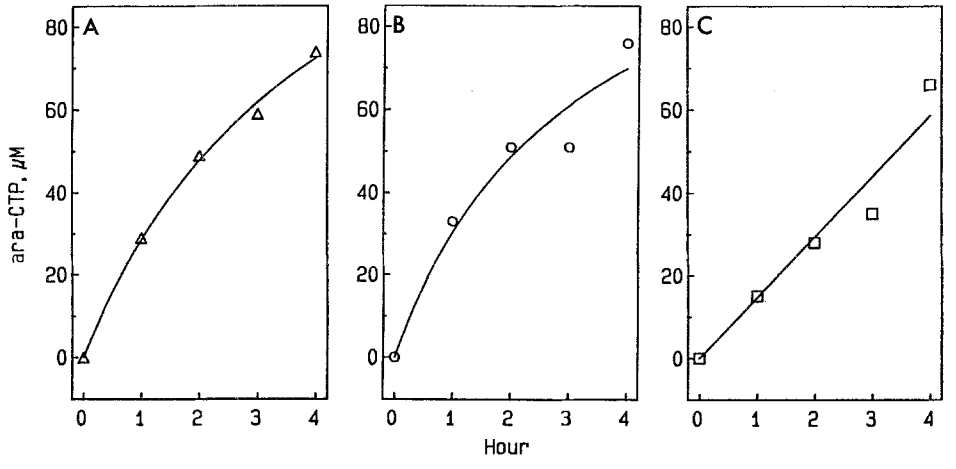

Fig. 2 A – C. Accumulation of ara-CTP by K562 cells in different phases of the cell cycle. **A** G_1 phase-enriched (65% G_1, 31% S, and 4% G_2+M), **B** S phase-enriched (17% G_1, 54% S, and 29% G_2+M), and **C** G_2+M phase-enriched (2% G_1, 25% S, and 73% G_2+M) subpopulations were incubated with 10 μM ara-C. At the indicated times, the intracellular concentration of ara-CTP was determined as described in Materials and methods. The curves for G_1 and S phase cells were plotted using a nonlinear regression-rectangular hyperbola to fit the data points, whereas the curve for G_2+M phase cells was plotted by linear regression

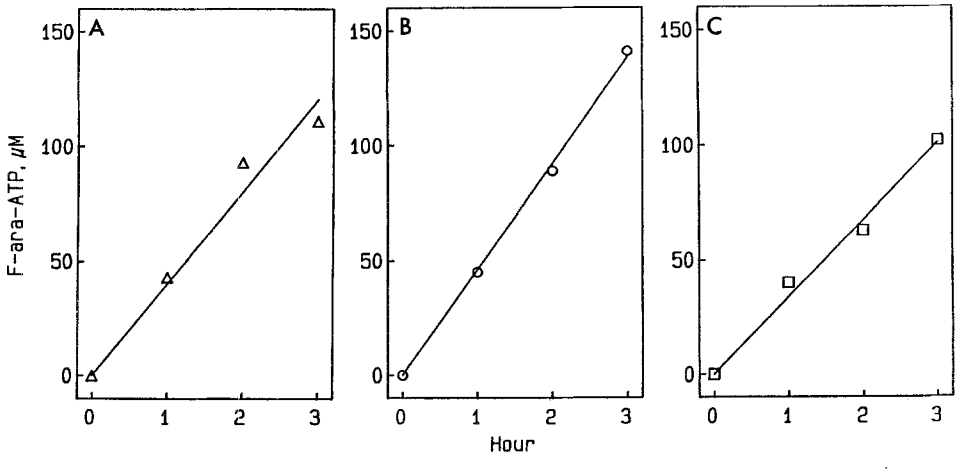

Fig. 3 A – C. Accumulation of F-ara-ATP by K562 cells in different phases of the cell cycle. **A** G_1 phase-enriched, **B** S phase-enriched, and **C** G_2+M phase-enriched populations were incubated with 300 μM F-ara-A. At the indicated times, the intracellular concentration of F-ara-ATP was determined as described in Materials and methods. The data points in all cell cycle phases were plotted by linear regression

Table 1. Effect of F-ara-A and ara-C on dNTPs

Cell cycle phase	Drug	dNTP (μM)			
		dCTP	dTTP	dATP	dGTP
G_1	No drug	7.0	11.5	3.6	4.5
	F-ara-A	1.7	4.8	2.5	1.3
	Ara-C	6.6	14.9	4.1	5.0
S	No drug	12.6	21.8	16.4	5.6
	F-ara-A	3.3	22.8	9.9	4.3
	Ara-C	13.9	20.1	19.3	4.9
$G_2 + M$	No drug	6.7	23.4	13.7	6.3
	F-ara-A	4.1	16.6	3.3	4.1
	Ara-C	ND	ND	ND	ND

K562 cells enriched for G_1 phase (81% G_1, 16% S, and 3% G_2+M), S phase (22% G_1, 60% S, and 18% G_2+M), and G_2+M phase (7% G_1, 12% S, and 81% G_2+M) subpopulations, respectively, were incubated with 300 μM F-ara-A or 10 μM ara-C for 2 h, and nucleotide pools were extracted. After removal of the ribonucleotides from the extract, deoxynucleotides were separated and quantitated by HPLC as described in Materials and methods. The data are representative of three separate experiments. ND, Not determined; dTTP, deoxythymidine triphosphate; dGTP, deoxyguanosine triphosphate

Elimination of ara-CTP and F-ara-ATP

To determine whether there were interphasic differences in the catabolism of these arabinosyl triphosphates, G_1, S, and G_2+M phase-enriched populations were incubated with 10 μM ara-C or 300 μM F-ara-A for 3 h, washed, and incubated in drug-free medium. Aliquots were taken at regular intervals for up to 3 and 5 h for ara-CTP and F-ara-ATP determinations, respectively. The $t_{1/2}$ values for ara-CTP elimination were 1.7, 1.5, and 1.9 h in the G_1, S, and G_2+M phases, respectively. The rates of F-ara-ATP degradation during these phases were 3.0, 4.5, and 3.0 h, respectively (data not shown).

Intracellular deoxynucleotide pools

Because dCyd kinase is regulated by endogenous dNTPs, interphasic variations in these pools would influence the phosphorylation of ara-C. To investigate the fluctuations, G_1, S, and G_2+M phase-enriched subpopulations were analyzed for deoxynucleotides. Cells were treated with ara-C or F-ara-A for 3 h, and the dNTPs were quantitated to determine the effect of ara-C and F-ara-A on these pools during different phases of the cell cycle. A comparison of dNTP pools in the G_1, S, and G_2+M phases of the cell cycle indicated an increase in the dNTP pools during the S and G_2+M phases (Table 1). The maximal increase occurred in the dATP pool followed by the deoxythymidine triphosphate (dTTP) pool. The dCTP pool showed a 1.8-fold increase during the S phase as compared with the G_1 or G_2+M phase, whereas levels of deoxyguanosine triphosphate (dGTP) changed only slightly. The increase in dNTP pools would appear even greater if the data were expressed as picomoles per number of cells because of the 1.5-fold and 2.2-fold increases in volume as cells progressed from the G_1 to the S phase and from the G_1 to the G_2+M phase, respectively. F-ara-A incubations caused an overall decline in the dNTP pools; dCTP (during all three phases) and dATP (during S and G_2+M phases) pools were affected the most. Similar incubations with ara-C, nucleotides of which do not inhibit ribonucleotide reduction, did not significantly change the dNTP pools in G_1 and S phase cells (Table 1). Ribonucleotide pools remained unaffected after treatment with either F-ara-A or ara-C (data not shown).

Table 1 contains two additional observations that appear paradoxical but are important to cell cycle regulation. First, during the G_1 phase, the dNTP pools decreased after treatment with F-ara-A. The triphosphate of F-ara-A is a known inhibitor of ribonucleotide reductase and thus lowers the dNTP levels. However, ribonucleotide reductase has been believed and shown to be inactive during the G_1 phase [27]. Also, the concentration of dATP, a global negative effector of the catalytic activity of ribonucleotide reductase [36], was the lowest of all dNTPs during the G_1 phase.

Effect of F-ara-A on ara-C metabolism and action

Since there was a variation in the dNTP pools during the cell cycle that could further be influenced by F-ara-A incubations as shown in Table 1, we studied the accumulation of ara-CTP after F-ara-A incubation during different phases of the cell cycle. The cells were incubated with 300 μM F-ara-A for 2 h so as to bring about the accumulation of F-ara-ATP and to affect the dNTP levels. They were then washed into drug-free medium and were incubated with ara-C. The accumulation of ara-CTP (shown in Fig. 4) was potentiated in all three phases of the cell cycle. The respective linear rates of ara-CTP accumulation for the first 2 h of incubation were 43, 37, and 26 μM/h in the G_1, S, and G_2+M phase-enriched populations, corresponding to a 1.7-, 1.5-, and 1.9-fold increase, respectively, over the values found for cells incubated with ara-C alone.

To determine whether the potentiation of ara-CTP accumulation by F-ara-A would result in greater cell kill, the cell populations enriched for different cell-cycle phases were incubated in the absence of drug, in the presence of ara-C or F-ara-A alone, or with ara-C following F-ara-A exposure and were then washed and cloned in agar. A 3-h period of incubation with F-ara-A decreased clonogenicity by 30%–40% in cell culture, whereas ara-C alone failed to affect cell viability significantly (Fig. 5). A sequential combination of F-ara-A and ara-C, however, exerted more than additive cytotoxicity (presented as dashed lines in Fig. 5). Furthermore, the cytotoxic effect of the combination schedule was maximal in S phase cells.

Activity of dCyd kinase

Dialyzed extracts of cells in different cell cycle phases were analyzed for ara-C phosphorylating activity for the investigation of cell cycle-specific fluctuations in the activity of dCyd kinase. The mean rates of ara-C phosphorylation were 12.4 ± 0.8, 24.5 ± 5.5, and 25.3 ± 1.6 pmol

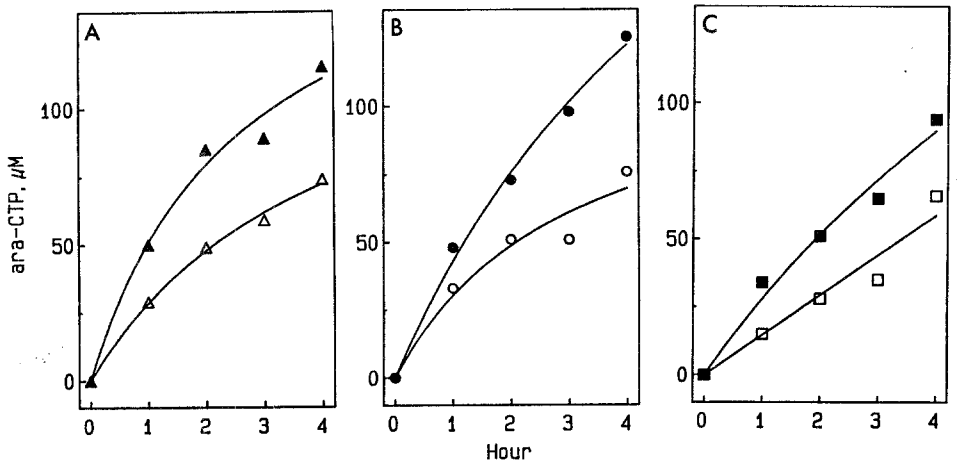

Fig. 4 A–C. Effect of F-ara-A incubation on the accumulation of ara-CTP by K562 cells in different phases of the cell cycle. **A** G_1 phase-enriched (65% G_1, 31% S, and 4% G_2+M), **B** S phase-enriched (17% G_1, 54% S, and 29% G_2+M), and **C** G_2+M phase-enriched (2% G_1, 25% S, and 73% G_2+M) subpopulations were incubated in the absence of drug (*open symbols*) or in the presence of 300 μM F-ara-A (*solid symbols*) for 2 h, washed into drug-free medium, and then incubated with 10 μM ara-C. The accumulation of ara-CTP was measured as described in Materials and methods. The curves were drawn using a nonlinear regression-rectangular hyperbola

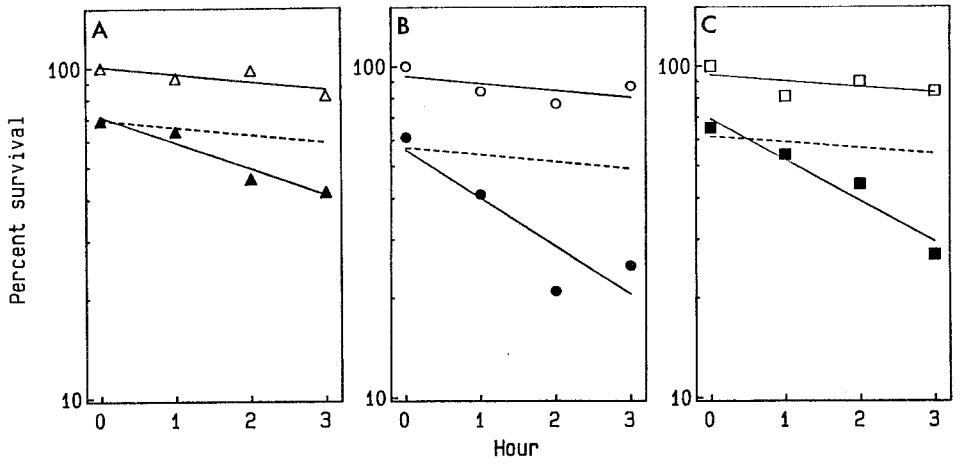

Fig. 5 A–C. Effect of F-ara-A and ara-C on the clonogenicity of K562 cells in different phases of the cell cycle. **A** G_1 phase-enriched (81% G_1, 16% S, and 3% G_2+M), **B** S phase-enriched (22% G_1, 60% S, and 18% G_2+M), and **C** G_2+M phase-enriched (7% G_1, 12% S, and 81% G_2+M) subpopulations were incubated in the absence drug (*open symbols*) or in the presence of 300 μM F-ara-A (*solid symbols*) for 2 h, washed, and then incubated with 10 μM ara-C. After appropriate dilution, cells were plated on agar and analyzed for clonogenicity after 10 days. The *dashed line* represents the expected survival (additive) for cells treated with a combination of F-ara-A and ara-C

min^{-1} mg $protein^{-1}$ in G_1, S, and G_2+M phase cells, respectively. The normalization of activity to the total amount of protein was necessary because the total protein content per cell was different during the various phases. Similar mechanical procedures to lyse the cells (10^6) resulted in the recovery of 103, 275, and 285 μg protein from the G_1, S, and G_2+M phase-enriched populations, respectively.

Discussion

The phosphorylation of ara-C in intact cells, presented as the rate of ara-CTP accumulation, is the result of the activity of dCyd kinase and the influence of deoxynucleotide levels on this activity. We postulated that the rate of intracellular accumulation of ara-CTP would be influenced by fluctuations in the activity of dCyd kinase and in the levels of dNTP pools during the cell cycle. We sought to analyze these two parameters to identify the cell cycle-specific regulation of ara-CTP accumulation.

The generation of ara-CTP is solely dependent on the salvage pathway, in which the activity of dCyd kinase is rate-limiting. Our results and previous observations demonstrate only 2- to 3-fold differences in dCyd kinase activity in populations enriched by centrifugal elutriation for different phases of the cell cycle [25, 37]. The activity of dCyd kinase in synchronized cultures of normal rat-kidney cells doubled during the S phase. Interestingly, the cells maintained in low-serum medium (presumably in the G_0 phase) showed dCyd kinase activity [43]. Similarly, Arner et al. [3] reported that the activity of dCyd kinase in resting lymphocytes showed only a 2-fold increase after phytohemagglutinin (PHA) stimulation. On the basis of their observations, they concluded that resting lymphocytes have a high capacity to phosphorylate dCyd. This finding is consistent with the in vivo situation, where in lymphocytes or blasts isolated from peripheral blood accumulate high levels of ara-CTP during intermediate- or high-dose ara-C therapy [34, 35]. The activity of thymidine kinase, in contrast to dCyd kinase, increases 10–100 times when cells pass from the G_1 to the S phase [4, 37].

Another important factor in the regulation of ara-CTP production is the fluctuation in dNTP pools during the cell cycle [25]. The activity of dCyd kinase is inhibited by dNTPs, particularly by dCTP [12, 29]. This effect of nucleotide pools on the activity of dCyd kinase in whole cells has complicated efforts to understand these interactions in cell-free extracts. For an analysis of this effect, dNTP pools were quantitated in G_1, S, and G_2+M phase-enriched subpopulations. A comparison of dNTP pools during different cell cycle phases indicated an increase in the dNTP pools during the S and G_2+M phases. The greatest increase oc-

curred in the dATP pool followed by the dTTP and dCTP pools. Thus, although the activity of dCyd kinase doubled in extracts of S phase cells, this may not be translated into a 2-fold increase in the activity of dCyd kinase in intact cells because the dCTP pool was increased proportionally in these cells. In intact cells, our results show that there was no increase in the rate of ara-CTP accumulation between G_1 and S phase-enriched cells, indicating that G_1 cells are capable of synthesizing ara-CTP as efficiently as cells in the S phase. Recently, Richel et al. [37] showed that the highest levels of ara-CTP measured during the cell cycle occurred in the G_1 phase. Similar results have been obtained in late G_1 phase cells [42]. dCTP accumulated from radioactive dCyd reached a 13-fold higher specific activity in murine G_1 cells than in S-phase cells [27]. This indicates that G_1 cells can efficiently phosphorylate dCyd or ara-C and accumulate their triphosphates. The ability of cells in the G_1 phase to phosphorylate ara-C is likely to be an important factor in the treatment of leukemia.

Cellular pharmacology studies of intermittent ara-C infusions have demonstrated that although the half-life of ara-C in plasma is short, that of ara-CTP in blasts is much longer; ara-CTP can be detected in leukemic cells at as late as 8–14 h after ara-C infusion [34, 35]. Although the percentage of cells in the S and G_2+M phase is low in this disease (less than 18% [42]), cells in the G_1 phase will accumulate ara-CTP. Due to the relatively long half-life of ara-CTP, when these cells enter the S phase, they may yet contain ara-CTP that may be incorporated into DNA, resulting in cytotoxicity. Therefore, although the cells are not in the S phase, they may be considered susceptible targets for nucleoside analogues as long as they are actively in cycle. As a corollary, consider that the fraction of cells in the quiescent stage (G_0) represent a critical factor regarding the sensitivity of ara-C. Although these cells most likely accumulate ara-CTP, they do not proceed through the cell cycle to the S phase, in which they would incorporate ara-CTP into their DNA [2], and they therefore represent a kinetically resistant population.

If resting and proliferating cells in different phases of the cell cycle accumulate ara-CTP, can accumulation be potentiated by preincubation with F-ara-A as demonstrated earlier in exponentially growing cells? To answer this question, we enriched K562 cells in different phases of the cell cycle by centrifugal elutriation. These subpopulations were incubated with F-ara-A, washed, and incubated with ara-C, and the accumulation of ara-CTP was then quantitated. The results presented in Fig. 4 indicate that the accumulation of ara-CTP was potentiated by 1.5–1.9 times in these subpopulations. Two main factors control the potentiation of ara-CTP accumulation by F-ara-ATP in exponentially growing K562 cells [14]. First, a direct effect of F-ara-ATP on the activity of dCyd kinase has been observed [14]. Second, the indirect effect mediated by F-ara-ATP inhibition of ribonucleotide reductase [41, 44], which reduces the dNTP pools, has been associated with an increase in ara-C phosphorylation [14, 25]. The inhibition of ribonucleotide reductase by F-ara-ATP and subsequent decrease in dNTP pools was observed during different phases of the cell cycle (Table 1). Subsequently, the F-ara-ATP-mediated reduction in the dNTP pool potentiates the activity of dCyd kinase by removing feedback inhibition, resulting in a higher rate of ara-C phosphorylation of G_1, S, and G_2+M phase cells.

The present study focused on cells in different phases of the cell cycle, but our previous work using freshly isolated lymphocytes obtained from patients with chronic lymphocytic leukemia demonstrated that these quiescent cells accumulate ara-CTP in vitro at a higher rate after F-ara-AMP infusion as compared with the lymphocytes obtained before therapy [18]. Peripheral blood lymphocytes contain relatively low levels of dNTPs [10]. Furthermore, the activity of ribonucleotide reductase in these cells is below the limit of detection [10]. If this is also true of lymphocytes from patients with chronic lymphocytic leukemia, the indirect effect of F-ara-ATP on the dNTP pool would be minimal. These findings highlight the likelihood that F-ara-ATP may directly affect the activity of dCyd kinase [20]. Additional evidence for an increase in dCyd kinase activity in cells containing F-ara-ATP was obtained by studying the metabolism of 2′,2′-difluorodeoxycytidine, a nucleoside analogue that also requires dCyd kinase for phosphorylation [16]. Lymphocytes isolated from patients with chronic lymphocytic leukemia loaded with F-ara-ATP accumulated 2′,2′-difluorodeoxycytidine triphosphate at twice the rate observed for untreated lymphocytes (unpublished results). This indicates that the effect of F-ara-ATP is not restricted to ara-C; rather, it potentiates the anabolism of nucleoside analogues that use dCyd kinase for phosphorylation [15].

Because ara-CTP accumulation can be potentiated in different phases of the cell cycle and in quiescent lymphocytes, the sequential combination of fludarabine (clinical F-ara-A) and ara-C would be expected to potentiate the rate of ara-CTP synthesis by leukemic lymphocytes and myeloblasts from patients with chronic lymphocytic leukemia [20] and acute myelogenous leukemia [19], respectively. This combination therapy may be synergistic for the metabolic enhancement of ara-CTP accumulation (Fig. 4) by increasing the phosphorylation of ara-C by dCyd kinase and for the antileukemic action of ara-C (Fig. 5). These hypotheses are currently being investigated in leukemia cells obtained from patients during treatment with the combination regimen [19, 20].

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