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Modulation of the cellular pharmacokinetics of ara-CTP in human leukemic blasts by dipyridamole*

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Summary. The effect of dipyridamole (DP) on the cellular retention of 1-β-D-arabinofuranosylcytosine (ara-C) and its metabolites was examined in leukemic blasts that had been isolated directly from bone marrow aspirates from patients afflicted with acute myeloid leukemia (AML). When AML cells were loaded for 2 h with 1 μM [^{3}H]-ara-C and then transferred to ara-C-free medium, total intracellular concentrations of radiolabel and [^{3}H]-ara-C 5′-triphosphate [^{3}H]-ara-C-CTP rapidly declined. After 8 h, total intracellular levels of tritium were 4.4 times higher if 10 μM was included in the washout medium; however, the majority of this intracellular radiolabel corresponded to [^{3}H]-uridine arabinoside ([^{3}H]-ara-U) and [^{3}H]-ara-C. DP significantly increased the mean $t_{1/2}$ for [^{3}H]-ara-CTP from 102 to 136 min ($P < 0.01$), but this effect was much less pronounced than that obtained for total tritium and the increase was quite variable (0–70%; median, 19%). The presence of DP in the washout medium also increased the incorporation of ara-C into DNA and the formation of ara-CDP-choline. The level of ara-CDP-choline continued to increase in both DP-containing and DP-free media for the first 4 h following drug removal and the formation of ara-CDP-choline continued during the first few hours in ara-C-free medium. At the end of the 8-h wash in DP-containing medium, the cellular concentration of ara-CDP-choline was equivalent to that found at the beginning of the washout period. Although statistically significant, the effect of DP on ara-CTP retention in AML blasts was much less pronounced than that previously observed in L5178Y leukemia. The former cells exhibited only 10% as many nucleoside transport carriers as did the L5178Y cells as measured by their capacity to bind [^{3}H]-nitrobenzylmercaptopurine riboside (NBMPR). The effect of DP in prolonging ara-CTP retention was proportional to the number of [^{3}H]-NBMPR binding sites. This suggests that in patients cells that exhibit extremely low transport capacity, most of the net catabolism occurs via deamination, and further inhibition of transport by DP in an effort to improve cellular retention of ara-C has little effect on ara-CTP catabolism.

Introduction

1-β-D-Arabinofuranosylcytosine (ara-C) is among the most effective drugs used for the treatment of acute myeloid leukemia [2]. Although the exact mechanism by which ara-C causes cell death remains unclear, its cytotoxicity has been shown to correlate most strongly with the extent of ara-C incorporation into DNA [4–7], which in turn correlates with the intracellular level of ara-C 5′-triphosphate (ara-CTP) [5]. Steady-state intracellular ara-CTP levels are determined by the balance between anabolism and catabolism; when cells that have been incubated with ara-C are transferred to ara-C-free medium or when an infusion of ara-C in patients is terminated, cellular concentrations of ara-CTP begin to decline. Prolonged retention of ara-CTP by human leukemia cells studied ex vivo as been shown to correlate with a positive response to treatment with both standard-dose [10] and high-dose ara-C [9]. Thus, any means of selectively enhancing ara-C retention by leukemia cells may enhance the drug's therapeutic potential.

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Abbreviations: ara-C, 1-β-D-Arabinofuranosylcytosine, cytosine arabinoside; ara-CMP, ara-C 5′-monophosphate; ara-CTP, ara-C 5′-triphosphate; ara-C-DNA, ara-C incorporated into DNA; DP, dipyridamole, Persantine, 2,2′,2″,2‴-[4,8-dipiperidinopyrimido(5,4-d)-pyrimidine-2,6-dinitrilo] tetraethanol; NBMPR, nitrobenzylmercaptopurine riboside

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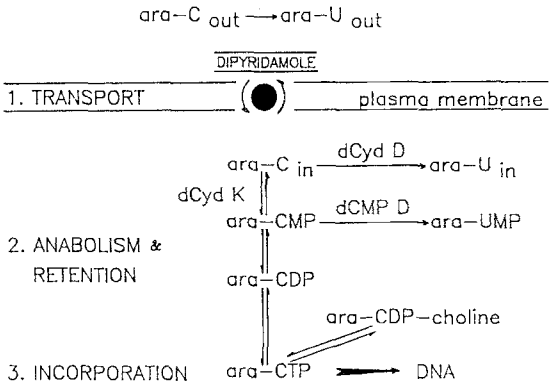

Fig. 1. Pathways of ara-C metabolism. *dCyd K*, deoxycytidine kinase; *dCyd D*, deoxycytidine deaminase; *dCMP D*, deoxycytidine monophosphate deaminase

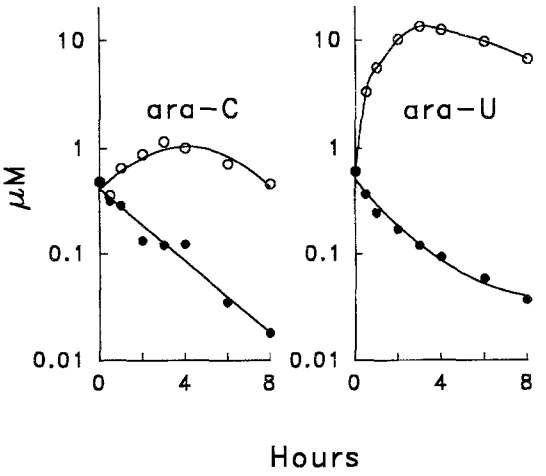

Fig. 2. Effect of DP on intracellular concentrations of ara-C and ara-U. Cells were incubated for 2 h with 1 μM [^{3}H]-ara-C and then washed and transferred to ara-C-free medium ± 10 μM DP. At intervals, cells were centrifuged through oil into 10% trichloroacetic acid, and the acid-soluble extracts were analyzed for [^{3}H]-ara-C (*left*) and [^{3}H]-ara-U (*right*). Data points represent the mean values for 8 populations of human leukemic blasts (control medium, ●; + 10 μM DP, ○). Within 1 h and throughout the 8-h time course, values were higher in the DP-treated cells from all 8 patients for both ara-C (P <0.05, Student's paired t-test) and ara-U (P <0.01)

The pathways for the net catabolism of ara-CTP are illustrated in Fig. 1. Ara-CTP may be successively dephosphorylated to ara-C 5′-monophosphate (ara-CMP) and then to ara-C. The ara-C resulting from ara-CTP catabolism may be rephosphorylated by dCyd kinase or may exit the cell (efflux) via the nucleoside carrier. Blocking efflux with a transport inhibitor would therefore increase the fraction of intracellular ara-C that is reused rather than lost. Our laboratory has demonstrated that sequential exposure to ara-C followed by dipyridamole (DP), a potent inhibitor of nucleoside transport [8, 15, 20], enhances the retention of ara-CTP in L5178Y cells [16, 19]. Similar effects of DP on ara-CTP retention by HL-60 and cultured human ovarian cells have been reported [1]. The present study using leukemia cells that were taken directly from patients represents an extension of these preclinical observations in an attempt to discover the potential clinical utility of the sequential use of dipyridamole following cellular loading with ara-C. A preliminary report of this work has appeared elsewhere [18].

Materials and methods

Unlabelled ara-C was obtained from the Upjohn Co. (Kalamazoo, Mich.) [^{3}H]-ara-C was obtained from Amersham International, Ltd. Other chemicals were supplied by Sigma Chemical Co. (St. Louis, Mo.). Cultured L5178Y cells were grown in Fischer's medium supplemented with 10% horse serum. Human leukemia cells were isolated from bone marrow aspirates obtained from patients exhibiting acute myeloid leukemia (AML) as previously described [11]. The number of specific binding sites for [^{3}H]-NBMPR were also determined as described elsewhere [11].

For uptake and efflux experiments, cells were suspended at 10^7/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cell suspension (5 ml) was incubated at 37° C in 25-cm^2 culture flasks in an incubator containing 5% CO_2. After incubation with 1 μM [^{3}H]-ara-C for 2 h, the cells were divided into two aliquots and centrifuged. The pellets were washed, resuspended in medium ± 10 μM DP, and incubated for an additional 8 h. At intervals, 0.5-ml samples were transferred to 1.5-ml microcentrifuge tubes containing 100 μl 10% trichloroacetic acid overlaid with 300 μl silicone oil (D = 1.03). After centrifugation at 12,500 g for 30 s, the medium and oil layers were aspirated and the trichloroacetic acid was extracted with 150 μl 0.6 M trioctylamine in Freon. The acid-insoluble fraction was washed twice with 100 μl 10% trichloroacetic acid and then dissolved in 100 μl dimethylsulfoxide for scintillation counting. Acid-insoluble tritium is described below as [^{3}H]-ara-C-DNA. Metabolites of [^{3}H]-ara-C were separated as previously described [19]. Briefly, 50-μl aliquots of the acid-soluble extract were loaded onto small (bed volume, 0.5 – 1 ml) columns of Sephadex A25 anion exchanger. [^{3}H]-ara-C and [^{3}H]-ara-U were eluted with H_2O. An aliquot of this fraction was applied to a similar small column of Dowex 50, which retained only the positively charged [^{3}H]-ara-C. Phosphorylated [^{3}H]-metabolites were successively eluted from the Sephadex A-25 columns with increasing concentrations of triethanolamine formate (pH 6.5) [17]. Half-life ($t_{1/2}$) values for ara-CTP retention were determined from the slopes of plots of ln[ara-CTP] vs time, where $t_{1/2}$ = ln(2)/slope. Semilogarithmic plots of all other metabolites were nonlinear.

Results

Leukemic blasts from the bone marrow of eight patients presenting with AML were incubated with 1 μl [^{3}H]-ara-C for 2 h and then resuspended in ara-C-free medium in the presence or absence of 10 μM DP. DP had a major effect on the rate of loss of total intracellular radiolabel. At 8 h after resuspension, the intracellular concentration of total tritium was 4.4 times greater if 10 μM DP was present in the medium; however, most of this intracellular radiolabel occurred in the form of nucleosides, especially ara-U, as shown in Fig. 2. This major accumulation of ara-U indicates that deamination plays a major role in the net catabolism of ara-CTP in these cells.

Retention of ara-CTP was also increased by DP, but the magnitude of the effect was much less dramatic than that observed for total tritium, and the increase varied among different AML cell populations. Table 1 summarizes the effects of DP on the $t_{1/2}$ value for ara-CTP in all eight AML cell populations. The increase in the half-life of ara-CTP following treatment with DP ranged from 0 to 70% and

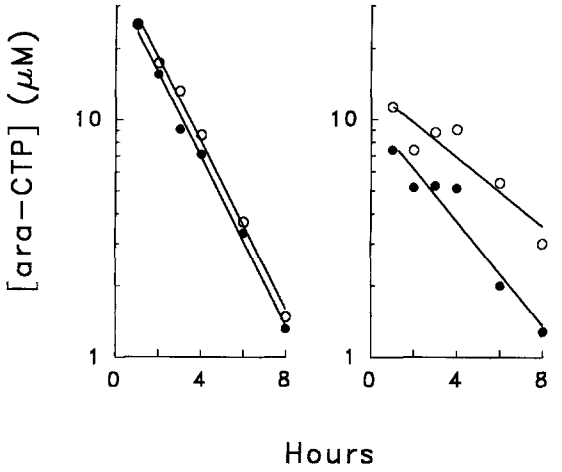

Fig. 3. Effect of DP on ara-CTP retention. In all, 8 populations of leukemia cells from patients were incubated as described in the legend to Fig. 2 and cell extracts were analyzed for ara-CTP. These graphs illustrate the populations that were least affected (*left*, $P < 0.2$) or most affected (*right*, $P < 0.05$) by the presence of DP in the efflux medium (control medium, ●; + 10 μM DP, ○)

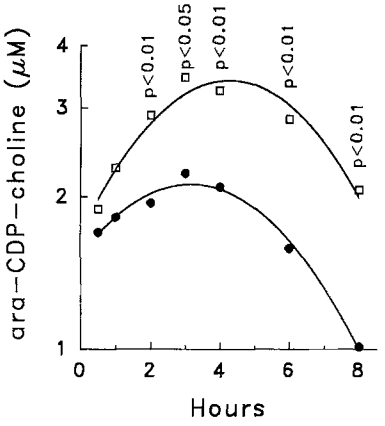

Fig. 4. Effect of DP on ara-CDP-choline levels. Patients' leukemia cells were incubated as described in Fig. 2 and cell extracts were analyzed for [^{3}H]-ara-CDP-choline. Data points represent the mean values ± SD for 8 populations of human cells (control medium, ●; + 10 μM DP, ○). The *p* values shown were determined using Student's paired *t*-test

Table 1. Effect of DP on the half-life of ara-CTP

Patient	$t_{1/2}$ (min)		Ratio	*P value*
	-DP	+DP		
ST	102	103	1	NS[a]
JM	53	57	1.07	NS
MA	76	81	1.07	NS
AW	116	136	1.17	NS
JC	109	131	1.2	$P < 0.1$
FV	57	74	1.3	$P < 0.1$
EW	164	258	1.57	$P < 0.1$
SF	143	243	1.7	$P < 0.05$
Mean ± SD	102 ± 37	136 ± 71	1.26 ± 23	$P < 0.01$[b]

Patients' cells were incubated with 1 μM [^{3}H]-ara-C for 2 h and then transferred to medium ± 10 μM DP; $t_{1/2}$ values were determined from plots of ln[ara-CTP] vs time. NS, Not significant

[a] Test for parallel lines

[b] Student's paired *t*-test

Table 2. Effect of DP on ara-C-DNA

Patient	ara-C-DNA(pmol/10^6 cells)		Ratio
	-DP	+DP	
ST	0.111	0.177	1.59
JM	0.131	0.082	0.63
MA	0.117	0.167	1.43
AW	0.049	0.067	1.37
JC	0.045	0.053	1.18
FV	0.138	0.204	1.48
EW	0.126	0.184	1.46
SF	0.024	0.062	2.58
Mean ± SD	0.093 ± 0.043	0.125 ± 0.06	1.46 ± 0.51
P-value (paired data) <0.05			

Patients' cells were incubated with 1 μM [^{3}H]-ara-C for 2 h and then transferred to medium ± 10 μM DP; [^{3}H]-ara-C-DNA was determined after 8 h incubation in ara-C-free medium

was statistically significant in only half of the samples. Fig. 3 compares the retention of ara-CTP in the two AML cell populations that were least or most affected by the inclusion of 10 μM DP in the efflux medium. The average increase in the $t_{1/2}$ value for ara-CTP was 26%. The effectiveness of DP did not correlate with the control value. DP also increased the incorporation of [^{3}H]-ara-C into DNA (Table 2) and ara-CDP-choline (Fig. 4). Although cellular levels of ara-CTP declined immediately following ara-C removal, the levels of ara-C-DNA and ara-CDP-choline continued to increase during the first few hours of the washout period. At 8 h after the resuspension of cells into ara-C-free medium, ara-D-DNA was 46% higher in the DP-treated cells. DP has a relatively greater effect on ara-CDP-choline levels, and the concentration found after 8 h in ara-C-free medium was higher than that measured at the beginning of the study.

Although statistically significant, the effects of DP on ara-CTP and ara-C-DNA in AML were quantitatively much less pronounced than those we previously reported for murine L5178Y leukemia cells [16, 19]. A major distinction between patient's leukemic cells and murine or cultured human leukemic cells is their capacity for nucleoside transport [11]. Table 3 contrasts the capacity for nucleoside transport as measured by the number of [^{3}H]-NBMPR binding sites exhibited by L5178Y cells vs 95 AML cell populations isolated directly from patients. As compared with the value found for L5178Y cells, the mean for patients cells was 10 times lower, although the range of values was broad and the standard deviation was high. Figure 5 reveals that the increase in the $t_{1/2}$ value for ara-CTP that was achieved by DP correlated with the number of NBMPR binding sites on the AML cells. This result indicates that DP would most effectively enhance ara-CTP retention in patients cells that exhibit a relatively high capacity for nucleoside transport.

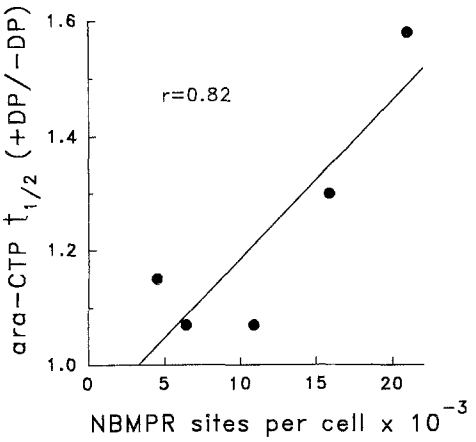

Fig. 5. Correlation of the effect of DP on the $t_{1/2}$ value for ara-CTP retention with the number of binding sites for [^{3}H]-NBMPR per cell. Data on [^{3}H]-NBMPR binding were not obtained for 3 of the 8 populations of AML leukemia cells used in the present study

Table 3. NBMPR sites in L5178Y vs patients' leukemic blasts

Cells	Sites/cell
L5178Y	74,000
AML	7,490±8,080 (mean ± SD)
	500–38,000 (range)
	(n = 95)

Discussion

The pathways for ara-CTP catabolism involve both dephosphorylation, which is reversible through rephosphorylation, and deamination, which is irreversible (Fig. 1). Intracellular ara-C resulting from the dephosphorylation of ara-CMP may exit the cell via the nucleoside carrier or may be phosphorylated again by deoxycytidine kinase. We have previously reported that blocking ara-C efflux with DP enhanced ara-C rephosphorylation and increased the $t_{1/2}$ value for ara-CTP catabolism. This was associated with an increase in the cytotoxicity of ara-C for L5178Y cells [16, 19]. These murine cells lack cytidine deaminase and exhibit the high capacity for nucleoside transport that is typical of experimental tumor cell lines [11]. Similar effects of DP on other cultured cell lines have been observed [1]. In contrast, Jamieson et al. [3] reported that DP had no perceivable effect on the $t_{1/2}$ value for ara-CTP in leukemic cells from patients.

The results we obtained using leukemic blasts that had been isolated from the bone marrow of eight patients presenting with AML show that DP increased the half-life of ara-CTP in most AML cell populations, resulting in a median increase of 26% (range 0–70%). This contrasts with our previous observation of a 250% increase in the $t_{1/2}$ value for ara-CTP in murine leukemia cells [19]. The lack of a major effect on the ara-CTP half-life in cells from patients despite a significant increase in total cellular tritium from [^{3}H]-ara-C indicates that deamination of either ara-C or ara-CMP clearly plays a major role in the net catabolism of ara-CTP in these cells. This was confirmed by the observed accumulation of high levels of ara-U in DP-treated cells. However, our results do not necessarily indicate that these cells exhibit a lower capacity for ara-CMP dephosphorylation than do L5178Y cells. Leukemia cells from patients are distinct from experimental murine and human cells in that the former show a greatly reduced capacity for nucleoside transport [11–14]. Deamination may play a relatively greater role in the *net* catabolism of ara-CTP in patients' cell because their inherently low transport capacity limits the loss of ara-C resulting from the dephosphorylation of ara-CMP. Inhibition of nucleoside transport by DP may be superfluous in patients' cells exhibiting an especially low transport capability; however, in those displaying a relatively high transport capacity, direct loss of ara-C across the cell membrane may be quantitatively more important, and blockade of this route would significantly extend the half-life of ara-CTP. The correlation of the effect of DP on ara-CTP retention with the number of [^{3}H]-NBMPR binding sites strongly supports this view.

Although the effect of DP on the ara-CTP half-life in cells from patients is less pronounced than that observed in experimental cell lines, this action might nevertheless be significant in a selected group of patients whose cells exhibit above-average transport capacity, since a 50% increase in the $t_{1/2}$ value would result in a >3-fold increase in ara-CTP at 8 h following the elimination of ara-C (based on an average control $t_{1/2}$ value of 1.6 h). Analysis of [^{3}H]-NBMPR binding in patients' cells prior to therapy would identify the patients who would be likely to benefit most from sequential treatment with ara-C followed by DP. However, in the absence of special selection procedures, the average effect of DP on ara-CTP retention is too slight to warrant a clinical trial.

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