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Enhanced retention of cytosine arabinoside and its metabolites and synergistic cytotoxicity by sequential treatment with dipyridamole in L5178Y leukemia*

Jin-Long Yang, J. Courtland White, and Robert L. Capizzi**

Cancer Center of Wake Forest University, Winston-Salem, NC 27103, USA

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Summary. Sequential treatment of murine leukemia L5178Y with cytosine arabinoside (ara-C) followed by dipyridamole (DP) resulted in synergistic cytotoxicity. Viability of cells exposed to 1 μ*M* ara-C for 4 h was 88% of control values, but if DP was included in the cloning medium, cell viability was reduced to only 30%. When cells exposed to 1 μ*M* ara-C were resuspended in ara-C-free medium containing 10 μ*M* DP, intracellular ara-C and its metabolites were retained for a significantly longer period than when cells were resuspended in drug-free medium. At 4 h after resuspension in ara-C-free medium, total intracellular [^{3}H] was 1.9 pmol/10^6 cells in control cells but amounted to 6.2 pmol/10^6 cells in DP-treated cells. Unchanged ara-C was 5.5-fold higher in the DP-treated cells. Presumably because of its effect on the concentration of intracellular ara-C, DP increased the half-life for ara-CTP from 97 to 250 min. Ara-CDP-choline declined with a half-life of 76 min on the transfer of cells to control medium, but levels of this metabolite remained constant or increased slightly in cells transferred to medium containing DP. After 4 h in ara-C-free medium with DP, [^{3}H]-ara-C incorporated into the acid-insoluble fraction was 140% of the level attained when cells were transferred to control medium. The increased levels of ara-C metabolites presumably represent the basis for the enhancement of ara-C cytotoxicity by sequential DP treatment.

Introduction

Although ara-C is one of the most effective drugs presently available for the treatment of acute myeloid leukemia, many patients either fail to achieve a complete remission or have remissions of short duration [4]. The cytotoxic action of ara-C on leukemic cells is dependent on its anabolism to ara-CTP and its incorporation into DNA [6–8, 10, 11]. Patient response to therapy and the remission duration have been correlated with the ability of leukemic cells to accumulate high levels of ara-CTP and, especially, to the cells' ability to retain ara-CTP following the removal of extracellular ara-C [16, 17, 21].

One means to increase the retention of ara-CTP would be to prevent the escape of ara-C resulting from ara-CTP dephosphorylation [19]. Trapped intracellular ara-C could then be rephosphorylated to ara-CTP. Ara-C is translocated across cell membranes via the facilitated diffusion system for nucleosides [13]. This carrier is sensitive to inhibition by a variety of compounds of diverse structure. Among the most potent of these (K_i = 20–50 n*M* [5]) and the most logical candidate for a clinical trial is dipyridamole (DP). There is considerable clinical experience in the use of this drug as an antithrombotic. Additionally, by blocking the uptake of nucleosides from serum, DP has been shown to potentiate the action of certain antimetabolites such as methotrexate (MTX) [12, 18] and acivicin [3], which inhibit the de novo synthesis of pyrimidine nucleotides. In this report we demonstrate that sequential administration of DP can increase the cytotoxicity of ara-C against L5178Y murine leukemia cells through increased retention of ara-CTP and increased incorporation of ara-C into DNA. A preliminary report has previously been presented [19] and confirmed [2].

Materials and methods

Drugs and chemicals. Ara-C was provided by the Upjohn Co. (Kalamazoo, Mich). Ara-U, DP, CDP-choline, dCMP, dCDP, and dCTP were obtained from the Sigma Chemical Co. (ST. Louis, Mo). [^{3}H]-Ara-C was

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** Leukemia Society of America Scholar.

Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine, cytosine arabinoside; ara-CTP, ara-C 5'triphosphate; ara-CDP-choline, ara-C diphosphocholine; ara-C-DNA, ara-C incorporated into DNA; DP, dipyridamole, Persantin, 2,2',2'',2'''-(4,8-dipiperidinopyrimido (5,4-d) pyrimidine-2,,6-dinitrilo) tetraethanol; PBS, phosphate-buffered saline

Offprint requests to: R. L. Capizzi

Table 1. Effect of DP on the cytotoxicity of ara-C in L5178Y cells

Group	Schedule[a]:			"Expected"[b]:		
	ara-C → wash with DP → clone with DP			% Viability	viability	P value[c]
	(*M*)	(*M*)	(*M*)			
1	–	–	–	100 ± 11.4[c]	–	>0.05
2	10^{-6}	–	–	88.3 ± 5.9	–	>0.05
3	–	10^{-5}	–	97.3 ± 14.6	–	>0.05
4	–	10^{-5}	2×10^{-6}	90.8 ± 14.5	–	>0.05
5	10^{-6}	10^{-5}	–	68.3 ± 7.0	85.9	<0.001
6	10^{-6}	10^{-5}	2×10^{-6}	30.4 ± 5.3	80.2	<0.001*

[a] Cells were incubated in control medium (group 1) or with 1 μM ara-C for 3.5 h (groups 2, 5, and 6). Then 10 μM DP was added to some cultures as indicated and the incubation was continued for an additional 0.5 h. The cells were then washed and cloned in soft agar without or with 2 μM DP for 7 days

[b] The "expected" viability from the combined treatment was the product of the viabilities resulting from ara-C alone and DP alone

[c] All data compared with those for group 1

* $P < 0.001$ when group 6 was compared with group 5

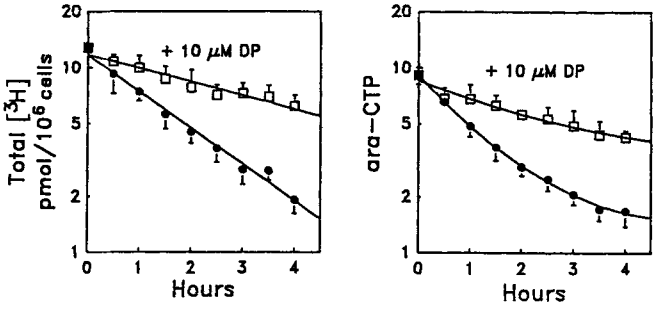

Fig. 1. Effect of DP in the retention of total [^{3}H] (*left*) or of [^{3}H]-ara-CTP (*right*) after treatment with [^{3}H]-ara-C. Cells were exposed to 1 μM [^{3}H]-ara-C for 60 min, then washed and resuspended in control medium (●) or medium containing 10 μM DP (□). Data points represent the average ± SD of 3 separate experiments

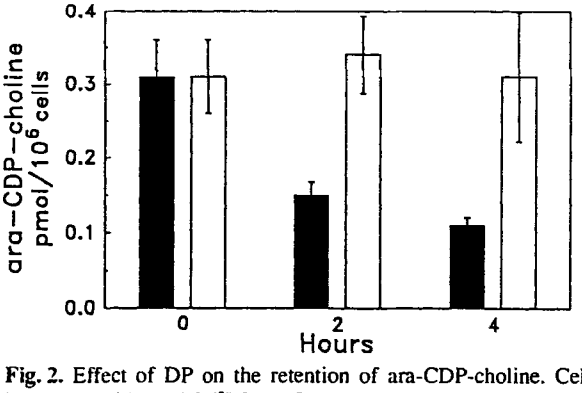

Fig. 2. Effect of DP on the retention of ara-CDP-choline. Cells were incubated with 1 μM [^{3}H]-ara-C, then transferred to control medium (*solid bars*) or medium containing 10 μM DP (*open bars*). Error bars represent the SD for 3 experiments

purchased from Amersham International Ltd. All drugs and chemicals except DP were dissolved in water or phosphate-buffered saline [0.14 *M* sodium chloride, 0.01 *M* potassium phosphate (pH 7.4), PBS]; DP was dissolved in dimethylsulfoxide (DMSO).

Cell culture and clonogenic assay. The methods of propagation of murine leukemia L5178Y in cell culture and the measurement of cell viability by clonogenic assay have previously been described [1]. All studies were carried out using cells in exponential growth.

Retention of ara-C and its metabolites by L5178Y cells. Cells were treated with either [^{3}H]-ara-C alone or sequential ara-C followed by DP. Following this treatment, the cells were washed three times with cold, drug-free medium and resuspended in medium warmed to 37°C. Cells previously treated with DP were resuspended in DP-containing medium. At intervals, 1-ml aliquots were transferred to 1.5-ml microfuge tubes containing 100 μl 10% trichloroacetic acid (TCA) overlaid with 300 μl silicone oil (density, 1.03 g/ml). After centrifugation and aspiration of the medium and oil, the TCA was extracted with 150 μl 0.6 *M* trioctylamine in freon. The acid-insoluble precipitate was washed with 100 μl 10% TCA, then dissolved in 100 μl DMSO for scintillation counting. A 10-μl aliquot of the acid-soluble extract was placed in scintillation cocktail for determination of total acid-soluble radioactivity. [^{3}H]-Ara-C metabolites were separated as previously described [20]. Briefly, a 50-μl aliquot was applied to a 1-ml mini-column of DEAE Sephadex-A25. The columns were eluted sequentially with H_2O and increasing concentrations of triethylammonium formate (pH 6.5).

Results

The effect of sequential DP on the cytotoxicity of ara-C against L5178Y murine leukemia cells was examined (Table 1). When cells were exposed to 1 μ*M* ara-C for 4 h prior to cloning in control medium, viability was 88.3% of control values. Exposure of cells to 10 μ*M* DP for 30 min prior to cloning in DP-free medium or medium containing 2 μ*M* DP also had a minimal cytotoxic effect; viability was 97% and 91%, respectively. Therefore, the "expected" viability for cells treated sequentially with ara-C followed with DP would be similar to that for ara-C alone (88%). In contrast, when cells were treated with ara-C for 3.5 h followed by 10 μ*M* DP for 30 min prior to cloning in medium containing 2 μ*M* DP, only 30% of cells remained viable. Continuous exposure to DP in the cloning medium was necessary to obtain maximal synergy, since viability declined to only 65% of control values when cells were exposed to sequential ara-C and DP as above but were cloned in DP-free medium.

The effect of 10 μ*M* DP on the retention of ara-C and its metabolites was examined in L5178Y cells that had been preincubated for 1 h with 1 μ*M* [^{3}H]-ara-C. The half-life for total intracellular radiolabel was increased 2.7-fold

Table 2. Effect of DP on ara-C incorporation into the acid-insoluble fraction of L5178Y cells

Post-ara-C	Ara-C level (pmol/10^7 cells): –DP	+DP	+DP/–DP
2 h	6.03 ± 1.13	7.78 ± 0.89	1.29
4 h	8.23 ± 1.15	11.5 ± 1.85	1.40*

Cells were treated with 1 μM [^{3}H]-ara-C for 60 min and then washed with fresh medium and resuspended in control medium or medium with 10 μM DP. The cells were collected at 2 and 4 h post-wash. Results represent the mean ± SD for 3 experiments
* $P < 0.05$

by DP, from a control value of 91 min (first-order rate constant = 0.0076 min^{-1}) to 244 min (K = 0.0028 min^{-1}) (Fig. 1). DP concentrations from 2 to 50 μM produced similar effects. Fractionation of radiolabel in cell extracts revealed that 4 h after resuspension in ara-C-free media, intracellular ara-C (as the nucleoside) was increased from 0.02 to 0.11 pmol/10^6 cells if DP was added to the efflux medium. Presumably as a result of its effect on intracellular ara-C, DP also extended the half-life of the principal metabolite, ara-CTP, to 250 ± 56 min as compared with 96 ± 6 min in control cells ($P < 0.001$, $n = 3$) (Fig. 1); however, the decline in ara-CTP was biphasic in control cells. The concentration of ara-CDP-choline remained essentially constant or rose slightly over a 4-h period in cells treated with DP, but ara-CDP-choline declined in untreated cells, with a half-life of 176 min (Fig. 2).

As a result of the increased retention of [^{3}H]-ara-CTP, DP enhanced the incorporation of [^{3}H]-ara-C into the acid-insoluble fraction (Table 2). The level was 140% of that in control cells after 4 h. Several laboratories have demonstrated that ara-C is incorporated exclusively into DNA [6, 10, 20].

Discussion

The duration of complete remissions following treatment of patients with acute myeloid leukemia with standard-dose ara-C protocols has been shown to correlate with the ability of patient leukemic blasts studied ex vivo to retain high levels of ara-CTP after the removal of extracellular drug [14, 16, 21]. Based on these observations, an important research goal is to find a means to increase ara-CTP retention in patient cells with high rates of catabolism.

The present studies demonstrate that using DP to block the transmembrane exit of ara-C (i.e., of the nucleoside) via the nucleoside carrier potentiated ara-C's cytotoxicity. The action of DP was rapidly reversible, since the effect was minimal if DP-treated cells were cloned in medium without DP. DP slowed the net loss of ara-CTP from L5178Y murine leukemia cells in vitro. It probably has no direct effect on the dephosphorylation of ara-CTP per se; however, by preventing the efflux of ara-C resulting from complete dephosphorylation, DP enables the rephosphorylation of this ara-C back to ara-CTP. This was manifested by an increased half-life for ara-CTP, which in turn resulted in an increase in ara-C incorporation into DNA, which has been shown to correlate with cytotoxicity [7, 10]. DP also prolonged the retention of ara-CDP-choline, presumably by the same mechanism. Ara-CTP is converted to ara-CDP-choline by phosphorylcholine synthetase [9]. We have previously shown that ara-CDP-choline can serve as a phosphorylcholine donor in phosphatidylcholine synthesis and that ara-C treatment increases [^{32}P]O_4 incorporation into phosphatidylcholine, resulting in changes in membrane structure [15]. Therefore, ara-CDP-choline may contribute to the cytotoxicity of ara-C, especially at high ara-C doses, and the maintenance of high levels of ara-CDP-choline by DP may be of therapeutic significance.

Studies are in progress in our laboratories to evaluate the effects of DP on the cellular pharmacokinetics of ara-C in blasts from patients with acute leukemia to discern the potential therapeutic utility of this pharmacologic strategy.

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