Cancer Chemother Pharmacol (1989) 24: 203–210

Cancer Chemotherapy and Pharmacology
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Pharmacodynamic and DNA methylation studies of high-dose 1-β-D-arabinofuranosyl cytosine before and after in vivo 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia*

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Summary. The primary development of clinical resistance to 1-β-D-arabinofuranosyl cytosine (ara-C) in leukemic blast cells is expressed as decreased cellular concentrations of its active anabolite. Correlations exist between the cellular concentrations of 1-β-D-arabinofuranosyl cytosine 5'-triphosphate (ara-CTP) in leukemic blast cells and inhibition of DNA synthetic capacity with the clinical response to high-dose cytosine arabinoside (HDara-C). 5-Azacytidine (5-Aza-C) and its congeners are potent DNA hypomethylating agents, an action closely associated with the reexpression of certain genes such as that for deoxycytidine kinase (dCk) in ara-C-resistant mouse and human leukemic cells. Reexpression of dCk could increase the cellular ara-CTP concentrations and the sensitivity to ara-C. A total of 17 pediatric patients with refractory acute lymphocytic leukemia (ALL) received a continuous infusion of 5-Aza-C at 150 mg/m^2 daily for 5 days after not responding to (13/17) or relapsing from (4/17) an HDara-C regimen (3 g/m^2 over 3 h, every 12 h, ×8 doses). Approximately 3 days after the end of the 5-Aza-C infusion, the HDara-C regimen was given again with the idea that the induced DNA hypomethylation in the leukemic cells may have increased the dCk activity and that a reversal of the tumor drug resistance to ara-C could have occurred. Deoxycytidine kinase (expressed as cellular ara-CTP concentrations) in untreated blasts, DNA synthetic capacity (DSC), and the percentage of DNA methylcytidine levels were determined before and after 5-Aza-C administration. Cellular ara-CTP was enhanced to varying degrees in 15 of 16 patients after 5-Aza-C treatment. The average cellular concentration of ara-CTP determined in vitro by the sensitivity test was 314±390 μ*M*, 2.3-fold higher than the average value before 5-Aza-C treatment. In 12 patients in whom the DNA methylation studies were completed before and after 5-Aza-C treatment, the average DNA hypomethylation level was 55.6%+15.8% of pretreatment values (n = 13; mean ± SD). DSC showed a profound decline in 2/9 evaluable patients who achieved a complete response (CR) after this regimen. The data suggest that treatment with a cytostatic but DNA-modulatory regimen of 5-Aza-C causes DNA hypomethylation in vivo, which is associated with dCk reexpression in the patients' leukemic blasts. The partial reversal of drug resistance to ara-C by 5-Aza-C yielded two CRs in this poor-prognosis, multiply relapsed patient population with refractory ALL. The data indicate that the 5-Aza-C plus HDara-C regimen may have a profound effect on controlling leukemia refractory to ara-C in patients.

Introduction

Cytosine arabinoside (ara-C) is a pyrimidine analog with proven antitumor activity against animal and human leukemias [3, 9, 19, 50]. An earlier study of high-dose ara-C (HDara-C) in pediatric patients showed that pharmacological parameters of the nucleoside pro-drug ara-C did not correlate with clinical response [3]. The inhibition of DNA synthetic capacity (DSC) and some parameters of intracellular 1-β-D-arabinofuranosyl cytosine 5'-triphosphate (ara-CTP) concentrations appeared to correlate with response [3]. Patients who did not respond to HDara-C and thus could be considered to be resistant to this regimen had relatively lower cellular concentrations of ara-CTP and higher DSC. Clinical trials of HDara-C in adult patients have shown that correlations exist between the cellular pharmacokinetics of ara-CTP in circulating blasts and the subsequent response to HDara-C therapy and that the determination of ara-CTP pharmacokinetics added significant prognostic information [18, 46].

The development of acquired resistance to ara-C by tumor cells is the major impediment to the treatment of pediatric cancers and leukemias [3, 4]. The causes of such resistance include (a) defective or nonexistent metabolism to the active metabolite, ara-CTP; (b) altered intracellular pools of nucleotides, specifically dCTP; (c) increased drug inactivation by deamination; and (d) altered DNA polymerase α [44]. Several approaches are now available to overcome drug resistance [11]. The HDara-C regimen may

* Supported by NIH-NCI research grant CA 38905, by a grant from The UpJohn Co., and by The T. J. Martell Foundation
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Abbreviations used: ara-C, 1-β-D-arabinofuranosyl cytosine; ara-CTP, 1-β-D-arabinofuranosyl cytosine 5'-triphosphate; 5-Aza-C, 5-Azacytidine; HDara-C, high-dose ara-C; mC, 5-methyl cytosine; PCA, perchloric acid; PBS, phosphate-buffered saline; dCk, deoxycytidine kinase; SAX, strong anion exchange; SCX, strong cation exchange; DSC, DNA synthetic capacity; $t_{1/2}$, elimination half-life; BM, bone marrow; PBC, peripheral blast cells; dThd, thymidine; ALL, acute lymphocytic leukemia

be able to increase intracellular ara-CTP pools sufficiently (in comparison with to conventional ara-C therapy) to overcome resistance due to the above mechanisms b, c, or d. Limited activation of ara-C (mechanism a) may be overcome by treatments that can alter the expression of genes such as that for deoxycytidine kinase (dCK) [4].

The enzymatically mediated posttranscriptional formation of 5-methylcytosine (5-mC) represents the only modified base known to occur in mammalian DNA [17, 32]. Although the biological function of DNA methylation is not clearly understood, methylation is considered to be one of several mechanisms involved in cell differentiation [47, 54] and regulation of gene transcription [1, 16, 48].

5-Azacytidine (5-Aza-C), a nitrogen bioisostere of cytidine, is an antimetabolite with antineoplastic, leukopenic, and mitogenic activity [12, 25, 34, 36, 43, 53]. 5-Aza-C is incorporated into DNA, inhibiting its synthesis and blocking cytosine methylation by noncompetitive inhibition of DNA methyltransferase, thus causing DNA hypomethylation [1, 15, 20, 42]. Studies using methylation-sensitive restriction endonucleases have shown that many genes are hypomethylated in their 5' flanking regions when they are being actively transcribed and hypermethylated in the nontranscribed chromatin conformation [31, 41, 43, 51]. Examples include the β-globin gene [51], the hypoxanthine phosphoribosyl transferase gene [41], and the dCk gene [1].

The therapeutic usefulness of ara-C is hindered by the frequent development of drug resistance in tumor cell populations after repeated exposure to this agent [24, 49]. The resistance is often associated with increased drug deamination in vivo [2] and a profound decrease in dCk activity in murine and human leukemia cell lines in vitro as well as in leukemic cells obtained from patients in relapse after treatment with HDara-C [3, 4]. Reexpression of dCk can be induced in mouse and human leukemic cells after pretreatment with 5-Aza-C or its congeners [1, 4]. These results formed the basis of an attractive hypothesis that drug resistance to ara-C could be reversed by the biochemical induction of DNA hypomethylation at the dCk gene locus. Therefore, we developed a clinical protocol to test whether pretreatment with 5-Aza-C can cause hypomethylation in leukemic cell DNA in vivo and possibly induce responses with HDara-C in pediatric patients with acute lymphocytic leukemia (ALL) refractory to ara-C.

Materials and methods

Chemicals and drugs. RPMI 1640 and heat-inactivated fetal calf serum were purchased from Irvine Scientific (Irvine, Calif). Ara-C was purchased from UpJohn Co. (Kalamazoo, Mich). Radioactive thymidine (methyl-[^{3}H]dThd) was obtained from Moravek Biochemicals, Inc. (Brea, Calif). Tritiated [5,6-^{3}H]ara-C was purchased from Moravek Biochemicals, Inc., and was purified by passage through a μC18 reverse-phase high-performance liquid chromatographic (HPLC) column equilibrated with 5% methanol in ammonium acetate (0.5 *M*, pH 6.50) to >99.2% radioisotopic purity. Specifically labeled [6-^{3}H]deoxycytidine for DNA methylation studies was purchased from Moravek Biochemicals, Inc. 5-Aza-C for the clinical studies was provided by NIH/NCI. Ficoll-Hypague with a specific density of 1.083 g/ml (Histopaque 1083) was purchased from Sigma Chemicals Co. (St. Louis, Mo). All other chemicals for extractions and HPLC assays were reagent or HPLC grade.

Patients. During the last 2 years, 17 pediatric patients (10 boys and 7 girls) with refractory ALL were entered into the study of 5-Aza-C and HDara-C. Of the 17 patients, 4 obtained remissions on HDara-C plus asparaginase induction and relapsed on an HDara-C maintenance regimen; 13/17 patients did not respond to HDara-C plus asparaginase induction [3]. All patients were considered to be clinically resistant to HDara-C at study entry. The age ranged from 4 to 19 years, with a median of 11 years and a mean of 12 years of age.

Only 9/17 patients were fully evaluable for response; 3 expired early in the treatment regimen, not completing it, and 5 patients expired after prolonged marrow aplasia and before documentation of bone marrow response or persistent disease could be done. All of the patients had previously received extensive chemotherapy and cranial irradiation. All patients were in bone marrow relapse at their entry into the study, which was approved by the Clinical Investigations Committee at Children's Hospital of Los Angeles. The patients were hospitalized and all underwent the surgical implantation of a multiport central venous catheter, through which intravenous fluids were infused and blood specimens were obtained.

The criterion for complete remission (CR) consisted of the complete disappearance of peripheral blast cells and a decrease in the bone marrow blasts to 0–5% of the total number of marrow cells, accompanied by the return of normal hematopoietic elements. Patients were followed as a minimum through day 28, when the diagnostic bone marrow aspirate was carried out.

For induction therapy, 150 mg/m^2 i.v. 5-Aza-C was given daily as a continuous infusion for 5 days. All patients were then treated with 3 g/m^2 i.v. HDara-C over 3 h and every 12 h beginning 2–5 days after the end of the 5-Aza-C infusion. The first two patients received eight doses of ara-C; due to the prolonged pancytopenia, subsequent patients received only four doses. Each cycle of ara-C was followed by *Escherichia coli* or *Erwinia* asparaginase (6,000 IU/m^2) given i.m. On day 8, another four-dose cycle of ara-C plus asparaginase was given if the lymphoblast count was >500/mm^3.

The responding patients were treated with maintenance therapy consisting of 2 days' infusion of 5-Aza-C plus three doses of HDara-C at 3 g/m^2 over 3 h and every 12 h followed by 6,000 IU/m^2 asparaginase. If the neutrophil count was >1,000/mm^3 this therapy was repeated every 4–6 weeks until relapse occurred. The nonresponding patients and relapsed patients were entered on alternative protocols or given supportive care at home.

Isolation of human lymphoblasts and determination of DNA synthesis. Prior to the 1st day of treatment with 5-Aza-C, heparinized peripheral blood samples (5 ml) were obtained from the patients' central venous catheter with a syringe containing preservative-free heparin, as previously reported [3, 46]. 5-Aza-C or HDara-C was given through a double-lumen central venous catheter. Bone marrow specimens were obtained by posterior iliac crest aspiration, placed in heparinized test tubes, which were then placed in an ice-bath and transported to the laboratory. The blood samples were handled as previously described [3].

To determine the DNA synthetic rate, duplicate samples of 1×10^7 cells were placed in sterile test tubes and incubated with 5 μCi [^{3}H]dThd in RPMI 1640 + 10% FCS

for 30 min at 37° C. At the end of the incubation, the cells were washed and extracted with perchloric acid (PCA, 0.4 *N*); the acid-insoluble fraction of the cells was washed again, transferred into a scintillation vial, and counted in a scintillation counter for [^{3}H]-DNA radioactivity. The results were calculated as the percentage of pretreatment values for each patient. The remaining lymphoblasts were extracted twice with 0.4 *N* PCA [3, 46]. The acid-soluble extracts were combined, chilled to 0° C in an ice bath, and neutralized with 10 *N* KOH. The $KClO_4$ formed was removed by centrifugation. The supernatant was brought to a volume of 2 ml with PBS and was either assayed immediately on an HPLC strong anion exchange (SAX-10) column or stored at −20° C until analyzed by HPLC as described below.

Sensitivity testing. Multiple sets of 2×10^7 blast cells freshly isolated from either peripheral blood or bone marrow specimens were placed in sterile test tubes with 2 ml RPMI 1640 growth medium enriched with 10% heat-inactivated fetal calf serum and 1% HEPES buffer solution. Purified [5,6-^{3}H]ara-C premixed with cold drug was added to the cell suspension to obtain 200 μ*M* and 1 m*M* concentrations. These concentrations were selected as the average peak plasma ara-C levels achieved in pediatric patients after an HDara-C regimen [3]. The cells were incubated for 1 h in a humidified incubator in the presence of 5% CO_2. After the incubation, the cells were placed in an ice bath to stop the reaction and were extracted with PCA [1, 3, 46]. The neutralized PCA extracts were assayed on SAX-10 HPLC columns for nucleotides and nucleotide analogs.

Determination of DNA methylation levels. The leukemic blast cells were incubated with [6-^{3}H]deoxycytidine for 24 h and processed as previously described [1]. The samples were assayed on strong cation exchange resin (SCX-10) HPLC columns and the data were calculated as previously described [1].

HPLC determination of nucleotides and ara-CTP in cellular acid extracts. The ara-CTP was separated from other cellular nucleotides by a modified HPLC C procedure and quantitated by either UV absorbance and/or the specific activity of the radioactive ara-C as previously described [2, 3, 46].

Determination of DSC in blast cells. The DSC was determined by the relative levels of thymidine incorporated into cellular DNA before and after treatment with the drug, as previously described [3].

Results

Pharmacodynamic studies

Pharmacodynamic studies were carried out in 15 of 17 patients with refractory ALL before and after 5-Aza-C treatment, all of whom had a high peripheral blast count. The cellular concentrations of ara-CTP in the untreated leukemic blasts was determined after in vitro incubations with ara-C as described in *Materials and methods.* The ara-CTP cellular concentrations ranged from <5.0 to 408 μ*M* before 5-Aza-C treatment, with an average value of 123 ± 117 μ*M* (mean ± SD; $n = 18$) and a median of 87 μ*M* (Table 1).

Table 1. Cellular concentrations of ara-CTP in leukemic cells from patients responding and not responding to the 5-Aza-C + HDara-C regimen

Patient	[ara-CTP][a] before 5-Aza-C (μ*M*)	[ara-CTP][a] after 5-Aza-C (μ*M*)	Increased expression of dCk	[ara-CTP][b] peak, 3 h (μ*M*)	[ara-CTP][b] through, 24 h (μ*M*)	Outcome
Responders:						
1. R. G.	84.0	229.0	2.96-fold	–	573.4	CR
2. L. L. (BM)[c]	78.0	208.0	2.67-fold	–	359.4	CR
L. L. (PBC)[d]	42.0	216.0	5.14-fold	–	–	
3. K. W.	90.0	108.0	1.20-fold	–	–	PR
Nonresponders:						
1. D. K.	329.6	1,187.0	3.60-fold	1,259.5	864.0	NR
2. R. G.	97.4	171.0	1.76-fold	460.0	120.5	NR
3. K. K.	86.8	94.3	1.10-fold	191.6	65.6	NR
4. S. S.	223.4	1,365.0	6.11-fold	–	1,025.0	NR
5. L. M.	92.0	142.0	1.54-fold	–	182.0	NR
6. O. M.	<5.0	30.3	6.00-fold	–	20.0	NR
7. H. K.	70.0	78.0	1.12-fold	–	99.0	NR
8. E. H.	29.0	168.0	5.79-fold	471.4	60.3	NR
9. U. P. (BM)[c]	408.0	573.4	1.41-fold	–	687.0	NR
U. P. (PBC)[d]	13.6	14.0	1.02-fold	–	–	NR
10. L. R.	297.0	–	–	–	–	NE
11. J. R.	168.0	143.0	0.85-fold	–	186.0	NR
12. D. W.	86.0	147.0	1.72-fold	–	389.0	NR
13. D. P.	10.0	465.0	46.50-fold[e]	–	579.3	NR
	$X_{18} = 122.7 \pm 116.8$	$X_{17} = 314.1 \pm 390.0$	$X_{16} = 2.75\text{-fold} \pm 1.95$		$X_{14} = 372.2 \pm 325.3$	

[a] Ara-CTP concentration data from the sensitivity test of blasts in vitro, 1 m*M* ara-C for 1 h
[b] Ara-CTP concentration data after in vivo HDara-C regimen
[c] Data in bone marrow blasts
[d] Data in perpheral blast cells
[e] Statistical outlier not used for the estimation of the mean

Approximately 3 days (range, 2–5 days) after the 5-day continuous infusion of 5-Aza-C, the patients' leukemic cells were tested in vitro under identical conditions; the average cellular ara-CTP concentration was 314±390 μ*M* (mean ±SD; $n = 17$) and the median, 171 μ*M*. The value of the mean was 2.8-fold higher than before 5-Aza-C treatment. At the same time, 3 days after the 5-Aza-C infusion ended, all but one of the patients received intermittent HDara-C at 3 g/m^2 over 3 h every 12 h for four or eight doses. The trough cellular ara-CTP concentrations at 24 h, just prior to the third dose of ara-C, averaged 372±325 μ*M* (mean ±SD; $n = 14$), with a median of 272 μ*M*. This average value was higher than that obtained from the sensitivity testing. The dCk specific activity of leukemic blasts from three patients who had high peripheral blast counts was increased in an identical manner with the cellular ara-CTP concentrations after the 5-Aza-C treatment.

In patient R. G. (Table 1), the partially purified dCk activity increased by 2.05-fold before and after 5-Aza-C treatment, and that of patient L. L. increased by 2.45-fold; both of these patients achieved a CR. The dCk activity in patient E. H. increased by 1.65-fold, and the K_m of ara-C declined from 35.9 to 9.2 μ*M*. Apparently, this increase was not sufficient for response.

Figure 1 depicts the cellular ara-CTP concentrations in blast cells from one patient before and 3 and 9 days after 5-Aza-C treatment. It is apparent that the cellular concentrations of ara-CTP and, presumably, dCk reexpression declined by day 9 compared with the results from day 3 after the 5-Aza-C treatment. Figure 2 shows the cellular ara-CTP concentrations in peripheral blast cells from the same patient after the HDara-C regimen. The intracellular peak ara-CTP concentration reached 1.2 m*M*, which compares favorably with the value obtained from the sensitivity testing.

A linear relationship was obtained between the concentrations of ara-CTP from the sensitivity testing and the in vivo through cellular ara-CTP concentrations in blasts after the HDara-C regimen was given to the patients (Fig. 3). On average, the in vitro sensitivity testing underestimated the concentrations obtained after in vivo treatment with HDara-C, but the difference was not statistically significant.

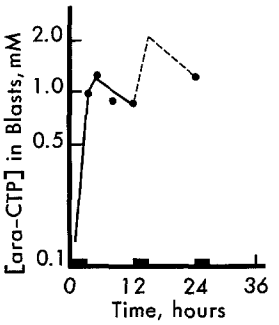

Fig. 2. Kinetics of cellular ara-CTP concentrations in circulating blast cells from the same patient as in Fig. 1 after two doses of HDara-C administration, 9 days after the 5-Aza-C treatment. The graph is the best-fit line for a one-compartment open model. *Dashed line*, predicted increase and elimination of ara-CTP concentrations after the second dose of ara-C; ●, determined concentrations of ara-CTP at 3, 5, 8, 12 and 24 h. The in vivo ara-CTP peak was 1,259 μ*M*, which compares favorably with the in vitro estimated peak after 5-Aza-C treatment; the elimination half-life of ara-CTP was 13.5 h

Studies on the inhibition of the DSC of blast cells

DNA synthesis was determined in vitro after 1 h incubation with 1 m*M* ara-C in leukemic blasts obtained before and after 5-Aza-C treatment and at 24 h after the HDara-C regimen was started. The DSC of blasts before 5-Aza-C treatment averaged 46.2%±40.6% of the respective controls (mean ±SD; $n = 8$), with a median of 27% (Table 2). The DSC values in the nonresponding patients 24 h after the HDara-C treatment began averaged 89.6%±71.3% (mean ±SD; $n = 4$), with a median of 40%, in comparision with the values in untreated controls. The DSC value in two patients who achieved a CR was <7% of that of their respective controls.

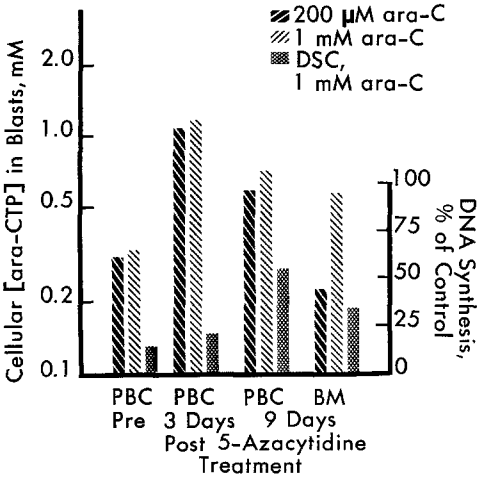

Fig. 1. Cellular ara-CTP concentrations in peripheral (*PBC*) and bone marrow (*BM*) blast cells from a patient after 1 h incubations with 0.2 and 1 m*M* ara-C. The sensitivity tests were conducted before, 3 days after, and 9 days 5-Aza-C in vivo treatment

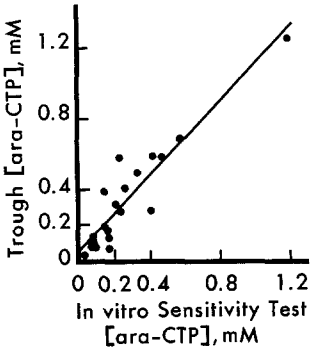

Fig. 3. Linear relationship between the through cellular concentration of ara-CTP determined in vivo in the patients' blast cells and the cellular ara-CTP concentration determined in vitro in the same patients after the sensitivity test as described in *Materials and methods*. The correlation coefficient was 0.86 ($n = 20$), and the equation describing the best-fit line is $y = 51.22 + 1.066x$

Table 2. DNA synthetic capacity studies in patient leukemic cells before and after 5-Aza-C followed by HDara-C

Patient	Pretreatment control	Pretreatment +1 m*M* ara-C[a] (% of control)	Posttreatment control	Posttreatment +1 m*M* ara-C[a] (% of control)	24 h post HDara-C[b] (% of control)	Outcome
1. R. G.	100%	–	57.1%	–	6.9%	CR
2. L. L.	100%	26.4%	100%	4.8%	3.8%	CR
3. K. W.	100%	72.5%	–	–	–	PR
4. U. P.	100%	25.8%	100%	51.0%	–	NR
5. J. R.	–	–	100%	314.6%	–	NR
6. D. W.	100%	45.0%	–	–	–	NR
7. E. H.	100%	28.2%	100%	25.0%	31.6%	NR
8. L. M.	100%	136.0%	100%	52.6%	112.0%	NR
9. D. K.	100%	13.7%	100%	21.6%	34.0%	NR
10. O. M.	100%	21.8%	100%	28.0%	180.7%	NR
		$X_8 = 46.2\%$[c] $\pm 40.6\%$[c]		$X_6 = 82.1\%$[d] $\pm 115.0\%$[d]	$X_4 = 89.6\%$[d] $\pm 71.3\%$[d]	

[a] 1 m*M* ara-C × 1 h incubation as described in *Materials and methods*
[b] DSC values at 24 h after the HDara-C regimen was begun and before the 3rd dose of ara-C, corresponding to trough ara-CTP cellular concentrations
[c] Mean ± SD of all patient data
[d] Mean ± SD of the nonresponding patient 4–10 data

Table 3. DNA methylation studies in patient leukemic cells before and after 5-Aza-C treatment

Patient	Percentage of 5-mC in DNA before 5-Aza-C (control methylation)[a]	Percentage of 5-mC in DNA after 5-Aza-C	DNA (% hypomethylation in leukemic cells)	Outcome
1. R. G.	3.37% ± 0.4%	1.20% ± 0.2%	61.3%	CR
2. L. L.	3.26% ± 0.3%	1.29% ± 0.4%	60.3%	CR
3. K. W.	3.22% ± 0.3%	1.16% ± 0.3%	63.9%	PR
4. U. P.	4.49% ± 0.4%	1.17% ± 0.6%	73.9%	NR
5. J. R.	3.29% ± 0.02%	1.57% ± 0.2%	52.3%	NR
6. D. W. (BM)	3.79% ± 0.2%	1.26% ± 0.1%	66.8%	NR
D. W. (PBC)	3.63% ± 0.2%	2.61% ± 0.4%	28.1%	NR
7. E. H.	3.54% ± 0.2%	2.44% ± 0.1%	31.1%	NR
8. L. M.	4.04% ± 0.3%	1.59% ± 0.1%	60.4%	NR
9. O. M.	3.94% ± 0.6%	2.36% ± 0.3%	40.0%	NR
10. D. P.	3.96% ± 0.2%	2.23% ± 0.5%	43.2%	NR
11. L. R.	3.01% ± 0.8%	0.59% ± 0.5%	80.4%	NE
12. K. K.	2.47%	0.97%	60.8%	NE
			$X_{13} = 55.6\% \pm 15.8\%$	

[a] Mean ± SD ($n = 4$)

DNA methylation studies

The methylation levels of total genomic DNA in blast cells from 12 patients were measured before and after 5-Aza-C in vivo treatment (Table 3). In all patients tested there was a significant decline in DNA methylation levels, which ranged from 28.1% to 80.4% hypomethylation in comparision with values obtained in the respective controls, with a mean of 55.6% ± 15.8% (mean ± SD; $n = 13$). There were no differences in the methylation levels between responding and nonresponding patients. The methylation levels 24 h after the HDara-C regimen was started were higher than but not significantly different from those measured before ara-C treatment.

Discussion

The problem of acquired drug resistance by tumor cells has been called the most serious problem in current cancer chemotherapy [1, 4]. Studies in classic genetics suggest that the evolution of resistant clonal populations of cells during tumor progression would occur by mutational mechanisms. Recent advances in our understanding of the control of eukaryotic gene expression, however, suggest that epigenetic mechanisms may also play an important role in the diversification of tumor cell populations during tumor progression [21, 28, 33]. One of these epigenetic phenomena is the loss of stable patterns of cell-specific DNA methylation [22]. Methylation, along with Z-DNA-binding proteins, may enhance the stabilization of the unstable, nontranscriptional Z-DNA form from the B_1 configuration [8, 23, 39]. Evidence supporting a direct relationship between DNA hypomethylation and active gene transcription has come from studies using hypomethylating agents such as 5-Aza-C and its congeners [1, 31, 40, 45]. Furthermore, the methylation of d(GpC) sequences in the 5'-flanking region of hemoglobin [10] or adenovirus [35] genes is ade-

quate to inactivate gene expression in transfection experiments.

Cells derived from several different species respond to 5-Aza-C treatment by the expression of previously suppressed genetic information, which may lead to differentiation [29]. In particular, considerable excitement was generated by the finding that this drug could induce the expression of genes located on inactive X chromosomes in a variety of hybrid systems [14, 30]. Studies using methylation-sensitive restriction endonucleases have shown that many genes are hypomethylated in their 5'-flanking regions when they are being actively transcribed and they are hypermethylated in the nontranscribed chromatin conformation [31, 41, 43, 51].

It is very important to establish whether the remarkable effects of 5-Aza-C or its congeners on cell differentiation are mediated through the perturbation of methylation patterns within the genomic DNA. Some evidence suggesting that 5-Aza-C acts through a DNA modulation mechanism includes the following: biologically active concentrations of 5-Aza-C inhibit DNA methylation [1, 14, 29, 30]; the differentiation effect is specific for the 5-position of the cytosine ring [30]; the change in expression is heritable after many generations in the absence of further 5-Aza-C treatment or similar selective conditions [17, 30]; hypomethylation is induced at specific sites within the expressed genes [13, 14, 38]; 5-Aza-C reactivates DNA function in DNA-mediated transfection of hypoxanthine hosphoribosyl transferase and the activation frequencies [17, 26, 37, 52].

In a previous study we reported that pediatric patients with ALL in relapse showed tremendous heterogeneity in cellular ara-CTP concentrations after in vivo treatment with HDara-C [3]. The cellular ara-CTP concentrations in some of these patients showed a gradual decline upon repeated dosing with the drug regimen and the patients were considered to be clinically resistant to the therapy. Similar heterogeneity in the cellular ara-CTP concentrations are seen in both in vivo and in vitro results. In both this and our previous studies, the cellular ara-CTP concentrations did not correlate with the clinical response to HDara-C [3]; this has been documented by other investigators as well [18, 27, 46].

In the current study, in vivo 5-Aza-C treatment had a cytostatic effect, and only 1 of 17 patients achieved a partial remission after 5-Aza-C alone. Nevertheless, this regimen of 5-Aza-C was sufficient to induce DNA hypomethylation in the leukemic cells of all 12 patients studied at a level similar to those determined in CEM human leukemic cells after in vitro treatment [1]. The cellular concentrations of ara-CTP in the patients' leukemic cells showed on average a nearly 3-fold increase after 5-Aza-C treatment in comparision with the pretreatment values, indicating that the induced DNA hypomethylation could cause the reexpression of dCk in leukemic cells.

The time lag of approximately 3 days in giving the HDara-C regimen was derived from our earlier studies showing that the maximal DNA hypomethylation occurred 48 h after treatment with 5-Aza-C [1] as well as from one patient in whom the cellular ara-CTP in the blasts was determined on days 3 and 9 after 5-Aza-C treatment. This patient's blast cells showed the highest cellular concentrations of ara-CTP 3 days after Aza-C treatment [4, 6]. The trough cellular ara-CTP concentrations 24 h after the HDara-C treatment were on average higher than the values determined by the sensitivity assay in vitro, although the two parameters were linearly related (Fig. 3). This suggested that the minimal concentration of the active anabolite ara-CTP to which the leukemic cells were exposed in vivo was higher after than before 5-Aza-C treatment.

The specific activity of purified dCk showed a similar increase, the cellular ara-CTP concentrations ranging from 1.65- to 2.45-fold in three patients, suggesting that hypomethylation of DNA is associated with the increased expression of dCk in leukemic cells in patients after 5-Aza-C treatment [5]. This increase in cellular ara-CTP concentrations may have been responsible for the profound decline of DNA synthesis in the two patients who responded to the combination chemotherapy regimen.

The level of hypomethylation of total genomic DNA was similar in responding and nonresponding patients, thus not permitting an association between this biochemical parameter and clinical efficacy. Furthermore, DSC did not decline in most patients, although the cellular ara-CTP concentrations increased. The latter observations suggest that some other mode of resistance to ara-C may be involved in many of these patients, possibly including elevations in dCTP pools and alterations of DNA polymerase α to such an extent that it is not inhibited by ara-CTP.

Since considerable toxicity was seen in these patients, our efforts were concentrated or reducing the toxicity to the host after the combination regimen. We have investigated a biochemically optimal regimen of a loading bolus plus continuous infusion of ara-C, with manageable toxicity [7]. An identical regimen of ara-C is currently given after 5-Aza-C to reduce the toxicity to the host. Two patients who had not responded to the HDara-C regimen alone clinically achieved a CR after the modulatory regimen of 5-Aza-C plus HDara-C [6]. Further studies are under way to investigate whether the degree of hypomethylation of the dCk gene is related to higher ara-CTP cellular concentrations, inhibition of DNA synthesis, and clinical response.

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Received 20 June 1988/Accepted 8 February 1989