Anti-Cancer Drugs 1995, **6**, pp. 303–310

Biochemical pharmacology and DNA methylation studies of arabinosyl 5-azacytidine and 5,6-dihydro-5-azacytidine in two human leukemia cell lines PER-145 and PER-163

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1-β-D-arabinofuranosyl-5-azacytosine (ara-AC) and 5,6-dihydro-5-azacytidine (DHAC) are two new antitumor agents under clinical investigations, which exhibit the chemical similarities found in the tumoricidal drug cytosine arabinoside (ara-C) and the nitrogen substitution in the 5 position of the pyrimidine ring found in 5-azacytidine (5-aza-C). The cellular anabolism of ara-AC and DHAC and their effect on DNA methylation have been examined in two new human leukemia cell lines, which are sensitive (PER-145) and resistant (PER-163) to ara-C. The triphos-phate anabolite of ara-AC, ara-ACTP, was the major cellular anabolite in the cellular extracts of the PER-145 cells, reaching a cellular saturation concentration of 64.1±3.2 μM using 25 μM of the drug. Only trace levels of ara-ACTP were detected in the PER-163 cell line, which lacks deoxycytidine kinase, after exposure to a similar concentration. Notably, after 1 mM, the ara-ACTP concentration averaged 12±3 μM. DHAC was anabolized by both cell lines to a similar degree but required much higher nucleoside concentrations (100 μM or higher) to achieve similar cellular concentrations of its triphosphate, DHACTP. Although the deoxy-derivative, DHAdCTP, was detected in both cell lines, it was detected at 1–2 $\log_{10}$ lower concentrations than DHACTP. DNA methylation studies showed that DHAC had a profound effect in inducing DNA hypomethylation in both cell lines, with nadir values of 27.3 and 29.2% of control. Ara-AC induced 45% DNA hypomethylation in PER-145 cells, but did not alter the DNA methylation pattern in PER-163 cells, except when they were exposed to 1 mM of the drug for 24 h. These results could be explained by the differential biochemical activation of these drugs in the human leukemia cell lines.

Key words: Cell lines, DNA methylation, human leukemia, nucleoside analogs.

Supported by a research grant, CA 38905 from the National Institute of Health, NCI, the Neil Bogart Memorial Laboratories by the TJ Martell Foundation for Cancer, Leukemia and AIDS Research, and The Children's Leukemia and Cancer Research Foundation, Western Australia.

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Introduction

5-Azacytidine (5-aza-C) was first described in 1964 and has been found to be clinically useful in the treatment of acute myelocytic leukemia.[1–3] 1-β-D-arabinofuranosyl-5-azacytosine (ara-AC) is a congener of 5-aza-C, which has the nitrogen substitution in the 5 position of the pyrimidine ring found in 5-aza-C and the 2′-β-arabinosyl configuration found in the tumoricidal drug ara-C.[4] 5,6-dihydro-5-azacytidine (DHAC) is a hydrolytically stable analog of 5-aza-C that has antileukemic activity against experimental leukemias.[5–7] Both ara-AC and DHAC, like 5-aza-C, are prodrugs that must be activated in a sequential manner to their respective triphosphate and deoxytriphosphate which are considered to be the active anabolites.[5,7–9] These anabolites induce DNA hypomethylation in murine and mammalian tumor cell lines. The drug-induced DNA hypomethylation has been associated with deoxycytidine kinase (dCk) re-expression in many cell lines and in leukemic cells from patients.[9–11] Nucleoside drug activity is hampered to some extent by the fact that in aqueous solutions, ara-AC undergoes rapid degradation similar to that of 5-aza-C; consequently, the stability of the drug solution is short-lived.[12,13] However, ara-AC has been shown to cause DNA hypomethylation in L1210/0 and the human lymphoid cell line CCRF/CEM/0, but not in the deoxycytidine kinase (dCk) mutant cell lines L1210/dCk(−) and CCRF/CEM/dCk(−).[8,14]

On the other hand, DHAC is far more stable, with a degradation half-life of about 3 months in aqueous solutions, than 5-aza-C or any of the other congeners.[15–17] DHAC is more efficacious against the ara-C-resistant murine cell line, L1210/ara-C, than against L1210/0, thus this murine leukemia model exhibits collateral sensitivity to ara-C.[5,7,15,16] DHAC is activated by both CCRF/CEM/0 cells and dCK

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mutant CCRF/CEM/dCk(−) cells, where it induces DNA hypomethylation and dCk re-expression.[1,5,14]

Two new human lymphoblastoid cell lines have been isolated without the application of a mutagen or retrovirus, from a pediatric patient with acute lymphocytic leukemia (ALL) before and after the patient was successfully treated for 6 weeks with ara-C as a high dose regimen (HDara-C)[18]. The PER-163 cells are resistant to ara-C as a result of a selection process *in vivo*, in contrast to the other cell lines CCRF/CEM/dCk(−), which was adapted to grow in the presence of the drug by selection *in vitro*. Both PER cell lines express markers present in common ALL, and have been characterized to be sensitive (PER-145) or resistant (PER-163) to ara-C, as determined by inhibition of growth, DNA synthesis and by examining dCk enzymatic activity.[18,19] A clinical interest exists for both drugs, ara-AC and DHAC, but DHAC seems to exhibit severe non-medullary toxicity (NCI, DHAC Working Group report), thus limiting its potential use in the clinic. Ara-AC was recently studied as a phase I drug and the clinical results are pending.

In light of the fact that DHAC and ara-AC are anabolized by different kinases,[5,8] the latter requiring dCk activity for activation, we investigated DHAC and ara-AC cellular anabolism and their effect on DNA methylation in the PER human leukemic cell lines. The purpose of these investigations was to investigate whether these compounds were activated by the resistant PER-163 cell line. This was especially pertinent because most patients entered in phase I trials are usually refractory to ara-C, probably due to lack of expression of dCk,[20] thus rendering ara-AC biochemically ineffective. The cellular anabolism studies were performed by determining the intracellular anabolites of these drugs in both cell lines after exposure to a wide range of concentrations. The amount of drug anabolites incorporated into DNA and RNA was also determined. Lastly, the DNA methylation patterns in these cell lines were investigated and correlated with the amount of cellular anabolites formed. The present study extends our previous findings of the cellular anabolism of these compounds in murine and CCRF/CEM human cell lines.

Materials and methods

Materials

DHAC and ara-AC were generously provided by the Investigational Drug Branch, NIH/NCI (Bethesda, MD). [5,6-^{3}H]DHAC and [^{3}H]ara-AC were custom tritiated and purchased from Moravek Biochemicals (Brea, CA). All other materials and chemicals were of analytical HPLC grade.

Cell culture and cytotoxicity studies of DHAC and ara-AC in PER-145 and PER-163 cell lines

Drug uptake of [5,6-^{3}H]DHAC and [^{3}H]ara-AC were tested in the two human leukemia cell lines. PER-145 is a human leukemia line isolated from a pediatric patient with ALL before treatment with HD-araC. The PER-163 line was isolated from the same patient after he achieved a complete remission with HDara-C and relapsed 6 weeks later. This line was shown to be more than 1000-fold
more resistant to ara-C than the PER-145 line as determined by inhibition of growth and DNA synthesis.[18] Further examination revealed that this resistance was due to the absence of dCk activity.[19]

The two cell lines were established and cultured as described earlier,[18,19] and both lines represent common ALL cells, negative for Epstein–Barr virus antigens with the following markers: CALLA$^+$, HLA-DR$^+$, slg$^-$, clg$^-$. The culture medium was RPMI 1640 (Gibco, Grand Island, NY), which was supplemented with L-glutamine (2 mM final concentration), 2-mercaptoethanol (10^{-5} M), pyruvate (1 mM), non-essential amino acids (Flow, Irvine, UK) and 10% heat-inactivated fetal calf serum (Flow Laboratories). The cell lines were plated at a cell concentration of 8×10^5/ml in 24-well multidishes (Nunc, Denmark; no. 143-982) and were incubated in a humidified 37°C incubator containing 5% CO_2. The two cell lines showed similar doubling times (72–79 h) and regular tests for the presence of *Mycoplasma* using an agar culture technique were negative.

The concentrations of DHAC and ara-AC that induced 50% growth inhibition as compared with untreated control (IC_{50}) were determined by incubating the cells for 24 h in the presence or absence of the drugs at concentrations ranging for both drugs from 10^{-9} to 10^{-2} M. The cells were washed thoroughly, replated in fresh medium and the cell numbers were determined after a further 72 h incubation period. The IC_{50} values were obtained from linear regression analyses of the cell numbers (means from three independent experiments).

Biochemical pharmacology studies of [5,6-^{3}H]DHAC and [^{3}H]-ara-AC in PER-145 and PER-163 cell lines

Ten million PER-145 and PER-163 cells were treated for 1 h with various concentrations of the two drugs ranging from 0.25 to 1000 μM *in vitro*. The drugs were premixed with highly purified [^{3}H]ara-AC or [^{3}H]DHAC to a known specific activity in the range of 5×10^4 c.p.m./nmol. After the incubations, the cells were extracted with perchloric acid (PCA) and neutralized. The extracts were assayed by HPLC using a strong anion exchange column (SAX-10) to separate the nucleosides, mono-, di- and triphosphate anabolites of the drugs. The radioactivity associated with the chromatographic peaks was identified as described previously[5,7,8] and the amount of radioactivity was used to calculate the cellular concentrations of each species of anabolites. The amount of radioactive anabolites incorporated into nucleic acids was determined after purification of the cellular RNA and DNA as described earlier.[5,7,8,14]

DNA methylation studies of DHAC and ara-AC in PER-145 and PER-163 cell lines

Quadruplicate aliquots of 4×10^5 cells from these cell lines were treated with ara-AC and DHAC at IC_{50} concentrations for up to 24 h in a similar manner as described previously.[5,7] Specifically labeled [6-^{3}H]Uridine, 10 μCi per test tube, was then utilized to determine the amount of intracellular [^{3}H]dCyt converted from [^{3}H]Uridine incorporated into DNA, including the portion of DNA that becomes methylated. Afterwards the DNA was isolated, purified and hydrolyzed by formic acid, as described earlier.[5,7] The DNA was then assayed by an HPLC strong cation exchange column (SCX-10) to separate the nucleoside bases. Thus, the radioactivity associated with the peaks of dC and 5-methyl-dC (5-mC) was determined,[5,7,14] and the percent of methylated dC in the DNA was calculated as:

$$\%5\text{-mC} = [5\text{-mC}/(5\text{-mC} + \text{dC}) \times 100.$$

Identical studies in untreated control cell cultures were also conducted.

Incorporation of [5,6-^{3}H]DHAC and [^{3}H]ara-AC anabolites in the DNA of PER-145 and PER-163 cell lines

The PCA insoluble pellet from each sample, treated with radioactive drugs for the determination of the cellular anabolites, was reacted with 0.1 N KOH at 37°C for 2 h to hydrolyze the RNA.[5,7] The samples were then centrifuged at 800 *g* for 5 min and the supernatant was removed for scintillation counting of radioactivity associated with RNA. The remaining pellet containing the DNA was suspended in 0.5 ml of 50 mM K_2HPO_4, pH 7.45. This suspension was digested with 4 mU phosphodiesterase type VII (*Crotalus atrox*; Sigma, St Louis, MO) and 200 U DNAse I (type II from bovine pancreas; Sigma) at 37°C for 18 h. Then the suspension was assayed with an HPLC reverse phase μC_{18} column (Millipore, Milford, MA) for nucleosides and nucleoside analogs of [5,6-^{3}H]DHAC or [^{3}H]ara-AC incorporated into DNA.[5,7,21]

Results

Determination of IC_{50} concentrations of DHAC and ara-AC in PER-145 and PER-163 cell lines

The inhibitory concentrations of DHAC and ara-AC that induced 50% growth compared with untreated control (IC_{50}) on PER-145 and PER-163 were 2.4×10^{-5}, 6.5×10^{-5} M and 2.8×10^{-5}, $\geq 10^{-3}$ M, respectively, indicating that ara-AC is not very active in inhibiting cell growth in PER-163 cells.

Intracellular anabolism of [5,6-^{3}H]DHAC and [^{3}H]ara-AC in PER-145 and PER-163 cell lines

The cellular pharmacology of [^{3}H]DHAC and [^{3}H]ara-AC was studied in PER-145 and PER-163 cell lines after treatment with various concentrations of the drugs, ranging from 0.25 to 1000 μM. [^{3}H]ara-AC was accumulated in these cell lines at 2:1 and 4:1 ratios in favor of the intracellular concentration of the drug. The accumulation appeared to be present in a log-linear manner up to 25 μM extracellular concentration, reaching saturation after 50 μM. [^{3}H]DHAC was accumulated log-linearly up to 100 μM, but only at a ratio of 0.61:1 to 0.65:1 extracellular drug concentration. There were no differences in the amount of DHAC accumulated in the two cell lines.

[^{3}H]ara-AC was phosphorylated to mono-, di- and triphosphate anabolites in a concentration-dependent manner from 0.25 and up to 25 μM of extracellular concentration in PER-145 cells. Incubations with higher drug concentrations did not increase the intracellular concentrations of the phosphorylated anabolites (Figure 1). The plateau cellular concentrations of ara-ACMP, ara-ACDP and ara-ACTP achieved after incubation with 25–100 μM in the PER-145 cell line averaged 41.1 ± 9.4, 5.3 ± 0.6 and

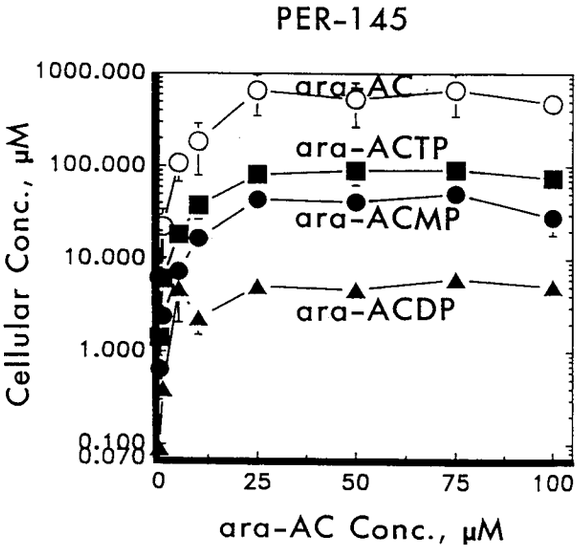
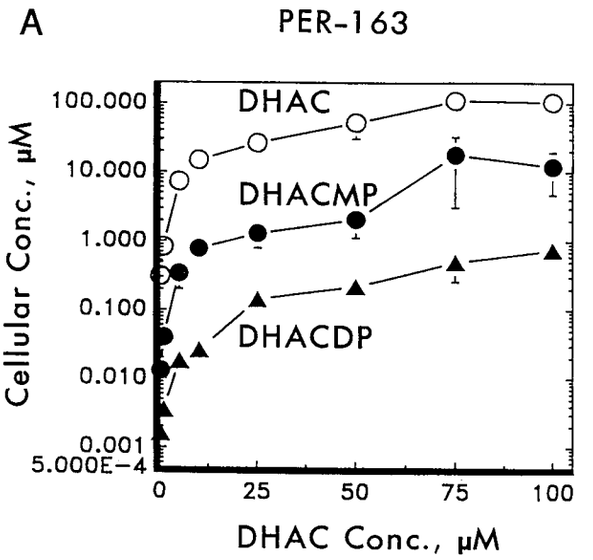


Figure 1. Intracellular concentrations of ara-AC and its phosphorylated anabolites in the human leukemia cell line PER-145, expressing dCk. Saturation of ara-AC phosphorylation occurs after exposure to 25 μM of the drug.



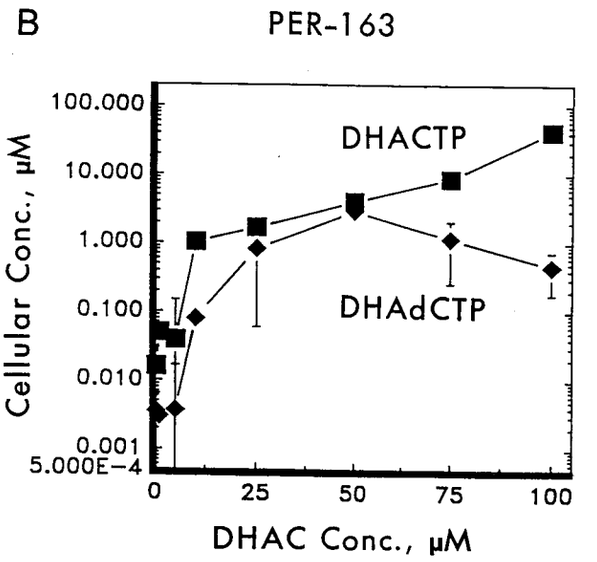


Figure 2. Intracellular concentrations of DHAC and its phosphorylated anabolites in the human leukemia cell line PER-163, which does not express dCk. Saturation of ara-AC phosphorylation appears to take place after exposure to 50 μM of the drug.

84.6 ± 8.0 μM, respectively. Only trace levels of ara-ACTP, the most abundant anabolite of ara-AC in the PER-145 cell line, were detectable in the PER-163 cell line after treatment with 28 μM of the drug, whereas after incubation with 1 mM, the ara-ACTP concentrations averaged 12 ± 3 μM. The IC_{50} concentrations of ara-AC in the PER-145 and PER-163 cell lines were 28 μM and ≥1 mM, respectively.

DHAC was anabolized by both cell lines but at a generally lower level. The plateau cellular concentrations of DHACMP, DHACDP, DHACTP and DHAdCTP in the PER-145 cell line averaged 5.0 ± 2.8, 0.5 ± 0.4, 5.8 ± 2.5 and 0.35 ± 0.08 μM, respectively. These anabolites accumulated at 10.6 ± 7.9, 0.5 ± 0.26, 18.5 ± 11.1 and 1.5 ± 1.3 μM, respectively, in the PER-163 cell line (Figure 2).

Incorporation into nucleic acids in PER-145 and PER-163 cell lines

The amount of drug anabolites of both ara-AC and DHAC incorporated into DNA of PER-145 and PER-163 cells gradually increased with increasing concentrations of the substrates to which the cells were exposed. There is an apparent log-linear relationship between drug concentration, up to 10 μM, and amount incorporated into DNA (Figure 3). At drug concentrations higher than 25 μM a plateau was reached at 2.65 ± 1.6 pmol/10^7 cells (mean ± SD, $n = 4$) in DNA for ara-AC in the PER-145 cell line, but was non-detectable in the DNA of the PER-163 cell line. The anabolite of DHAC as incorporated at similar levels in the DNA of PER-145 and PER-163 cell lines, averaging 0.54 ± 0.17 and 0.43 ± 0.14 pmol/10^7 cells, respectively (Figure 3). These averages are approximately 10-fold lower compared to the ara-AC values in the

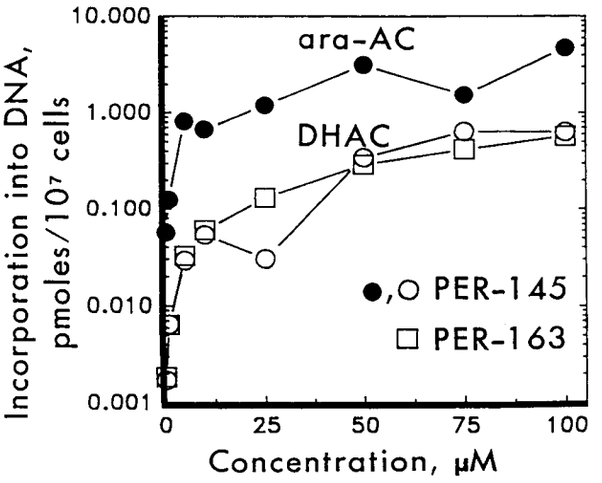


Figure 3. Amount of ara-AC and DHAC anabolites incorporated into DNA of human leukemic cell lines PER-145 and PER-163 after exposure to various concentrations of drugs. Incorporation into cellular DNA reached saturation at 25 μM with both drugs.

PER-145 cell line and follow the same pattern of reduced cellular concentrations displayed by respective triphosphate anabolites. Both drug anabolites appear to be significantly incorporated in the RNA, again following a similar pattern as in the DNA (data not shown).

Effect of DHAC and ara-AC on DNA methylation in PER-145 and PER-163 cell lines

The total genomic DNA methylation results in the PER-145 and PER-163 cell lines are shown in Tables 1 and 2. The average DNA methylation levels in control PER-145 and PER-163 cells were 4.79 ± 0.16 and $4.90 \pm 0.36\%$ methyl-C (mean ± SD, n = 5), respectively. The nadir methylation levels obtained after exposure to IC_{50} concentrations (for 24 h) of ara-AC were 77% of control in PER-145. A 12 h incubation of PER-163 cells with ara-AC yielded a DNA methylation level of 112.2% of untreated control. Thus, ara-AC exerted no effect on DNA methylation in the cell line lacking dCk. The nadir methylation levels obtained after exposure to IC_{50} concentrations (for 24 h) of DHAC were 27.3 and 29.2% of control in PER-145 and PER-163 cell lines. The DNA methylation levels appeared to recover to near control levels as early as 3 days post end of treatment with the drugs (Figure 4). The long-term methylation studies, up to 8 days post treatment, showed that the DNA methylation patterns remained hypomethylated for up to 2 days and then recovered to near control levels.

Discussion

Previous studies have determined the cellular anabolism of DHAC and ara-AC in murine and human leukemic cell lines both *in vivo* and *in vitro*.[5,7,8] This study confirms that DHAC is metabolized by both PER-145 and the dCk lacking PER-163 human cell line, both isolated from a pediatric patient with ALL.[18] Ara-AC is only readily anabolized by the PER-145 line and not by the PER-163 line, a pattern which is identical to that noted in the CEM/0 and CEM/dCk(−) cell lines.[8] Similarly, DNA methylation levels were reduced in both PER-145 and PER-165 cell lines after exposure to IC_{50} concentrations of

Table 1. DNA methylation in PER-145 and PER-163 after treatment with ara-AC

Time post RX (h)	DNA methylation (% 5-methyl-C)		
	PER-145 (2.8×10^{-5} M)	PER-163 (2.8×10^{-5} M)	PER-163 (1 mM)
0 (control)	4.79 ± 0.16	5.76 ± 0.82	5.76 ± 0.82
1	4.02 ± 0.16	—	5.93 ± 1.36
2	3.90 ± 0.15	7.08 ± 3.40	5.47 ± 0.95
4	3.83 ± 1.90	4.38 ± 0.29	4.76 ± 0.63
6	3.81 ± 0.26	5.05 ± 0.14	5.48 ± 1.14
9	3.78 ± 0.22	5.23 ± 0.36	4.92 ± 1.59
12	3.74 ± 0.30	6.46 ± 1.36	4.44 ± 2.68
18	3.70 ± 0.18	—	3.94 ± 0.91
24	3.73 ± 0.23	—	3.47 ± 1.63

Table 2. DNA methylation in PER-145 and PER-163 after treatment with DHAC

Time post RX (h)	DNA methylation (% 5-methyl-C)			
	PER-145 (2.4×10^{-5} M)	% control	PER-163 (6.5×10^{-5} M)	% control
0 control	4.92 ± 0.32	100	4.90 ± 0.36	100
1	—		5.68 ± 1.05	115.9
2	4.90 ± 0.36	99.6	5.55 ± 1.32	113.3
4	4.52 ± 0.21	91.9	4.01 ± 1.66	81.8
6	4.10 ± 0.55	83.3	2.28 ± 0.90	46.5
9	2.54 ± 0.18	51.6	1.43 ± 0.47	29.2
12	1.34 ± 0.61	27.3	1.76 ± 0.05	35.9
18	2.57 ± 0.41	52.3	2.53 ± 0.74	51.6
24	3.13 ± 0.53	63.6	2.42 ± 0.23	49.4

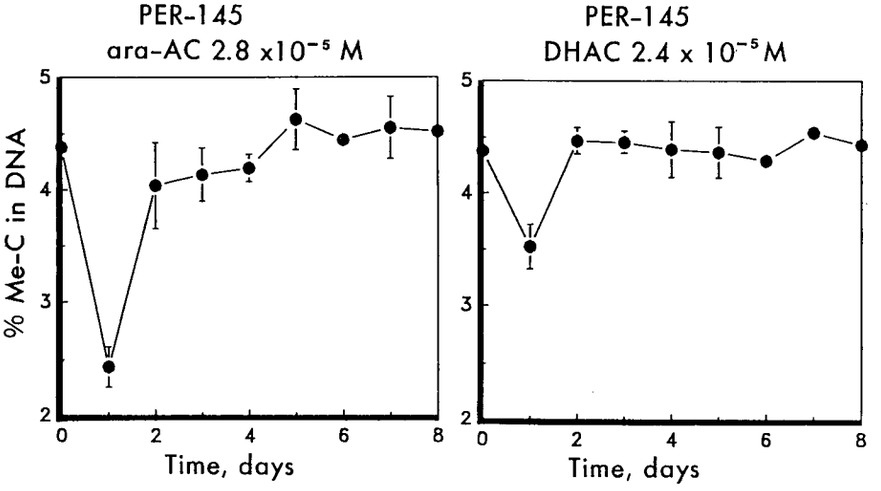


Figure 4. DNA methylation levels in the human leukemic cell line PER-145 after exposure to IC_{50} concentrations of ara-AC and DHAC for 24 h. DNA methylation patterns appear to recover to near control (pre-treatment) levels by day 3 post treatment.

DHAC but only in the PER-145 line after treatment with 2.8×10^{-5} M ara-AC. Exposure of PER-163 cells to an extremely high concentration of ara-AC induced some drug activation and subsequent DNA hypomethylation.

The triphosphate of DHAC is incorporated in a concentration-dependent manner into RNA of both PER-145 and PER-163 cells in significant quantities. This has been observed in the CEM cell system.[7] A greater than 4-fold higher cellular concentration of DHAdCTP was determined in PER-163 cells as compared with PER-145 cells. However, this difference did not result in higher levels of incorporation into DNA of these cell lines (Figure 3). The near identical level of DHAC anabolite incorporated into DNA may explain the similar inhibition of cell growth as it is expressed by the IC_{50} values, 2.4×10^{-5} versus 6.5×10^{-5} M in these cell lines, respectively. In addition, there was a similar level of DNA hypomethylation achieved in both cell lines after treatment with the IC_{50} values of DHAC.

In this study we report that ara-AC is taken up into both the PER-145 and the PER-163 cell lines to a similar extent. The uptake in these cell lines was in favor of 2:1 to 4:1 for the extracellular concentration of the drug. The drug is then phosphorylated to the mono-, di- and triphosphate anabolites, primarily in PER-145 cells. In murine and human CEM leukemia cell systems, resistance to ara-AC has been associated with a reduction or lack of dCK activity.[7,8,21]

PER-163 cells are resistant to ara-C due to lack of dCk; in addition, this cell line showed resistance to ara-AC at low extracellular concentrations, a phenomenon not seen with DHAC or 5-aza-C.[7,20,21] The biochemical basis for this difference is that DHAC and 5-aza-C are activated by nucleoside (U–C) kinase, whereas ara-AC is primarily activated by dCk. There is an apparent saturation of activation of both DHAC and ara-AC in the cell lines in which they are readily activated (Figures 1 and 2). This limitation is also seen in the anabolism of ara-C by CEM/0 cells and may be related to the affinity of these drugs to the respective activating enzymes, i.e. U–C kinase, dCk.

The triphosphate of ara-AC, ara-ACTP, is incorporated into both RNA and DNA in the PER-145 cell line in a drug concentration-dependent manner. Ara-ACTP is also incorporated into RNA and DNA of CEM cells.[8] The incorporation of the ara-AC anabolite into DNA appears to be log-linear at low extracellular concentrations and reaches a plateau after 5 μM in PER-145 cells (Figure 3). Since 5 μM is close to the IC_{50} value of the drug, this suggests that incorporation of fraudulent nucleotides into nucleic acids may be quantitatively associated with cellular cytotoxicity. The saturation of ara ACTP formation occurred at higher concentrations, 25 μM or more of the prodrug ara-AC. Since the saturation of ara-ACMP incorporation into newly synthesized DNA strands occurred at 5 pM and since this is approximately the IC_{50} value for this cell line for ara-AC, it is deduced that the incorporation into newly synthesized DNA is the biological event that confers cellular toxicity. The cellular concentration of ara-ACTP was approximately 8-fold lower after exposure to an extracellular concentration of ara-AC of 5 μM compared with 25 μM, but the amount of this anabolite incorporated into DNA was similar. Hence, this verifies that the incorporation of the fraudulent nucleotide into DNA strands is the toxic event in biological terms.

In earlier studies, we have shown that we could not reduce the methylation levels in previously hypomethylated, cellular DNA and that there is a limit to the hypomethylation of DNA that may be reached with treatments using nucleoside analogs.[5,8,14] This phenomenon may be explained by the achievement of the plateau in the amount of drug anabolite incorporated into DNA in the PER-145 and PER-163 cell lines. The similarities of cellular anabolism of these drugs in both murine and human leukemia cell lines as well as primary human leukemia cells suggests the universality of these pathways.

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(Received 10 August 1994; received in revised form 26 December 1994; accepted 19 January 1995)