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Cellular metabolism of 5,6-dihydro-5-azacytidine and its incorporation into DNA and RNA of human lymphoid cells CEM/O and CEM/dCk(−)*

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Summary. 5,6-Dihydro-5-azacytidine (DHAC) is a hydrolytically stable analog of 5-azacytidine (5-aza-C) that has antileukemic activity against experimental leukemias and, like 5-aza-C, causes DNA hypomethylation. We report the cellular metabolism of DHAC and its incorporation into nucleic acids in the CCRF/CEM/O and deoxycytidine kinase mutant CCRF/CEM/dCk(−) human lymphoid cell lines. The cells were incubated with their respective IC_{50} concentrations for 24 h, then aliquot samples were removed at predetermined intervals and extracted for nucleotides. The acid-soluble extracts of the cells were assayed on HPLC for nucleotides of DHAC. The major anabolite of [^{3}H]DHAC, [^{3}H]DHACTP, peaked at 110.3 ± 30.7 μ*M* in CEM/O and at 96.3 ± 41.9 μ*M* in CEM/dCk(−) cells at 9 and 12 h, respectively. The intracellular concentrations of the deoxyribonucleoside triphosphate, [^{3}H]DHAdCTP, peaked at 13.5 ± 7.7 μ*M* at 4 h in CEM/O and at 80.8 ± 13.8 μ*M* at 12 h, a 6-fold greater cellular concentration, in the dCk mutant cell line. The amount of DHAC anabolites incorporated into CEM/O nucleic acids reached a plateau in RNA at 552.6 ± 7.8 pmol/10^7 cells and in DNA at 64.55 ± 10.0 pmol/10^7 cells. In CEM/dCk(−) cells, DHAC anabolites reached a plateau in RNA and DNA at $4{,}256.3 \pm 631.0$ and 395.5 ± 145.4 pmol/10^7 cells, respectively. Thus, with equitoxic treatments of DHAC, the incorporation of its analog anabolites into RNA and DNA was 8- and 6-fold greater in CEM/dCk(−) cells. DNA methylation levels were depressed equally despite a 6-fold greater incorporation of the analog in DNA in the CEM/dCk(−) cells indicating that hypomethylation may be saturated after DHAC treatment. The DNA methylation levels reached a nadir of 0.19% and 0.20% methyl-C (percentage of methylation) in the two cell lines at 6 and 12 h after the beginning of drug treatment and remained relatively constant for the duration of the 24-h treatment. A curve-linear relationship was obtained between the DNA methylation levels in both cell lines and the amounts of DHAC anabolite incorporated into DNA.

Introduction

5,6-Dihydro-5-azacytidine (DHAC) is a hydrolytically stable analog of 5-azacytidine (5-aza-C) that has antileukemic activity against experimental leukemias and, like 5-aza-C, causes DNA hypomethylation [1, 3, 5, 11, 19, 24]. 5-Aza-C, a nucleoside analog of cytidine, was first described in 1964 by Piskala and Sorm [17] and has been found to be useful in the treatment of acute myelocytic leukemia [13, 15]. DHAC was far more stable (degradation half-life, ≈3 months) than 5-aza-C in aqueous solutions [5, 12, 14]. The decomposition of 5-aza-C is a hydrolytic attack at neutral pH of the 5,6 double bond, which leads to the opening of the triazine ring, producing compounds of unknown therapeutic efficacy [12]. DHAC is more efficacious against the L1210/ara-C and ara-C-resistant murine leukemia cell lines, than against the parent L1210/0 cell line, thus making this drug collaterally sensitive to ara-C in the murine leukemia model [3, 19, 23]. DHAC is also cross-resistant to a 5-aza-C-resistant murine cell line, L1210/aza-C [24]. The results of biochemical pharmacologic studies of DHAC in the murine leukemia lines L1210/0 and L1210/dCk(−) after in vivo and in vitro administration have previously been reported [18, 19, 25].

DHAC has been shown to cause DNA hypomethylation in the murine leukemia cell lines L1210/0 and L1210/dCk(−) and the human lymphoid CCRF/CEM/O and deoxycytidine kinase (dCk) mutant CCRF/CEM/dCk(−) cell lines [1, 19]. The observed DNA hypomethylation has been closely associated with dCk re-expression in L1210/dCk(−) and CCRF/CEM/dCk(−) lines after in vivo and in vitro treatments, respectively [1, 3, 19].

The purpose of this study was to investigate the cellular metabolism of DHAC and determine whether there is collateral sensitivity with ara-C in the human leukemia cell lines CCRF/CEM/O and CCRF/CEM/dCk(−), hereafter called CEM/O and CEM/dCk(−) [20]. This was accomplished by determining the intracellular kinetics of DHAC and its metabolites in both cell lines, and then determining the amount of drug anabolites incorporated into

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Abbreviations used: 5-aza-C, 5-azacytidine; DHAC, 5,6-dihydro-5-azacytidine; DHACTP, 5,6-dihydro-5-azacytidine 5′-triphosphate; DHAdCTP, 5,6-dihydro-5-azacytidine 5′-deoxy-triphosphate; dCk, deoxycytidine kinase; PCA, perchloric acid; SAX, strong anion exchange; PBS, phosphate-buffered saline

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the cellular DNA and RNA. Lastly, the DNA methylation studies in these cells were investigated and correlated with the amount of drug anabolites incorporated into the DNA.

The present study extends our previous findings of the cellular metabolism of DHAC in tumor-bearing mice after in vivo administration and compares the level of activation with the DNA hypomethylation determined in the human lymphoid cell lines.

Materials and methods

Materials. DHAC was generously provided by the Investigational Drug Branch, NIH/NCI (Bethesda, Md). [5,6-^{3}H]DHAC was purchased from Moravek Biochemicals, Inc. (Brea, Calif). All other chemicals were of analytical or HPLC grade.

Cell culture and cytotoxicity studies of DHAC. CEM/O and CEM/dCk(−) human lymphoid cells were provided by Dr. Arnold Fridland, St. Judes Hospital (Memphis, Tenn) and were maintained in suspension culture in RPMI 1640 (Irvine Scientific, Santa Ana, Calif) enriched with 10% fetal calf serum (Irvine Scientific, Santa Ana, Calif) and 1% HEPES buffer (Whittacker Bioproducts, Walkersville, Md) in the absence of antibiotics. The cells were subcultured every 3–4 days at 37° C in a humidified incubator with 5% CO_2. The doubling time of the cell lines was 22–24 h. The determination of the inhibitory concentration (IC_{50}) of DHAC was carried out by separately incubating subcultures of the two cell lines with a 5-log range of concentrations of the drug [1]. The linear portions of the sigmoidal growth inhibition curves were fit on an exponential regression analysis program. The 50% inhibition concentration (IC_{50}) was determined from the equation of the best fit line in the "log-linear" portion of the sigmoidal curve [1, 20].

Biochemical pharmacologic studies of DHAC in CEM/O and CEM/dCk(−) cells. The [^{3}H]DHAC was added to the cell suspension at IC_{50} concentrations for each cell line. Triplicate aliquots of 1×10^7 cells were taken at 0 (control), 1, 2, 4, 6, 9, 12, 18 and 24 h from both CEM/O and CEM/dCk(−) lines. These aliquots were then extracted for nucleotides by using 0.4 *N* perchloric acid (PCA) as described elsewhere [4, 19]. The supernatant was neutralized and assayed by high-performance liquid chromatography (HPLC) on a strong anion exchange column (SAX-10) with a gradient elution for mono-, di-, and triphosphates of nucleosides and the anabolites of [^{3}H]DHAC, as previously described [18–20]. [^{3}H]DHACTP was observed to elute in the triphosphate region 2 min before CTP [19]. Fractions were collected by a programmable fraction collector (ISCO Retriever III, Lincoln, Neb) and counted on a scintillation counter for all possible metabolites of [^{3}H]DHAC.

DNA methylation studies of DHAC in CEM/O and CEM/dCk(−) cells. Methylation levels in CEM/O and CEM/dCk(−) cellular DNA were determined as previously described [1, 19]. Briefly, cells were treated with their respective IC_{50} concentrations and at various time points quintuplicate aliquots of 3×10^5 cells were removed, washed twice with phosphate-buffered saline (PBS), placed in 2 ml culture media in the presence of 10 μCi specifically labeled [6-^{3}H]uridine, and incubated further for 24 h at 37° C. At the end of this incubation period, the cells were washed once with PBS and lysed in 0.3 *N* NaOH + 0.1% sodium dodecyl sulfate (SDS) at 37° C for 24 h. The DNA was then separated via centrifugation and hydrolyzed to its bases in 88% formic acid at 180° C for 1 h. After evaporation and reconstitution with 0.2 ml PBS, the bases were separated by HPLC on a strong cation exchange column (SCX-10; Custom LC, Inc., Houston, TX) at room temperature. The separation of 5-methyl-C (5-mC) and C was achieved by an isocratic elution with 60 m*M* KH_2PO_4 (pH 2.50) at a flow rate of 0.7 ml/min. The eluates were monitored at 280 nm, collected in 1-min fractions with a fraction collector, and counted in a scintillation counter for tritium radioactivity. The percentage of degradation per minute (dpm) of 5-mC was calculated as:

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

Separation of DNA and RNA from the PCA-insoluble fraction of cellular extracts. The PCA-insoluble pellet from each sample was reacted with 1.0 *N* KOH at 37° C for 2 h to hydrolyze the RNA. The samples were then centrifuged at 800 *g* for 5 min, and the supernatant was removed for scintillation counting of RNA-associated radioactivity. The remaining pellet containing the DNA was resuspended in 0.5 ml 0.005 *M* K_2HPO_4 (pH 7.45). This suspension was digested with phosphodiesterase (0.4 units, type VII, *Crotalus atrox*; Sigma Chemical Co.) at 37° C for 18 h. The final hydrolysis product was assayed by HPLC on a reverse-phase μC18 column for nucleosides and nucleoside analogs of [^{3}H]DHAC incorporated into the cellular DNA [6, 20].

Results

Determination of the IC_{50} concentrations and cellular uptake studies of DHAC in CEM/O and CEM/dCk(−) cells

The two human lymphoblastoid cell lines CEM/O and its dCk mutant were used in the determination of the cytotoxic characteristics of DHAC as described in *Materials and methods.* The IC_{50} concentrations of DHAC for the CEM/O and CEM/dCk(−) cells were 100 and 200 μ*M*, respectively. The log-linear portions of the two sigmoidal curves of the plot between the percentage of cell lethality vs the log of DHAC concentration were nearly superimposable, thus not statistically different from each other. However, the best-fit lines in the semilogarithmic graph of 5 cycles indicated a 2-fold difference in the IC_{50} values, although the distance between them was very small.

The uptake of [^{3}H]DHAC by CEM/O and CEM/dCk(−) cells was investigated at 6 h after drug incubation with their respective IC_{50} values. Both cell lines accumulated similar amounts of total radioactivity from the drug, averaging 34.2 ± 0.2 and 33.9 ± 0.1 nmol/10^7 cells, respectively. When these amounts were expressed as cellular concentrations, they exceeded 4.7 and 4.5 m*M* DHAC in CEM/O and CEM/dCk(−) cells, respectively. These concentrations were 47- and 27-fold higher than the those in the respective growth media in which the cells were incubated. These results indicate that both cell lines attained equal influx of the drug after exposure to 2-fold different concentrations of DHAC. The first and second washings of the

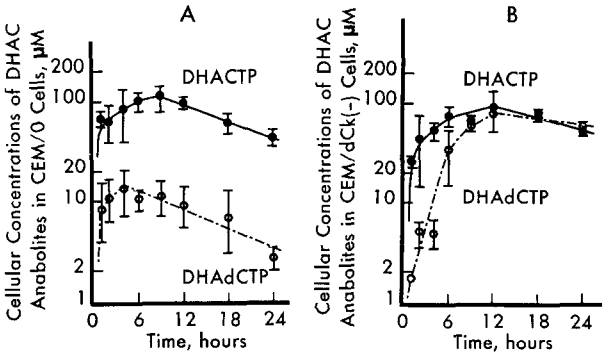

Fig. 1. Kinetics of intracellular concentrations of [^{3}H]DHACTP and [^{3}H]DHAdCTP in CEM/O (*A*) and CEM/dCk(−) (*B*) cells. The cells were treated with their respective IC_{50} concentrations of [^{3}H]DHAC, extracted with PCA, and assayed by HPLC on a strong anion exchange column (SAX-10) as described in *Materials and methods*. Symbols represent the means ±SD (n = 3)

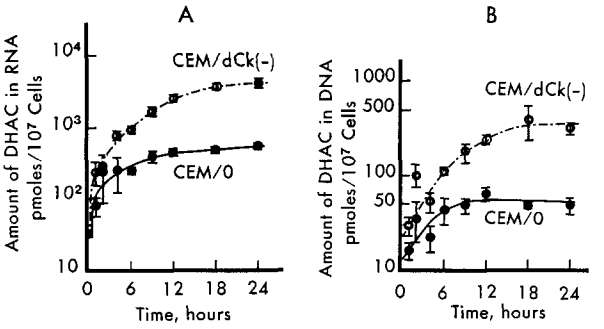

Fig. 2. Incorporation of [^{3}H]DHAC anabolites into the RNA and DNA of CEM/O (*A*) and CEM/dCk(−) (*B*) cells over time. Symbols represent the means ±SD (n = 3)

cell pellets released approximately 1.5 and 0.1 nmol/10^7 cells or, 5% and 0.2%, respectively, in comparison with control values.

Intracellular kinetics of [^{3}H]DHAC triphosphate anabolite [[^{3}H]DHACTP] in CEM/O and CEM/dCk(−) cells

[^{3}H]DHACTP was the major anabolite of [^{3}H]DHAC in PCA extract of both CEM/O and CEM/dCk(−) cells after in vitro treatment with the respective IC_{50} concentrations for each cell line. A gradual accumulation of the [^{3}H]DHACTP intracellular concentrations was observed over time, reaching a peak of 110.3±30.7 μ*M* (±SD; n = 3) at 9 h in the CEM/O cells and 96.3±41.9 μ*M* (±SD; n = 3) at 12 h in CEM/dCk(−) cells after treatment (Fig. 1, *A, B*). [^{3}H]DHACTP intracellular concentrations appeared to decline over time, with elimination half-lives of 11.2 and 14.8 h in CEM/O and CEM/dCk(−) cell lines, respectively. Since the cells were exposed to a relatively constant extracellular concentration of [^{3}H]DHAC, this apparent elimination phenomenon may be attributable to a number of possible mechanisms, including depletion due to the use of the nucleoside triphosphate anabolite, the reduction in synthesis of the anabolite due to drug-induced cell death, the feedback inhibition of the kinase by normal and analog nucleotide pools, and the induction of catabolic enzymes.

Intracellular kinetics of [^{3}H]DHAdCTP in CEM/O and CEM/dCk(−) cells

A new anabolite of [^{3}H]DHAC was observed to elute in the triphosphate region approximately 3 min after [^{3}H]DHACTP and was identified as the 2′-deoxy-derivative, [^{3}H]DHAdCTP, due to its resistance to $NaIO_4$ oxidation and the relative elution characteristic on the HPLC column. The intracellular concentrations of this anabolite peaked at 13.5±7.7 μ*M* (±SD; n = 3) at 4 h and 80.8±13.8 μ*M* (±SD; n = 3) at 12 h after the drug treatment in CEM/O and CEM/dCk(−) cells, respectively (Fig. 1, *A, B*). There was an apparent decline in cellular [^{3}H]DHAdCTP concentrations, with rather long elimination half-lives of 9.45 h in CEM/O and 31 h in CEM/dCk(−) cells.

Incorporation into nucleic acids

The amount of drug anabolite incorporated into the RNA of CEM/O and CEM/dCk(−) cells gradually increased throughout the 24 h drug treatment. The drug reached a plateau at 552.6±7.8 pmol/10^7 cells (±SD; n = 3) in RNA in the CEM/O cells and a substantially higher plateau at 4,256.3±631 pmol/10^7 cells (±SD; n = 3) in RNA in the CEM/dCk(−) cells (Fig. 2, *A*). The amount of drug anabolite incorporated into the DNA peaked at 64.55±10 pmol/10^7 cells (±SD; n = 3) 12 h after the beginning of drug treatment in the CEM/O cells. In the DNA of CEM/dCk(−) cells, the anabolite peaked at 395.5±154.4 pmol/10^7 cells (±SD; n = 3) 18 h after the beginning of treatment (Fig. 2, *B*).

Effect of DHAC treatment on DNA methylation in CEM/O and CEM/dCk(−) cells

The methylation results in the CEM/O and CEM/dCk(−) cells are shown in Table 1. The average DNA methylation levels in control CEM/O and CEM/dCk(−) cells were 3.72±0.05% and 2.97±0.07% methyl-C (±SD; n = 4), respectively. The nadir methylation levels obtained after exposure to IC_{50} concentrations of DHAC were 0.19% and 0.20% at 6 and 12 h in the CEM/O and CEM/dCk(−) cells, respectively. When the amount of [^{3}H]DHAC anabolite incorporated into the DNA of CEM/O and CEM/

Table 1. DNA methylation levels in CCRF/CEM/0 and CCRF/CEM/dCk(−) cells after treatment with IC^{50} concentrations of DHAC

Time (h)	CCRF/CEM/0:		CCRF/CEM/dCk(−):	
	% methyl-C[a]	1% of control	% methyl-C[a]	% of control
0, control	3.72±0.05	100.0%	2.97±0.07	100.0%
1	2.57±0.41	69.2	1.75±0.08	58.9
2	1.44±0.08	38.8	1.26±0.04	42.5
4	0.23±0.05	6.1	0.68±0.49	22.9
6	0.19±0.0[b]	5.1	0.22±0.04	7.5
9	0.22±0.04[b]	5.9	0.21±0.03[b]	6.9
12	0.22±0.06	5.9	0.20±0.04	6.7
18	0.37±0.11	10.0	0.31±0.05	10.4
24	0.36±0.05	9.7	0.23±0.03	7.6

[a] Mean ± SD; n = 4

[b] Average of duplicate determinations ± range

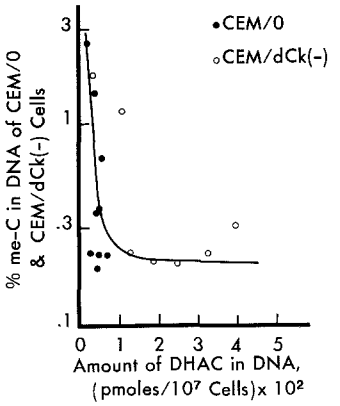

Fig. 3. Relationship between the amount of [^{3}H]DHAC anabolites incorporated into DNA and methylation levels in CEM/O and CEM/dCk(−) cells

dCk(−) cells was plotted vs the log of the percentage of methylation level, a log-linear relationship was obtained for the CEM/O and a curve-linear relationship, for the CEM/dCk(−) cell data (Fig. 3). This indicates that once a certain level of DNA hypomethylation is achieved, an increase in the cellular concentration of the deoxyribonucleoside analog triphosphate has no effect on the further hypomethylation of the DNA.

Discussion

In an effort to understand the relative importance of the biochemical and pharmacologic parameters that may be important in the development and preclinical testing of new nucleoside analog drugs with different biological and biochemical activities, we investigated the cellular metabolism of DHAC, a hydrolytically stable congener of 5-aza-C, in human lymphoid cell lines. The most prominent pyrimidine nucleoside analog drug for the treatment of acute leukemias, ara-C, has been shown (either alone in in combination regimens) to induce complete remission rates in a majority of patients and has produced a small but significant number of long-term "cures" [4, 8]. However, the leukemic cells of patients who relapse after ara-C treatment are often refractory to this drug [4]. Experimental leukemia cell lines resistant to ara-C due to a lack of the activating enzyme deoxycytidine kinase have been used to investigate the possible mechanisms of reversal of tumor resistance to this important antileukemic drug. We have reported that the reversal of drug resistance takes place to some extent by the induction of a hypomethylation state on the genomic DNA in CEM/dCk(−) cells after treatment with 5-aza-C and DHAC [1, 20]. 5-aza-C and its congeners are incorporated into DNA in place of dCMP, and the 5-aza-C-DNA segments are potent inhibitors of cytosine methyltransferase. This covalent interaction is formed between DNA containing 5-aza-C residues and DNA-cytosine methyltransferase [22].

DHAC is an excellent hypomethylating agent that is activated primarily by enzyme uridine-cytidine kinase (U-Ck) and can thus be successfully used to exert its cytotoxic and hypomethylating effects on cell lines that lack dCk and are thus resistant to ara-C [1, 3, 19]. We have previously reported the metabolic activation of DHAC in tumor-bearing mice, which included the isolation and identification of the triphosphate anabolite as well as the DNA hypomethylation induced in L1210/0 and L1210/dCk(−) cells [18, 19].

In the present study we report the uptake of DHAC to similar cellular levels by both CEM/O and CEM/dCk(−) cells, where it is phosphorylated to mono-, di-, tri-, and deoxyribonucleoside triphosphate anabolites. The ara-C-resistant human cell line showed partial resistance to DHAC, a phenomenon not seen in the murine leukemia system, where DHAC and ara-C show collateral sensitivity [19, 23]. The biochemical basis for this discrepancy between murine and human leukemia cell lines is not known. The intracellular concentrations of the triphosphate anabolite were detected at approximately equal levels in the ara-C-sensitive and -resistant human cell lines after treatment with equitoxic concentrations (100 and 200 μM) of the nucleoside drug (Fig. 1, *A, B*).

Detectable degradation of DHACTP from the cells was observed in the presence of exogenous DHAC. This phenomenon of elimination of DHACTP may primarily be due to drug-induced death, the use of the anabolite as it is incorporated into RNA, or the possible induction of catabolic enzymes [10]. Another possibility is that because U-Ck is feedback-inhibited by both CTP and UTP, the initial phosphorylation rate of DHAC by this enzyme is inhibited by normal and analog nucleotide pools. DHAC is also used at the diphosphate level as a source for interconversion to the deoxyribonuceloside diphosphate derivative by ribonucleotide reductase. The deoxyribonucleoside triphosphate anabolite of DHAC was detected in quantities approximately 10% of those of DHACTP in CEM/O cells, but DHAdCTP cellular concentrations were 6-fold greater in CEM/dCk(−) than in CEM/O cells. Therefore, nucleotide analog levels per se are not key determinants of growth inhibition.

In contrast to the difference in DHAdCTP levels is the fact that both cell lines accumulated similar cellular concentrations of DHACTP after equitoxic treatments with the pro-drug DHAC. The enzymatic basis for the increased accumulation of DHAdCTP in dCk-deficient cells is not known. A possible explanation for the higher cellular concentrations of DHAdCTP in CEM/dCk(−) cells is that these cells may have higher ribonucleotide reductase levels, because their cellular dCTP pools must be formed via the interconversion of CDP to dCDP. The nucleoside diphosphate, CDP, is the product of de novo pyrimidine nucleoside biosynthesis. Identification of [^{3}H]DHAdCTP was achieved by its delayed retention time in relation to that of the ribonucleoside triphosphate and its resistance to periodate oxidation. Periodate ($NaIO_4$) treatment of the evaporated HPLC column eluates corresponding to the anabolite, which cleaves the 2′, 3′-*cis*-OH configuration of the furanoriboside ring but not the 2′-deoxy configuration, did not oxidize the anabolite carrying the radiolabel of the parent drug, thus verifying the 2′-deoxy character of the pentose moiety [3].

The triphosphate of DHAC is gradually incorporated into the RNA of both CEM/O and CEM/dCk(−) cells in significant quantities. The results show that there was an 8-fold greater incorporation of the DHAC anabolite into RNA and a 6-fold greater incorporation into DNA in CEM/dCk(−) than in CEM/O cells when they were treated at equitoxic levels. This suggests that the incorporation of fraudulent nucleotides into nucleic acids per se is not

quantitatively associated with cytotoxicity; a corollary is that incorporation into nucleic acids is not proportional to the cellular concentrations of nucleotides in these cell lines. However, the DNA methylation levels were equally inhibited in the cells after treatments with DHAC. DNA hypomethylation reached similar levels despite a 6-fold greater incorporation of the analog anabolite in the DNA of CEM/dCk(−) cells, indicating that hypomethylation may be saturated after DHAC treatment and may be associated with cytotoxicity. The DNA hypomethylation results compare favorably with those obtained in murine leukemia cell lines sensitive and resistant to ara-C [19].

In an earlier study, we have shown that the methylation levels in previously hypomethylated cellular DNA could not be further reduced and that there is a limit to the hypomethylation state of DNA that can be obtained with treatments of nucleoside analog [1, 19]. This phenomenon may be explained by the achievement of a plateau in the amount of drug anabolite incorporated into DNA in these cell lines (Fig. 2). The apparent differences in DNA methylation levels obtained in the present studies vary by $< 10\%$ in comparison with the same cell lines treated with DHAC and grown for 24 h in drug-free media, probably because of the two cell divisions that occurred after drug exposure [1]. Each cell division reduces the DNA methylation levels by 50% [11, 21]: two of four daughter cells in which the genomic material was never hypomethylated, the two cells whose DNA is derived from the original, fully methylated DNA strands will give rise to two fully methylated DNA cells [21].

Another plausible mechanism of cytotoxicity of DHAC is the reported DNA strand breaks after treatment with the drug [7]. We showed in this study that the deoxyribonucleoside triphosphate of DHAC is incorporated in substantial amounts into the DNA of both cell lines, which could be responsible for the DNA double-strand breaks. There appears to be an association between the time at which DNA methylation levels reached a plateau at 10% of control levels, 6–12 h after drug exposure, and the time at which the plateau of incorporation of DHAdCTP into DNA was achieved in CEM/O and CEM/dCk(−) cell lines (Table 1, Fig. 2). Therefore, the amount of anabolite incorporated into DNA and the subsequent hypomethylation are suppressed, probably due to the inhibition of DNA synthesis after DHAC treatment.

There was a curve-linear relationship between the amount of DHAC anabolite incorporated into DNA and the percentage of methylation levels determined at the same time points in both cell lines. The results from the CEM/O cell line alone show a log-linear relationship (Fig. 3, *solid symbols*). The first four data points from the CEM/dCk(−) cells show linearity between the two parameters (Fig. 3), *open symbols*), indicating that the percentage of methylation of total genomic DNA is correlated with the amount of hypomethylating drug that is incorporated into DNA. The curve-linear shape of the curve in a semilogarithmic graph between these two parameters indicates that the second-order relationship could be the net result of the linear rate of incorporation and the subsequent saturation of accumulation into DNA.

The present studies confirm that DHAC, unlike ara-C [9, 20], produces a profound decline in the methylation levels of DNA in both sensitive and resistant human lymphoid cells lines, reproducing the results obtained in murine leukemia lines [19]. This has been attributed to the similarity of the anabolic pathway of DHAC in the human and murine leukemia lines. On the other hand, the drug synergism that was seen in the murine lines was not observed in the human lines. The results of these experiments suggest that DHAC has unique biochemical properties, such as stability and DNA-hypomethylating ability, making it an attractive drug for combination chemotherapy with ara-C, especially where ara-C treatment has previously been unsuccessful. The fact that so many questions remain unanswered indicates that further experimentation is required before the relationship between growth inhibition and DHAC metabolism can be completely elucidated.

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