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Cellular metabolism of 1-β-D-arabinofuranosyl-5-azacytosine and incorporation into DNA and RNA of human lymphoid CEM/0 and CEM/dCk(−) cells*

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Summary. 1-β-D-arabinosyl-5-azacytosine (ara-AC) is a relatively new antitumor agent under clinical investigation, which has the 2′-β arabinosyl configuration found in the tumoricidal drug ara-C and the nitrogen substitution in the 5-position of the pyrimidine ring found in 5-azacytidine (5-aza-C). The present study examined the cellular metabolism and the effect on DNA methylation of ara-AC in human CCRF/CEM cells sensitive and resistant to ara-C. The triphosphate anabolite of the drug, ara-ACTP, was the major anabolite in the CEM cellular extracts, peaking at 50.6 ± 23 μ*M* 4 h after incubation with IC_{50} concentrations (0.25 μ*M*) of [^{3}H]ara-AC. The mono- and diphosphate anabolites accumulated 10-fold lower cellular concentrations than ara-ACTP. The nucleoside triphosphate (NTP) pools and, especially, cellular ATP declined significantly by 9 h after the initiation of drug treatment and remained depleted for the 24-h treatment. The drug anabolite was gradually incorporated into both RNA and DNA, peaking in CEM/0 at 3.44 and 0.14 nmol/10^7 cells, respectively. The DNA methylation levels in these cells declined rapidly after treatment with ara-AC, attaining a nadir plateau at 29% of control methylation value. The deoxycytidine kinase (dCk) mutant CEM cell line [CEM/dCk(−)] neither activated ara-AC at appreciable levels nor induced DNA hypomethylation at low concentrations (0.25–1 μ*M*). However, the drug was activated at 0.2–1 μ*M* extracellular concentrations of ara-AC, probably by an as yet unknown nucleoside kinase at approximately 10% of the amount in CEM/0 cells. Ara-AC appears to mediate its cytotoxic action through the accumulation of its triphosphate anabolite, ara-ACTP, and the subsequent incorporation into nucleic acids. DNA methylation may also contribute to its cytotoxicity.

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Abbreviations: 5-aza-C, 5-azacytidine; ara-AC, 1-β-D-arabinofuranosyl-5-azacytosine; ara-ACTP, 1-β-D-arabinofuranosyl-5-azacytosine 5′-triphosphate; dCk, deoxycytidine kinase; PCA, perchloric acid; SAX, strong anion exchange; PBS, phosphate-buffered saline

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Introduction

Arabinosyl-5-azacytosine (ara-AC) is a congener of 5-azacytidine (5-aza-C), which has the nitrogen substitution in the 5-position of the cytosine ring found in 5-aza-C and the 2′-β arabinosyl configuration found in the tumoricidal drug cytosine arabinoside (ara-C) [10]. Ara-AC has been shown to have potent antitumor activity against experimental leukemias and, like 5-aza-C, causes DNA hypomethylation [4, 7, 12, 15, 17, 19, 25, 26]. 5-Aza-C, a nucleoside analog of cytidine, was first described in 1964 by Piskala and Sorm [20] and has been found to be useful in the treatment of acute myelocytic leukemia [5, 6, 17, 18]. Initial data suggest that ara-AC exhibits less host toxicity in experimental animals than either 5-aza-C or ara-C [10]. In aqueous solutions, ara-AC undergoes rapid degradation of an analogous type to 5-aza-C; consequently, the drug solution has a shortlived stability [9, 19].

Ara-AC has been shown to cause DNA hypomethylation in the murine leukemia cell lines L1210/0 and the human lymphoid cell lines CCRF/CEM/0, but not in the deoxycytidine kinase (dCk) mutant lines L1210/dCk(−) and CCRF/CEM/dCk(−) [2, 21, 22]. DNA hypomethylation by 5-aza-C and its congenes has been closely associated with dCk re-expression in L1210/dCk(−) and CCRF/CEM/dCk(−) after in vivo and in vitro treatments, respectively [2, 3, 6, 7].

Studies of the initial velocity of ara-AC uptake have shown only minor differences between sensitive and resistant P388 murine leukemia lines, whereas the nucleotide formation rates from both ara-AC and deoxycytidine were significantly depressed in the latter cells [1]. However, drug accumulation and incorporation into DNA were only investigated at a single time point after a pulse treatment of 15 min. Other studies have shown that a multiple treatment with ara-AC in tumor (L1210)-bearing mice resulted in increased efficacy as determined by the percentage of increase in life span (%ILS), and the effect was produced at a total dose lower than that achieved using a q 4 d treatment [12].

The purpose of this study was to investigate the cellular metabolism of ara-AC in the human leukemia cell lines CCRF/CEM/0 and CCRF/CEM/dCk(−) after a 24-h continuous exposure to simulate the continuous infusion regimens used clinically. This was accomplished by determining the intracellular kinetics of ara-AC and its metabolites in both cell lines, then determining the amount of the drug anabolite incorporated into the cellular DNA and

RNA. Finally, the DNA methylation studies in these cells were investigated and correlated with the amount of drug anabolites incorporated into the DNA. The present study extended our earlier findings on the cellular metabolism of ara-AC in these cell lines [23] and compared the level of activation with the DNA hypomethylation determined in the human lymphoid cell lines.

Materials and methods

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

Cell culture and cytotoxicity studies of DHAC. CEM/0 and CEM/dCk(−) human lymphoid cells were provided by Dr. Arnold Fridland (St. Jude's 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 of ara-AC was carried out by separately incubating subcultures of the two cell lines in a 5-log range of concentrations of the drug [2]. The linear portions of the sigmoidal growth-inhibition curves were fit on an exponential regression analysis program. The 50% inhibitory concentration (IC_{50}) was determined from the equation of the best-fit line in the log-linear portion of the sigmoidal curve [2, 7].

Biochemical pharmacologic studies of ara-AC in CEM/0 and CEM/dCk(−) cells. [^{3}H]Ara-AC 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/0 and CEM/dCk(−) lines. These aliquots were then extracted for nucleotides by using 0.4 *N* perchloric acid (PCA) as described elsewhere [4, 23]. The supernatant was neutralized and assayed by 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]ara-AC, as described earlier [22, 23]. [^{3}H]Ara-ACTP was observed to elute in the triphosphate region 2 min after ATP [23]. 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]ara-AC.

DNA methylation studies of ara-AC CEM/0 and CEM/dCk(−) cells. Methylation levels in CEM/0 and CEM/dCk(−) cellular DNA were determined as previously described [2, 7]. Briefly, cells were treated with their respective IC_{50} concentrations and, at various time points, quadruplicate 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 on a strong cation exchange HPLC column (SCX-10; Custom, LC, Inc., Houston, Tex) 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 fractions of 1 min with a fraction collector, and counted in a scintillation counter for tritium radioactivity. The percentage 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 hydrolize 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]ara-AC incorporated into the cellular DNA [22, 23].

Results

Determination of the IC_{50} concentrations of ara-AC in CEM/0 and CEM/dCk(−) cells

The two human lymphoblastoid cell lines, CEM/0 and its dCk mutant, were used in the determination of the cytotoxic characteristics of ara-AC as described in *Materials and methods.* The IC_{50} concentrations of ara-AC in the CEM/0 and CEM/dCk(−) cells were 0.25 and >1,000 μ*M*, respectively. The log-linear portions of the two sigmoidal curves of the plot between the percentage of cell lethality vs log of ara-AC concentration were statistically different from each other, and the best-fit lines in the semilogarithmic graph of five cycles indicated the 4-lod difference in IC_{50} values. When CEM/0 and CEM/dCk(−) cells were incubated with 0.25 μ*M* [^{3}H]ara-AC for 1 h, no apparent differences were detected in the uptake of the prodrug by either cell line, as determined by HPLC assay of the nucleoside in the PCA cellular extract.

Intracellular kinetics of the [^{3}H]ara-AC triphosphate anabolite in CEM/0 and CEM/dCk(−) cells

[^{3}H]Ara-ACTP was the prominent anabolite of [^{3}H]ara-AC in the PCA extract of both CEM/0 and CEM/dCk(−) cells after in vitro treatment with their respective IC_{50} concentrations. A graudal accumulation of th [^{3}H]ara-ACTP intracellular concentrations was observed over time reaching a peak of 50.6 ± 23 μ*M* (mean; *N* = 4 ± SD) at 4 h post treatment in CEM/0 cells and that of 0.2 ± 0.02 μ*M* (mean; *n* = 3 ± SD) at 4 h in CEM/dCk(−) cells (Fig. 1). However, at higher extracellular concentrations of ara-AC (0.25–1 m*M*), the CEM/dCk(−) cells only accumulated

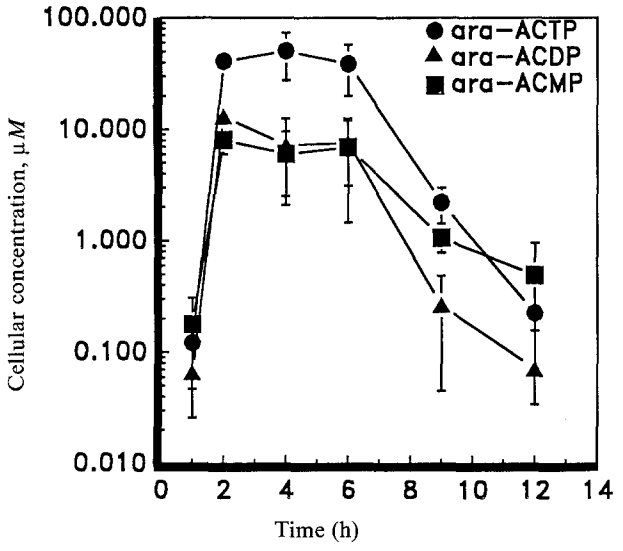

Fig. 1. Kinetics of intracellular concentrations of mono-, di-, and triphosphate anabolites of [^{3}H]ara-AC in CEM/0 cells. The cells were treated with 0.25 μ*M* (IC_{50}) concentrations of the drug, and aliquots of 10^7 cells were extracted with PCA and assayed on an SAX-10 HPLC column as described in *Materials and methods.* The *symbols* represent the means of 4 experiments ± SD

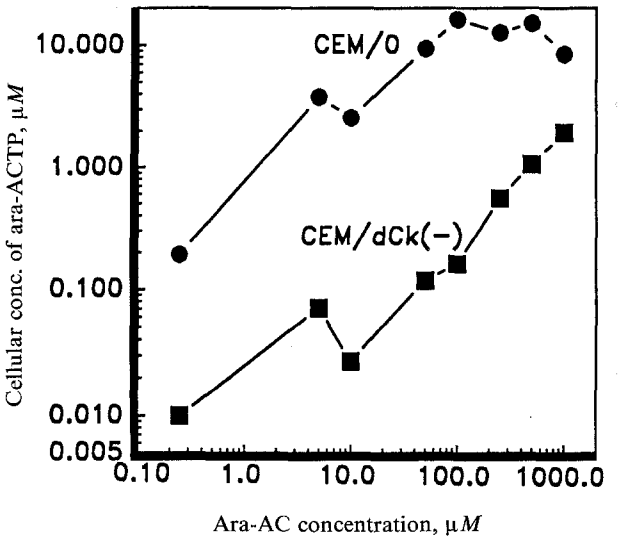

Fig. 2. Effect of ara-AC concentration on metabolism by CEM/0 and CEM/dCk(−) cells. The cells were treated with various concentrations for 1 h and then extracted with PCA and assayed as described in *Materials and methods*

10-fold lower in molarity cellular ara-ACTP concentrations than those in CEM/0 cells (Fig. 2). The phosphorylation of ara-AC at the higher concentrations cannot be attributed to dCk, which activates the drug at low concentrations. It appears that the drug can be activated by an as yet unknown nucleoside kinase, possibly uridine-cytidine kinase.

[^{3}H]Ara-ACTP intracellular concentrations in CEM/0 cells reached a plateau in up to 6 h, from which they declined over time, with an elimination half-life of 0.8 h in the CEM/0 cell line (Fig. 1). Since the cells were exposed to a constant extracellular concentration of [^{3}H]ara-AC, this apparent elimination may be attributed to a number of

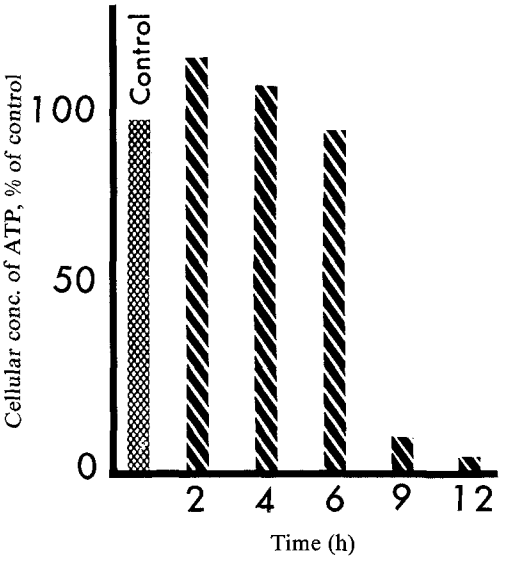

Fig. 3. Effect of ara-AC treatment on CEM/0 ATP cellular concentrations. The *bars* represent the means of 4 experiments

possible mechanisms, including depletion due to the use of the nucleoside triphosphate (NTP) anabolite, a reduction in the synthesis of the anabolite due to drug-induced cell death or decreased ATP, the feedback inhibition of dCk by normal and analog nucleotide pools, and the induction of catabolic enzymes.

Effect of ara-AC treatment on cellular NTP pools

NTP cellular concentrations declined rapidly in the cells after exposure to ara-AC; by 9 h they reached an average through of 10%, and at 12 h they were reduced to <5% of control values (Fig. 3). The depletion of NTP and, especially, ATP was associated with a reduction in phosphorylation of ara-AC to ara-ACMP, to ara-ACTP, which declined 13- and 110-fold at 9 and 12 h respectively in comparison with peak cellular concentrations.

Incorporation of the [^{3}H]ara-AC anabolite into nucleic acids

The amount of radioactive drug anabolite incorporated into the RNA and DNA of CEM/0 cells gradually increased throughout the 18-h treatment. The drug anabolite reached a peak in RNA and DNA at 3.44 and 0.14 nmol/10^7 cells, respectively (mean; $n = 4$), in CEM/0 cells (Fig. 4). In CEM/dCk(−) cells, where the cellular concentrations were 2–3 logs lower than in CEM cells after treatment at the same drug concentration (0.25 μ*M* ara-AC), the drug anabolite peaked in DNA at 10.1 ± 3.2 pmol/10^7 cells ($n = 3$; ± SD) 4 h after the drug treatment started.

Effect of ara-AC treatment on DNA methylation in CEM/0 and CEM/dCk(−) cells

The methylation results in CEM/0 and CEM/dCk(−) cells are shown in Fig. 5. The average DNA methylation levels in control CEM/0 cells were 3.72% ± 0.05% methyl-C, respectively ($n = 4$; ± SD). The nadir methylation levels obtained after exposure to 0.25 μ*M* ara-AC were 29% at 12 h after the start of drug treatment in CEM/0 cells. The plateau achieved in DNA hypomethylation indicates that although the incorporation of the ana-

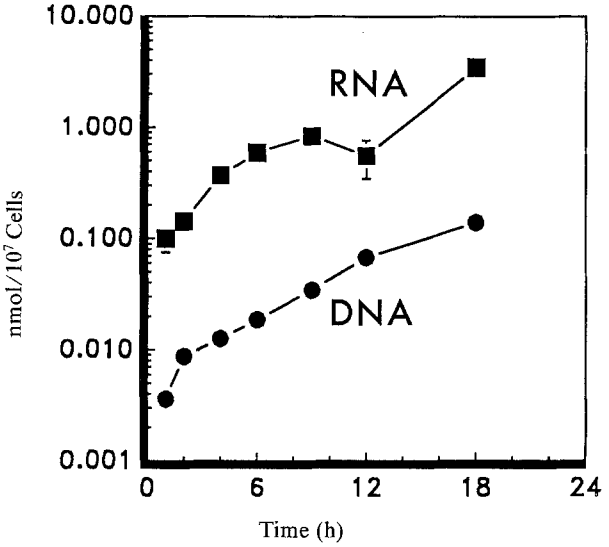

Fig. 4. Incorporation of the [^{3}H]ara-AC anabolite into RNA and DNA of CEM/0 cells over time. The *symbols* represent the means of 4 experiments.

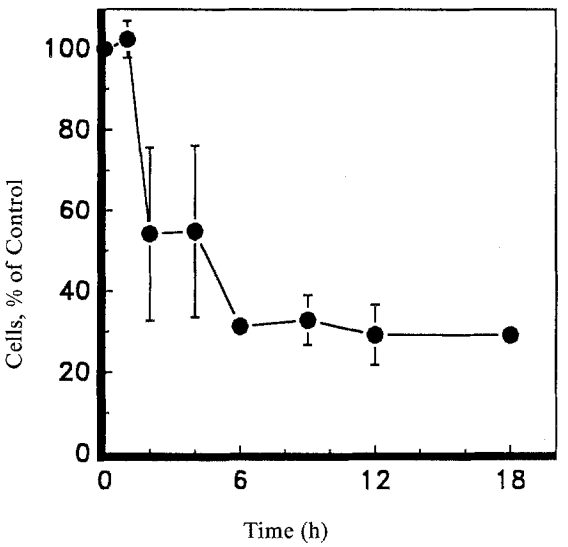

Fig. 5. Effect of ara-AC treatment on total genomic DNA methylation in CEM/0 cells over time. The *symbols* represent the means of 5 experiments ± SD

log anabolite increased up to 18 h in cellular DNA, this had no effect on further hypomethylation of the DNA. The depleted NTP pools may play an inhibitory role in this respect as well. No effect on DNA methylation was observed in CEM/dCk(−) cells after treatment with 0.5 μ*M* ara-AC for 24 h.

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 ara-AC, a 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 to induce, either alone or in combination regimens, complete remission rates in a majority of patients and has produced a small but significant number of long-term "cures" [4, 6, 13].

However, the leukemic cells of patients who relapse after ara-C treatment are often refractory to this drug [6]. 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 drug resistance to this important antileukemic drug. We have previously determined that reversal of drug resistance takes place to some extent by inducing a hypomethylation state on the genomic DNA of CEM/dCk(−) cells after treatment with 5-aza-C and DHAC [2, 6, 7, 10, 21]. 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 [24].

We have previously reported the metabolic activation of ara-AC in tumor cells, including the isolation and identification of the triphosphate anabolite as well as induced DNA hypomethylation in L1210/0 and L1210/dCk(−) cells [22, 23].

In the present study we report that ara-AC is taken up to similar extents by both CEM/0 and CEM/dCk(−) cells, where it is phosphorylated to mono-, di- and triphosphate anabolites, primarily by CEM/0 cells. In murine leukemia cells, resistance to ara-AC has been associated with a reduction in or lack of dCk activity [1, 22]. The human cell line resistant to ara-C due to a lack of dCk showed resistance to ara-AC at low extracellular concentrations, a phenomenon not seen with DHAC or 5-aza-C [7, 22, 25]. The biochemical basis for this difference between these two drugs is the fact that DHAC and 5-aza-C are activated by uridine-cytidine kinase and ara-AC, primarily by dCk.

The intracellular concentrations of the triphosphate anabolite of ara-AC were detected at levels approximately 2–3 logs lower in the ara-C-resistant vs sensitive human cell lines after treatment with the IC_{50} concentration (0.25 μ*M*) of the nucleoside drug. However, at higher extracellular concentrations, a difference of only 1 log could be observed (Fig. 2).

Detectable degradation of ara-ACTP from the CEM/0 cells was observed in the presence of exogenous ara-AC. This phenomenon of ara-ACTP elimination may be primarily due to drug-induced death, to use of the anabolite as it is incorporated into DNA and RNA, or to possible induction of catabolic enzymes (Fig. 4) [15]. Another possibility is that since dCk is feedback-inhibited by both dCTP and dTTP, the initial phosphorylation rate of ara-AC by this enzyme is inhibited by normal and analog nucleotide pools.

Ara-AC treatment had a profound effect on the NTP pools in CEM cells. NTPs declined linearly with time, reaching an average of 10% of control values by 9 h post-treatment (Fig. 3). Naturally, the depletion of NTPs, in particular ATP, could have played a considerable role in the inhibition of ara-AC phosphorylation after 9 h to its phosphorylated products. We did not have sufficient cellular extract to assay for the cellular concentrations of the deoxyribonucleoside triphosphates; however, a dramatic decline would be expected. The depletion of NTP and,

possibly, dNTP may potentiate the pharmacologic role of ara-ACTP in a dCTP-depleted cell.

The triphosphate of ara-AC is gradually incorporated into RNA and DNA of both CEM sublines in significant quantities; CEM/dCk(−) cells accumulated much lower amounts. This study showed that there was an 8- to 10-fold greater incorporation of ara-AC anabolite into RNA than into DNA of CEM/0 cells. The peak levels of ara-AC anabolite incorporated into RNA and DNA were similar to those of DHAC anabolites after equitoxic treatments [7]. This suggests that incorporation of different, fraudulent nucleotides into nucleic acids may be quantitatively associated with cytotoxicity. Of interest is the observation that the peak intracellular concentrations of the respective triphosphates varied at least 2-fold [7, 22]. A corollary is that incorporation into nucleic acids is not proportional to the cellular concentrations of nucleotides in these cell lines.

The DNA methylation levels were similarly decreased in CEM/0 cells treated with ara-AC and DHAC. DNA hypomethylation reached 28% after ara-AC treatment, a value slightly higher than that determined in the same cells after DHAC [7]. The attainment of a plateau of DNA hypomethylation after treatment of CEM/0 cells with either drug indicates that DNA hypomethylation may be saturable and may be associated with cytotoxicity. The results on DNA hypomethylation compare favorably with those obtained in murine leukemia cell lines both sensitive and resistant to ara-C [21].

In an earlier study, 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 reacted with treatments using nucleoside analogs [2, 7]. 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. 4). Another plausible mechanism of ara-AC cytotoxicity may be similar to that reported for DHAC, in that DNA double-strand breaks have been detected after treatment with the drug [11]. In the present study we showed that the triphosphate of ara-AC is incorporated in substantial amounts into DNA in CEM/0 cells, which could account for such DNA double-strand breaks. There appears to be an association between the time at which DNA methylation levels reached a plateau at 28%–30% of control, 6–12 h after drug exposure, with the time at which the plateau of ara-ACTP incorporation into DNA was achieved in CEM/0 (Figs. 4, 5). Therefore, the amount of anabolite incorporated into DNA and the subsequent hypomethylation are suppressed, probably due to the inhibition of DNA synthesis and the NTP depletion seen after ara-AC treatment.

The present studies confirmed that ara-AC, unlike ara-C [2, 6, 14], produces a profound decline in the methylation levels of DNA in human lymphoid cell lines sensitive to ara-C, reproducing the results obtained with DHAC in murine leukemia cells [21]. This has been attributed to the similarity of the anabolic pathway of ara-AC in the human and murine leukemia lines. The results of these experiments suggest that in addition to its potent antileukemic efficacy in murine tumor systems, ara-AC has unique biochemical properties such as DNA hypomethylating ability, making it an attractive drug for combination chemotherapy with ara-C, especially where ara-C treatment has previously been unsuccessful. A number of questions as to the mechanism of ara-AC cytotoxicity still remain unanswered, indicating that further experimentation is required before the relationship between growth inhibition and DHAC metabolism can be completely elucidated.

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