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Biochemical pharmacology of 5,6-dihydro-5-azacytidine (DHAC) and DNA hypomethylation in tumor (L1210)-bearing mice*

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Summary. Dihydro-5-azacytidine (DHAC) is a hydrolytically stable congener of 5-azacytidine, which retains antileukemic activity against experimental leukemias. The biochemical pharmacology of DHAC was studied in tumor-bearing mice in order to elucidate the mode of action of this drug. We found that after an LD_{10} dose of DHAC, the plasma peak concentration achieved was 317 μM and was eliminated biexponentially, with a $t_{1/2}\alpha$ of 1.03 h and a $t_{1/2}\beta$ of 5 h. By 4 h, an unidentified metabolite of [^{3}H]DHAC peaked and was eliminated biexponentially, with a $t_{1/2}\alpha$ of 1.06 h and a $t_{1/2}\beta$ of 10.6 h. [^{3}H]DHACTP was the major anabolite in the L1210/0 cells, and was also eliminated biexponentially, with a $t_{1/2}\alpha$ of 4.3 h and a $t_{1/2}\beta$ of 12.2 h. An unknown anabolite of [^{3}H]DHAC that eluted 5 min after [^{3}H]DHACTP, between UTP and ATP, peaked at 3 h and could possibly be the deoxy-derivative [^{3}H]DHAdCTP. A tissue distribution study revealed that the liver, L1210/0, and lung accumulate the most radioactivity per gram of wet tissue. Methylation studies showed that an LD_{10} dose of [^{3}H]DHAC resulted in a 25.06% hypomethylation of DNA in L1210/0 cells and a 46.32% hypomethylation in a deoxycytidine kinase mutant cell line L1210/dCK(–), compared with their respective controls.

Introduction

5-Aza-C was first described in 1964 and has been found to be clinically useful in the treatment of acute myelocytic leukemia [8, 11, 13]. However, the instability of 5-aza-C in aqueous solutions is well documented [6, 9, 10]. It is decomposed by hydrolytic action at neutral pH values of the 5,6 double bond, which leads to the opening of the triazine ring, producing compounds of unknown therapeutic efficacy [12]. Dihydro-5-azacytidine (DHAC) is a hydrolytically stable analog of 5-aza-C with antileukemic activity against experimental leukemias and, like 5-aza-C, causes DNA hypomethylation [1, 4, 7, 18]. DHAC is more effective against L1210/ara-C, the ara-C-resistant murine leukemia line, than the parent L1210/0 and is cross-resistant to a 5-aza-C-resistant line, L1210/aza-C [4, 16, 17]. Although the optimal dose of DHAC is greater in molar equivalents than that of 5-aza-C, its antitumor activity in experimental murine leukemias is comparable to that of 5-aza-C [5].

The purpose of this study was to investigate the metabolism of DHAC in tumor-bearing mice, to identify and to quantitate the plasma and intracellular kinetics of DHAC and its metabolites, and to determine whether DHAC causes hypomethylation of cellular DNA in L1210/0 and the deoxycytidine kinase mutant L1210/dCK(–) cells after in vivo treatment. We report here the plasma kinetics of the parent DHAC and an unidentified metabolite; we also report the cellular (L1210/0) kinetics and tissue distribution in mice after in vivo treatment; finally, we report the DNA methylation studies in L1210/0 and L1210/dCK(–) murine leukemia lines after an in vivo treatment with DHAC.

Materials and methods

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

Methods. Healthy, male $BD2F_1$ mice were inoculated i.p. on day 0 with 1×10^5 L1210/0 cells; the mice were then divided into eight groups of four mice each. On day 6 all groups were injected with an LD_{10} dose (1500 mg/kg) of [^{3}H]DHAC (20 μCi/mouse). At 1, 2, 4, 6, 9, 12, 18, and 24 h, three mice per time point were sacrificed and the blood and L1210/0 cells harvested. The blood was centrifuged and the plasma removed and stored frozen at $-20°$ C until it was assayed by HPLC on a μC18 reverse-phase column for [^{3}H]DHAC as described below. Fractions of eluates were collected and counted on a scintillation counter for [^{3}H]DHAC. The L1210/0 cells were counted on a Coulter counter and their size was determined with a Coulter Channelyzer (Hialeah, Fla). Triplicates of (3×10^5) cells were removed and incubated with [^{3}H]uridine for the DNA methylation study. The remaining L1210/0 cells

Abbreviations used: 5-aza-C, 5-azacytidine; DHAC, 5,6-dihydro-5-azacytidine; DHACTP, dihydro-5-azacytidine 5'-triphosphate; DHAdCTP, dihydro-5-azacytidine 5'-deoxytriphosphate; LD_{10}, lethal dose 10% of animals; UTP, uridine 5'-triphosphate; ATP, adenosine 5'-triphosphate; TCA, trichloroacetic acid; PCA, perchloric acid; RBC, red blood cells; i.p., intraperitoneally; SAX, strong anion exchange; SCX, strong cation exchange

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were acid-extracted using 0.4 *N* PCA as previously described elsewhere [2, 3, 14, 15]. The supernatant was neutralized and assayed on HPLC with a strong anion-exchange column (SAX-10), with a gradient elution for mono-, di-, and triphosphates of nucleosides as described below. Fractions were again collected and counted on a scintillation counter for quantiation of all possible metabolites of [^{3}H]DHAC.

HPLC assay of [^{3}H]DHAC. A Waters Associates liquid chromatograph (Milford, Mass) was used to assay DHAC in plasma. The HPLC consisted of two M-510 pumps, a U6K injector, M481 UV/VIS detector, Data Module (#730), and a system controller (#720). A reverse-phase μC18 column (Waters Associates, Milford, Mass) was used, and the elution buffer was ammonium acetate 0.5 *M* (pH 6.50) at a flow rate of 0.5 ml/min. The variable wavelength detector was set at 234 nm/0.01 OD (λ max of DHAC). The calibration curve was linear over a wide concentration range of DHAC, and the average reference factor (RF) was entered into the data module for automatic integration and quantitation of the DHAC peak. [^{3}H]DHAC eluted at 7.7 min.

HPLC assay of [^{3}H]DHAC anabolites from cellular acid extracts. The above-described HPLC system was employed to assay the anabolites of [^{3}H]DHAC in cellular PCA extracts using a gradient elution [14, 15]. A strong anion-exchange column (SAX-10, Custom LC, Inc., Houston, Tex) was used to separate the mono-, di-, and triphosphate anabolites of nucleosides of [^{3}H]DHAC. The buffers used were ammonium phosphate monobasic: for pump A, 0.005 *M* (pH 2.80), and for pump B, 0.750 *M* (pH 3.50). The linear gradient was from 0% to 100%, pump B, at a flow rate of 2 ml/min. The column eluates were collected by a fraction collector every 0.5 min and were counted for radioactivity in a scintillation counter.

Determination of methylation levels in tumor [L1210/0, L1210/dCK(−)] DNA. Samples containing 3×10^5 leukemic cells were incubated in vitro in 2 ml RPMI with 10% fetal calf serum and 10 μCi [6-^{3}H]uridine at 37° C for 24 h. At the end of the incubation period, the cells were pelleted and washed once with PBS. The washed cells were suspended in 1 ml 0.3 *M* NaOH containing 0.1% SDS, and the samples were incubated in a humidified atmosphere at 37° C for another 24 h. During this incubation, the cells were lysed and the RNA was hydrolyzed. The samples were neutralized by the addition of 150 μl 2*N* HCl and 400 μl 0.5 *M* TRIS-HCl (pH 7.6) containing 150 μg proteinase K, were incubated at 65° C for 1 h, and then placed on ice for 10 min. In order to percipitate the DNA, 0.5 ml 50% trichloroacetic acid (TCA) was added, the samples were further incubated on ice for 30 min and then centrifuged, and the pellets were washed consecutively with 4 ml 5% TCA and 4 ml 70% ethanol. After the last wash, the tubes were carefully dried and 1 ml 88% formic acid was added; the samples were then hydrolyzed at 180° C for 50 min, dried, and resuspended in 300 μl PBS. The samples were finally analyzed by HPLC on a SCX-10 column (Custom L. C., Inc., Houston, Tex), equilibrated, and developed in 60 m*M* KH_2PO_4 buffer (pH 2.5) at a constant flow rate of 0.7 ml/min at room temperature. The eluate was monitored at 280 nm and collected in fractions of 0.7 ml. The fractions were counted for radioactivity in the peaks corresponding to cytosine and the 5-methyl-cytosine was estimated and the percentage of methylated cytosine was calculated [1].

Results

[^{3}H]DHAC kinetics in mouse plasma

[^{3}H]DHAC concentrations peaked in mouse plasma 1 h after the i.p. injection at 317 μ*M*. The elimination of the total radioactivity of [^{3}H]DHAC from plasma was biexponential in nature, with a $t_{1/2}\alpha$ of 1.45 h and a $t_{1/2}\beta$ of 22.5 h. By 4 h, an unidentified metabolite of [^{3}H]DHAC carrying radioactivity from the parent drug was clearly separated from [^{3}H]DHAC (Fig. 1). The plasma elimination kinetics of the parent drug, [^{3}H]DHAC, were biexponential, with a $t_{1/2}\alpha$ of 1.03 h and a $t_{1/2}\beta$ of 5 h (Fig. 2). The plasma elimination kinetics of the unknown [^{3}H] metabolite were biexponential, with a $t_{1/2}\alpha$ of 1.06 h and a $t_{1/2}\beta$ of 10.6 h (Fig. 2). The unknown metabolite was not detected in the early samples, and it peaked at 4 h at 57.4 μ*M*. The [^{3}H]DHAC plasma concentration at the same time (4 h) was 33.4 μ*M* and the total radioactivity was 90.8 μ*M*.

[^{3}H]DHACTP kinetics in L1210/0 leukemic cells

[^{3}H]DHACTP was identified eluting in the triphosphate region, 2 min before CTP (Fig. 3), and after isoltaion and dephosphorylation with alkaline phosphatase, the radioactive hydrolysis product coeluted with authentic DHAC

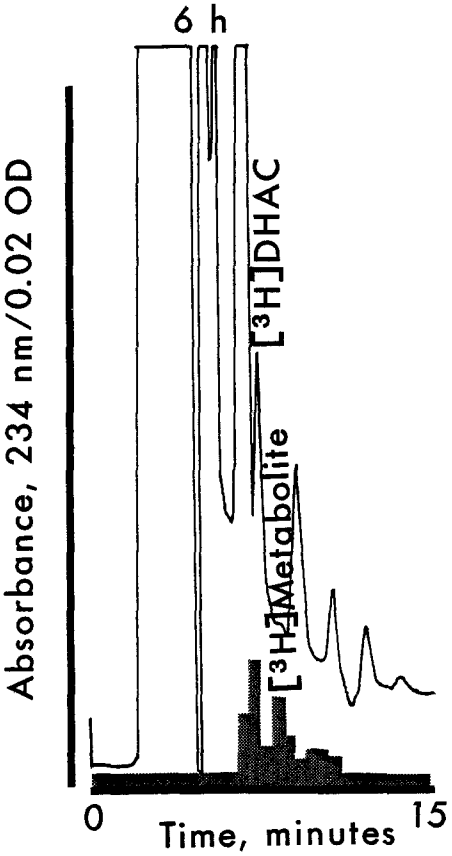

Fig. 1. High-pressure liquid chromatogram of mouse plasma 6 h post-DHAC administration (see *Materials and methods*)

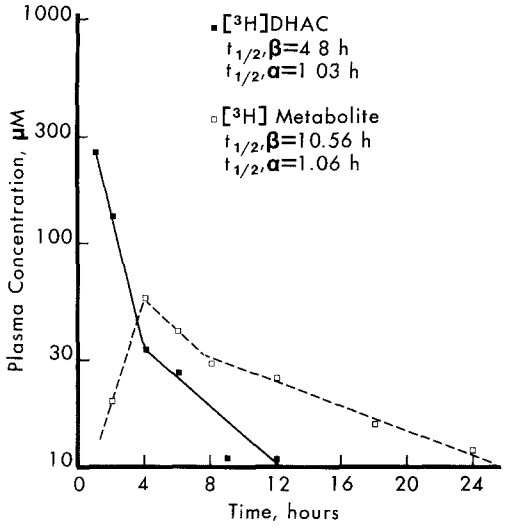

Fig. 2. Plasma kinetics of [^{3}H]DHAC and [^{3}H] metabolite in mouse plasma over time. Symbols are the means of $n = 3$ mice

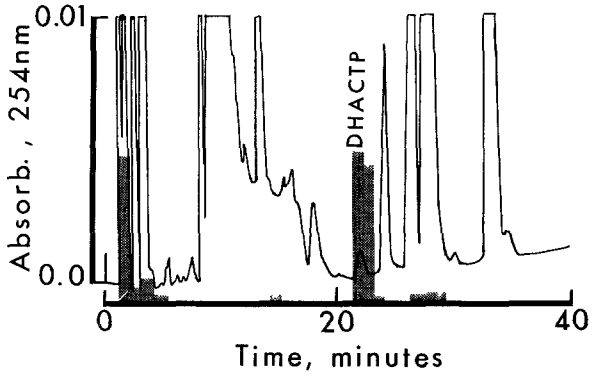

Fig. 3. High-pressure liquid chromatogram of L1210/0 PCA extract on a SAX-10 column. DHAC elutes before CTP at 22 min. The sequence of the other triphosphate peaks are: CTP, UTP, ATP, and GTP

[14]. The isolated product was sensitive to $NaIO_4$ oxidation, and thus it was a 2',3' riboside [3]. [^{3}H]DHACTP was the major anabolite (65% of total radioactivity) in L1210/0 cell PCA extracts (Fig. 3). The other peaks of radioactivity 1 h after treatment were associated with mono (14.5%), di- (2.3%), and at the solvent front (14.6%), which could be [^{3}H]DHAC or tritiated water. From 2 h after the i.p. treatment, a new unidentified peak carrying radioactivity eluted 5–6 min after [^{3}H]DHACTP and between UTP and ATP. This anabolite could possibly be the deoxy-derivative [^{3}H]DHAdCTP [14, 15]. The percentage of this anabolite ranged from 4.2% of total radioactivity at 3 h (peak time) to less than 1.5% at later times. The [^{3}H]DHACTP in the same samples ranged from 47% to less than 34% of total radioactivity.

The peak [^{3}H]DHACTP cellular concentration was 944 μM ±353 ($n = 3$, ± SD), and it was eliminated biexponentially from L1210/0 cells, with a $t_{1/2}\alpha$ of 4.3 h and a $t_{1/2}\beta$ of 12.2 h (Fig. 4).

[^{3}H]DHAC in DNA hydrolysate of L1210/0 cells

DNA from 1×10^5 L1210/0 cells was purified and hydrolyzed with concentrated HCOOH at 180° C for 2 h. The hydrolysis product was evaporated under nitrogen, reconstituted with PBS, and analyzed on a cation-exchange column for the bases of nucleosides. The aglycon of [^{3}H]DHAC was then detected in the eluates of the DNA hydrolysate. There were no major variations in the amount of [^{3}H]DHAC in the L1210/0 DNA over the 24-h period. The relative amount in the tumor DNA was approximately 1/1000 of the peak amount of [^{3}H]DHACTP (nmol/10^7 L1210/0 cells).

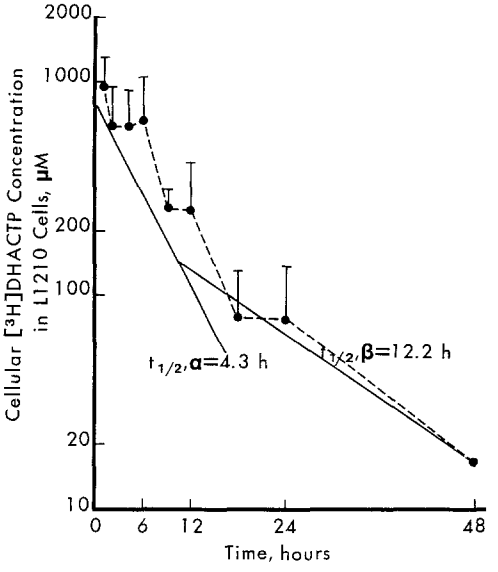

Fig. 4. Intracellular concentration kinetics of DHACTP in L1210/0 cells over time. Symbols are the means of $n = 3 \pm$ SD

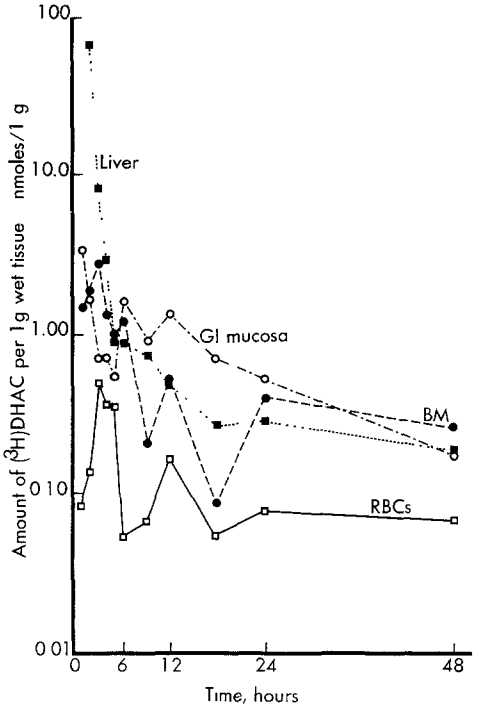

Fig. 5. Tissue distribution of total radioactivity of [^{3}H]DHAC in mouse liver, GI mucosa, bone marrow, and RBCs (see *Materials and methods*)

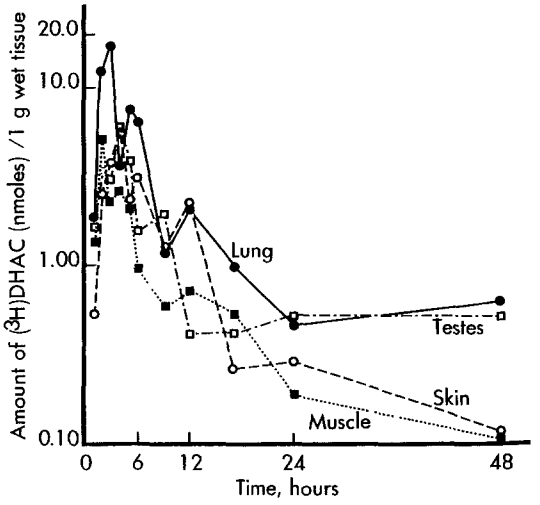

Fig. 6. Tissue distribution of total radioactivity of [^{3}H]DHAC in mouse lung, testes, skin, and muscle

Tissue distribution of [^{3}H]DHAC in tumor-bearing mice

The amount of total radioactivity was determined in twelve tissues (RBC, liver, gastrointestinal mucosa, bone marrow, heart, muscle, kidney, spleen, brain, lung, skin, and testes) and L1210/0 cells after an LD_{10} dose of [^{3}H]DHAC to tumor-bearing mice. The samples were obtained from one mouse per time point at 1, 2, 3, 4, 5, 6, 9, 12, 18, 24, and 48 h after the i.p. injection of [^{3}H]DHAC into eleven mice. Liver L1210/0, and lung accumulated the highest amounts of total radioactivity per gram of wet tissue. The peak amounts at 2 and 3 h were 66.95, 105.9, and 17.6 nmol [^{3}H]DHAC/g wet tissue in liver, L1210/0, and lung, respectively. RBC and muscle accumulated the lowest amounts of [^{3}H]DHAC (10- to 100-fold less than liver). The elimination of the total radioactivity of [^{3}H]DHAC from most tissues could be described as triexponential, and in the others as biexponential (Figs. 5–7).

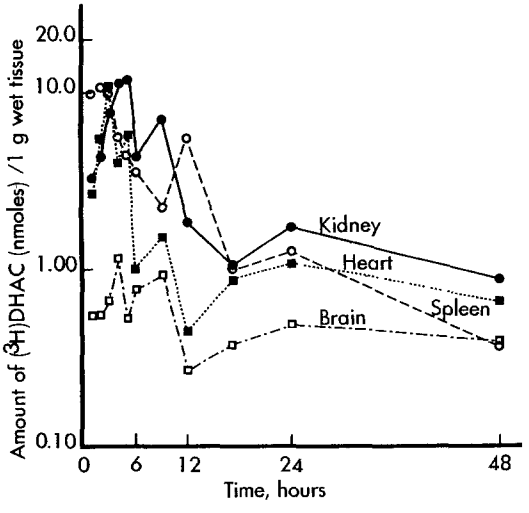

Fig. 7. Tissue distribution of total radioactivity of [^{3}H]DHAC in mouse kidney, heart, spleen, and brain

Table 1. Hypomethylation studies on L1210/0 cells after in vivo treatment with an LD_{10} dose of DHAC

Cell line	% Methylation*	% Control methylation	% Hypo-methylation
L1210/0	3.50 ± 0.02		
	3.96 ± 0.11		
	3.27 ± 0.23		
	3.57 ± 0.35	100.00%	0.00%
L1210/0 + DHAC[a]	2.70 ± 0.10		
	3.02 ± 0.19		
	2.31 ± 0.16		
	2.68 ± 0.36	74.94%	25.06%

* Mean, $n = 3 \pm$ SD
[a] DHAC, LD_{10} dose, × 1, i.p., 48 h earlier

Table 2. Hypomethylation studies on L1210/dCK(–) cells after in vivo treatment with an LD_{10} dose of DHAC

Cell line	% Methylation*	% Control methylation	% Hypo-methylation
L1210/dCK(–)	3.70 ± 0.20		
	3.85 ± 0.26		
	3.46 ± 0.08		
	3.67 ± 0.20	100.00%	0.00%
L1210/dCK(–) + DHAC[a]	1.90 ± 0.20		
	1.64 ± 0.21		
	2.37 ± 0.08		
	1.97 ± 0.37	53.68%	46.32%

* Mean, $n = 3 \pm$ SD
[a] DHAC, LD_{10} dose, × 1, i.p., 48 h earlier

DNA hypomethylation studies in L1210/0 and L1210/dCK(–). Cells after in vivo treatment with an LD_{10} dose of DHAC

The hypomethylation results in L1210/0 cells are depicted in Table 1. The average DNA methylation level in L1210/0 control cells was 3.57% ±0.35%, and after DHAC in vivo treatment it was reduced to 2.68% ±0.36%. When these results are expressed as percentage of control, the DNA hypomethylation after DHAC treatment was 25.06% in L1210/0 cells.

Table 2 summarizes the hypomethylation results in L1210/dCK(–) cells after an LD_{10} treatment of DHAC in vivo. The control DNA methylation was 3.68% ±0.20% in the L1210/dCK(–) cells, and after treatment with DHAC it decreased to 1.97% ±0.37%, or 46.32% hypomethylation. This hypomethylation level can be correlated with the reexpression of deoxycytidine kinase (dCK) in these cells, and thus it can be correlated with the increase in ara-CTP accumulated when these cells were treated with ara-C in our earlier studies [1].

Discussion

This study confirms that DHAC is metabolized in mouse plasma to an as yet unknown metabolite, which carries the radioactivity of the parent drug. This metabolite is elimi-

nated biexponentially with a terminal half-life double that of the parent drug. This metabolite is probably responsible for the rather prolonged half-life of elimination of the total radioactivity (>20 h) from the host [14]. DHAC is taken up by the leukemic cells, where it is phosphorylated by uridine-cytidine kinase to the monophosphate and by other kinases to the di- and triphosphate anabolite, DHACTP [1, 14, 15]. A single LD_{10} dose of DHAC is therapeutically effective in L1210/0 and L1210/dCK(–) murine leukemia-bearing mice [4]. The same dose produces cellular concentrations of DHACTP that on average approached 1 m*M* and another cellular triphosphate anabolite, which could be the deoxy-derivative, DHAdCTP [15]. The DHACTP has a very long half-life of elimination from the leukemic cells, where it is found in considerable cellular concentrations 24 h after the dose has been given. This anabolite, however, has been completely eliminated from the bone marrow and the gastrointestinal mucosa tissues by 18 h [14]. Our experiments with human lymphoid cells in culture (CCRF/CEM) demonstrated that the anabolic pathway of DHAC is identical between mouse and human leukemic cells [1, 15]. It appears that these principles may be applied to the in vivo situation as well. DHAC is well distributed in all soft tissues of the mouse, with greater affinity of accumulation in the L1210, liver, and lung.

DHAC induced DNA hypomethylation in both the L1210/0 and the L1210/dCK(–) cell lines after in vivo treatment to a substantial hypomethylation level. These levels were 25% and 46% of the respective controls and were similar to the DNA hypomethylation levels achieved in the human lymphoid cell lines after in vitro treatments with the same drug [1]. The amounts of drug accumulated in the purified DNA were also similar in the two cell lines. This hypomethylation of total genomic DNA and, presumably, of the dCk gene can reactivate its expression in a small portion of the resistant cell population [4]. Confirmation of this hypothesis has been achieved in the CCRF/CEM/dCK(–) cells [1]. DHAC exerts its antitumor activity against both the L1210/0 and the dCK mutant cell line and has been used successfully to reverse the resistance of the L1210/dCK(–) and the CCRF/CEM/dCK(–) cells to ara-C [1, 4].

Finally, in addition to its antileukemic activity, the successful attempts to restore drug sensitivity due to DNA hypomethylation in the ara-C resistant clones of murine and human cell lines both in vivo and in vitro, make DHAC an attractive candidate for future studies in humans.

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