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DNA-methylation in HL-60 cells treated with 3-deaza-(±)-aristeromycin and 3-deazaadenosine

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5-mCyt* occurs as a minor, modified base in the DNA of eukaryotes. Methylation of Cyt in DNA may be important in controlling gene expression, and alterations in 5-mCyt levels have been found in the DNA of carcinogen treated tissues and transformed cells (for reviews on the subject, see Refs. 1–4). Several reports indicate that 5-mCyt may be involved in the control of cellular differentiation [5–8].

The adenosine analogues c^3Ado and c^3Ari share the capacity to induce leukemia cell differentiation, inhibit AdoHcy hydrolase (EC 3.3.1.1), and perturb levels of transmethylation reactions in cells [9–11]. Hence the perturbation of transmethylation could be related to hypomethylation of DNA and alteration of gene expression in leukemic cells induced to functional maturation by the analogues [9, 10]. The level of DNA methylation in cells induced to differentiation by these compounds is therefore of interest. c^3Ado has multiple cellular targets [9, 12–15], and cytostasis and toxicity occur at concentrations lower than those needed for perturbation of transmethylations [9]. In order to study effects of the adenosine analogues on viable cells, the present investigation was undertaken using a dose schedule that caused cell cycle perturbation, cytostasis and differentiation but no overt toxicity [10, 11, 16, 17].

Materials and methods

Cells. HL-60 cells (a promyelocytic cell line) were grown in RPMI 1640 medium (Gibco, Paisley, U.K.) supplemented with 10% horse serum and 100 units of streptomycin/penicillin per ml, in an atmosphere of 5% CO_2 and 95% air. Cell counts were determined by the use of a hemocytometer chamber. Cell viability was assessed by exclusion of Trypan Blue.

* Abbreviations: c^3Ado, 3-deazaadenosine; c^3Ari, 3-deaza-(±)-aristeromycin; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; Adn, adenine; Cyt, cytosine; 5-mCyt, 5-methylcytosine, Thym, thymine; Gua, guanine; TFA, trifluoroacetic acid.

Drugs. c^3Ado and c^3Ari, kindly supplied by Dr Peter K. Chiang, were dissolved in RPMI 1640 medium to ten-fold of final concentration before they were added to the cell suspension.

DNA isolation. Cells were harvested after 36 hr of logarithmic growth. DNA was isolated by a modification of reported methods [18, 19]. Cell pellets of 10^7 cells were dissolved in 1 ml 20 mM Tris, pH 8, 100 mM EDTA and treated with 100 μg/ml Proteinase K (Sigma Chemical Co., St Louis, MO) and 0.5% SDS at 37° overnight. The samples were sonicated for 3 min in a MSE sonicator adjusted to minimum output, allowed to cool, and subsequently extracted with buffer-saturated phenol [20, 21], phenol/chloroform/isoamylalcohol (25:24:1), and chloroform/isoamylalcohol (24:1). The aqueous phase was transferred to 15 ml Corex tubes, 100 μl of 3M potassium acetate was added, and DNA was precipitated with six volumes of cold (−20°) 96% ethanol. The precipitate was collected by centrifugation at 11,000 *g* for 30 min at 4°, resuspended in 1 ml of cold 70% ethanol, re-centrifuged and the supernatant decanted.

Samples were dissolved in 10 mM Tris, pH 8, 1 mM EDTA and treated with 100 μg/ml RNAase A (Sigma Chemical Co.), heated to 100° for 15 min before use) and 100 U/ml RNAase T_1 (Boehringer–Mannheim) for 60 min at 37°. DNA samples were re-precipitated as above.

Hydrolysis of DNA was performed by concentrated TFA [22]. DNA-precipitates were dissolved in 1 ml TFA and the sample was incubated at 100° for 1 hr in a sealed ampule. The liquid was blown off with gaseous nitrogen. Control experiments showed that this method of acid hydrolysis did not affect the 5-mCyt/Cyt ratio.

Chromatographic analysis. HPLC analysis was performed by a Spectraphysics SP 8770 isocratic solvent delivery system equipped with a Spectroflow 773 (Kratos) detector at 280 nm, 0.01 aufs. The DNA-bases were separated on a strong cation-exchange (Partisil PXS 10/25 SCX) column (supplied with a Supelguard LC-SCX, 5 μm, 2 cm column) with 30 mM ammonium formate, pH 3.5 and 3% methanol at a flow rate of 1.3 ml/min.

The data were processed by a Spectraphysics SP 4270 integrator and calculated by using peak height standard curves of Cyt and 5-mCyt (Sigma) dissolved in the mobile phase. Measurement of peak heights gave good quantitation for 5-mCyt, which was shown by determination of standards. Concentrations of 5-mCyt and Cyt in DNA hydrolysates were determined by linear regression analysis using values supplied by standard curves. Percent methylation was calculated by the ratio 5-mCyt/5-mCyt plus Cyt multiplied by 100.

Results and discussion

Figure 1(a) shows the elution profile of a 5 μM standard mixture of the five DNA-bases Thym, Gua, Adn, Cyt and 5-mCyt. Figure 1(b) shows the chromatogram of the DNA hydrolysate from HL-60 control cells. Ion-exchange

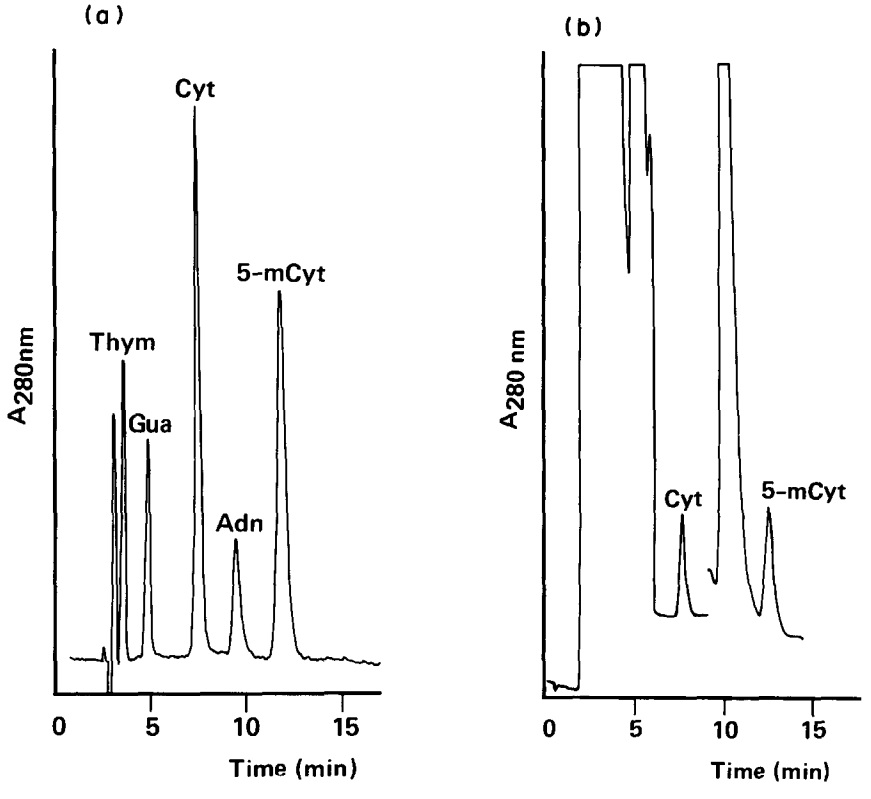

Fig. 1. Elution profile of the DNA bases. (a) Standard mixture containing 5 μM each of thymine (Thym), guanine (Gua), cytosine (Cyt), adenine (Adn) and 5-methylcytosine (5-mCyt). (b) DNA hydrolysate from control HL-60 cells. The sensitivity of detection was increased by a factor of 32 after 9 min. Chromatographic conditions: Partisil PXS 10/25 SCX column eluted with 30 mM ammonium formate, pH 3.5 and 3% methanol at a flow rate of 1.3 ml/min.

chromatography was effective in separating the five DNA bases and minimum detection level of 5-mCyt was less than 5×10^{-12} mole of 5-mCyt injected. Both figures illustrate the high resolution by the method of 5-mCyt and Cyt from other bases and from impurities in the DNA extract. The method showed a high degree of reproducibility with a coefficient of variation of 3.2% for both within, and between, day runs.

The cell-doubling time (Table 1) is shorter (18 vs 40 hr), and the percentage of 5-mCyt in control HL-60 cells (Table 2) is higher (5.3 ± 0.3% vs 3.94 ± 0.08%) than previously reported [11].

Cell counts of control and drug-treated cells after 36 hr (Table 1), indicate that no DNA-synthesis took place when cells were treated with 50 μM c³Ado for 36 hr; whereas with 25 μM c³Ado and 25 μM c³Ari, the cells have proliferated (1.6 and 2.2 times the initial cell concentration, respectively).

Table 2 shows the content of 5-mCyt in the DNA of control, c³Ado- and c³Ari-treated HL-60 cells. Since the cell counts indicate no cell proliferation, it is uncertain whether the data obtained for 50 μM c³Ado (% 5-mCyt = 5.5 ± 0.5) gives a basis for evaluation of the effect on DNA methylation. After treatment with 25 μM c³Ado and c³Ari, the percentage of 5-mCyt was 5.09 ± 0.15 and 5.40 ± 0.26, respectively. In untreated cells the percentage was 5.3 ± 0.3, which was not significantly different from the values obtained for drug-exposed cells.

We have previously reported that 5 μM c³Ari reduced the incorporation of [³H]methyl from labelled methionine into 5-mCyt of HL-60 cell DNA, and that no difference was noted in the overall levels of 5-mCyt measured by HPLC and UV detection [11]. Since we did not include in our experiments correction for rate of DNA synthesis, the reduction in incorporation could be explained by a lower level of maintenance methylation reflecting the slower rate of proliferation observed in c³Ari-treated cells compared to control cells [11]. Correction for rate of DNA synthesis has traditionally been carried out by measuring incorporation of radioactively labelled thymidine. This technique is not, however, without severe shortcomings [23], and adenosine analogues may affect cellular pools of nucleotides [24]. Therefore we have assessed overall DNA methylation by a sensitive, non-radioactive assay.

Table 1. Effect of c³Ari and c³Ado on the growth of HL-60 cells*

	Control	c³Ari (25 μM)	c³Ado (25 μM)	c³Ado (50 μM)
0 hr	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
36 hr	3.7 ± 0.5	1.9 ± 0.3	2.6 ± 0.3	0.9 ± 0.13

* Cells 10^5/ml. Three experiments, counted in duplicate.

Table 2. Effect of c³Ari (25 μM) and c³Ado (25 μM, 50 μM) on the 5-mCyt ratio in HL-60 cells after 36 hr*

	% 5-mCyt/5-mCyt + Cyt	Rel. SD
Control	5.3 ± 0.3*	6%
c³Ari (25 μM)	5.40 ± 0.26	5%
c³Ado (25 μM)	5.09 ± 0.15	3%
c³Ado (50 μM)	5.5 ± 0.5	9%

* Mean ± SD for three experiments, two analysed in triplicate, one in quadruplicate.

* Data not shown.

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c³Ado and c³Ari differ in a number of important aspects [9, 12–17]. Previous experiments have shown that treatment with 25 μM of the analogues for 2 cell doublings in HL-60 cells caused a change in the levels of the transmethylation metabolites AdoHcy and AdoMet only for c³Ari [10, 11]; and a pronounced and a minor perturbation of the cell cycle for both c³Ari [17] and c³Ado*, respectively. The lack of change in the level of 5-mCyt observed after treatment with c³Ari could be due to intracellular compartmentalization of AdoHcy [25, 26]. Only separate intracellular pools may be available for some methyltransferases, and thus the response of an AdoMet-dependent methylation reaction to changes in AdoMet/AdoHcy may be modulated [25].

However, an alternative interpretation is provided by the investigations of Liteplo [27]. Pronounced effects upon intracellular ratios of AdoMet/AdoHcy in a L3 murine tumor cell line and in B16 murine melanoma cells were revealed after 24 hr treatment with 50 μM c³Ari or c³Ado, respectively, while no significant influence on the levels of DNA methylation were observed. The data indicated that the level of DNA methylation was relatively insensitive to changes in the ratio of AdoMet/AdoHcy.

In summary, the present study revealed no significant changes in overall DNA methylation of HL-60 cells treated with 3-deaza-(±)-aristeromycin or 3-deazaadenosine in doses that cause cell cycle perturbation, cytostatis and differentiation.

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