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# The effects of cladribine and fludarabine on DNA methylation in K562 cells

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**Abstract**

The effects of the antileukemic adenosine analogues, 2-chloro-2′-deoxyadenosine (cladribine) and 9-β-D-arabinosyl-2-fluoroadenine (fludarabine), on DNA methylation were studied in a cell line K562. It was previously found that both drugs inactivated SAH hydrolase, an enzyme which participates in the ''active methyl'' cycle. The study examined the effects of these drugs on three aspects of DNA methylation: (i) activity of endogenous C-5 DNA methyltransferase; (ii) capacity of genomic DNA (gDNA) to accept methyl groups, transferred from *S*-adenosylmethionine by the bacterial methyltransferase, *Sss*I; (iii) estimation of changes of methylated cytosine levels in gDNA, using methylation-dependent restriction analysis. Cladribine and fludarabine inhibited C-5 DNA methyltransferase, with $ED_{50}$ values of 3.5 and 47.0 μM, respectively, after 24 hr cell growth in the presence of the drugs. After 48 hr growth of cells with cladribine (0.1 μM) or fludarabine (3 μM), the capacity of DNA to accept methyl groups, in the presence of exogenous bacterial *Sss*I methylase, increased by approximately 1.8 and 1.6 times, respectively, compared to control DNA. Digestion of gDNA with endonucleases *Hpa*II and *Bss*HII followed by *Sss*I DNA methylation, indicated that cladribine (0.1 μM) reduced the level of methylated cytosines in both CpG islands and CCGG sequences, sensitive to *Hpa*II restriction enzyme. Inhibition of DNA methylation by fludarabine was observed mainly in CpG dinucleotide located within sequences sensitive to *Hpa*II. The perturbation of DNA methylation was considered as a complex process. Our findings for cladribine and fludarabine should be regarded as an extra element of their antileukemic efficacy.


*Keywords:* DNA methylation; Cladribine; Fludarabine; Decitabine

## 1. Introduction

Methylation as a post-replicative modification of DNA is one of the important elements of epigenetic mechanisms which regulate gene expression, cell differentiation and cellular transformation, particularly during tissue carcinogenesis [1–3]. We have previously undertaken investigations of abnormal DNA methylation observed in cancer cells, with respect to modification of this process by drugs [4–6]. Three components of methylation, gDNA, *S*-adenosylmethionine (SAM) which is the methyl donor compound, and C-5 DNA methyltransferase (C-5 MT-ase, EC 2.1.1.37), can be targets for pharmacological intervention [7]. Attention was focused on compounds which are either analogues of SAM or can indirectly modulate the endogenous SAM pool through inactivation of *S*-adenosylhomocysteine (SAH) hydrolase [8–10]. SAH hydrolase participates in the ''active methyl'' cycle, and the enzyme plays an important regulatory role in this cycle. Previous studies have confirmed that some antiviral adenosine analogues inhibit SAH hydrolase [8], and hence may act as modulators of DNA methylation [7,11,12]. These studies have suggested that the differentiation induced in myeloid leukemic cell lines by the deoxyadenosine analogues, neplanocin A, pentostatin and vidarabine, in combination with all-*trans* retinoic acid or 1α,25-dihydrovitamin $D_3$, act through inhibition of SAH hydrolase activity and consequent perturbation of SAM-dependent methylation reactions [12]. Although a causal relationship between SAH hydrolase inhibition and altered DNA methylation has been suggested by different authors [9–12], however, there is little experimental evidence to support this hypothesis.

The adenosine analogues, cladribine and fludarabine, of therapeutic benefit in a variety of lymphoproliferative

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*Abbreviations:* SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; C-5 MT-ase, C-5 DNA methyltransferase; gDNA, genomic DNA; cladribine, 2-chloro-2′-deoxyadenosine; fludarabine, 9-β-D-arabinosyl-2-fluoroadenine; decitabine, 5-aza-2′deoxycytidine; $ED_{50}$, effective dose.

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disorders, are also used in the treatment of indolent lymphoid malignancies [13–15]. The efficacy of these drugs results from inhibition of DNA synthesis by their nucleotide derivatives, leading to DNA fragmentation at internucleosomal sites [16]. In early clinical studies we observed decreased SAH hydrolase activity in erythrocytes of patients treated with cladribine [17]. Our *in vitro* studies with the murine leukemic cell line L1210 and human stimulated T cells incubated with cladribine (0.1 μM) and fludarabine (10 μM) showed marked inhibition of SAH hydrolase as well as C-5 MT-ase activity after 72 hr [18,19]. Moreover, methylation-dependent restriction analysis of gDNA isolated from L1210 cells treated with fludarabine, showed decreased methylated cytosine levels [20].

The aim of the present studies is to examine the inhibitory effects of cladribine and fludarabine on the activity of C-5 MT-ase and its relation to changes in the methylation status of CpG sequences in DNA of the human erythroleukemic cell line K562. DNA methylation-dependent restriction analysis, by the specific endonucleases *Hpa*II and *Bss*HII which digest DNA containing unmethylated cytosine only, were used to estimate changes of DNA methylation level. These results were compared to DNA methylation changes induced by decitabine (5-aza-2′-deoxycytidine), a potent inactivator of C-5 MT-ase.

These investigations show that the perturbation of DNA methylation is implicated in the molecular mechanisms of cytotoxicity of cladribine and fludarabine. Incubation with either leads to inhibition of C-5 MT-ase activity and a decrease in methylated cytosines in CpG sequences. The results indicate that hypomethylation is non-random and affects mainly CpG rich islands or CCGG sequences. The alteration of DNA methylation may be an important finding which may point to a hitherto unrecognised therapeutic mechanism against haematological malignancies in which epigenetic gene silencing is a feature.

## 2. Materials and methods

### 2.1. Cell culture and DNA purification

K562 cells were cultured in 6-well plates for 0, 1, 2 or 3 days in RPMI 1640 medium supplemented with 10% foetal bovine serum and gentamicin, in the absence or presence of nucleoside analogues. The total number of viable cells was determined by trypan blue staining and by tetrazolium salt (XTT) reduction [21].

To purify gDNA, cells were washed twice in ice-cold PBS, then resuspended in lysis buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, 0.5% (w/v) SDS, proteinase K 0.2 mg/mL) and incubated at 37° for 20 hr. DNA was then extracted with phenol–chloroform–isoamyl alcohol (Sigma–Aldrich) and precipitated with ethanol. Purified gDNA was used for both the methylation assay and methylation-dependent restriction endonucleases assay.

### 2.2. Methylation assay

Endogenous C-5 MT-ase activity was analysed strictly according to the method of Issa *et al*. [22], in which methyl groups were transferred from [methyl-$^3$H]SAM to cytosine of the acceptor groups poly[dI-dC]·poly[dI-dC]. Homogenates of K562 cells, in lysis buffer, were used as a source of enzyme. The reaction mixture contained: cell homogenates (5 μg protein), poly[dI-dC]·poly[dI-dC] (0.5 or 0.25 μg) and 3 μCi of [methyl-$^3$H]SAM in a total volume of 20 μL, was incubated at 37° for 2 hr. The reaction was stopped by adding 300 μL buffer which enables isolation and purification of DNA, followed by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. RNA was removed by incubation with alkali [22] and purified gDNA was spotted onto Whatman GF/C filter discs. These were washed with 5% (v/v) trichloroacetic acid followed by 70% (v/v) ethanol and counted in the scintillation counter (1600 TR Packard Instrument Comp.). Determination of C-5 MT-ase activity in K562 cell homogenates, from cells cultured in the presence/absence of the analogues, was made at 24-hr intervals, and used to estimation $ED_{50}$ value (i.e. effective dose of drug which reduces C-5 MT-ase activity to 50% of control cell homogenates). $ED_{50}$ values were calculated from Dixon plots, in which the reciprocal of the velocity is a function of inhibitor concentration, at two fixed substrate concentrations of poly[dI-dC]·poly[dI-dC], 0.5 and 0.25 μg, respectively [23]. Protein was measured by Sedmak's method with Coomassie brilliant blue reagent [24].

### 2.3. DNA methyl-accepting capacity

The capacity of gDNA to accept methyl groups in the presence of exogenous bacterial *Sss*I methylase is inversely proportional to the initial DNA methylation status. Using the method of Pogribny *et al*. [25], purified gDNA (0.5 μg), bacterial *Sss*I methylase (2 U) and [methyl-$^3$H]SAM (3 μCi per sample) were incubated in buffer containing 50 mM NaCl, 10 mM Tris–HCl, pH 8.0, 10 mM EDTA (final volume 25 μL) for 2 hr at 37°. The reaction was stopped by placing tubes on ice. Then 10 μL aliquots from each reaction mixture were applied onto GF/C filter discs, washed with 5% trichloroacetic acid followed by 70% (v/v) ethanol and processed for the scintillation counting. The results were expressed as [methyl-$^3$H] (cpm) incorporated into 0.5 μg DNA.

### 2.4. Estimating DNA methyl-accepting capacity by restriction analysis

The method of Pogribny *et al*. [25], modified in our laboratory, was used to estimate the methyl-accepting capacity of gDNA measured as the loss of unmethylated cytosine after digestion with methylation-sensitive endonucleases. Two restriction endonucleases, which recognise

unmethylated cytosine, were used: *Hpa*II, specific for C$^{\downarrow}$CGG sequences and *Bss*HII which digests CpG rich islands (C$^{\downarrow}$GCGCG). Purified gDNA (5 μg), isolated from K562 cells (grown with or without the nucleoside analogues), was digested overnight with excess *Hpa*II or *Bss*HII according to the manufacturer's protocol. Samples of undigested gDNA, isolated from the cells grown with the drug as well as samples of digested gDNA of control cells cultured with no nucleoside, served as controls. Undigested and digested gDNA was treated with phenol–chloroform–isoamyl alcohol and precipitated with ethanol. Purified gDNA (0.5 μg) was used for DNA methyl-accepting capacity assay according to the earlier protocol.

### 2.5. Chemicals

Cladribine, fludarabine, decitabine, poly[dI-dC]·poly-[dI-dC] and phenol–chloroform–isoamyl alcohol were purchased from Sigma Aldrich Chemical Co; restriction endonucleases *Hpa*II and *Bss*HII were products of Promega; *Sss*I methylase (CpG methylase) was the product of New England BioLabs; [methyl-$^{3}$H]SAM was from Amersham Life Science.

## 3. Results

### 3.1. K562 cells and cytotoxicity of adenosine analogues

Fig. 1 shows the effects of cladribine (A), fludarabine (B) and decitabine (C), on K562 cell growth at 24 and 48 hr. Expressing the number of viable cells as a percentage of the number of untreated control cells and plotting these against drug concentration allows the calculation of the $IC_{50}$ values (i.e. inhibitory concentration of the drug required to reduce K562 cell growth by 50% of control) shown in Table 1. The $IC_{50}$ value for cladribine was below 100 nM, and over 60-fold lower than for fludarabine. The results are consistent with earlier studies which showed *in vitro* cladribine and fludarabine cytotoxicity against the murine leukemic cell line L1210 [20], as well as against lymphoma and myeloid cell line models [13].

Table 1
Values of $ED_{50}$ and $IC_{50}$

| Drugs | $ED_{50}$ (μM) | $IC_{50}$ (μM) | |
|---|---|---|---|
| | | 24 hr | 48 hr |
| Cladribine | 3.5 ± 0.3 | 0.09 | 0.07 |
| Fludarabine | 47.0 ± 2.1 | 5.5 | 2.3 |
| Decitabine | 0.6 ± 0.05 | 1.9 | 1.8 |

$ED_{50}$ represents the drug concentration which reduces activity of C-5 MT-ase to 50% after 24 hr growth of K562 cells in the presence of the drugs. Each value (in μM) represents the mean ± SEM of three experiments. $IC_{50}$ represents the drug concentration resulting in a 50% inhibition of cell growth after 24 or 48 hr cell growth in the presence of the drugs.

### 3.2. C-5 MT-ase activity

The change of methylation activity by C-5 MT-ase in homogenates of control K562 cells (i.e. without inhibitors) during 72 hr cells growth is shown in Fig. 2. The maximum

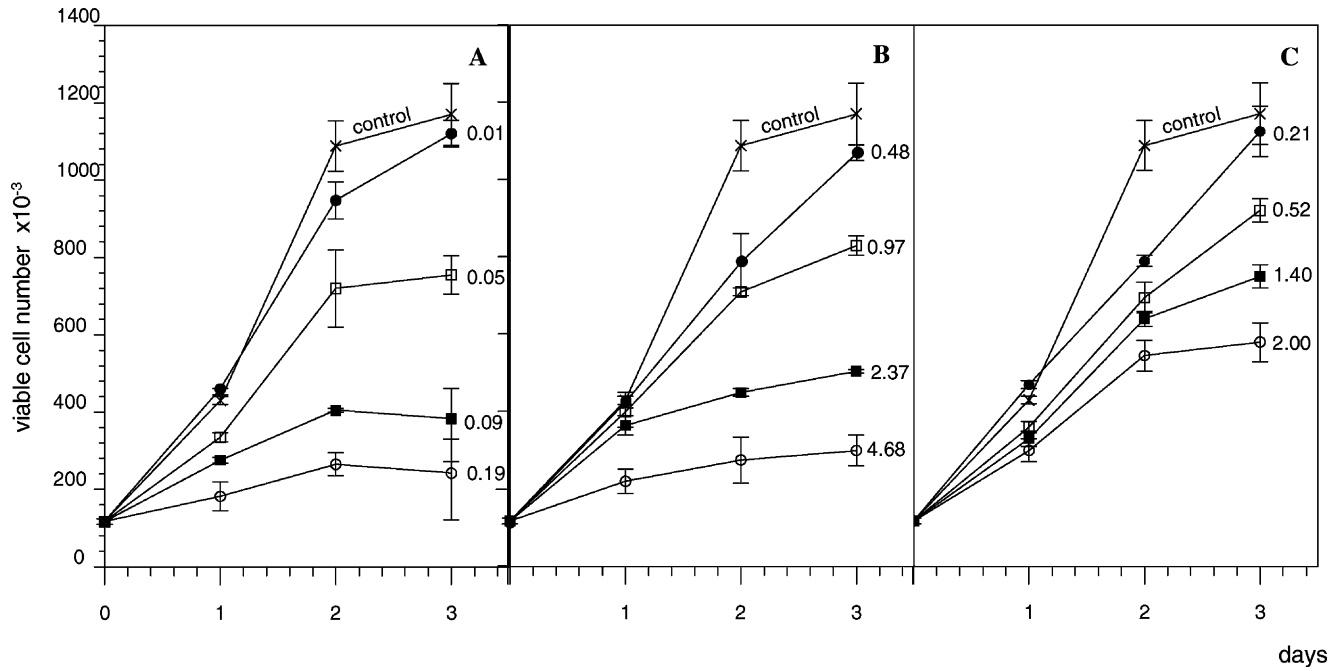


Fig. 1. Viability of K562 cells in the presence of cladribine (A), fludarabine (B) and decitabine (C). The cells were grown at the indicated drugs concentration for 24, 48 and 72 hr and total viable cells were counted. Each cell count represents the mean ± SD for three independent assays. The drug concentrations are shown in micromoles.

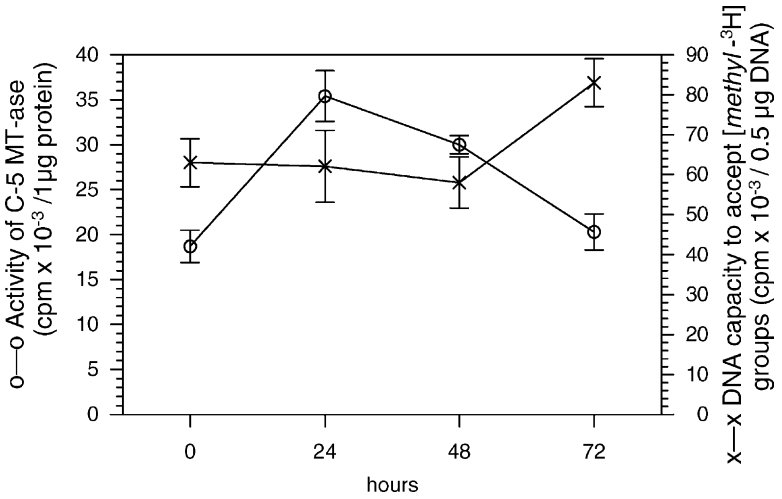


Fig. 2. Activity of C-5 MT-ase and gDNA capacity to accept methyl groups during 72 hr growth of control K562 cell line. Activity of C-5 MT-ase is expressed as the amount of incorporated [methyl-$^3$H] group into poly[dI-dC]·poly[dI-dC] by enzyme, present in cell homogenate (cpm × $10^3$ per 1 μg protein). DNA methyl-accepting capacity is expressed as the amount of [methyl-$^3$H] group incorporated into gDNA (cpm × $10^3$ per 0.5 μg DNA). The data are presented as the mean ± SD for four independent experiments with three simultaneous samples per each.

methylation activity was at 24 hr, which was similar to previous *in vitro* studies measuring C-5 MT-ase in L1210 cell homogenates [18] and NIH 3T3 cells [26]. Table 1 shows the $ED_{50}$ values of the drugs inhibiting endogenous C-5 MT-ase of K562 cell homogenates at 24. The $ED_{50}$ values for cladribine, fludarabine and decitabine were 3.5, 47 and 0.6 μM, respectively. This indicates that, in the case of cladribine and fludarabine, inhibition of C-5 MT-ase is achieved at concentration which completely block proliferation of K562 cells. In contrast, the decitabine concentration to reduce cell growth to 50% of untreated cells was three time higher than the $ED_{50}$ for C-5 MT-ase activity reduction.

### 3.3. Methyl-accepting capacity of gDNA

Fig. 2 shows that the amount of gDNA methylation sites, available for the *Sss*I methylase over 72 hr growth of control K562 cells, declined insignificantly up to 48 hr, after which there was an increase. The incorporation of methyl groups and the initial methylation status of gDNA are inversely proportional. The observed decrease of methylation level of gDNA of control cells cultured over 48 hr, which corresponds with the increase of incorporated methyl groups by bacterial methylase, may thus result from slowing of cell growth in impoverished culture medium. Fig. 3 shows the effects of the cladribine (0.1 μM), fludar-

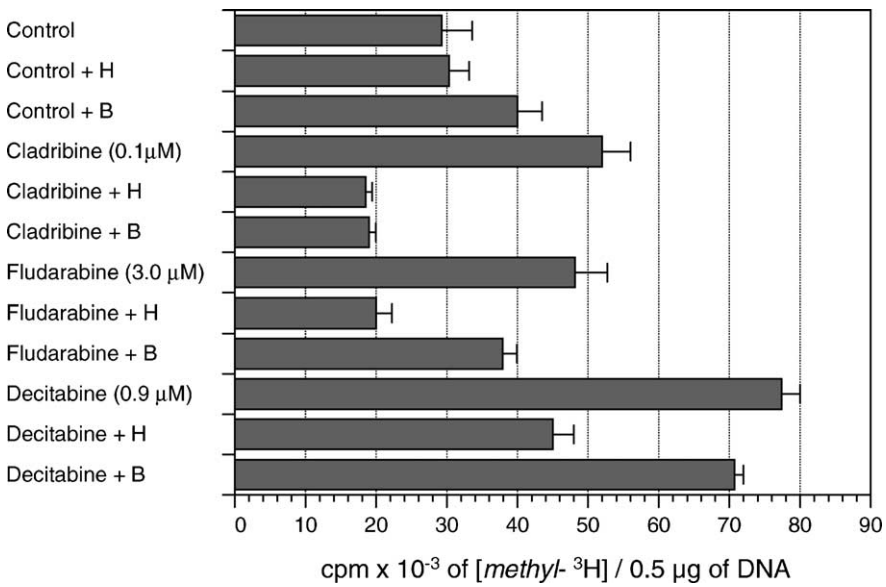


Fig. 3. Capacity of gDNA, undigested and digested with *Hpa*II (H) or *Bss*HII (B) endonucleases, to accept methyl groups after 48 hr cell growth in the presence of the drugs. Each value, expressing the number of incorporated methyl groups into DNA (cpm × $10^3$ [methyl-$^3$H] per 0.5 μg DNA), represents the mean ± SD for six experiments with three simultaneous samples per each.

abine (3.0 μM) and decitabine (0.9 μM) on the methyl-accepting capacity of gDNA prepared from cells cultured for 48 hr. The drug concentrations used were close to the $IC_{50}$ values. This allowed the K562 cell culture to remain viable while simultaneously inhibiting cell proliferation. gDNA isolated from cells exposed to cladribine or fludarabine exhibited (respectively) 1.8 and 1.6 times higher levels of methyl acceptance than gDNA of untreated cells. The effect of decitabine was the most potent, the gDNA methyl acceptance being 2.6 times higher than gDNA from the control cells.

### 3.4. DNA methylation-dependent restriction analysis

Digestion with *Hpa*II or *Bss*HII restriction endonucleases results in the destruction and hence loss of potential methylation sites. Fig. 3 shows that a decline in methylation of gDNA follows digestion with these restriction enzymes. The greatest decrease in potential methylation sites in gDNA was noted in the case of decitabine where digestion with *Hpa*II (digests the sequence CCGG) resulted in a 42% reduction in methylation compared to undigested DNA derived from decitabine-treated cells. However, decitabine inhibition of cytosine methylation in CpG islands was much less; approximately 8% of CpG sequences were eliminated by digestion with *Bss*HII (digests the sequence CGCGCG), compared to undigested DNA derived from decitabine-treated cells. About 50% of cytosine methylated by *Sss*I occurred in the single CpG dinucleotide situated within the DNA sequences unrecognised by either of the used restriction enzymes.

In the case of cells growing with cladribine, gDNA methylation was 27% lower than for cells grown in the presence of decitabine. Restriction analysis with endonucleases indicated that cladribine affects methylation at specific sites, cytosines in sequences sensitive to *Hpa*II and in CpG islands were affected.

The effect of fludarabine on gDNA methylation differed from cladribine, effecting mainly CCGG sequences sensitive to *Hpa*II. In the case of fludarabine, the decline in methylated cytosines, located in CpG islands was 2-fold lower than that with cladribine, and 2-fold higher than that caused by decitabine (Fig. 3).

## 4. Discussion

Our experiments demonstrated that cladribine and fludarabine inhibit methylation of cellular DNA. This effect may contribute to the anticarcinogenic activity of these drugs. It must be remembered that the mechanisms of cytotoxicity of these adenosine analogues involve mainly inhibition of DNA synthesis by their nucleotide metabolites [13–15]. Adenosine analogues may also inhibit SAH hydrolase, as demonstrated using in erythrocyte lysates [27], L1210 cell homogenates [18] and human liver [8].

The current studies were aimed at verifying whether adenosine analogues which inactivate SAH hydrolase can directly cause an alteration of DNA methylation (as measured by C-5 MT-ase activity and gDNA cytosine methyl acceptance). We also tried to answer two questions: (i) Is there inhibition of C-5 MT-ase under physiological conditions? (i.e. with drug levels expected during therapy); and (ii) Can these adenosine analogues cause a decrease of methylated cytosines in gDNA, at concentrations which do not inhibit C-5 MT-ase?

C-5 MT-ase in K562 cells was inhibited to a similar degree by both adenosine analogues, but at different concentrations ($ED_{50}$). It is probable that inhibition of C-5 MT-ase is the result of SAH hydrolase inactivation and disturbance of the ''active methyl'' cycle. After intravenous administration of cladribine, the mean maximum plasma concentration is approximately 0.2 μM while the intracellular cladribine nucleotides reach approximately 11.0 μM [13], and based on the pharmacodynamic properties of fludarabine, similar results would be expected [15]. Once inside the cells the nucleoside analogue is immediately phosphorylated to form nucleotides, so intracellular nucleoside concentrations would not be expected to reach levels which exceed the $ED_{50}$ for C-5 MT-ase activity reduction.

On the other hand, the decrease of C-5 MT-ase activity may be due to down-regulation of gene expression. The drug nucleotides may achieve a cellular response by either inhibition of DNA synthesis [13,14] or by slowing down DNA replication [28], as well as ''switching'' cellular signals that regulate expression of the C-5 MT-ase gene [29]. The latter hypothesis, that alteration of DNA methylation is a consequence of cellular changes caused by drug nucleotides acting on gene expression, seems consistent with other authors' suggestions. Thus, adenosine nucleosides which are resistant to adenosine deaminase or are its inhibitors must first be phosphorylated in order to affect DNA methylation [30]. In addition, a report on cells treated with deoxyadenosine analogues in combination with non-steroid compounds, i.e. all *trans*-retinoic acid or Vitamin $D_3$, indicated that the disturbance of SAM-dependent methylation reactions is associated with induction of cell differentiation due to the combined treatment [12].

The results of experiments with bacterial DNA methylase provided clear evidence that the presence of either cladribine or fludarabine in cell growth medium leads to diminution of levels of methylated CpG sequences. The results, shown in Fig. 3, are consistent with our previous polyacrylamide gel (PAGE) analyses of DNA fragments end-labelled with [$\alpha$-$^{32}$P]dCTP after digestion with *Hpa*II, which showed a decrease in the methylated cytosine level in DNA isolated from cell lines L1210 and K562 grown in cladribine [31] or fludarabine [20]. However, extensive fragmentation of gDNA isolated from L1210 cells grown with cladribine (3 μM) confused interpretation of these results. In the case of decitabine, the present results are

consistent with the well-documented inhibitory effect of the drug on gene methylation and reactivation of silenced genes [32–34]. The decrease of methylated cytosines in gDNA probably cannot be attributed to changes of DNA conformation or properties after incorporation of the nucleotide analogues, because the same cellular DNA showed a high capacity to accept methyl groups in the presence of bacterial *Sss*I methylase.

The assays which combined the gDNA capacity to accept methyl groups and digestion by specific endonucleases, enabled the characterisation of the CpG sequences hypomethylated in cells treated with the nucleoside analogues. It appeared that action of decitabine was non-specific to cytosine localisation, because we noted decrease of methylated cytosines in isolated CpG sequences, in CpG islands and in CCGG sequences sensitive to *Hpa*II. In contrast, cladribine effects on gDNA methylation was limited mainly to cytosines in CpG rich islands and CCGG sequences. The inhibitory effect of fludarabine on DNA methylation is less specific and lower in relation to CpG islands than that of cladribine.

We interpret these results as follows. Decitabine acted as an irreversible inactivator of C-5 MT-ase; in cells, the drug was bound to the majority of the methyltransferase, hindering both maintenance and *de novo* gDNA methylation. It should be noted firstly that the majority of CpG islands are found in promoters regions of genes [35]. Secondly, *de novo* methylation of CpG islands in genes involved in cell differentiation is associated with intensive tumour cell growth as well as with cell ageing [36]. Thus, decitabine, by binding to C-5 MT-ase inactivates most methylation reactions, independent of type—maintenance or *de novo* methylation. The predicted non-specific action of decitabine thus seems to be consistent with *in vitro* studies which showed decitabine does not cause demethylation of gDNA. This may result from insufficient drug concentration to bind all of the C-5 MT-ase [36].

The action of adenosine analogues seems to be different than decitabine. Because of the indirect nature of cladribine or fludarabine effects on C-5 MT-ase activity (i.e. *via* disturbance of ''active methyl'' cycle, or decrease in gDNA synthesis), we speculate that when the growth of K562 cells is inhibited by these drugs, the *de novo* methylation of CpG islands and CCGG sequences is either diminished or is not initiated. Further studies of the role of adenosine analogues in altering methylated cytosines levels, in promoter regions of certain suppressor genes, are currently being undertaken.

In summary, the results clearly demonstrate that the antileukemic adenosine analogues, cladribine and fludarabine, affect DNA methylation in K562 cells. The disturbance can result from a decrease of both C-5 MT-ase activity and the level of methylated CpG sequences in gDNA. The latter effect is particularly important in the case of cladribine which leads to a substantial drop in methylated cytosine in CpG rich islands in our cell culture model. This fact may be significant for therapy of cancers that are associated with gene silencing due to hypermethylation of their regulatory regions. The reduction of DNA methylation by cladribine and fludarabine is a new, hitherto unreported element of their cytotoxic mechanisms and it should be taken into consideration in understanding the therapeutic and antileukemic action of these drugs.

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