AZT-induced hypermethylation of human thymidine kinase gene in the absence of total DNA hypermethylation

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Abstract Genome-wide DNA hypermethylation induced by 3′-azido-3′-deoxythymidine (AZT) has been suggested to be involved in the development of AZT resistance. We used a CD4 T-lymphoblastoid CEM line and its AZT-resistant MT500 variant with reduced thymidine kinase activity. Evaluation of total DNA methylation, after AZT treatment, failed to show an increase in the 5-methylcytosine level in both parental and AZT-resistant cells. The effect was instead observed at a more specific gene level, on the three *Hpa*II sites present in exon 1 of the human thymidine kinase gene. These results suggest that AZT treatment can induce site-specific hypermethylation, even in the absence of a more general DNA hypermethylating effect.

Key words: 3′-Azido-3′-deoxythymidine; Drug resistance; DNA methylation; Thymidine kinase gene; *Hpa*II/PCR assay

1. Introduction

The clinical benefits of 3′-azido-3′-deoxythymidine (AZT) treatment in AIDS patients are often reduced, especially after prolonged therapy, by the appearance of drug resistance [1–3]. Mutations in the reverse transcriptase (RT) gene of human immunodeficiency virus (HIV-1) [4] and cell-mediated mechanisms [5,6] have both been suggested as possible factors underlying the development of clinical resistance. Antiviral AZT efficacy depends on its initial transformation to AZT monophosphate (AZT-MP) by cellular thymidine kinase (TK), followed by further phosphorylation steps to AZT diphosphate and AZT triphosphate (AZT-TP), catalyzed by thymidylate kinase and pyrimidine nucleoside diphosphate kinase, respectively. The last product inhibits HIV-1 replication by interrupting the viral DNA chain elongation [7]. Low levels of TK activity could therefore induce a decrease in the amount of AZT-MP available for AZT-TP synthesis, thus reducing the therapeutic efficacy of AZT.

Enzymatic DNA methylation at CpG dinucleotides is considered to be one of the mechanisms involved in the control of gene expression in eukaryotic cells [8]. Consequently, a genome-wide DNA hypermethylation has been suggested as a major mechanism in the development of resistance by inhibition of transcription of the TK gene [5,9], although only under conditions severely affecting cell viability. Moreover, only scanty information is available about the methylation status of specific sites of the TK gene after exposure to AZT.

This paper reports data concerning the effects of AZT on human CD4 T-lymphoblastoid CEM cell line and on its derived AZT-resistant cell variant, where a reduced TK activity has been previously reported [10]. In particular, we investigated the effects of AZT on the total DNA methylation as well as on specific methylation sites present in the TK gene. A hypermethylating effect was found, in the absence of both overall DNA hypermethylation and of any great loss of cell viability, at the analyzed TK sites, after AZT treatment of CEM cells and of its derived AZT-resistant cell variant. This phenomenon, which was more marked in the resistant cells, could be the molecular basis for the transcriptional inactivation of the TK gene.

2. Materials and methods

2.1. Cell cultures

The human CD4 T-lymphoblastoid CEM line was kindly provided by Dr. W.T. Beck (Memphis, TN, USA). The AZT-resistant cell line variant was obtained by exposure of the parental CEM cells to increasing concentrations of AZT (Sigma Chemical Co., St. Louis, MO, USA). To this purpose, CEM cells were initially propagated in the presence of 1 μM AZT. 2-fold increasing concentrations of AZT were then added to the culture medium and the cells were allowed to grow until they reached a cell density of 10^6 cells/ml. After approx. 2 months a stable CEM cell variant actively proliferating in the presence of 32 μM AZT was isolated. An additional 2 months of drug selection produced an AZT cell variant resistant to 2 mM AZT, named MT500. The cells were maintained in RPMI-1640 medium supplemented with 10 mM HEPES, 2×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 10% fetal calf serum (FCS) and antibiotics. MT500 were routinely cultured in the presence of AZT (2 mM). Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay [10].

2.2. Determination of total DNA methylation

Cells were exposed to various AZT concentrations for 48 h in the presence of 2 μCi/ml [6-^{3}H]uridine (Du Pont-New England Nuclear, Bad Homburg, Germany, 24 Ci/mmol). 5-Methylcytosine content was determined by HPLC analysis, after isolation and acid hydrolysis of DNA, as previously described [11].

2.3. DNA extraction and restriction endonuclease treatment

Genomic DNA, extracted from the various samples using a standard procedure [12], was treated separately with the following restriction endonucleases: (i) *Eco*RI, which has no recognition site internal to the amplified fragment; (ii) *Hpa*II, which has three recognition sites internal to the amplified fragment and is methylation sensitive (i.e. it does not cut if the CCGG recognition sequence is methylated at any C); (iii) *Ava*I, which has two recognition sites internal to the amplified product and is methylation insensitive. In each case, 1.5 μg of ge-

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; TK, thymidine kinase; RT, reverse transcriptase; AZT-MP, AZT monophosphate; AZT-TP, AZT triphosphate; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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nomic DNA were digested overnight, at 37°C, with 3 units of enzyme, and then with a further 3 units for an additional 6 h, in a final volume of 40 μl, with the buffer provided by the manufacturer.

2.4. HpaII/PCR assay

For amplification of the human thymidine kinase gene (GenBank, accession numbers M15205, M15206) [13], the following primers were used: forward primer HSTKP3, 5′-GCGGGACCAGGGGCTTACT-GC-3′, which is complementary to exon 1 (nt 451–471); reverse primer HSTKM1, 5′-GGACACAGGCTATCACCACGACC-3′ which is complementary to intron 1 (nt 624–646). This couple of primers was expected to produce, from uncut DNA, an amplified fragment of 196 bp. About 20 pmol of each primer were included in the reaction mixture. PCR was performed on 50 ng of DNA, in a final volume of 50 μl containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% (w/v) gelatin, 200 μM dNTPs with 1 unit of SuperTaq (HT Biotechnology LTD, UK). After an initial denaturation for 3 min at 94°C, a scalar number, from 20 to 35, of cycles (1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C) and a final extension of 5 min at 72°C were performed in a Perkin Elmer 480 Thermal Cycler. By this *Hpa*II/PCR assay [14,15] an amplified product could be obtained only if the DNA fragment to be amplified by the specific couple of primers, failed to be cut by a restriction endonuclease. The analysis of the amplified products obtained from the *Hpa*II-treated samples allows the determination of the methylation status of three CCGG sites in exon 1 of TK gene. The *Eco*RI-treated samples were used as positive controls and the *Ava*I-treated samples as negative ones.

2.5. Gel electrophoresis and analysis of PCR products

The PCR products were evaluated by electrophoresis, in 1.5% agarose gel, of 15 μl of the PCR reaction. Each gel was scanned by a CCD camera and acquired on a Bio Image (Millipore, USA) computerized densitometer. Fragment size and relative abundance (expressed as optical density) were calculated by the system. The amplified products were recovered from the gel using Amicon filters (Micropure 0.22+Microcon 50) and the specificity of the fragments was assessed by restriction analysis. The intensity of amplified products obtained after *Hpa*II treatment of the samples was normalized to the intensity of amplified products obtained after *Eco*RI treatment, both measured densitometrically and expressed as optical density units.

3. Results and discussion

Human lymphoblastoid CEM cells and the AZT-resistant MT500 variant cell line were exposed for 48 h to increasing concentrations of AZT, ranging from 5 μM to 1 mM for the parental drug-sensitive and from 5 to 150 μM for the resistant variant. The incubation was performed in the presence of [^{3}H]uridine in order to measure the percentage of 5-methylcytosine. There was little or no difference, between CEM cells and MT500 resistant cell variant, in total DNA methylation in either the presence or absence of AZT (Table 1). Under these conditions, cell viability of CEM sensitive cells was reduced (after 48 h) to approx. 50%, and to even lower values, after 72 h at the highest drug concentration (Fig. 1).

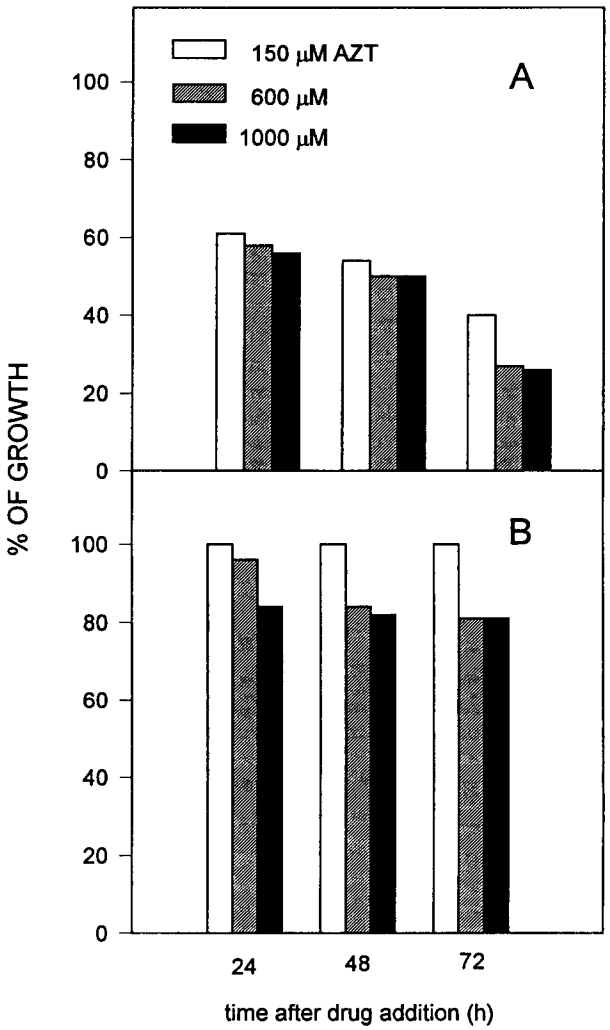

Fig. 1. AZT effect on the growth of CEM (A) and MT500 (B) cells exposed to different AZT concentrations for the indicated times. Cell viability was determined by MTT assay. Percentages are referred to the growth of cells incubated in the absence of AZT. White bars: 150 μM AZT, grey bars: 600 μM AZT, black bars: 1000 μM AZT.

Table 1
Effect of AZT treatment on DNA methylation of CEM and MT500 cells

AZT (μM)	% methylcytosine	
	CEM	MT500
0	3.32 ± 0.08	3.29 ± 0.10
5	3.30 ± 0.10 (99.4%)	3.28 ± 0.15 (100.0%)
75	3.38 ± 0.12 (101.8%)	3.91 ± 0.1 (118.8%)
150	3.40 ± 0.25 (102.4%)	3.21 ± 0.12 (0.98%)
300	3.38 ± 0.15 (101.8%)	N.D.
600	3.16 ± 0.09 (95.2%)	N.D.
1000	3.42 ± 0.34 (103.0%)	N.D.

Data are expressed as the mean of percentages of methylcytosine over total cytosine from three independent experiments ± S.D. In parentheses are reported the percentages with respect to untreated cells. N.D., not determined.

We have also assessed the effect, in CEM cells, of some compounds known to induce, in other cell lines [16], different levels of DNA hypermethylation. As reported in Table 2 only cisplatinum caused, after 48 h, a 143% increase in methylation, while none of the other drugs significantly affected 5-methylcytosine levels.

Considering that HPLC analysis of total DNA methylation levels could be not sensitive enough to detect small variations in the methylation status of specific sites, but that even minor alterations might be highly effective in modulating the expression of genes to which these sites are associated, we have used the *Hpa*II/PCR assay in order to analyze the methylation

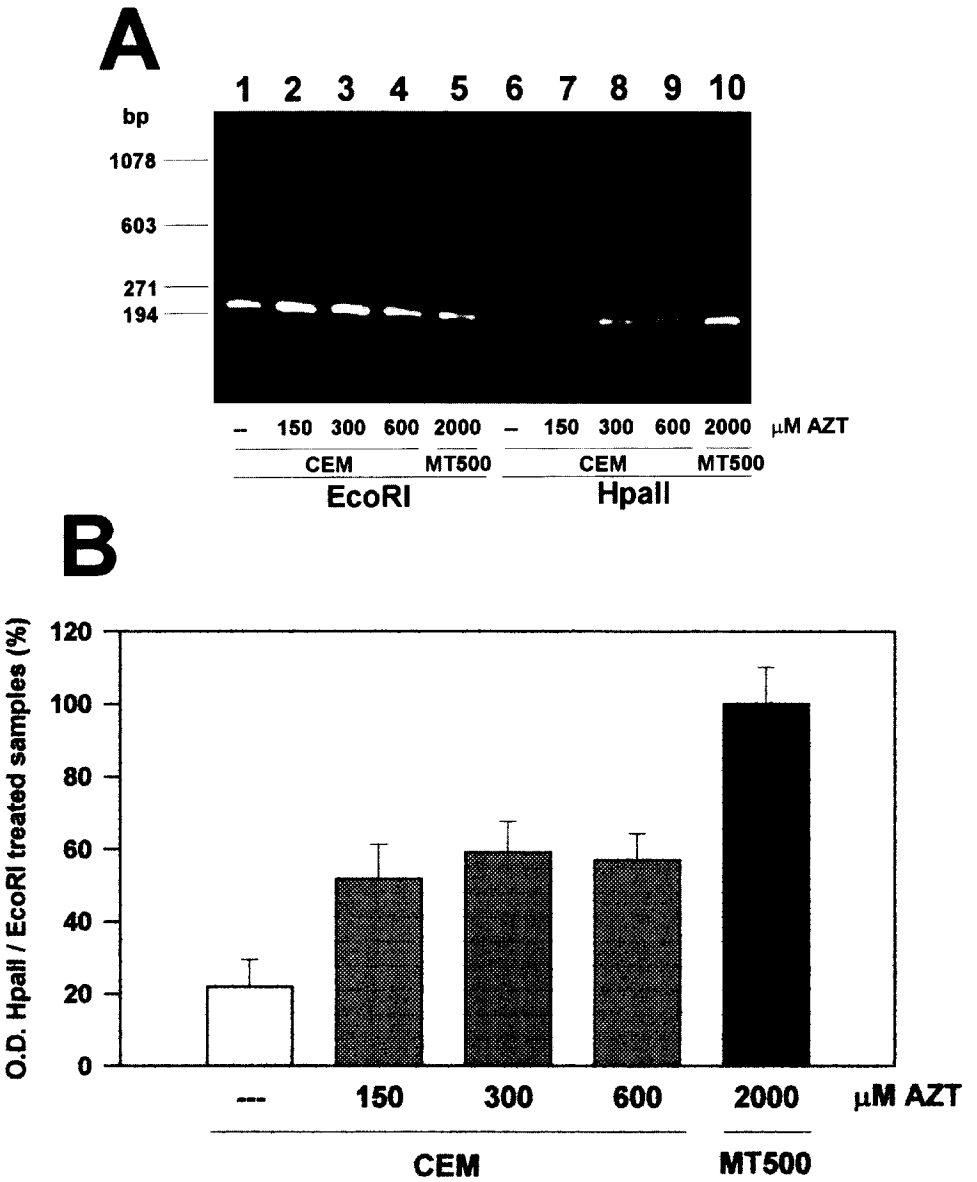

Fig. 2. *Hpa*II/PCR assay performed on the three CCGG sites of exon 1 of human TK gene. (A) Amplified products from *Eco*RI-treated DNAs (lanes 1–5) or from *Hpa*II-treated DNAs (lanes 6–10), extracted from CEM cells not treated (lanes 1,6) or treated with 150 μM (lanes 2,7), 300 μM (lanes 3,8) and 600 μM (lanes 4,9) AZT. In lanes 5 and 10 the results relative to MT500 cell line variant exposed to 2 mM AZT are shown. (B) Semiquantitative output obtained by normalization of the optical density (O.D.) of signals relative to *Hpa*II-treated samples with that of signals relative to *Eco*RI-treated samples. (The value relative to the MT500 cell line variant was set to 100%.)

status of three specific CCGG sites in exon 1 of TK gene. Fig. 2A shows the amplified products obtained after *Hpa*II treatment of DNA extracted from untreated (lane 6) and AZT-treated (lanes 7–9) CEM cells. The amplified product obtained

Table 2
Effect of various compounds on DNA methylation of CEM cells

Compound	Duration of treatment (h)	% methylcytosine
None	24	3.24
	48	3.27
1 mM 5-fluorouracil	24	3.17
	48	3.30
100 μM cisplatinum	24	3.09
	48	4.43
1 mM 1-β-D-arabinofuranosylcytosine	24	3.93
	48	3.12
100 μM 2′,3′-dideoxyinosine	24	3.04
	48	3.18

Data are expressed as in Table 1.

from *Hpa*II-treated DNA extracted from MT500 cells exposed to 2 mM AZT for 48 h is shown in lane 10. Lanes 1–5 show the amplified products obtained from DNA treated with *Eco*RI, which has no recognition site internal to the sequence to be amplified, and is therefore used as a positive internal control of the amplification. It can be noted that the *Eco*RI-treated samples produced similar quantities of amplified products, whereas, among the *Hpa*II-treated samples, the intensity of the amplified product was lowest in the control CEM sample, highest in the MT500 resistant cell line variant and of intermediate level in the AZT-treated CEM samples. The linearity range of the assay was assessed performing amplifications with a scalar number of cycles (from 20 to 35), and we verified that, within the linearity range, the amount of amplified products was proportional to the amount of initial DNA target in the *Eco*RI-treated samples. In the *Hpa*II-treated samples, the level of amplified products was proportional to the initial amount of uncut target, i.e. to the initial amount of methylated sequences (data not shown). It can be concluded that methylation is highest in the resistant MT500 and lowest in the sensitive CEM parental line. AZT-treated CEM cells showed intermediate levels of methylation. No amplified products were present in *Ava*I-treated samples (data not shown). A semiquantitative output can be obtained by referring the optical density of the amplified products relative to the *Hpa*II-treated samples to that of the corresponding *Eco*RI-treated samples (Fig. 2B).

MT500, an AZT-resistant cell line obtained in our laboratory and able to grow in the presence of 2 mM AZT, has been found to have an approx. 20-fold reduction in TK activity [10]. A similar defect in TK activity has been suggested to occur, in other cell lines [5,9], as a consequence of AZT-induced genome-wide DNA hypermethylation. Drug-induced DNA hypermethylation has indeed been observed as part of the response of various cell lines to the cytotoxic effect of several antitumor agents [16]. However, with these compounds and with AZT, the global hypermethylation effect was found only under conditions of marked inhibition of DNA synthesis and 90–100% cell death, determined by concentrations of drugs unsuitable for clinical practice. The conditions and the cell lines we used for AZT treatment, showed instead, even in the parental CEM cells, no severe immediate loss of cell viability, although the changes occurring within the first 48 h of culture will, later on, result in the block of replication and finally in cell death. No genome-wide DNA hypermethylation was found to take place during this time interval. A highly significant alteration in the methylation pattern of exon 1 of the TK gene could instead be detected in CEM cells treated with AZT and even more, always in the absence of a more generalized hypermethylation, in the resistant clone MT500. These results support the hypothesis that a fairly specific hypermethylation of the TK gene is associated with both short-term cell survival in the presence of AZT and with selection of a stable AZT resistance.

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