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ZIDOVUDINE (AZT) RESISTANCE IN H9 CELLS DUE TO DECREASED TK EXPRESSION IS ASSOCIATED WITH HYPERMETHYLATION OF TK GENE

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ABSTRACT

AZT resistant human T-lymphoid H9 cells, deficient in TK gene expression, re-expressed TK mRNA and regained the ability to metabolize AZT by exposure to the demethylation agent azacytidine (AzaCd). Cytotoxic and anti-HIV-1 effects of AZT were increased in H9 AZT resistant cells treated with AzaCd when compared to untreated cells. This leads to the assumption that drug induced DNA hypermethylation was involved in the TK gene-silencing mechanism. Our results suggest approaches using modulation of gene methylation for increasing antiviral efficiency of drugs.

The aim of current treatment strategies in HIV-1 infection includes the suppression of viral replication as strong as possible by using a combination of nucleoside reverse transcriptase inhibitors (NRTIs), non nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) (1). These therapy strategies are associated with reduced morbidity and prolonged life in patients with HIV-1 infection. However, an increasing common problem in the treatment of HIV-1 infected patients is the failure of a drug regimen after a period of success. It has been shown that in many cases this is due to viral resistance, which means that HIV-1 virus mutants evolve and become resistant to drugs (2). Different other factors, such as

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poor tolerability, difficulties with pharmacokinetic of drugs and persistent virus in sanctuary sites as well as development of cellular (pharmacological) resistance of NRTIs and PIs may also influence therapy success (3). Cellular resistance mechanisms are characterized by insufficient intracellular concentration of the active form of drug and result in failure of antiretroviral effects (4). Since NRTIs belong to the class of 2',3'-dideoxynucleosides the efficiency of these substances strongly depends on their intracellular conversion to the corresponding 5'-triphosphate form catalyzed by cellular nucleoside kinases (5). Only in triphosphate form the compounds are potent terminators of the growing viral nucleic acid. Cellular nucleoside kinases which catalyse phosphorylation of deoxynucleosides are cell type specific and cell cycle dependent (6) and represent limiting factors for the effective conversion of the compounds (7). Previously, it has been reported, that continuous treatment of cell lines with AZT or ddC results in lower gene expression and activity or even lack of enzymes responsible for drug activation, which results in loss of antiretroviral efficiency of the drugs in AZT or ddC resistant cells (8,9). The aim of our study was to get more insights in the molecular mechanisms of cellular resistance in an AZT resistant *in vitro* cell system.

Selection of AZT-resistant cell line: AZT-resistant H9 cells were established by continuous cultivation of the cells in IMDM medium supplemented with 10% FCS containing increasing concentrations of AZT (10). The resistant cell subline, grown for more than one year in medium containing 2000 μ M AZT designated as H9^rAZT²⁰⁰⁰, was used in the experiments. **Antiviral agents:** AZT and AzaCd were obtained from Sigma (Deisenhofen, Germany). Drugs were dissolved in dimethylsulfoxide at a concentration of 10 mM and stored at -20°C . **Virus:** Virus stock of HIV-1 laboratory strain HTLV-III_{RF} was obtained from MRC AIDS Reagent Project (Hertfordshire, UK). **Determination of cytotoxicity:** Cytotoxic effects of drugs were determined by MTT assay as described previously (11). **Antiretroviral assay:** Anti-HIV-1 activity of drugs was determined by the reduction of HIV-1 p24 antigen in cell culture supernatant using a sandwich ELISA testsystem (ABBOT). **Determination of gene expression by RT-PCR:** RT-PCR was performed as described previously (11). Following primers were used: TK1: 5'-CAG GAT CCT CGG GTT CGT GAA C-3', TK2: 5'-TAG AAT TCG GCC CTT GCA GGT C-3' (12) (PCR product: 765 bp); TMPK1 5'-GTC TGT TCC TCC AGT TAC AGC T-3', TMPK2 5'-CAG CCT GCA GAT CTC TGC TG-3' (393 bp), NDK1 5'-ATG CAG TGC GGC CTG GTG GG-3', NDK2 5'-GAC CCA GTC ATG AGC ACA AGA C-3', (405 bp), GAPDH1 5'-TGG GGA AGG TGA AGG TCG GA-3', GAPDH2 5'-GAA GGG GTC ATT GAT GGC AA-3' (124 bp). The cycling parameters were: 95°C 120s, 25 cycles of 94°C 30s, 55°C 30s, 72°C 30s, hold 7 min 72°C . The PCR products were separated on a agarose gel and density of PCR products were quantified by EAS (Herolab GmbH, Wiesloch, Germany).

To elucidate the molecular resistance mechanisms induced in H9 cells by continuous AZT treatment the amount of mRNA levels of nucleoside kinases responsible for AZT phosphorylation, such as TK, thymidylate kinase (TMPK) and



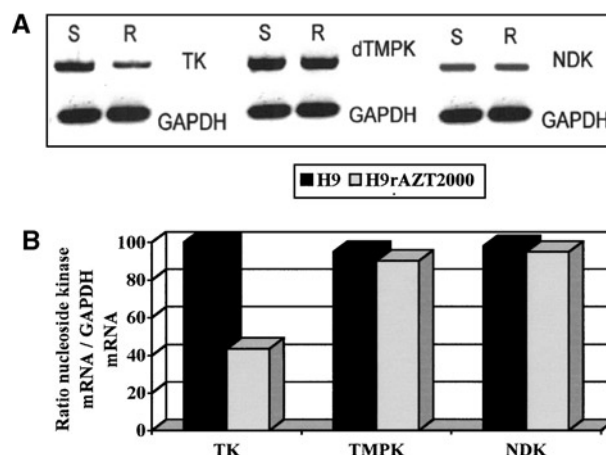


Figure 1. Specific PCR products from cDNA of TK, TMPK, NDK and GAPDH mRNA separated by gel electrophoresis (S: H9, R: H9^rAZT²⁰⁰⁰) (A) and ratio of nucleoside kinases and GAPDH mRNA levels (B).

nucleoside diphosphate kinase (NDK) was measured by semiquantitative RT-PCR. Comparable to results previously reported for H9^rAZT²⁵⁰ cells (8), a lower gene expression rate of TK gene (three fold) was observed in H9^rAZT²⁰⁰⁰ cells in comparison to parental cells. No differences in TMPK and NDK mRNA levels were seen between H9 and H9^rAZT²⁰⁰⁰ cells (Fig. 1A and 1B). Decreased mRNA levels for any gene may result from a variety of mechanisms, including decrease gene copy number, abnormal processing of mRNA, decreased stability of mRNA, or a decreased message-transcription rate (13). Several *in vitro* and *in vivo* studies demonstrated that drug-induced DNA hypermethylation constitutes one response of cells to cytotoxic levels of DNA synthesis inhibiting drugs (14,15). We hypothesize that TK gene expression in H9^rAZT²⁰⁰⁰ cells was down-regulated due to hypermethylation of CpG dinucleotides in TK promoter gene. Such epigenetic drug resistance mechanism which results in gene-silencing have been reported for AZT and other nucleoside analog antimetabolites (12,15). To test this hypothesis H9 parental and AZT resistant cells were treated with 1 μ M of demethylating agent AzaCd for 3 days. Following, TK and TMPK mRNA levels were determined by RT-PCR. Azacytidine treatment upregulates TK mRNA levels significantly in H9^rAZT²⁰⁰⁰ cells compared to untreated AZT resistant cells ($p < 0.05$, Student's t-test) (Fig. 2a and 2b). These results imply that drug induced DNA hypermethylation was involved in the TK gene-silencing mechanism in AZT resistant cells. Testing the cytotoxic and anti-HIV-1 effects of AZT in H9^rAZT²⁰⁰⁰ cells treated with the demethylation agent AzaCd, the results showed that these TK-revertants regained the susceptibility to cytotoxic and anti-HIV-1 effects of AZT showing decreased CC₅₀ and EC₅₀ values of AZT (Table 1 and 2). TK re-expression due to AzaCd treatment presumably lead to the ability of H9^rAZT²⁰⁰⁰ cells to



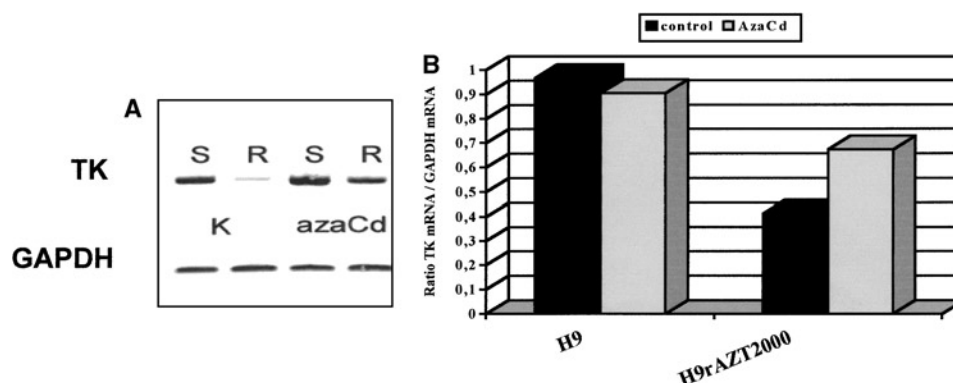


Figure 2. Specific PCR products from cDNA of TK, mRNA (A) and ratio of TK and GAPDH mRNA levels (B) in H9 (S) and H9^rAZT²⁰⁰⁰ (R) cells after treatment with 1 μ M AzaCd for 3 days.

metabolize AZT to the corresponding 5-mono-, di- and triphosphate form, which is necessary for cytotoxic and anti-HIV-1 efficiency of AZT. DNA methyltransferases 1, 3a and 3b are cellular enzymes responsible for DNA methylation (16). They possess the ability to catalyze covalent addition of a methyl group to cytosine within the context of CpG dinucleotides. Whereas DNMT 1 is responsible for maintenance of gene methylation during DNA synthesis (methylation of the second DNA strand), DNMT 3a and 3b catalyzes the newly formed *de novo* methylation patterns on double strand DNA (17). Further studies including measurement of DNMT 1, 3a and 3b mRNA levels as well as enzyme activity in H9 parental and H9^rAZT²⁰⁰⁰ cells should explain their role in cellular resistance mechanisms of AZT resistant cells, such as deficiency in TK gene expression.

In conclusion these results showed that DNA methylation of genes, which encodes for enzymes necessary for activation of drugs such as NRTIs, plays a major role in development of cellular resistance mechanisms against these drugs. The simultaneous application of demethylating agents without disturbing the physiological mechanisms of cells may be useful for overcoming resistance mechanisms and

Table 1. Cytotoxic Effects of AZT in H9 and H9^rAZT²⁰⁰⁰ Cells After Treatment with 1 μ M AzaCd for 6 Days

Substances	CC ₅₀ ^a	
	H9	H9 ^r AZT ²⁰⁰⁰
0	111.0 \pm 32.5 ^b	>2000
AzaCd	101.7 \pm 19.8	566 \pm 210.7

^aConcentration of drug, which inhibits cell growth by 50%.

^bResults represent mean value \pm SD of three different experiments.



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Table 2. Antiretroviral Activity of AZT in H9 and H9^TAZT²⁰⁰⁰ Cells After Treatment with 1 μ M AzaCd for 6 Days

Substances	EC ₅₀ ^a	
	H9	H9 ^T AZT ²⁰⁰⁰
0	0.1 \pm 0.03	>100
AzaCd	0.05 \pm 0.001	5.3 \pm 0.4

^aConcentration of drug, which inhibits HIV-1 p24 antigen production by 50%.

^bResults represent mean value \pm SD of three different experiments.

increase the therapeutic effects of NRTIs, especially in the case of deficiency of nucleoside kinases.

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