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3′-Azido-2′,3′-dideoxythymidine induced deficiency of thymidine kinases 1, 2 and deoxycytidine kinase in H9 T-lymphoid cells

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Abstract

Continuous cultivation of T-lymphoid H9 cells in the presence of 3′-azido-2′,3′-dideoxythymidine (AZT) resulted in a cell variant cross-resistant to both thymidine and deoxycytidine analogs. Cytotoxic effects of AZT, 2′,3′-didehydro-3′-deoxythymidine as well as different deoxycytidine analogs such as 2′,3′-dideoxycytidine, 2′,2′-difluoro-2′-deoxycytidine (dFdC) and 1-ß-D-arabinofuranosylcytosine (Ara-C) were strongly reduced in H9 cells continuously exposed to AZT when compared to parental cells (>8.3-, >6.6-, >9.1-, 5×10^4-, 5×10^3-fold, respectively). Moreover, anti-HIV-1 effects of AZT, d4T, ddC and 2′,3′-dideoxy-3′-thiacytidine (3TC) were significantly diminished (>222-, >25-, >400-, >200-fold, respectively) in AZT-resistant H9 cells. Study of cellular mechanisms responsible for cross-resistance to pyrimidine analogs in AZT-resistant H9 cells revealed decreased mRNA levels of thymidine kinase 1 (TK1) and lack of deoxycytidine kinase (dCK) mRNA expression. The loss of dCK gene expression was confirmed by western blot analysis of dCK protein as well as dCK enzyme activity assay. Moreover, enzyme activity of TK1 and TK2 was reduced in AZT-resistant cells. In order to determine whether lack of dCK affected the formation of the active triphosphate of the deoxycytidine analog dFdC, dFdCTP accumulation and retention was measured in H9 parental and AZT-resistant cells after exposure to 1 and 10 μM dFdC. Parental H9 cells accumulated about 30 and 100 pmol dFdCTP/10^6 cells after 4 hr, whereas in AZT-resistant cells no dFdCTP accumulation was detected. These results demonstrate that continuous treatment of H9 cells in the presence of AZT selected for a thymidine analog resistant cell variant with cross-resistance to deoxycytidine analogs, due to deficiency in TK1, TK2, and dCK.

Keywords: Cellular resistance; Zidovudine; Gemcitabine; Cytarabine; Thymidine kinase 1; Deoxycytidine kinase

1. Introduction

Chemotherapeutic agents belonging to the group of nucleoside analogs play a very important role in the treatment of many viral as well as tumoral diseases [12]. For example the current treatment strategy of HIV-1 infection includes the combination of nucleoside analogs inhibiting the viral reverse transcriptase (nucleoside reverse transcriptase inhibitors, NRTIs) with non-nucleoside reverse transcriptase inhibitors or protease inhibitors [13]. This highly active anti-retroviral therapy effectively suppresses viral replication resulting in reduced morbidity and prolonged life of HIV-1 infected patients [28]. After entering the target cells NRTIs, however, must undergo anabolic phosphorylation by intracellular deoxynucleoside kinases to their triphosphate forms. In this triphosphate form they act as competitive inhibitors or alternate substrates of the viral reverse transcriptase resulting in potent anti-HIV-1 activity [12,35]. Enzymes of the cellular pyrimidine salvage pathways are essential for the phosphorylation process of

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Abbreviations: AZT, 3′-azido-2′,3′-dideoxythymidine; d4T, 2′,3′-didehydrodeoxy-thymidine; ddC, 2′,3′-dideoxycytidine; dFdC, 2′,2′-difluoro-2′-deoxycytidine; Ara-C, 1-ß-D-arabinofuranosylcytosine; 3TC, 2′,3′-dideoxy-3′-thiacytidine; TK1, thymidine kinase 1; TK2, thymidine kinase 2; dCK, deoxycytidine kinase.

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thymidine and deoxycytidine analogs such as AZT, d4T, ddC, and 3TC [16,24,35]. Lack in enzymatic activity of intracellular deoxynucleoside kinases abolishes anti-retroviral activity of NRTIs and probably contributes to the development of resistant viral mutants [17,36].

Several studies elucidated the effects of continuous NRTI treatment *in vitro* which results in cells resistant to the therapeutic effects of the respective compound [1–4,7,10,14,17–21]. However, recent findings indicated that *in vitro* selection with nucleoside analogs results not only in cells resistant to a single substance but also to cross-resistance against different nucleoside analogs [1,21]. Agarwal *et al*. [1] established ddC resistant cells which were cross-resistant to AZT. In previous studies we showed that cytarabine induced resistance not only to deoxycytidine analogs but also to thymidine analog AZT [21].

In this study, we defined molecular mechanisms in T-lymphoid H9 cells cross-resistant to thymidine and deoxycytidine analogs. A cross-resistant cell variant was selected by continuous AZT treatment. Analysis of molecular mechanisms responsible for cellular resistance indicated that these cells exert beside decreased TK1 gene expression and enzyme activity lack of dCK gene and protein expression. Moreover, cytotoxic and anti-HIV-1 effects of several thymidine and deoxycytidine analogs markedly failed in H9 AZT-resistant cells.

2. Material and methods

2.1. Induction of AZT-resistance in H9 cells

H9 AZT-resistant cells, continuously growing in medium containing 250 μM AZT ($H9^{r}AZT^{250}$) were established as described previously [11]. Briefly, H9 cells were selected for AZT resistance by adding increasing amounts of AZT to the cell culture medium over a period of 12 months. Starting with 1 nM AZT, cells were resistant to 7.5 μM AZT after 6 months and resistant to 250 μM after 12 months. Cells used in these experiments were cultivated for more than 3 years in Iscove's modified Dulbecco's medium supplemented with 10% FCS containing 250 μM AZT. Before performing the experiments, cells were cultivated 3 weeks in drug-free medium.

2.2. Chemicals

AZT, d4T, ddC, Ara-C, and ddI were obtained from Sigma. 3TC was purchased from Hartmann Analytic GmbH. dFdC was kindly supplied by Eli Lilly and Co. The drugs were dissolved in dimethylsulfoxide and stored by -20°.

2.3. Drug sensitivity assay

Cytotoxic effects of different nucleoside analogs in parental H9 and AZT-resistant cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [10]. Cells were suspended in cell culture medium at a density of 10^5 cells/mL. Afterwards, cell suspension was brought into each well of 96-well microtiter plates and was incubated with medium containing various concentrations of the test compounds or medium without drug. Six days later MTT solution was added to each well and absorbance ($A_{620/690}$) of the solution was determined by photometry. CC_{50} values were calculated using the method of Chou and Talalay [9] describing the concentration reducing the amount of cell growth by 50%.

2.4. Virus

Virus stock of HIV-1 laboratory strain $HTLV\text{-}III_{RF}$ was obtained from the AIDS Reagent Project, National Institute for Biological Standards and Control. Infective dose ($TCID_{50}$) of virus stock was quantified by endpoint dilution and was determined by the method of Reed and Muench [27].

2.5. Antiretroviral assay

Antiretroviral activity of different NRTIs was determined by the measurement of reduction of HIV-1 p24 antigen in cell culture supernatant using an ELISA test system (HIV-1 Ag Monoclonal; ABOTT). Briefly, cells were infected with the $HTLV\text{-}III_{RF}$ strain at multiplicity of 0.1 $TCID_{50}$ per cell and incubated without or with different concentrations of the drugs for 6 days. Amount of HIV-1 p24 antigen in infected cultures were quantitatively determined according to the manufacturer's instructions. EC_{50} values were calculated using the method of Chou and Talalay [9] describing the concentration reducing the amount of HIV-1 p24 antigen in cell culture supernatant by 50%.

2.6. Semiquantitative RT–PCR

Semiquantitative RT–PCR was performed as described previously [21]. Briefly, total cellular RNA was extracted using Trizol™ (Life Technologies) according to the manufacturers instructions and quantified photometrically. cDNA was synthesized using the GeneAmp RNA–PCR kit (Perkin-Elmer). PCR was carried out using a PCR-mix of 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris–HCl, 0.2 mM each dNTP, 25 pmol each primer, and 1 U Amplitaq Polymerase. For the amplification of a region out of the dCK, TK1, thymidylate kinase (TMPK), nucleoside diphosphate kinase (NDK), ribonucleotide reductase (RR) and glycerine-aldehyd-phosphate-dehydrogenase (GAPDH) mRNA following primers were used: dCK-1 5′-aggtcaggatctggctagc-3′, dCK-2 5′-atctggaaccatttggctgc-3′ (926 bp), TK1-1 5′-caggatcctcgggttcgtgaac-3′, TK1-2 5′-tagaattcggcccttgcaggtc-3′ (765 bp) [38], TMPK-1 5′-gtctgttcctccagttacagct-3′,

TMPK-2 5′-cagcctgcagatctctgctg-3′ (393 bp), NDK-1 5′-atgcagtgcggcctggtggg-3′, NDK-2 5′-gacccagtcatgagcacaa-gac-3′ (405 bp), RR-1 5′-atgtgatcaagcgagatggc-3′, RR-2 5′-gtcagggtgcttagtagtca-3′ (219 bp), GAPDH-1 5′-tggggaagg-tgaaggtcgga-3′, and GAPDH-2 5′-gaaggggtcattgatggcaa-3′ (124 bp). The cycling parameters were: 25 cycles of 94° 30 s, 55° 30 s, 72° 30 s for amplification of deoxynucleoside kinases and 94° 30 s, 60° 30 s for the amplification of GAPDH mRNA. PCR products were separated on an agarose gel by electrophoresis and density of PCR products were quantified by the Enhanced-Analysis-System (Herolab GmbH). To ascertain that transcripts were specifically amplified, sequence analysis of PCR products was performed.

2.7. dCK, TK1 and TK2 activities in cell extracts

dCK, TK1, and TK2 activities were determined as described previously [5]. Briefly, cell extracts obtained from H9 parental and AZT-resistant cells (10,000 *g* supernatants) were used for enzyme activity measurements. Aliquots of cell extracts were taken to measure protein content using the Bio-Rad Bradford protein assay. dCK activity was measured after mixing the cold reaction buffer containing 5 mM Mg^{2+}/ATP and 10 mM NaF in Tris buffer (pH 7.6) and 50 μM [^{3}H]CdA as substrate with 10,000 *g* supernatant. TK1 and TK2 activities were measured after mixing the cold reaction buffer containing 2.5 mM Mg^{2+} and 5 mM ATP in Tris buffer, pH 7.6 and 11 μM [^{3}H]TdR as substrate with 10,000 *g* supernatant. dCTP was used as a TK2 inhibitor. The reaction mixtures were incubated at 37° for 30 min and terminated by heating at 95° for 3 min and subsequent addition of 10 μL 5 mM unlabeled dCyd. The substrates were separated from the phosphorylated product by thin layer chromatography on polyethylene imine cellulose layers, with distilled water as eluent. The spots were visualized, marked, and cut out. Radioactivity was estimated in a liquid scintillation counter after addition of 9 mL Optima Gold. Enzyme activities were expressed as nanomoles product formed per hour per 10^6 cell (nmol/hr/10^6 cells).

2.8. dCK protein expression by western blot analysis

Thirty microgram of total protein of H9 parental and AZT-resistant cells were loaded onto a 12.5% SDS–polyacrylamide gel and separated by electrophoresis. After transfer the proteins onto a nitrocellulose membrane, the sheet was blocked overnight, at 4°, in a solution of 1% BSA and 1% milk, in Tris Buffered Saline with 0.1% Tween 20 (TBST). After incubation with the first antibody (Rabbit anti-human dCK-pep, 1:5000) [23] and the secondary antibody (Goat Anti-Rabbit-PO, 1:40,000), the blot was incubated with ECL for 1 min. Subsequently an autoradiographic film was illuminated for 1 min.

2.9. Intracellular dFdCTP accumulation and nucleoside triphosphate (NTP) pools

Cells at a density of 5×10^5 cells per mL were incubated in 20 mL IMDM cell culture medium without or with dFdC at concentrations of 1 or 10 μM. After 4 hr of incubation (37°) cells were harvested and after washing proceeded for nucleotides extractions as previously described [25,31]. Briefly, cell pellets were resuspended in 150 μL cold PBS (pH 7.4), and subsequently 50 μL ice-cold 40% trichloroacetic acid (w/v) was added. The suspension was kept on ice for 20 min. Proteins were spun down (5 min, 12,000 *g*, 4°) and the supernatants were neutralized with 400 μL tri-octylamine/1,1,2-tri-chloro-trifluoroethane (v/v:1/4). Finally, dFdCTP and NTPs were analyzed on HPLC using a Partisphere SAX (Whatman) column with a linear gradient between 5 mM $NH_4H_2PO_4$ (pH 2,8; buffer A) and 0.5 M $NH_4H_2PO_4$/0.25 M KCl (pH 3.0; buffer B; 35–100% B over 30 min) at a flow rate of 1.5 mL/min [30]. For NTP determination, isocratic anion-exchange HPLC was used (0.25 M KH_2PO_4 containing 0.5 M KCl (pH 4.5). dFdCTP and nucleotides were detected at 254 nm [25].

2.10. Statistical analysis

Statistical significance of data were calculated using the Student's *t*-test. P values <0.05 were considered to indicate a statistically significant difference.

3. Results

3.1. Cytotoxic effects of nucleoside analogs in H9 parental and AZT-resistant cells

Cytotoxic effects of several nucleoside analogs determined by the MTT-assay and expressed as cytotoxic concentration inhibiting cell growth by 50% (CC_{50}) are shown in Table 1. Cytotoxicity of AZT was at least 8.3-fold decreased in H9 AZT-resistant cells compared to parental

Table 1
Cytotoxic effects of nucleoside analogs in H9 parental and AZT-resistant cells

Drug	CC_{50} (μM)[a]		
	H9	$H9^rAZT^{250}$	RI[b]
AZT	60.4 ± 7.8	>500	>8.3
d4T	75.2 ± 3.4	>500	>6.6
ddC	55.1 ± 5.5	>500	>9.1
3TC	>500	>500	n.d.[c]
dFdC	0.01 ± 0.0016	>500	5×10^4
Ara-C	0.1 ± 0.001	>500	5×10^3
ddI	1450.0 ± 307.4	1789.6 ± 545.6	1.2

[a] Results represent mean value ± SD of three different experiments.
[b] Resistance-index (ratio CC_{50} $H9^rAZT^{250}$:CC_{50} H9).
[c] n.d.: not detectable.

H9 cells. Moreover, d4T showed decreased cytotoxic effects in $H9^rAZT^{250}$ resistant cells (resistance index, RI > 6.6). Interestingly, AZT-resistant cells developed cross-resistance to deoxycytidine analogs such as ddC, dFdC, and Ara-C. CC_{50} values of these drugs were at least one to four orders of magnitude increased in AZT-resistant cells in comparison to the parental cells, which was demonstrated by RIs of >9.1, 5×10^4 and 5×10^3 for ddC, dFdC, and Ara-C, respectively (Table 1). ddI showed comparable cytotoxic effects in both parental and AZT-resistant cells.

3.2. Anti-HIV-1 effects of nucleoside analogs in H9 parental and AZT-resistant cells

Anti-HIV-1 activity of different NRTIs, expressed as the effective drug concentration reducing the amount of HIV-1 p24 antigen in cell culture supernatant by 50% (EC_{50}) in parental and AZT-resistant cells are summarized in Table 2. AZT completely failed to inhibit HIV-1 replication in $H9^rAZT^{250}$ cells (RI > 222). Moreover, anti-HIV-1 activity of d4T, ddC, and 3TC was significantly reduced in $H9^rAZT^{250}$ resistant cells (RIs: >25, >400, >200, respectively), whereas ddI showed comparable anti-HIV-1 activity in AZT-resistant as well as in parental cells (Table 2).

3.3. mRNA expression of deoxynucleoside kinases in H9 parental and AZT-resistant cells

Molecular mechanisms responsible for cross-resistance in AZT-resistant cells were elucidated by measuring the gene expression rates of thymidine and deoxycytidine metabolizing enzymes such as TK1, dCK, TMPK, NDK, as well as RR. Amount of mRNA expression in both cell lines was determined by semiquantitative RT–PCR. Fig. 1 depicts an agarose gel in which the amplified DNA products were separated by electrophoresis (Fig. 1). Comparison of TK1 mRNA expression in H9 parental and AZT-resistant cells revealed a 3-fold decrease in gene expression rate in AZT-resistant cells ($P < 0.05$). Furthermore, no dCK mRNA level was detectable in AZT-resistant cells (Fig. 1). Comparable gene expression rates of TMPK, NDK, and RR were seen in both cell lines. Equal amounts of mRNA-expression in each sample were detected by the measurement of GAPDH mRNA levels.

Table 2
Anti-HIV-1 effects of nucleoside analogs in H9 parental and AZT-resistant cells

Drug	EC_{50} (μM)[a]		
	H9	$H9^rAZT^{250}$	RI[b]
AZT	0.09 ± 0.01	>20	>222
d4T	0.8 ± 0.024	>20	>25
ddC	0.05 ± 0.002	>20	>400
3TC	0.1 ± 0.002	>20	>200
ddI	9.1 ± 0.5	12.5 ± 0.9	1.4

[a] Results represent mean value ± SD of three different experiments.
[b] Resistance-index (ratio EC_{50} $H9^rAZT^{250}$:EC_{50} H9).

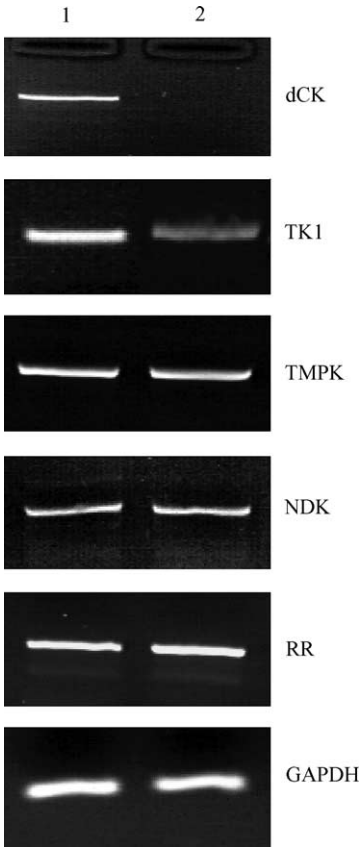

Fig. 1. Specific PCR products of dCK, TK1, TMPK, NDK, RR, and GAPDH mRNA received from H9 parental (1) and $H9^rAZT^{250}$ resistant (2) cells measured by RT–PCR and separated on an agarose gel by electrophoresis. mRNA expression levels were similar in at least three independent experiments of which one is shown in the figure as a representative experiment. Densitometric analysis of the bands was performed and following ratios between GAPDH and deoxynucleoside kinase mRNA levels were calculated.

3.4. Western blot analysis of dCK

To confirm lack of dCK gene expression in H9 AZT-resistant cells western blot analysis of dCK protein was performed. Using a dCK specific polyclonal antibody [23] a visible dCK specific band was seen for H9 parental cells, however, no dCK band was observed for AZT-resistant cells (Fig. 2).

3.5. dCK, TK1, and TK2 activities in H9 parental and AZT-resistant cells

Enzyme activities of dCK, TK1, and TK2 in H9 parental and AZT-resistant cells were measured using radio-labeled chlorodeoxyadenosine and thymidine as substrates. As shown in Table 3 TK1 and TK2 enzyme activities were significantly decreased in AZT-resistant cells, when compared to parental cells (5.6- and 3.9-fold, respectively; $P < 0.05$). Although RT–PCR and western blot analysis of

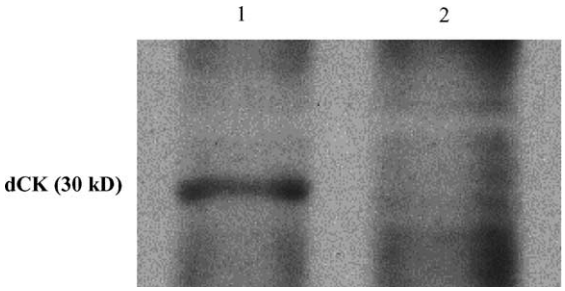

Fig. 2. Immunoblotting of dCK in H9 parental (1) and H9^{r}AZT^{250} resistant (2) cells was performed by using a dCK specific monoclonal antibody. Antibody binding was detected by chemoluminiscence and was similar in at least three independent experiments of which one is shown in the figure as a representative experiment.

dCK gene and protein expression showed no dCK specific product in AZT-resistant cells, a low amount of dCK enzyme activity was measurable in AZT-resistant cells when compared to parental cells. Difference in dCK activity between both cell lines was 9.9-fold ($P < 0.05$; Table 3).

3.6. dFdCTP accumulation and retention in H9 parental and AZT-resistant cells

In order to determine whether deficiency of dCK affected the formation of the active triphosphate form of deoxycytidine analogs, dFdCTP accumulation and retention in H9 parental and AZT-resistant cells were measured. After exposure of cells to 1 and 10 μM dFdC for 4 hr dFdCTP accumulation in parental H9 cells was about 30 and 100 pmol dFdCTP/10^6 cells (Table 4). dFdCTP was almost completely retained after 24 hr in H9 parental cells. However, no dFdCTP accumulation was detectable in H9^{r}AZT^{250} resistant cells (Table 4).

3.7. NTP pools in H9 parental and AZT-resistant cells

Since NTP pools play a role in resistance to nucleoside analogs, the amounts of NTPs such as CTP, UTP, ATP, and GTP in both H9 parental and AZT-resistant cells were measured by HPLC (Fig. 3A and B). NTP pools were almost 1.7-fold higher in H9^{r}AZT^{250} cells than in H9 parental cells ($P < 0.05$). Treatment of H9 cells with 1 μM dFdC for 4 hr decreased NTP pools for almost 1.6-fold ($P < 0.05$), while exposure of H9 cells with 10 μM dFdC increased all measured NTP pools significantly compared to untreated H9 cells (2.4-, 1.5-, 1.3-, 1.4-fold; $P < 0.05$, respectively; Fig. 3A). The exposure of H9^{r}AZT^{250} cells to 1 or 10 μM dFdC decreased significantly NTP levels for about 1.9-fold ($P < 0.05$; Fig. 3B).

Table 4
dFdCTP accumulation in H9 parental and AZT-resistant cells

dFdC (μM)	pmol ddFdCTP/10^6 cells	
	H9	H9^{r}AZT^{250}
1	30.7 ± 12.5	n.d.
10	100 ± 25.8	n.d.

Results represent mean value ± SD of three different experiments, n.d.: not detectable.

4. Discussion

In this study, we describe the characterization of H9 cells resistant to continuous AZT treatment, which were not only highly cross-resistant to a wide variety of thymidine analogs, but unexpectedly also to deoxycytidine analogs. Analysis of molecular mechanisms revealed that this cell line lacked expression of dCK gene at protein and mRNA level, which was evident in H9 parental cells. Lack of synthesis of dCK mRNA and protein correlated with the lack of dFdCTP accumulation in H9^{r}AZT^{250} resistant cells. Similar to other deoxycytidine analogs such as ddC or 3TC, dFdC is a deoxycytidine analog that requires phosphorylation by dCK for its activation [29]. The small amount of residual dCK activity in H9^{r}AZT^{250} cells might be related to the possibility that chlorodeoxyadenosine used in dCK activity assay as substrate was phosphorylated by deoxyguanosine kinase [40].

Moreover, TK1 gene expression rate as well as TK1 and TK2 enzyme activities were modified in AZT-resistant cells when compared to parental cells. TK occurs in eucaryotic species in two different TK isoenzymes. Cytoplasmic TK1 isoenzyme is expressed in cell cycle dependent manner while the other mitochondrial TK2 isoenzyme is expressed constitutively [22]. Cytoplasmic TK1 is considered to be the major isozymic form responsible for AZT activation [15]. Mitochondrial TK2 phosphorylates AZT to some extent and in addition phosphorylates deoxycytidine and gemcitabine. Gene expression of TK1 and the levels of thymidine kinase 1 and 2 activities were significantly decreased in the AZT-resistant cell variant compared to the parental cells. Since TK1, TK2, and dCK pathways are

Table 3
dCK, TK1, and TK2 enzyme activities in H9 parental and AZT-resistant cells

Cell line	dCK + dGK activities (nmol/hr/10^6 cells)	TK1 activity (nmol/hr/10^6 cells)	TK2 activity (nmol/hr/10^6 cells)
H9	1.59 ± 0.12	1.69 ± 0.07	1.21 ± 0.06
H9^{r}AZT^{250}	0.16 ± 0.04[a]	0.30 ± 0.04	0.31 ± 0.07

Results represent mean value ± SD of three different experiments.
[a] Value represents only dGK activity since no dCK mRNA or protein could be detected in H9^{r}AZT^{250} cells.

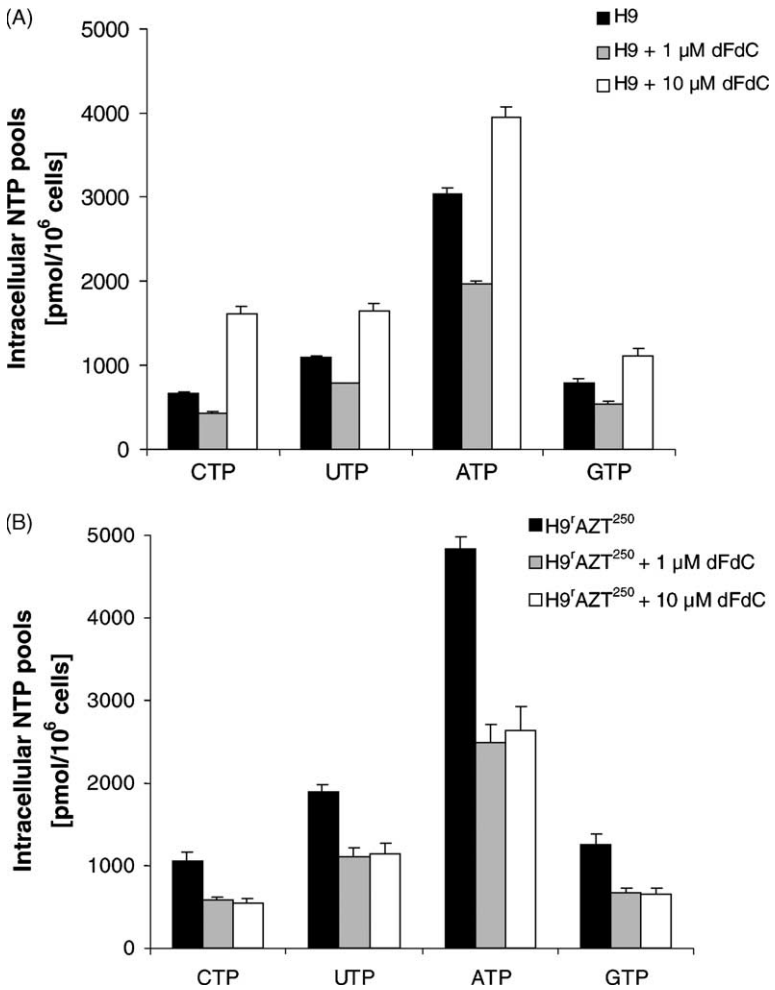

Fig. 3. NTP pools in H9 parental (A) and H9^{r}AZT^{250} resistant (B) cells after 4 hr incubation without (■) or with 1 μM (▨) or 10 μM (□) dFdC. Results represent mean values of three independent experiments ± standard variation.

not dependent on each other it seems possible that selection of H9 cells in the presence of AZT results in co-incidentially selection of a TK1, TK2, and dCK deficient cell variant. Moreover, mRNA levels of genes of different other enzymes involved in the pyrimidine salvage pathways such as TMPK, NDK, and RR were unaltered in AZT-resistant cells when compared with H9 cells.

Resistance to nucleoside analogs is often accompanied with an increase in intracellular nucleoside triphosphate levels [8]. Measurement of NTP pools in H9 parental and AZT-resistant cells revealed a statistically significant elevation of NTP levels in H9^{r}AZT^{250} cells, which may in part contribute to the resistance of cytotoxic and anti-HIV-1 effects of nucleoside analogs tested in these cells. It has been shown previously that dFdC has a profound effect on intrinsic NTP pools, which might play an important role in dFdCTP accumulation [32,37]. In our studies, incubation with 10 μM dFdC increased NTP pools in H9 parental cells significantly, especially in terms of ATP and UTP levels. These results are in accordance with the findings of Van Moorsel *et al*. [37], who demonstrated that besides the accumulation of dFdCTP, the CTP/UTP ratio was related to the sensitivity to dFdC. Furthermore, UTP levels and the CTP/UTP ratio after dFdC treatment were related to dFdCTP accumulation. On the other hand both 1 and 10 μM dFdC decreased significantly NTP pools in H9^{r}AZT^{250} resistant cells similar to the effect of a low concentration (1 μM) of dFdC in H9 cells.

The mechanisms for dCK and TK deficiency in H9 cells due to long-term exposure of cells to AZT are mainly unknown. Stegmann *et al*. [34] demonstrated that the exposure to Ara-C induces a resistant phenotype with marked functional dCK deficiency that may be the consequence of mutations in the dCK gene. Moreover, one potential mechanism of drug resistance in cells exposed to AZT involves drug-induced DNA hypermethylation resulting in transcriptional inactivation of cellular TK1 gene whose products are required for drug activation [26]. Wu *et al*. [38] reported that Jurkat E6-1 cells continuously treated with AZT showed decreased expression of TK1 mRNA which is associated with highly methylated 5′ end of the gene in the AZT-resistant cells when compared to wild-type cells. AZT-resistant human T-lymphoid H9 cells continuously exposed to 2000 μM AZT and deficient in TK1 gene expression,

re-expressed TK1 mRNA and regained the ability to metabolize AZT by the exposure to the demethylation agent azacytidine [20]. Further studies should show whether mutations or hypermethylation occurring in genes coding for TK1, TK2, or dCK might be responsible for deficiency in gene expression in $H9^rAZT^{250}$ resistant cells.

Although the exact mechanism of cross-resistance in $H9^rAZT^{250}$ resistant cells is not clear yet, deficiency in dCK, TK1, and TK2 caused drug resistance to thymidine and deoxycytidine analogs. All substances tested in this study were activated either by TK1, TK2, or dCK, thus loss of dCK and decreased TK1 gene expression is expected to be the main reason for resistance against nucleoside analogs in $H9^rAZT^{250}$ resistant cells. AZT-resistant cells were highly cross-resistant to cytotoxic and anti-HIV-1 effects of different thymidine and deoxycytidine analogs such as AZT, d4T, ddC, 3TC, Ara-C, and dFdC.

Additional mechanisms causing multidrug-resistance are characterized by the over-expression of the MDR-1 gene, which encodes P-glycoprotein (Pgp). Yusa *et al*. [39] showed that human multidrug-resistant K562/ADM cells overexpressing Pgp, were cross-resistant to AZT and ddC. The results suggest that anti-HIV nucleoside analogs could be transported by outward drug-transport system such as Pgp or multidrug resistance-associated protein 4 in multidrug-resistant cells [6,33]. However, in $H9^rAZT^{250}$ resistant cells described here no Pgp or multidrug resistance-associated protein 4 mRNA over-expression was detectable using RT–PCR analysis (data not shown).

In summary, these results demonstrate that *in vitro* selection of T-lymphoid cells with AZT resulted in a cell variant cross-resistant to different pyrimidine analogs, due to deficiency in TK1, TK2, and dCK. This was confirmed by decreased gene expression rate, protein expression and enzyme activities as well as lack of gemcitabine-triphosphate formation. We conclude that in $H9^rAZT^{250}$ cells decreased TK1, TK2, and failure in dCK gene expression is responsible for inefficient anabolization of different thymidine and deoxycytidine analogs, which results in failure of their anti-HIV-1 efficiency. Although the resistance mechanisms described in AZT-resistant cells do not reflect the clinical situation in HIV-1 infected patients, the AZT-resistant cell line provides an excellent cell system to test different nucleoside analogs, e.g. monophosphorylated nucleoside analogs, for their ability to inhibit HIV-1 replication without requirement of intracellular activation by cellular kinases such as thymidine kinase 1, 2 or deoxycytidine kinase.

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