ELSEVIER

Antiviral Research 53 (2002) 143–152

Antiviral Research

www.elsevier.com/locate/antiviral

S-acyl-2-thioethyl (SATE) pronucleotides are potent inhibitors of HIV-1 replication in T-lymphoid cells cross-resistant to deoxycytidine and thymidine analogs

B. Gröschel [a], J. Cinatl [b], C. Périgaud [c], G. Gosselin [c], J.-L. Imbach [c], H.W. Doerr [a], J. Cinatl Jr. [a,*]

[a] *Institute of Medical Virology, Johann Wolfgang Goethe University Frankfurt/M., Paul-Ehrlich-Str. 40, 60596 Frankfurt M., Germany*

[b] *Department of Hematology and Oncology, Center of Pediatrics, Johann Wolfgang Goethe University Frankfurt/M., Theodor-Stern-Kai 7, 60596 Frankfurt/M., Germany*

[c] *UMR CNRS 5625, Case Courrier 008, Université Montpellier II, Place Eugène Bataillon, 34095 Montpellier Cedex 5, France*

Received 21 March 2001; accepted 28 September 2001

Abstract

The biological evaluation of mononucleotide prodrugs (pronucleotides) of various nucleoside reverse transcriptase inhibitors (NRTIs) such as zidovudine (AZT), zalcitabine (ddC) and lamivudine (3TC) was reported in human T-lymphoid MOLT-4/8 cells which were grown continuously for more than 1 year in a medium containing cytarabine (Ara-C). In this cell line, expression of deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1) was decreased in comparison to parental cells (3.8 and 2.9-fold, respectively). The lower mRNA level of TK1 correlated significantly with lower enzyme activity, whereas no dCK activity was detectable. In Ara-C-resistant cells, anti-HIV-1 effects of ddC, 3TC and AZT were more than 100-fold lower compared with parental cells. In contrast, the corresponding mononucleoside phosphotriesters bearing *S*-acyl-2-thioethyl (SATE) groups as biolabile phosphate protection retained anti-HIV-1 activity due to their ability to bypass the first monophosphorylation step catalyzed by dCK or TK1. The results demonstrate that in vitro selection of T-lymphoid cells in the presence of Ara-C results in cross-resistance to deoxycytidine (ddC, 3TC) and thymidine (AZT) analogs and that these cellular resistance mechanisms can be bypassed by the use of bis(SATE) pronucleotides.

Keywords: HIV-1; Cellular resistance; AZT; Ara-C; Mononucleotide; Prodrug

1. Introduction

A common problem in the treatment of HIV-1 infected patients using a combination of nucleoside analog reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase in-

* Corresponding author. Tel.: +49-69-6301-6409; fax: +49-69-6301-4302.

E-mail address: cinatl@em.uni-frankfurt.de (J. Cinatl, Jr.).

0166-3542/02/$ - see front matter
PII: S0166-3542(01)00205-4

hibitors (NNRTIs) and protease inhibitors (PIs) (German and Austrian Guidelines, 2000) is the failure of a drug regimen after a period of success (Hammer et al., 1997; Martinez-Picado et al., 2000). It has been shown that in many cases this is due to virus-drug resistance, which means that HIV-1 virus mutants evolve rapidly and become resistant to drugs (Larder et al., 1989). Different other factors, such as poor tolerability, difficulties with compliance and persistent virus in sanctuary sites as well as development of cellular (pharmacological) resistance towards NRTIs and PIs may also influence therapy success (Back, 1999). In general, cellular resistance mechanisms account for an insufficient intracellular concentration of the active form of drugs, which results in lack of antiretroviral activity (Gröschel et al., 1997).

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 enzymes (Furman et al., 1986). In their triphosphate form the compounds are potent terminators of the growing viral nucleic acid. Most cellular enzymes which catalyze phosphorylation of deoxynucleosides are cell type-specific and cell cycle-dependent (Gao et al., 1994) and represent limiting factors for the effective conversion of the compounds (Lavie et al., 1997). It has been previously reported that continuous treatment of cell lines with nucleoside analogs results in lower expression and activity of enzymes responsible for activation of the respective substance (Cinatl et al., 1993, 1997; Gröschel et al., 1999a,b; Dianzani et al., 1994; Antonelli et al., 1996; Wu et al., 1995; Brandi et al., 1997; Agarwal et al., 1999).

To overcome these cellular resistance mechanisms, phosphorylated prodrugs (pronucleotides) were designed (Périgaud et al., 2000; Parang et al., 2000; Kukhanova et al., 2000). In this respect, mononucleoside phosphotriester derivatives bearing *S*-acyl-2-thioethyl (SATE) groups as transient phosphate protections were able to deliver the corresponding 5′-mononucleotide inside the cell through an esterase-mediated activation process (Périgaud et al., 1993; Lefebvre et al., 1995; Périgaud et al., 1997). On the basis of AZT as nucleoside model, it has also been demonstrated that the SATE pro-moieties, as well as their degradation products, did not induce additional cellular toxicity (Périgaud et al., 1996). Furthermore, in AZT-resistant MOLT-4/8 cells the phosphotriester derivative of AZT which incorporates the *S*-pivaloyl-2-thioethyl (tBuSATE) group showed comparable cytotoxic and antiretroviral activity as in parental cells (Cinatl et al., 1997). The effect of bypassing the first monophosphorylation step in a MOLT-4/8^{r}ddC^{250} dCK deficient cell line has also been demonstrated for ddC- and 3TC-bis(-SATE) pronucleotides (Gröschel et al., 1999a).

In the present study, we tested the in vitro cytotoxic and anti-HIV-1 effects of bis(SATE) phosphotriesters of different NRTIs in MOLT-4/8 T-lymphoid cells selected for Ara-C resistance. Resistance mechanisms induced by continuous Ara-C treatment were elucidated by measurement of gene expression rate and activity of nucleoside kinases, such as dCK and TK1 responsible for phosphorylation of deoxycytidine and thymidine analogs to their corresponding monophosphate form.

2. Material and methods

2.1. Selection of Ara-C-resistant cell line

Ara-C-resistant cell line was established by continuous cultivation of MOLT-4/8 cells in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS) containing increasing concentrations of Ara-C. Resistant cells, which have grown for more than 1 year in medium containing Ara-C at a concentration of 250 μM (MOLT4/8^{r}Ara-C^{250}), were used in the experiments.

2.2. Chemotherapeutic agents

Ara-C, ddC, 3TC, AZT, and didanosine (ddI) were obtained from Sigma (Deisenhofen, Germany). The synthesis of bis(SATE) phosphotriester derivatives of Ara-C [bis(tBuSATE)Ara-C-MP], AZT [bis(tBuSATE)AZT-MP], ddC [bis(-MeSATE)ddC-MP] and 3TC [bis(tBuSATE)3TC-

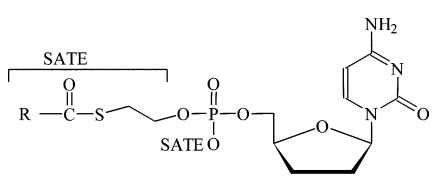
MP] were carried out according to general procedure already published (Lefebvre et al., 1995; Fig. 1). These compounds were found to be pure by rigorous high performance liquid chromatography (HPLC) analysis, high-field multinuclear magnetic resonance (NMR) spectroscopy, quantitative ultraviolet (UV) spectra, mass spectroscopy and elemental analysis. The drugs were dissolved in dimethylsulfoxide at an initial concentration of 10 mM.

2.3. Virus

Virus stock of HIV-1 laboratory strain HTLV-III_{RF} was obtained from the AIDS Reagent Project, National Institute for Biological Standards and Control (Hertfordshire, UK). Cell culture infective dose ($CCID_{50}$) of virus stock was quantified by endpoint dilution and was determined by the method of Reed and Muench (1938).

2.4. Determination of cytotoxicity

Cytotoxic effects of different nucleoside analogs and bis(SATE) phosphotriesters in parental MOLT-4/8 cells and the Ara-C resistant cell line MOLT-4/8^{r}Ara-C^{250} were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Cinatl et al., 1997). Cells were suspended in cell culture medium at a density of 10^5 cells per 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.



R = CH_3, *S*-acetyl-2-thioethyl, MeSATE

R = $(CH_3)_3C$, *S*-pivaloyl-2-thioethyl, tBuSATE

Fig. 1. General structure of a bis(SATE) pronucleotide illustrated on the basis of the dideoxycytidine as nucleoside analog.

2.5. Antiretroviral assay

Antiretroviral activity of different NRTIs was determined by the reduction of HIV-1 p24 antigen in cell culture supernatant using an enzyme linked immunosorbent assay (ELISA) test system (HIV-1 Ag Monoclonal; ABBOTT, Delkenheim, Germany). Briefly, cells were infected with the HTLV-III_{RF} strain at a multiplicity of 0.1 $CCID_{50}$ per cell and incubated without or with different concentrations of the drugs for 6 days. HIV-1 p24 antigen in infected cultures was quantitatively determined according to the manufacturer's instructions.

EC_{50} values were calculated using the method of Chou and Talalay (1984), the EC_{50} being the concentration reducing the amount of HIV-1 p24 antigen in the cell culture supernatant by 50%.

2.6. Determination of nucleoside kinase gene expression by RT-PCR

Total cellular RNA was extracted using Trizol™ (GibcoBRL, Eggenstein, Germany) according to the Manufacturer's instructions and quantified photometrically. cDNA was synthesized using the GeneAmp RNA-PCR kit (Perkin Elmer, Langen, Germany). Briefly, 1 μg of total cellular RNA per 20 μl reaction volume using 2.5 mM random hexamers, 1 mM each dNTP, 2.5 U/μl MuLV Reverse Transcriptase, 1 U/μl RNAse inhibitor in a buffer containing 50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris–HCL pH 8.3 were used. PCR was carried out with an amount of cDNA according to 500 ng total RNA in a total volume of 50 μl. PCR buffer was added to the cDNA to reach a final concentration in the PCR-mix of 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris–HCl, 0.2 mM each dNTP, 25 pM each primer and 1 U Amplitaq Polymerase. For the amplification of a region out of the dCK, TK1,

Table 1
Primer sequences of dCK, TK1, TMPK, NDK, RR and GAPDH mRNA

Name	Sequence	PCR product (bp)
dCK1	5′-AGGTCAGGATCTGGCT TAGC-3′	
dCK2	5′-ATCTGGAACCATTTGG CTGC-3′	926
TK1-1	5′-CAGGATCCTCGGGTTC GTGAAC-3′[a]	
TK1-2	5′-TAGAATTCGGCCCTTG CAGGTC-3′[a]	765
TMPK1	5′-GTCTGTTCCTCCAGTTA CAGCT-3′	
TMPK2	5′-CAGCCTGCAGATCTCT GCTG-3′	393
NDK1	5′-ATGCAGTGCGGCCTGG TGGG-3′	
NDK2	5′-GACCCAGTCATGAGCA CAAGAC-3′	405
RR1	5′-ATGTGATCAAGCGAGA TGGC-3′,	
RR2	5′-GTCAGGGTGCTTAGTA GTCA-3′	219
GAPDH1	5′-TGGGGAAGGTGAAGG TCGGA-3′	
GAPDH2	5′-GAAGGGGTCATTGATG GCAA-3′	124

[a] Wu et al. (1995).

thymidylate kinase (TMPK), nucleoside diphosphate kinase (NDK), ribonucleotide reductase (RR) and glycerine-aldehyd-phosphate-dehydrogenase (GAPDH) mRNA primers listed in Table 1 were used (Table 1). The cycling parameters were, 25 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 30 s for amplification of nucleoside kinases and 94 °C 30 s, 60 °C 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, Wiesloch, Germany).

2.7. dCK and TK1 activity assay

dCK and TK1 assay was performed with some minor modifications using methods described by Magnani et al. (1995) and Wu et al. (1995). Briefly, cell extracts obtained from MOLT-4/8 and MOLT4/8^{r}Ara-C^{250} cells were incubated at 37 °C for 30 min with different concentrations of [^{3}H]dCd or [^{3}H]dThd (ranging from 0.1 to 100 μM) in a reaction buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 6 mM ATP, 6 mM $MgCl_2$ and 6 mM NaF for dCK assay and 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 20 mM ATP, 10 mM $MgCl_2$ and 10 mM NaF for TK1 assay. EDTA was added to stop the reaction and 50 μl of incubation mixture was transferred onto a 25 mm DEAE paper (DE-81 paper disk; Whatman, Clifton, NJ). The disks were washed twice in an excess of 1 mM ammonium formiate, pH 5.6, in order to remove unconverted nucleosides and transferred in vials containing 10 ml scintillation fluid. The bounded tritium marked nucleotides were washed off with 100 μl 0.1 N HCl/KCl and radioactivity was counted by β-counter. Protein concentrations in cell extracts were measured using the Bio-Rad (Richmond, CA) protein assay with bovine serum albumin (BSA) as standard.

3. Results

3.1. Cytotoxic effects of bis(SATE) pronucleotides in parental and Ara-C-resistant MOLT-4/8 cells

Prolonged treatment of T-lymphoid cells with the antitumoral agent Ara-C induced resistance of the cells to deoxycytidine analogs such as Ara-C and ddC (Table 2). Cytotoxic effects of both substances, expressed as concentrations inhibiting 50% of cell growth (CC_{50}) were decreased in Ara-C-resistant cells when compared with parental cells. Resistance index (RI, ratio of CC_{50} in MOLT-4/8^{r}Ara-C^{250} and MOLT-4/8 cells) of Ara-C and ddC were 4.8×10^4 and > 42, respectively. To test whether Ara-C-resistant cells were cross-resistant to other deoxynucleoside analogs, cytotoxicity of AZT and ddI was tested in both cell lines. Ara-C-resistant cells were partially cross-resistant to the deoxythymidine analog AZT ($RI = 9.7$). No cross-resistance to ddI was observed in Ara-C-resistant cells. Cytotoxic effects of bis(SATE) pronucleotides of Ara-C, ddC and

Table 2
Cytotoxic effects of nucleoside analogs and corresponding bis(SATE) pronucleotides in MOLT-4/8 and MOLT-4/8^{r}Ara-C^{250}cells

Drug	CC_{50} (μM)[a]		
	MOLT-4/8	MOLT-4/8^{r}Ara-C^{250}	RI[b]
Ara-C	0.03 ± 0.005	1434.0 ± 267.6	4.8×10^4
Bis(tBuSATE)Ara-C-MP	17.0 ± 1.5	38.0 ± 11.6	2.2
DdC	47.5 ± 7.6	>2000	>42
Bis(MeSATE)ddC-MP	47.2 ± 5.6	65.7 ± 13.2	1.4
AZT	71.5 ± 16.5	693.5 ± 125.4	9.7
Bis(tBuSATE)AZT-MP	47.1 ± 2.3	113.5 ± 16.2	2.4
3TC	>150	>150	n.d.
Bis(tBuSATE)3TC-MP	>150	>150	n.d.
ddI	1446.0 ± 304.3	1957.2 ± 176.5	1.4

n.d., Not determined.
[a] Results represent mean values ± S.D. for three different experiments.
[b] RI (Ratio CC_{50} MOLT-4/8^{r}Ara-C^{250}, CC_{50} MOLT-4/8).

Table 3
Anti-HIV-1 effects of nucleoside analogs and corresponding bis(SATE) pronucleotides in MOLT-4/8 and MOLT-4/8^{r}Ara-C^{250} cells

Drug	EC_{50} (μM)[a]		
	MOLT-4/8	MOLT-4/8^{r}Ara-C^{250}	RI[b]
AZT	0.013 ± 0.01	>10	>770
Bis(tBuSATE)AZT-MP	0.6 ± 0.02	0.47 ± 0.2	0.8
ddc	0.17 ± 0.1	>20	>120
Bis(MeSATE)ddC-MP	0.16 ± 0.1	0.6 ± 0.2	3.8
3TC	0.12 ± 0.01	>20	>170
Bis(tBuSATE)3TC-MP	0.22 ± 0.03	0.18 ± 0.012	0.8
ddI	13.8 ± 2.9	27.4 ± 1.6	2

[a] Results represent mean values ± S.D. for three different experiments.
[b] RI (Ratio EC_{50} MOLT-4/8^{r}Ara-C^{250}, EC_{50} MOLT-4/8).

AZT were almost comparable in parental MOLT-4/8 and MOLT-4/8^{r}Ara-C^{250} resistant cells with RIs of 2.2, 1.4 and 2.4, respectively (Table 2). No cytotoxicity of 3TC and bis(SATE)3TC-MP up to a concentration of 150 μM (maximum concentration tested) was detectable in parental and Ara-C-resistant MOLT-4/8 cells (Table 2).

3.2. Anti-HIV-1 effects of nucleoside analogs and bis(SATE) pronucleotides in parental and Ara-C-resistant MOLT-4/8 cells

In MOLT-4/8^{r}Ara-C^{250} cells infected with the laboratory HIV-1 strain HTLV-III$_{RF}$ deoxycytidine analogs ddC and 3TC failed to inhibit virus replication at the concentrations tested (20 μM). In contrast, in parental MOLT-4/8 cells both ddC and 3TC inhibited HIV-1 at nanomolar concentrations. In addition to the resistance to deoxycytidine analogs, cross-resistance to the anti-HIV-1 activity of AZT in MOLT-4/8^{r}Ara-C^{250} cells was found (Table 3). Concentrations of ddC, 3TC and AZT required to inhibit HIV-1 replication in Ara-C-resistant cells were more than 120-, 170- and 770-fold increased, respectively, as compared with non-resistant cells. No cross-resistance to anti-HIV-1 activity of ddI was observed in MOLT-4/8^{r}Ara-C^{250} cells (Table 3). To show whether resistance mechanisms may be due to the deficiency of monophosphorylation process of nu-

cleoside analogs, bis(SATE) pronucleotides of ddC, 3TC and AZT were tested for their anti-HIV-1 effects in MOLT-4/8^{r}Ara-C^{250} resistant cells, compared with non-resistant parental cells (Table 3). These phosphotriester derivatives significantly inhibited the replication of HIV-1 in MOLT-4/8^{r}Ara-C^{250} resistant cells at concentrations at which the parental nucleoside analogs completely failed to do so. The RIs were 3.8, 0.8 and 0.8 for the bis(SATE) pronucleotides of ddC, 3TC and AZT, respectively. These results demonstrate that the SATE prodrugs are able to overcome resistance mechanisms in MOLT-4/8^{r}Ara-C^{250} cells.

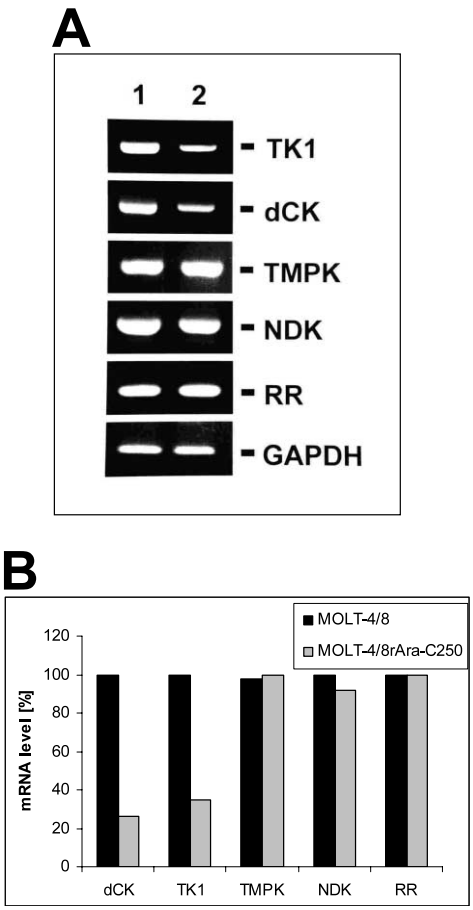


Fig. 2. (A) Specific PCR products of dCK, TK1, TMPK, NDK, RR and GAPDH mRNA obtained from parental MOLT-4/8 (1) and MOLT-4/8^{r}Ara-C^{250} (2) resistant cells separated on an agarose gel by electrophoresis. mRNA expression levels were similar in at least three independent experiments. (B) mRNA expression of dCK, TK1, TMPK, NDK, RR and GAPDH in parental MOLT-4/8 (■) and MOLT-4/8^{r}Ara-C^{250} (□) resistant cells measured by RT-PCR. Densitometric analysis of the bands was performed and ratios between GAPDH and nucleoside kinases mRNA levels were calculated.

3.3. Gene expression of nucleoside kinases in parental and Ara-C-resistant MOLT-4/8 cells

To determine resistance mechanisms in MOLT-4/8^{r}Ara-C^{250} cells continuously treated with Ara-C, mRNA levels of different pyrimidine metabolizing enzymes involved in conversion of nucleosides to their respective triphosphates were determined by RT-PCR. Fig. 2A and B, depicts mRNA levels of dCK, TK1, TMPK, NDK, RR, and GAPDH in parental MOLT-4/8 cells in comparison to MOLT-4/8^{r}Ara-C^{250} cells. RT-PCR products were separated on an agarose gel by electrophoresis (Fig. 2A) and densitometric analysis of the bands was performed calculating the ratio between GAPDH bands and specific PCR products (Fig. 2B). As shown in Fig. 2A and B GAPDH, TMPK, NDK and RR mRNA levels were unchanged in parental MOLT-4/8 and MOLT-4/8^{r}Ara-C^{250} cells, whereas dCK and TK1 mRNA-levels were significantly decreased in Ara-C-resistant cells (3.8- and 2.9-fold, respectively; $P < 0.05$, Student's t-test; Fig. 2A and B).

3.4. Enzyme activity of dCK and TK1 in parental and Ara-C-resistant MOLT-4/8 cells

To study enzyme activities of dCK and TK1 in parental and Ara-C-resistant MOLT-4/8 cells, cell extracts of both cell lines were incubated with ^{3}H-labeled deoxycytidine (dCd) and deoxythymidine (dThd) in different concentrations. The amounts of tritium labeled nucleoside phosphates were measured, and activity of both enzymes was calculated and expressed as pmol/mg per h. No dCK activity in MOLT-4/8^{r}Ara-C^{250} cells was observed, in contrast to parental cells (Fig. 3A). Although TK1 activity was measurable in Ara-C-resistant MOLT-4/8 cells, enzyme activity was 3-fold decreased in MOLT-4/8^{r}Ara-C^{250} cells when compared with parental cells (Fig. 3B).

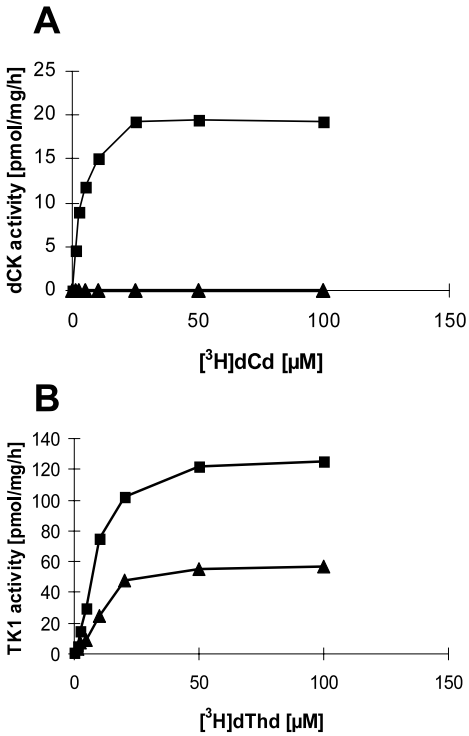


Fig. 3. Enzyme activity of dCK (A), and TK1 (B) in parental MOLT-4/8 (■) and MOLT-4/8^{r}Ara-C^{250} (▲) cells expressed as pmol/mg per h.

4. Discussion

The present study demonstrated that MOLT-4/8 cells continuously grown in the presence of Ara-C developed resistance to antiretroviral and cytotoxic effects of NRTIs. Interestingly, Ara-C-resistant cells showed resistance not only against deoxycytidine analogs such as ddC, 3TC and Ara-C but also cross-resistance to the thymidine analog AZT. Our findings of cross-resistance to pyrimidine analogs in MOLT-4/8^{r}Ara-C^{250} cells are consistent with a report from Agarwal et al. (1999), who found that a cell line resistant to cytotoxic effects of ddC was cross-resistant to AZT. However, these authors did not test anti-HIV-1 activity of nucleoside analogs in the resistant cells. Our study demonstrated for the first time, that in addition to ddC and 3TC also AZT failed to inhibit HIV-1 replication in Ara-C-resistant cells.

It has been shown that continuous treatment of cells with antitumoral agents such as Ara-C promotes resistance against Ara-C by different resistance mechanisms (Chabner, 1996). dCK is the rate-limiting enzyme for phosphorylation of deoxycytidine analogs such as Ara-C, ddC and 3TC (Gao et al., 1994). It has been demonstrated that decreased activity of dCK is the most frequent resistance mechanism found in cells resistant to these drugs (Drahovsky and Kreis, 1970; Agarwal et al., 1999; Brandi et al., 1997; Gröschel et al., 1999a). The cytoplasmatic nucleoside kinase TK1 is mainly responsible for AZT conversion to the monophosphate form (Munch-Petersen et al., 1991; Arnér et al., 1992). Phosphorylation to the di- and triphosphate form is catalyzed by TMPK and NDK (Furman et al., 1986). Whereas phosphorylation of AZT to the monophosphate form catalyzed by TK1 is facile and fast, the product, AZT-MP, is a very poor substrate for the enzyme thymidylate kinase. Therefore, the diphosphorylation of AZT-MP to AZT-DP catalyzed by TMPK is the rate limiting step in AZT activation (Furman et al., 1986; Lavie et al., 1997).

In Ara-C-resistant cells, decreased expression of dCK and lack of dCK activity was observed. However, lack of dCK activity correlated significantly with the very high resistance to deoxycytidine analogs such as Ara-C and ddC in Ara-C-resistant cells. Simultaneously, TK1 activity was significantly decreased in Ara-C resistant cells. The residual enzyme activity of TK1 in Ara-C-resistant MOLT-4/8 cells leads presumably to a minimal intracellular amount of AZT-MP which results in minor toxic effects of AZT in Ara-C-resistant cells. Our results on complete resistance to the antiviral effect of AZT in Ara-C-resistant cells lead to the assumption that AZTMP was not further phosphorylated to the corresponding di- and triphosphate to a marked extent. Although gene expression rate of TMPK and NDK is comparable in parental and Ara-C-resistant cells, enzyme activity or substrate affinity of these enzymes may be modified in Ara-C-resistant MOLT-4/8 cells. Future studies including the determination of enzyme activities as well as intracellular AZT metabolites are needed to clarify this topic.

In the MOLT-4/8 cells which we used for these experiments, continuous Ara-C treatment caused dCK deficiency and may have coincidentally co-selected partial TK1 deficiency, which resulted in resistance to the cytotoxic and anti-HIV-1 effects of deoxycytidine analogs and cross-resistance to the thymidine analog AZT. Cross-resistance to cytotoxic effects of Ara-C and AZT presumably caused by deficiency in dCK and TK1 has also been found in C8166 cells upon prolonged treatment with 5 or 250 μM Ara-C (data not shown).

Other authors have reported that in vitro induced Ara-C resistance may be caused by mutations within the dCK coding region of the dCK gene (Stegmann et al., 1995). Flasshove et al. (1994) found that point mutations in the coding region of dCK in patients may be responsible for Ara-C resistance in vivo. This was not confirmed in our Ara-C resistant cells as sequencing of dCK coding region did not show any mutations (data not shown).

In order to overcome the dependence of nucleoside analogs upon nucleoside kinase activation, various research groups have focused their attention on the study of mononucleotide prodrugs, namely pronucleotides. In this respect, mononucleoside phosphotriester derivatives bearing SATE groups as biolabile phosphate protections emerged as a promising pronucleotide series (Périgaud et al., 2000; Wagner et al., 2000). Applied to anti-HIV and anti-HBV nucleoside analogs which are hampered at the first phosphorylation step by a dependence on kinase-mediated phosphorylation or by a rate limiting step in the anabolism pathway, the use of the corresponding bis(SATE) pronucleotides leads in vitro to markedly enhanced antiviral activity (Périgaud et al., 1994; Girardet et al., 1995; Périgaud et al., 1999). Moreover, we have previously reported that cellular resistance mechanisms emerging in cells which were grown continuously for a prolonged time in a medium containing AZT or ddC were bypassed by the use of the SATE pronucleotide approach (Cinatl et al., 1997; Gröschel et al., 1999a,b).

Here, the bis(SATE) phosphotriesters of AZT, ddC and 3TC proved to be effective in MOLT-4/8^{r}Ara-C^{250} cells which were completely resistant to the anti-HIV-1 effects of the parental nucleosides. This result strongly supports the hypothesis that such pronucleotides are able to deliver the respective 5′-monophosphate forms inside the HIV-1-infected cells bypassing the first phosphorylation step catalyzed by dCK and TK1.

In conclusion, this study demonstrates that in vitro selection of T-lymphoid cells with Ara-C resulted in cross-resistance to different pyrimidine nucleoside analogs, due to deficiency in dCK and TK1 expression. Bis(SATE) pronucleotides represent a valuable approach to overcome resistance mechanisms due to deficiency in nucleoside kinases. One has to take into consideration that such cellular resistance mechanisms (deficiency in nucleoside kinases) may also occur in vivo (Jacobsson et al., 1995; Antonelli et al., 1996; Gröschel et al., 2000). Therefore, mononucleotide prodrugs offer the opportunity for treatment of HIV-1-infected patients, who do not respond to nucleoside analogs due to deficiency of cellular kinases. The newly established Ara-C-resistant MOLT-4/8 cell line provides a suitable cell culture system for testing substances for their ability to overcome cellular resistance mechanisms such as deficiency in dCK and TK1.

Acknowledgements

We thank Dr R. Bazzanini and Dr J.-L. Girardet for the synthesis of the bis(SATE)pronucleotides of Ara-C and 3TC, respectively. We are grateful to Cornelia Lang for her excellent technical assistance. This work was generously supported by the Foundations ‘Hilfe für krebskranke Kinder Frankfurt, e.V.’, ‘Frankfurter Stiftung für krebskranke Kinder’ and the Agence Nationale de Recherches sur le SIDA (ANRS).

References

Agarwal, R.P., Wang, W., Yo, W.P., Han, T., Fernandez, M., 1999. Cross-resistance of dideoxycytidine-resistant cell lines to azidothymidine. Biochem. Pharmacol. 58, 1603–1608.

Antonelli, G., Turriziani, O., Verri, A., Narciso, P., Ferri, F., D’Offizi, G., Dianzani, F., 1996. Long-term exposure to zidovudine affects in vitro and in vivo the efficiency of phosphorylation of thymidine kinase. AIDS Res. Hum. Retroviruses 12, 223–228.

Arnér, E.S., Valentin, A., Eriksson, S., 1992. Thymidine and 3′-azido-3′-deoxythymidine metabolism in human peripheral blood lymphocytes and monocyte-derived macrophages. J. Biol. Chem. 267, 10968–10975.

Back, D.J., 1999. Pharmacological issues relating to viral resistance. Infection 2, S42–S44.

Brandi, G., Puddu, P., Casabionca, A., Cianfriglia, M., Magnani, M., 1997. The inhibitory effect of ddC on human immunodeficiency virus replication diminishes in cells that are chronically exposed to the drug. Antiviral Chem. Chemother. 8, 113–119.

Chabner, B.A., 1996. Cytidine analogs. In: Chabner, B.A., Dan, L.L. (Eds.), Cancer Chemotherapy and Biotherapy. Lippincott-Raven, Philadelphia, pp. 213–233.

Chou, T.C., Talalay, P., 1984. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55.

Cinatl, J. Jr, Cinatl, J., Weber, B., Rabenau, H., Weissflog, A., Mainke, M., Kornhuber, B., Doerr, H.W., 1993. Decreased anti-human immunodeficiency virus type-1 activities of 2′,3′-dideoxynucleoside analogs in Molt-4 cell sublines resistant to 2′,3′-dideoxynucleoside analogs. Acta Virol. 37, 360–368.

Cinatl, J. Jr, Gröschel, B., Zehner, R., Cinatl, J., Périgaud, C., Gosselin, G., Imbach, J.L., Doerr, H.W., 1997. HIV resistance to AZT in MOLT-4/8 cells is associated with the lack of AZT phosphorylation and is bypassed by AZT-monophosphate SATE-prodrugs. Antiviral Chem. Chemother. 8, 343–352.

Dianzani, F., Antonelli, G., Turriziani, O., Riva, E., Simeoni, E., Signoretti, C., Strosselli, S., Cianfriglia, M., 1994. Zidovudine induces the expression of cellular resistance affecting its antiviral activity. AIDS Res. Hum. Retroviruses 10, 1471–1478.

Drahovsky, D., Kreis, W., 1970. Studies on drug resistance. II. Kinase patterns in P815 neoplasms sensitive and resistant to 1-β-D-arabinofuranosylcytosine. Biochem. Pharmacol. 19, 940–944.

Flasshove, M., Strumberg, D., Ayscue, L., Mitchell, B.S., Tirier, C., Heit, W., Seeber, S., Schutte, J., 1994. Structural analysis of the deoxycytidine kinase gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. Leukemia 8, 780–785.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Lehrman, S.N., Bolognesi, D.P., Broder, S., Mitsuya, H., 1986. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA 83, 8333–8337.

Gao, W.Y., Agbaria, R., Driscoll, J.S., Mitsuya, H., 1994. Divergent anti-human immunodeficiency virus activity and anabolic phosphorylation of 2′,3′-dideoxynucleoside analogs in resting and activated human cells. J. Biol. Chem. 269, 12633–12638.

German and Austrian guidelines, 2000. Deutsche AIDS-Gesellschaft (DAIG). Oesterreichische AIDS-Gesellschaft (OAG). German–Austrian guidelines for antiretroviral therapy in HIV infection. Eur. J. Med. Res. 5, 129–138.

Girardet, J.-L., Périgaud, C., Aubertin, A.-M., Gosselin, G., Kirn, A., Imbach, J.-L., 1995. Increase of the anti-HIV activity of D4T in human T-cell culture by the use of the SATE pronucleotide approach. Bioorg. Med. Chem. Lett. 5, 2981–2984.

Gröschel, B., Cinatl, J., Cinatl, J. Jr, 1997. Viral and cellular factors for resistance against antiretroviral agents. Intervirology 40, 400–407.

Gröschel, B., Himmel, N., Cinatl, J., Périgaud, C., Gosselin, G., Imbach, J.L., Doerr, H.W., Cinatl, J. Jr, 1999a. ddC- and 3TC-bis(SATE) monophosphate prodrugs overcome cellular resistance mechanisms to HIV-1 associated with cytidine kinase deficiency. Nucleosides Nucleotides 18, 921–926.

Gröschel, B., Meier, C., Zehner, R., Cinatl, J., Doerr, H.W., Cinatl, J. Jr, 1999b. Effects of cycloSal-d4TMP derivatives in H9 cells with induced AZT resistance phenotype. Nucleosides Nucleotides 18, 933–936.

Gröschel, B., Cinatl, J., Miller, V., Doerr, H.W., Cinatl, J. Jr, 2000. Activity of thymidine kinase 1 in PBMCs of HIV-1 infected patients: novel therapy marker. Infection 28, 209–213.

Hammer, S.M., Squires, K.E., Hughes, M.D., Grimes, J.M., Demeter, L.M., Currier, J.S., Eron, J.J. Jr, Feinberg, J.E., Balfour, H.H. Jr, Deyton, L.R., Chodakewitz, J.A., Fischl, M.A., 1997. A controlled trial of two nucleoside analogs plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. New Engl. J. Med. 337, 725–733.

Jacobsson, B., Britton, S., He, Q., Karlsson, A., Eriksson, S., 1995. Decreased thymidine kinase levels in peripheral blood cells from HIV-seropositive individuals: implications for zidovudine metabolism. AIDS Res. Hum. Retroviruses 11, 805–811.

Kukhanova, M., Krayevsky, A., Prusoff, W., Cheng, Y.C., 2000. Design of anti-HIV compounds: from nucleoside to nucleoside 5′-triphosphate analogs. Problems and perspectives. Curr. Pharm. Des. 6, 585–598.

Larder, B.A., Darby, G., Richmann, D.D., 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prologned therapy. Science 243, 1731–1734.

Lavie, A., Schlichting, I., Vetter, I.R., Konrad, M., Reinstein, J., Goody, R.S., 1997. The bottleneck in AZT activation. Nat. Med. 3, 922–924.

Lefebvre, I., Périgaud, C., Pompon, A., Aubertin, A.M., Girardet, J.L., Kirn, A., Gosselin, G., Imbach, J.L., 1995. Mononucleoside phosphotriester derivatives with *S*-acyl-2-thioethyl bioreversible phosphate-protecting groups: intracellular delivery of 3′-azido-2′,3′-dideoxythymidine 5′-monophosphate. J. Med. Chem. 38, 3941–3950.

Magnani, M., Brandi, G., Casabianca, A., Fraternale, A., Schiavano, G.F., Rossi, L., Chiarantini, L., 1995. 2′,3′-Dideoxycytidine metabolism in a new drug-resistant cell line. Biochem. J. 312, 115–123.

Martinez-Picado, J., DePasquale, M.P., Kartsonis, N., Hanna, G.J., Wong, J., Finzi, D., Rosenberg, E., Gunthard, H.F., Sutton, L., Savara, A., Petropoulos, C.J., Hellmann, N., Walker, B.D., Richman, D.D., Siliciano, R., D'Aquila, R.T., 2000. Antiretroviral resistance during successful therapy of

HIV type 1 infection. Proc. Natl. Acad. Sci. USA 97, 10948–10953.

Munch-Petersen, B., Cloos, L., Tyrsted, G., Eriksson, S., 1991. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. J. Biol. Chem. 266, 9032–9038.

Parang, K., Wiebe, L.I., Knaus, E.E., 2000. Novel approaches for designing 5′-*O*-ester prodrugs of 3′-azido-2′, 3′-dideoxythymidine (AZT). Curr. Med. Chem. 7, 995–1039.

Périgaud, C., Gosselin, G., Lefebvre, I., Girardet, J.L., Benzaria, S., Barber, I., Imbach, J.L., 1993. Rational design for cytosolic delivery of nucleoside monophosphates: 'SATE' and 'DTE' as enzyme-labile transient phosphate protecting groups. Bioorg. Med. Chem. Lett. 3, 2521–2526.

Périgaud, C., Aubertin, A.M., Benzaria, S., Pelicano, H., Girardet, J.L., Maury, G., Gosselin, G., Kirn, A., Imbach, J.L., 1994. Equal inhibition of the replication of human immunodeficiency virus in human T-cell culture by ddA bis(SATE)phosphotriester and 3′-azido-2′,3′-dideoxythymidine. Biochem. Pharmacol. 48, 11–14.

Périgaud, C., Girardet, J.-L., Lefebvre, I., Xie, M.-Y., Aubertin, A.-M., Kirn, A., Gosselin, G., Imbach, J.-L., Sommadossi, J.-P., 1996. Comparison of cytotoxicity of mononucleoside phosphotriester derivatives bearing biolabile phosphate protecting groups in normal human bone marrow progenitor cells. Antiviral Chem. Chemother. 7, 338–345.

Périgaud, C., Gosselin, G., Imbach, J.-L., 1997. Minireview: from the pronucleotide concept to the SATE phosphate protecting groups. In: Alexander, J.C. (Ed.), Current Topics in Medicinal Chemistry. Blackwell Science, Oxford, pp. 15–29.

Périgaud, C., Gosselin, G., Girardet, J.-L., Korba, B.E., Imbach, J.-L., 1999. The SATE pronucleotide approach applied to acyclovir. Part I. Synthesis and in vitro anti-HBV activity of bis(SATE)phosphotriester derivatives of acyclovir. Antiviral Res. 40, 167–178.

Périgaud, C., Gosselin, G., Imbach, J.-L., 2000. Anti-HIV phosphotriester nucleotides. Basis for the rational design of biolabile phosphate protection. In: Torrence, P.F. (Ed.), Biomedical Chemistry Applying Chemical Principles to the Understanding and Treatment of Disease. Wiley, New York, pp. 115–141.

Reed, L.I., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Ann. J. Hyg. 27, 493–497.

Stegmann, A.P., Honders, M.W., Willemze, R., Landegent, J.E., 1995. De novo induced mutations in the deoxycytidine kinase (dck) gene in rat leukemic clonal cell lines confer resistance to cytarabine (AraC) and 5-aza-2′-deoxycytidine (DAC). Leukemia 9, 1032–1038.

Wagner, C.R., Iyer, V.V., McIntee, E.J., 2000. Pronucleotides: toward the in vivo delivery of antiviral and anticancer nucleotides. Med. Res. Rev. 20, 417–451.

Wu, S., Liu, X., Solorzano, M.M., Kwock, R., Avramis, V.I., 1995. Development of zidovudine (AZT) resistance in Jurkat T cells is associated with decreased expression of the thymidine kinase (TK1) gene and hypermethylation of the 5′ end of human TK gene. J. Acquir. Immune Def. Syndr. Hum. Retrovirol. 8, 1–9.