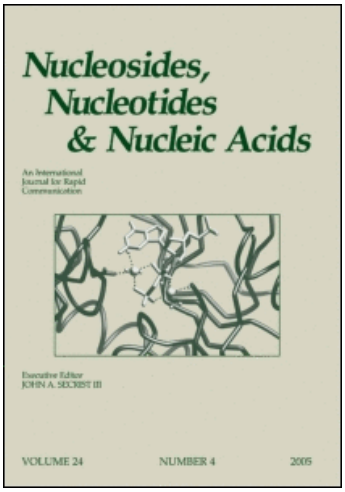

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DDC- AND 3TC-BIS(SATE) MONOPHOSPHATE PRODRUGS OVERCOME CELLULAR RESISTANCE MECHANISMS TO HIV-1 ASSOCIATED WITH CYTIDINE KINASE DEFICIENCY

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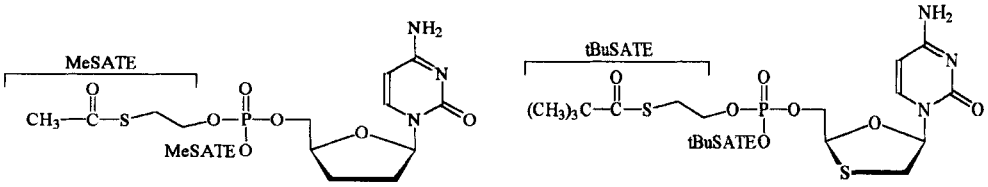
ABSTRACT: A 2',3'-dideoxycytidine (ddC)-resistant T-lymphoid cell line (MOLT-4/8^{r}ddC^{250}), in which deoxycytidine kinase (dCK) gene-expression was decreased when compared with parental cells, has been selected. Cytotoxic and antiretroviral activity of ddC and 3TC was significantly lower in MOLT-4/8^{r}ddC^{250} than in parental MOLT-4/8 cells. ddC- and 3TC-bis(SATE)phosphotriesters completely overcame cellular resistance mechanisms and showed comparable both cytotoxic and antiretroviral activity in parental and ddC-resistant cells.

Several nucleoside analogs such as 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-didehydro-3'-deoxythymidine (d4T), 2',3'-dideoxycytidine (ddC), 2',3'-dideoxy-3'-thiacytidine (3TC) and 2',3'-dideoxyinosine (ddI), which inhibit reverse transcriptase (RT) of *human immunodeficiency virus type 1* (HIV-1) are licensed for the clinical treatment of HIV-1 infected patients[1]. However, nucleoside analogs need to be phosphorylated intracellularly to their corresponding triphosphate form by cellular kinases[2]. In triphosphate form the compounds compete with natural nucleotides for binding the viral RT or incorporating in viral DNA, which causes inhibition of virus replication[3]. Several *in vitro* and *ex vivo* studies indicate that cellular mechanisms, induced by continuous treatment of cells with nucleoside analogs, may result in decreasing antiretroviral activity



bis(MeSATE)ddCMP bis(tBuSATE)3TCMP

FIG.1: Structures of the studied bis(SATE)phosphotriester derivatives

of the compounds[4]. These cellular resistance mechanisms involve decreased uptake, increased efflux or defective cellular enzymatic activation of nucleoside analogs. Nucleoside analog monophosphate prodrugs are an alternative strategy for overcoming cellular resistance mechanisms, which result from decreased catalytic activity of the first nucleoside kinases[5,6]. In this study we have selected a ddC-resistant cell line (MOLT-4/8^{r}ddC^{250}) by continuous growth, in medium containing increasing concentrations of ddC, for the investigation of cellular resistance mechanisms, which were induced by ddC treatment. Resistant MOLT-4/8^{r}ddC^{250} cell subline was characterized by measurement of dCK gene expression (dCK catalyses the first phosphorylation step of ddC to ddCMP[7]). Furthermore, cytotoxicity and antiretroviral activity of different bis(SATE) monophosphate prodrugs (FIG.1), which were designed to deliver the monophosphate form intracellularly were investigated in ddC-resistant cells in comparison to parental cells.

Selection of ddC-resistant cell line: MOLT-4/8 ddC-resistant cell subline was established by continuous cultivation of the cells in Iscove's modified Dulbecco's medium supplemented with 10% FBS containing increasing concentrations of ddC. The resistant cell subline, grown for more than one year in medium containing 250 μM ddC designated as MOLT4/8^{r}ddC^{250}, was used in the experiments. **Antiretroviral agents:** ddC and 3TC were obtained from Sigma (Deisenhofen, Germany). The synthesis of bis(*S*-acyl-2-thioethyl)phosphotriester derivatives (FIG. 1) of ddC [bis(MeSATE)ddCMP] and 3TC [bis(tBuSATE)3TCMP], was carried out according to general procedure already published[8]. These compounds were characterized on the basis of their physical and spectroscopic properties. Their purity was ascertained by high-pressure liquid

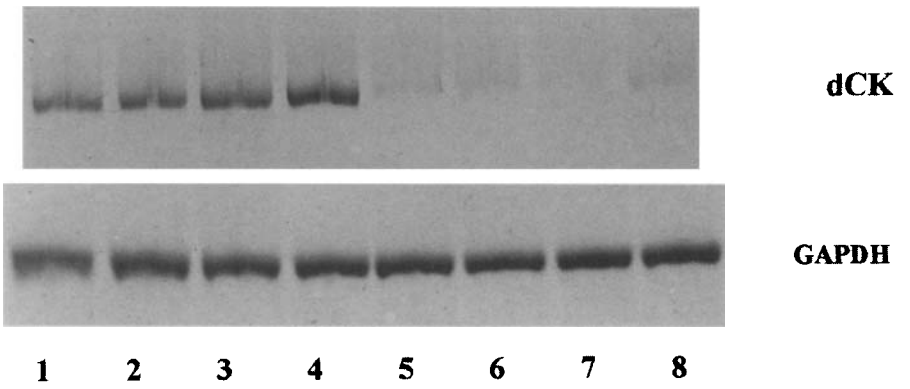


FIG.2: Specific PCR products from cDNA of dCK-mRNA (926 bp) and GAPDH-mRNA (126 bp) separated by polyacrylamide gel electrophoresis received from four different experiments. Lane 1-4 MOLT-4/8, lane 5-8 MOLT-4/8^{r}ddC^{250}

chromatography. The drugs were dissolved in dimethylsulfoxide at a concentration of 10 mM and stored at room temperature. **Determination of cytotoxicity:** Cytotoxic effects of different nucleoside analogs and bis(SATE) monophosphate prodrugs in MOLT4/8 and MOLT-4/8^{r}ddC^{250} cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[9]. The method was performed in 96 well plates as described previously[10]. **Antiretroviral assay:** Antiretroviral activity of different drugs was determined by the reduction of HIV-1 p24 antigen in cell culture supernatant using an ELISA test system (NEN Life Science Products, Boston, UK) as described previously[10]. **Determination of dCK gene expression by RT-PCR:** Total cellular RNA extraction and RT-PCR were performed as described previously[10]. For the amplification of a region out of the dCK mRNA following primers were used: dCK1: 5'-AGGTCAGGATCTGGCTTAGC-3' and dCK2: 5'-ATCTGGAACCATTTGGCTGC-3' (PCR product: 926 bp). The cycling parameters were: 25 cycles of 94°C 30s, 55°C 30s, 72°C 30s. The PCR products were separated on a polyacrylamid gel and density of PCR products were quantified by EAS (Herolab GmbH, Wiesloch, Germany).

To characterize a possible mechanism of resistance in MOLT-4/8^{r}ddC^{250} cell subline, dCK gene expression was determined. A region out of the dCK mRNA was amplified by RT-PCR (FIG. 2). MOLT-4/8^{r}ddC^{250} cells expressed dCK-mRNA to an 8-fold lower extent than in parental cells ($p<0.05$, Students-*t*-test). TABLE 1 shows the cytotoxic

TABLE 1. Cytotoxicity of different bis(SATE)phosphotriester derivatives in MOLT-4/8 and MOLT-4/8^{r}ddC^{250} cells

Substances	CC_{50}[a]		RI[b]
	MOLT-4/8	MOLT-4/8^{r}ddC^{250}	
ddC	68.4 ± 4.5	> 2000	> 30
bis(MeSATE)ddCMP	43.0 ± 2.9	91.4 ± 5.5	2
3TC	70.7 ± 1.4	> 2000	> 30
bis(tBuSATE)3TCMP	47.7 ± 9.5	30.9 ± 0.6	0.6

[a] Results represent mean value ± SD of three different experiments.
[b] Resistance-index (Ratio CC_{50} MOLT-4/8^{r}ddC^{250} : CC_{50} MOLT-4/8)

TABLE 2. Antiretroviral activity of different bis(SATE)phosphotriester derivatives in MOLT-4/8 and MOLT-4/8^{r}ddC^{250} cells

Substances	EC_{50}[a]		RI[b]
	MOLT-4/8	MOLT-4/8^{r}ddC^{250}	
ddC	0.048 ± 0.06	> 100	>2000
bis(MeSATE)ddCMP	0.93 ± 0.04	0.1 ± 0.05	0.1
3TC	0.25 ± 0.05	> 100	>400
bis(tBuSATE)3TCMP	0.008 ± 0.004	0.001 ± 0.005	0.1

[a] Results represent mean value ± SD of three different experiments.
[b] Resistance-index (Ratio EC_{50} MOLT-4/8^{r}ddC^{250} : EC_{50} MOLT-4/8).

effects of different bis(SATE)monophosphate prodrugs and the parental nucleoside analogs in ddC-resistant and parental MOLT-4/8 cells. ddC as well as 3TC have no cytotoxic effects in MOLT-4/8^{r}ddC^{250} resistant cells in contrast to parental cells represented by the very high resistance index (RI > 30 versus 2; $p<0.05$, Students-*t*-test). Bis(MeSATE)ddC- and bis(tBuSATE)3TC-monophosphate derivatives are designed to bypass the dCK catalysed activating step[8]. They showed comparable cytotoxicity in ddC-resistant as well as in parental cells (CC_{50} = 43.0 ± 2.9 μM versus 91.4 ± 5.5 μM for bis(MeSATE)ddCMP; CC_{50} = 47.7 ± 9.5 μM versus 30.9 ± 0.6 μM for bis(tBuSATE) 3TCMP) (TABLE 1). TABLE 2 represents the anti-HIV-1 activity of ddC, 3TC and the corresponding bis(SATE)phosphotriesters against the HTLV-III$_{RF}$ strain, as expressed by the drug concentration inhibiting 50% of HIV-1 p24 antigen (EC_{50}). ddC and 3TC did not have any antiretroviral effects in MOLT-4/8^{r}ddC^{250} resistant cells (EC_{50} >100 μM), while HIV-1 was inhibited potently in parental cells (EC_{50} = 0.048 ± 0.06 μM for ddC; EC_{50} =

0.25 ± 0.05 μM for 3TC). EC_{50} values for ddC- and 3TC-bis(SATE) monophosphate prodrugs were about 10-fold lower in ddC-resistant when compared with parental cells (TABLE 2). Bis(MeSATE)ddCMP showed lower antiretroviral activity in MOLT-4/8 cells (20-fold) than ddC, whereas bis(tBuSATE)3TCMP was much more efficient in MOLT-4/8 cells than 3TC (30-fold) (TABLE 2).

Cellular resistance mechanisms, such as insufficient phosphorylation of nucleoside analogs, in addition to viral factors may contribute to failure in treatment of HIV infected patients. In this study continuous ddC-treatment of T-lymphoid MOLT-4/8 cells induced cellular resistance mechanisms such as reduction of dCK gene expression. Similar observations have been reported in several AZT-resistant cell lines, in which thymidine kinase (TK) gene expression and TK activity were diminished after continuous treatment with AZT[4]. Only a few experiments have investigated the role of ddC-resistance *in vitro*[11-13]. In the present study we demonstrated, that MOLT-4/8^{r}ddC^{250} resistant cells developed cross-resistance to 3TC, probably due to the deficiency of dCK. These findings are in agreement with other investigations, which showed that dCK is the rate-limiting enzyme for anabolic phosphorylation of both ddC and 3TC[14]. Our results showed that bis(MeSATE)ddCMP and bis(tBuSATE)3TCMP derivatives overcome cellular resistance mechanisms in MOLT-4/8^{r}ddC^{250} resistant cell line, which is caused by the reduced expression of dCK gene and presumably by lower enzymatic activity. These prodrugs showed antiretroviral activity in MOLT-4/8^{r}ddC^{250} resistant cells, in which ddC and 3TC were completely ineffective. Interestingly, bis(tBuSATE)3TCMP was much more effective both in parental and ddC-resistant cells than bis(MeSATE)ddCMP. Previously, we showed that bis(tBuSATE)AZTMP is significantly more efficient antiretroviral agent than bis(MeSATE)AZTMP and bis(iPrSATE)AZTMP[10]. These results suggest that bis(tBuSATE) monophosphate derivatives provides more effective anti-HIV agents. In addition to dCK deficiency, other cellular resistance mechanisms may be induced in ddC-resistant cells. Therefore, MOLT-4/8^{r}ddC^{250} cells resistant against ddC provide optimal instruments to study the phenomenon of cellular resistance mechanisms against ddC and related nucleoside analogs, and also in order to test drugs designed to overcome such mechanisms.

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