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Cytosine arabinoside affects multiple cellular factors and induces drug resistance in human lymphoid cells

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Abstract

Continuous in vitro cultivation of human lymphoid H9 cells in the presence of 0.5 μM arabinosyl-cytosine (araC) resulted in cell variant, H9-araC cells, that was >600-fold resistant to the drug and cross resistant to its analogs and other unrelated nucleosides, e.g. dideoxycytidine (5-fold), thiacytidine (2-fold), 2-fluoro-adenine arabinoside (8.3-fold), and 2-chloro-deoxyadenosine (2.1-fold). Compared to the parental cell line, the resistant cells accumulated <1% araCTP, and had reduced deoxycytidine kinase (dCK) activity (31.4%) and equilibrative nucleoside transporter 1 (ENT1) protein. The expression of the dCK gene in araC resistant cells was reduced to 60% of H9 cells, which correlated with lower dCK protein and activity. Whereas, there was no difference in the expression of ENT1 mRNA between the cell lines, ENT1 protein content was much lower in the resistant cells than in H9 cells. The concentrative nucleoside transporter (CNT3) was slightly increased in H9-araC cells, but CNT2, and MDR1 remained unaffected. Although a definitive correlation remains to be established, the amount of Sp1 protein, a transcription factor, that regulates the expressions of dCK, nucleoside transporters and other cellular proteins, was found reduced in H9-araC cells. Like ENT1, the Sp1 mRNA levels remained unaffected in H9-araC whereas protein contents were reduced. These observations are indicative of differences in the production and/or turnover of ENT1 and Sp1 proteins in H9-araC cells. Since nucleoside transporters and dCK play an important role in the activity of potential antiviral and anticancer deoxynucleoside analogs, understanding of their regulation is important. These studies show that the exposure of cells to araC, in vitro, is capable of simultaneously affecting more than one target site to confer resistance. The importance of this observation in the clinical use of araC remains to be determined.

Keywords: Transcription factor; Sp1; Deoxycytidine kinase; Arabinosylcytosine; Nucleoside transport; Cellular resistance; Human lymphoid cells

1. Introduction

Nucleoside analogs represent a novel group of cytotoxic antimetabolites in the treatment of hematological malignancies, solid tumors and viral infections [1–4]. They mimic physiological nucleosides and share their metabolic pathways. Most nucleosides enter cells via a specialized plasma membrane NT[4] and phosphorylated by cellular kinases to their cytotoxic 5′-triphosphates, which affect RNA and DNA synthesis and other metabolic targets. araC, a deoxycytidine analog, is one of the most important antileukemic drugs currently available for the treatment of acute myeloid leukemia [5,6]. Following entering cells through ENT1, one of the several plasma membrane NTs, araC is sequentially phosphorylated to its 5′-triphosphate, araCTP, which interferes with the synthesis of DNA [7–9]. There is a correlation between the cellular accumulation and retention of araCTP and the manifestation of araC mediated cytotoxicity and clinical response [6]. The enzyme dCK is known to catalyze the first and the rate

Abbreviations: araC, 1-β-D arabinofuranosyl-cytosine (cytosine arabinoside); araCTP, 5′-triphosphate of araC; AZT, 3′-azido-2′,3′-dideoxythymidine (zidovudine); CdA, 2-chloro-2′-deoxyadenosine (cladribine); CNT2 and CNT3, concentrative nucleoside transporter 2 and 3; dCK, deoxycytidine kinase (EC 2.7.1.74); ddC, 2′,3′-dideoxycytidine; F-araA, 2-fluoro-arabinosyladenine; FUR, 5-fluoro-uridine; FUdR, 5-fluoro deoxyuridine; ENT1 and ENT2, equilibrative nucleoside transporter 1 and 2; NBMPR, nitrobenzyl-mercaptopurine ribonucleoside; NT, nucleoside transporter; 3TC, thiacytidine

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limiting step of the sequential phosphorylation of araC to araCTP.

Prolonged, in vitro and in vivo, treatment with araC has, however, resulted in the emergence of drug resistant cells with diminished sensitivity to the drug and ultimately contributing to treatment failures [6,10,11]. Although several mechanisms of resistance to araC, such as increased inactivation of araC by cytidine deaminase, decreased intracellular permeation, decreased cellular activation by dCK, increased degradation of araC-nucleotides by 5′-nucleotidase, imbalance of cellular deoxynucleotide pools, and increased capability of repair of damaged DNA, have been reported, most of the studies focused on a single mechanism of resistance[7,10–16]. We show here that in vitro cultivation of human lymphoid H9 cells in the presence of araC affects more than one target site, i.e. ENT1 and dCK, to confer araC resistance. We also present studies suggesting the molecular basis of the reduced ENT1 and dCK.

2. Materials and methods

2.1. Cells

H9 cells were maintained in RPMI 1640 medium (Gibco) containing 10% heat inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. H9-araC cells (araC resistant H9 cells) were developed by continuously exposing H9 cells to 0.5 μM araC for 3–4 weeks and characterized as described earlier [17].

2.2. Drug sensitivity assays

The cell growth inhibitory effects of nucleosides in H9 and H9-araC cells were determined as described earlier [17–19]. In brief, triplicate samples of 0.5–2.0 × 10^5 cells/ml, in 24-well costar plates (Cambridge, MA), were treated with increasing concentrations of the drugs and the trypan blue excluding cells were counted after 72 h of incubation at 37 °C. The IC_{50} values (inhibitory concentrations at 50% cell growth) were then calculated from the growth-inhibition curves.

2.3. Deoxycytidine kinase assay

For enzyme assays, the exponentially growing cells were harvested, washed and suspended in a buffer consisting of 50 mM Tris–HCl (pH 7.5), 2 mM DTT, 5 mM benzamidine, 0.5 mM PMSF, 20% (v/v) glycerol, and 0.5% IGEPAL CA-630 (Sigma). The cell-free extracts prepared by freeze thawing (4-cycles) were used as a crude source of the enzyme. The reaction mixture (0.2 ml total) contained: 37.6 mM Tris (pH 7.5), 3.8 mM $MgCl_2$, 7.5 mM NaF, 1.5 mM dithiothreitol, 3.7 mM ATP, 1 μmol creatine phosphate, 1 unit creatine kinase, 50 μM [^{3}H]-deoxycytidine and cell extract (about 60 μg protein). After incubation for 0–60 min at 37 °C, 20 μl of aliquots were withdrawn and spotted on DE81 discs (Whatman). The discs were washed three times with 1 mM ammonium formate and once with water, dried and counted. Protein concentration was determined using Biorad protein assay reagent.

2.4. Immunoblotting of deoxycytidine kinase, ENT1, ENT2, CNT2, CNT3 and Sp1

Total cell-lysates were prepared using M-PER (Pierce) according to the manufacturer′s protocol. Identical amounts of H9 and H9-araC cell lysate proteins (60 μg) were loaded onto 10% SDS-PAGE and transferred onto PVDF membrane (Biorad) using the semidry transfer method. The blots were blocked with 5% nonfat dry milk in 1 X TBS having 0.1% Tween 20 and probed with dCK antibody (1:1000), a generous gift from Dr. S. Eriksson of Sweden, and washed three times for 10 min each. Horseradish peroxidase conjugated anti-rabbit IgG (1:5000) was used as a secondary antibody and chemiluminescence detection was followed using ECL plus (Amersham).

The immunoblotting process of ENT1, ENT2, CNT2 and CNT3 was essentially similar to that described above except that in place of cell lysates the crude membrane preparation (10 μg) and monoclonal antibodies to ENT1, ENT2, CNT2, and CNT3 were used [20–22].

A similar procedure was followed for Sp1 except that 30 μg of nuclear and cytoplasmic proteins were used. Anti Sp1 was obtained from Sigma Chemical Co.

2.5. Real time quantitative PCR for dCK and Sp1

Total cellular RNA was prepared from 5 × 10^6 cells using Tri reagent (Sigma) as per manufacturers protocol and checked for its integrity by electrophoresis on 1% agarose. Total RNA (2 μg) was used for first strand synthesis using M-MLV reverse transcriptase (Promega) and the second strand synthesis was carried out in a reaction mixture containing 200 nM each of dCK gene specific primers and 25 μl of QuantiTect SYBR Green PCR Master Mix (Qiagen) in a final reaction volume of 50 μl. RT-PCR was performed in a 96-well plate on GENEAmp Sequence Detection System 5700 (Applied Biosystem). The mean normalized expression (MNE) was calculated using β-actin gene as an internal reference. The primer sequences of dCK, Sp1 and β-actin used for amplification were:

dCK (forward): 5′-AGA AGC TGC CCG TCT TTC TCA-3′
dCK (reverse): 5′-CTG AAG AAC ATT CCC ACC AT-3′
Sp1 (forward): 5′-AGT TGG TGG CAA TAA TGG GGG CAA-3′
Sp1 (reverse): 5′-TTG GCA CCC TGT GAA AGT TGT GT-3′

β-Actin (forward): 5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′
β-Actin (reverse): 5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′

2.6. Preparation of nuclear extracts

Nuclear extracts were prepared according to Schreiber et al. [23] with slight modifications. Exponentially growing 1×10^7 cells were washed twice in 20 ml of PBS and collected by centrifugation at $1500 \times g$ for 3 min. The pellets were suspended in 400 μl of ice-cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and protease cocktail inhibitor (Sigma). After the cells were allowed to swell on ice for 15 min, 25 μl of 10% Nonidet NP-40 was added and vigorously vortexed for 10 s at room temperature. The lysates were centrifuged at $1500 \times g$ for 5 min at 4 °C. The nuclear pellets were suspended in 50 μl ice-cold buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and protease cocktail inhibitor). Following incubation on ice for 15 min with intermittent shaking, the extracts were centrifuged at $2000 \times g$ for 5 min at 4 °C and the supernatants were stored at −70 °C.

2.7. Electrophoretic mobility shift assay

The assay was carried out according to Chen et al. [24]. The Sp1 probe, 5′-CGC GCG GCC CCG CCC CGG CC-3′, and its complementary strand were labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs) and annealed. Preincubation of 5 μg of nuclear extract with 2 μg of poly dI-dC in buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 0.6 mM DTT, 1 mM EDTA, 12% glycerol, 1 mM $MgCl_2$) was carried out for 10 min at room temperature followed by addition of 1 μl (4 ng) of labeled probe and further incubation for 20 min at room temperature. The complex was immediately loaded onto 4.5% native PAGE and run at 4 °C using TGE as running buffer till bromophenol reached one third of the gel. The gel was dried and autoradiographed. For super shift assay, the incubation of nuclear extracts with 1 μg of anti Sp1 was carried out for 10 min at room temperature prior to the addition of the probe.

3. Results

3.1. Cytotoxic effects of araC and other related and unrelated nucleosides in H9 and H9-araC cells

Cytotoxic effects of several nucleoside analogs including that of araC determined by cell growth inhibition assays and expressed as IC_{50} values are presented in Table 1. Compared to H9 cells, the cytotoxicity of araC, ddC and 3TC in the resistant cells was decreased by >600-fold, 5-fold and 2.0-fold, respectively. H9-araC cells were also cross-resistant to two deoxyadenosine analogs tested, F-araA (8.3-fold) and CdA (2.1-fold).

Table 1
Cytotoxic effects of nucleoside analogs, araC accumulation, araCTP, deoxycytidine kinase activity and NBMPR binding sites in H9 and H9-araC cells

Nucleoside analogs	H9 cells	H9-araC cells	RI[a]
IC_{50} values (μM)			
araC	0.006	3.5	>600
ddC	6.0	30.0	5
3TC	300	600	2
FaraA	0.6	5.0	8.3
CdA	0.6	1.2	2.1
FUR	0.03	8.0	267 [17]
FdUR	0.01	0.8	80 [17]
AZT	42.6	>2000	>47 [18]
Accumulation of [^{3}H]-araC (pmol/10^6 cells)			
Total	343 ± 3	6.6 ± 0.3	
araCTP	100.3	Undetectable	
dCK activity (pmol/min/mg protein)	252 ± 96	79 ± 14.3	
NBMPR binding sites (fmol/10^6 cells)	24 ± 4.3	12.8 ± 0.4	[17]

[a] Resistance index.

3.2. Deoxycytidine kinase and araCTP formation

The values of dCK and cellular accumulation of araC and araCTP formation in H9 and H9-araC cells are presented in Table 1. Compared to the parental cell line, the dCK activity, determined in cell-free extracts, was about 31.4%. The total araC accumulation in H9-araC cells (6.6 pmol/10^6 cells) was 1.9% of the accumulation in H9 cells (343 pmol/10^6 cells). araCTP was almost undetectable in the resistant cells, compared to 100.3 pmol/10^6 cells in the parental cell line (Table 1).

3.3. Deoxycytidine kinase mRNA and protein expression in H9 and H9-araC cells

In order to examine the molecular mechanisms of reduced dCK activity, immunoblot and quantitative RT-PCR analyses were performed. Immunoblots revealed that dCK protein expression was decreased in the resistant cells (Fig. 1). Densitometric analysis of the bands suggested that

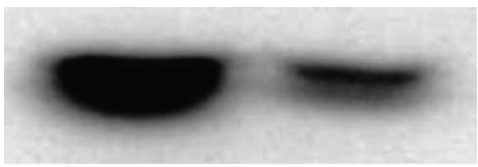

Fig. 1. Immunoblots of dCK in H9 and H9-araC cells. The bands were compared by densitometric analysis.

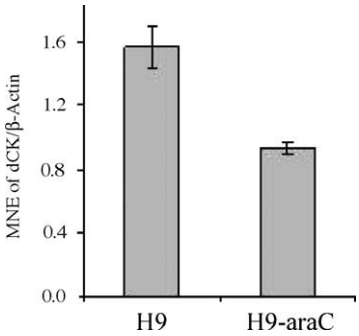

Fig. 2. Quantitative RT-PCR for dCK. Mean normalized expression (MNE) of dCK gene against β-actin.

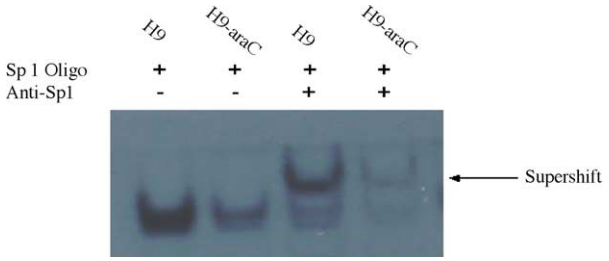

Fig. 4. Electrophoretic mobility shift assays. Gel mobility shift and super shift analysis of the DNA binding affinity of Sp1. The band in lane 2 shows a reduced binding efficiency of Sp1 in H9-araC cells compared to H9 cells (lane 1), the bands in lanes 3 and 4 confirm the specificity of binding.

dCK protein was reduced to about 40%. The mean normalized expression of dCK mRNA in H9-araC cells was about 60% of the parental cell line (Fig. 2).

3.4. ENT1, ENT2, CNT2, and CNT3 protein expression in H9 and H9-araC cells

NBMPR is a tight binding inhibitor of ENT1, one of the membrane proteins that facilitate nucleoside transport across the cell membrane. We reported earlier that H9-araC cells had about 53.3% NBMPR binding sites compared to H9 cells (Table 1) [17]. In order to determine the molecular basis of the lower NBMPR binding sites, ENT1 protein expression was studied. As shown in Fig. 3a, ENT1 protein expression in the resistant cells was reduced, which is consistent with the lower binding sites of NBMPR in these cells. Semi-quantitative RT-PCR analysis showed no difference in the mRNA of ENT1 in H9 and H9-araC cells (data not shown).

Probing the cell membranes with antibodies to ENT2, CNT2, and CNT3 revealed a slight over-expression of CNT3 in H9-araC cells (Fig. 3b), but there were no detectable bands of ENT2 and CNT2 in either cell line (data not presented).

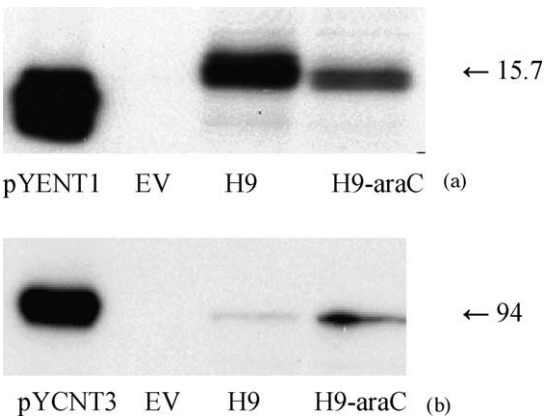

Fig. 3. (a) Immunoblots of ENT1; (b) CNT3 in H9 and H9-araC cells. EV, empty vector; pY, positive control from yeast. Due to lower glycosylation the yeast ENT1 has lower molecular weight, hence greater mobility.

3.5. Activity, immunoblot analysis and quantitative RT-PCR for Sp1

Decreased transcription of the dCK gene could have been due to hypermethylation of DNA and/or reduced amount and binding efficiency of the transcription factors. The analysis of binding efficiency by the gel shift assay showed that the binding of Sp1 protein in H9-araC cells was specific (Fig. 4, lanes 3,4) but lower than that in H9 cells (lane 2).

To determine whether the reduced binding of Sp1 to GC oligonucleotides was due to a decreased amount of protein itself and/or defective localization, western blot analyses of Sp1 in the nuclear as well as cytoplasmic fractions were carried out. As shown in Fig. 5, there is no evidence of Sp1 in the cytoplasm of either cell line. It is also evident here that in H9 cells the Sp1 appeared as a clear band in the nuclear fraction but it was almost undetectable in H9-araC cells. However, the mRNA expression of Sp1 was not significantly different between the cell lines (Fig. 6).

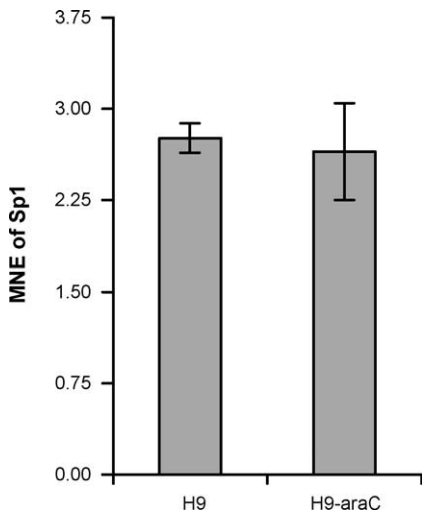

Fig. 5. Quantitative RT-PCR for Sp1. Mean normalized expression (MNE) of Sp1 gene in H9 and H9-araC cells.

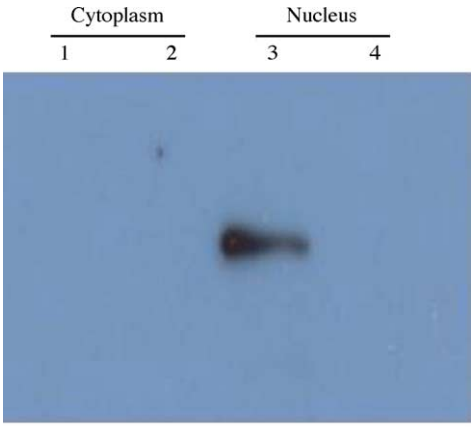

Fig. 6. Immunoblots of Sp1. Thirty micrograms each of cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) proteins of H9 and H9-araC cells, respectively, were electrophoresed and probed with Sp1 antibody as described in Section 2.

4. Discussion

In this study, we describe a human lymphoid cell line variant that became highly resistant to araC (>660-fold) following continuous drug treatment in vitro. This finding is consistent with other reports showing that, in vivo and in vitro, prolonged treatment with araC results in drug resistance with diminished drug efficacy [6,10,11]. araCTP is considered the cytotoxic metabolite of the drug [6]. An almost complete lack of araCTP accumulation in the resistant cells correlated well with the degree of resistance. Since the low dCK activity alone in H9-araC cells was insufficient to explain the extent of decrease in araCTP accumulation and the degree of resistance, studies to identify additional factors revealed diminished ENT1 sites and protein expression in the resistant cells. Although the cellular nucleoside influx is mediated through a complex array of plasma membrane NT system, araC is believed to permeate through ENT1 [25–29]. Therefore, the combined effect of reduced ENT1 and dCK activity may have been responsible for the high degree of resistance to the drug in H9-araC cells. Reduced dCK and ENT1 have been reported as a major contributing factors to araC resistance, but most of these studies focused on one mechanism of resistance, e.g. dCK or ENT1[7,10,29]. The important finding of the present studies and the studies reported earlier on the cross resistance of H9-araC cells to FUR, FUdR is that araC can simultaneously affect more than one target site to confer resistance to its own analogs as well as to other unrelated nucleosides [17,18]. Nucleosides affecting multiple sites and conferring resistance to unrelated nucleosides has also been shown recently by Groschel et al. [30], thus supporting our thesis.

Examining other possible mechanisms of araC resistance, we found no difference in the expression of efflux proteins (MDR1) between the H9 and araC cells [18], suggesting their insignificant role in the drug resistance of H9-araC cells. The cytidine deaminase and 5′-nucleotidase activity and the cellular dCTP levels were not examined here.

Since a reasonable correlation between cellular influx and dCK activity and clinical response to a number of antiviral and anticancer nucleosides listed in Table 1 has been reported [31–36], it is likely that low NT and dCK activity contributed to their cross resistance in H9-araC cells. The degree of resistance to these nucleosides, however, varied, which could be explained on the basis of differences in the cellular transport of these nucleosides even though some of them are phosphorylated by dCK.

In order to improve intervention by nucleoside analogs, it is therefore important to understand the molecular mechanisms of araC-induced deficiency of dCK and ENT1, which remain relatively unknown. The regulation at pre-translational and/or post-translational levels can alter the activities of these processes. Gene methylation [37–39], mutation affecting gene transcription and processing [40] and alternatively spliced dCK mRNA products [41] have been implicated in araC resistance. The reports also suggest that at the post-translational level the activity may be regulated by protein phosphorylation [42] and other cellular factors that alter nucleotide pools [43,44]. A significantly reduced dCK mRNA and protein in H9-araC cells suggested that araC affect at the transcriptional level may have reduced synthesis of the dCK protein. araC is known to hyper-methylate DNA and down regulate gene expression [37–39]. Although our preliminary study of methylation specific PCR of dCK failed to demonstrate a difference between the H9 and H9-araC cells, the presence of minor differences in methylationcould not be detected under the present conditions. The molecular studies of ENT1, revealed that the drug had no effect on the expression of its mRNA but reduced the amount of ENT1 protein in H9-araC cells. These differences suggest the difference in the translation and/or the turnover of ENT1. This important observation needs further exploration.

Another interesting finding was that the H9-araC cells had reduced Sp1 protein. This transcription factor is known to bind at the promoter sites and regulate the expression of NT, dCK and thymidine kinase [45–48], which were lower in H9-araC cells (this study and [18]). Although regulation of dCK by Sp1 has been demonstrated [45], to our knowledge, there is no report on the effect of the cellular levels of Sp1 protein in araC resistant cells [45]. It will be interesting to examine in the future if increasing Sp1 protein reverts the resistant cells to araC sensitive cells. It should be noted, however, that other transcription factors (USF1 and E2F) also bind at the dCK promoter sites and are affected in resistant cells [45,46]. Therefore, the establishment of the exact causal role of low Sp1 in H9-araC cells remains to be determined.

In summary, observations presented here significantly extend the earlier knowledge of araC-induced resistance in

lymphoid cells. Our observations show that araC treatment can, at the same time, affect more than one mechanism to induce resistance, while most other studies have focused on only one mechanism of resistance. Although a causal relationship has yet to be established, this is the first report to our knowledge that shows reduced levels of Sp1 in araC resistant cells, which also have low NT and dCK expression. A deeper understanding of the major determinants of NT and dCK regulation may, therefore, help modify cellular activities of these processes and enhance the antiviral and anticancer potential of deoxynucleosides analogs.

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