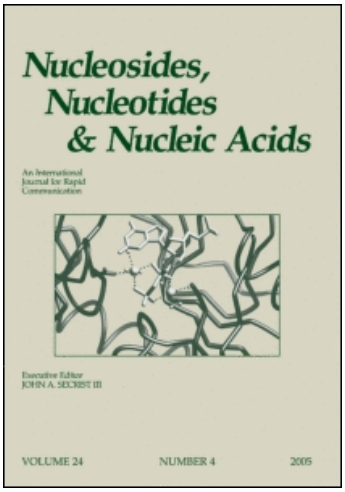

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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To cite this Article Fyrberg, Anna , Albertioni, Freidoun and Lotfi, Kourosh(2008) 'RNAi Depletion of Deoxycytidine and Deoxyguanosine Kinase in Human Leukemic CEM Cells', Nucleosides, Nucleotides and Nucleic Acids, 27: 6, 712 — 719
To link to this Article: DOI: 10.1080/15257770802145231
URL: http://dx.doi.org/10.1080/15257770802145231

Nucleosides, Nucleotides, and Nucleic Acids, 27:712–719, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802145231

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RNAi DEPLETION OF DEOXYCYTIDINE AND DEOXYGUANOSINE KINASE IN HUMAN LEUKEMIC CEM CELLS

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□ *Resistance toward nucleoside analogues is often due to decreased activities of the activating enzymes deoxycytidine kinase (dCK) and/or deoxyguanosine kinase (dGK). With small interfering RNA (siRNA), dCK and dGK were downregulated by approximately 70% in CEM cells and tested against six nucleoside analogues using the methyl thiazol tetrazolium assay. SiRNA-transfected cells reduced in dCK activity were 3- to 6-fold less sensitive to CdA, AraC, and CAFdA. The sensitivity to AraG and FaraA was unchanged, while the sensitivity toward gemcitabine was significantly increased. dGK depletion in cells resulted in lower sensitivity to FaraA, dFdC, CAFdA, and AraG, but slightly higher sensitivity to CdA and AraC.*

Keywords Deoxycytidine kinase; deoxyguanosine kinase; nucleoside analogue; resistance; RNA interference

INTRODUCTION

The cytosolic enzyme deoxycytidine kinase (dCK, E.C.2.7.1.74) is the major activating enzyme for several nucleoside analogues used clinically and experimentally, but the mitochondrial deoxyguanosine kinase (dGK, E.C.2.7.1.113) contributes to some of the activation as well.[1] Nucleoside analogues are prodrugs commonly used in the treatment of haematological malignancies, requiring intracellular phosphorylation to become active. Resistance against nucleoside analogues is a common phenomenon and

This work was supported by grants from the Swedish Cancer Foundation, Signe and Olof Wallentin Foundation, Capio's Research Foundation,Swedish Fund for Research without Animal Experiments, and the County Council of Östergötland.

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downregulation of the activating enzymes is considered one of the explanations for cellular resistance against these compounds.[2,3] Once activated the cytotoxic effects of nucleoside analogues are carried out by the nucleotides (mono-, di, and triphosphates) involving disruption of DNA and RNA synthesis, inhibition of enzymes vital for the biosynthesis of nucleotides, as well as disruption of mitochondrial integrity leading to the release of the pro-apoptotic mitochondrial proteins cytochrome c and apoptotic protease activating factors.[4]

Cell lines deficient in dCK and/or dGK can be generated by several months of exposure of cells to increasing concentrations of a nucleoside analogue activated by these two enzymes. This will generate a cell line cross-resistant not only to other nucleoside analogues also activated by these enzymes [3,5] but to completely different types of drugs as well. [6] However, this technique is time consuming and it has been shown that compensatory mechanisms become present in the cells when they develop resistance.[3,5] To generate dCK- and dGK-deficient cells without prior exposure to drugs we chose to use the technique of RNA interference. Small interfering RNA (siRNA) with a length of 21–22 nucleotides can, if transfected into a cell, mediate a sequence-specific degradation of mRNA, and is, therefore, a useful tool to study a specific gene function and its protein product.[7] The aim of this study was to transfect the human leukemic cell line CCRF-CEM (CEM) with siRNA against dCK and dGK and to study the impact of downregulation on the activity of different nucleoside analogues and to determine their dCK and/or dGK dependency.

MATERIALS AND METHODS

Cell Culture

CEM cells (acute T-lymphoblastic leukaemia, American Type Culture Collection, Rockville, MD) were kept in RPMI-1640 medium with 10% foetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Subculturing took place weekly, and cells were grown in a humidified atmosphere, 37°C, and 5% CO_2.

siRNA Transfection

Cells were washed in RPMI-1640 medium, counted and resuspended at a concentration of 4×10^6 cells/ml in RPMI-1640 medium containing 2 mM L-glutamine. Dimethyl sulfoxide (DMSO) was added to a concentration of 1.25% for cell membrane stability and siRNA was added to a concentration of 250 nM (*Silencer* Validated siRNA #70 for dCK and #1606 for dGK, Ambion, Inc., Huntington, Cambridge, UK). Four hundred μl CEM cell suspension (4×10^6 cells/ml) was electroporated in 0.4 cm cuvettes (Bio-Rad

Laboratories, Hercules, CA) using square waves from a Gene Pulser Xcell Electroporation System (Bio-Rad) at 340 V twice for 10 ms. After transfection, cells were resuspended in RPMI-1640 medium containing 2 mM L-glutamine and 20% FCS. A negative control siRNA (Ambion Inc.) that did not bind to any human mRNA sequence was used and transfected into control cells, and *Silencer* GAPDH siRNA (Ambion, Inc.) was used as a positive control.

RNA Extraction, Reverse Transcriptase PCR, and Real-Time PCR

RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Solna, Sweden). Complementary DNA was produced using the High Capacity Archive kit (Applied Biosystems, Stockholm, Sweden) according to the manufacturer's instructions. PCR primers and Taqman probes designed for dCK, dGK, and the housekeeping gene β_2-microglobulin were from Applied Biosystems and have been previously published.[8] The reactions were run in an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Protein Extraction and Enzyme Activity Measurements

Proteins were extracted and enzyme activities measured as previously described.[9+] Substrates used were 10 μM deoxycytidine ([8-^{3}H]-2′-deoxycytidine, specific activity 8.3 Ci/mmol) for dCK and 40 μM deoxyguanosine ([6-^{3}H]-2′-deoxyguanosine, specific activity 10 Ci/mmol) for dGK. All activities were expressed as pmol product formed per minute per mg of protein. Protein content (mg/ml) was determined by the method of Lowry [10] using the DC protein assay (Bio-Rad) employing bovine serum albumin as standard.

Cytotoxicity Assay

Cellular sensitivity toward different drugs was assessed using the methyl thiazol tetrazolium (MTT) assay, which measures cell proliferation. After siRNA transfection, cells were allowed to recover for 24 hours before MTT assay. The MTT assay was performed as described earlier but with 48 hours of drug incubation instead of 72 hours.[9]

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Data were expressed as means and standard deviations and were analysed with the Student's t-test with a p value of ≥ 0.05 considered significant.

RESULTS AND DISCUSSION

The expression and enzymatic activities of dCK and dGK were determined over a time course of 96 hours and were reduced up to at least 72 hours after transfection, which was also confirmed at the protein expression level (not shown). At some time-points the reduction was approximately 70% at mRNA and activity level compared to control cells. The downregulation of dCK was most prominent at 24 hours after transfection both at the mRNA and activity level and lasted up to 96 hours (Figure 1A). The downregulation of dGK was most efficient 24 hours after transfection at the mRNA level while at the activity level downregulation was seen at 72 and 96 hours after transfection (Figure 1B). dGK expression and activity were measured in the cells with reduced dCK expression and vice versa to determine possible off-target effects of the siRNA or unspecific binding to similar targets. When dCK had been downregulated the expression and activity of dGK were unchanged up to 72 hours after transfection but at 96 hours dGK expression became elevated (Figure 2A) potentially as a late compensatory effect of the low dCK expression. No apparent effects on dCK expression or activity were observed when dGK had been downregulated (Figure 2B).

A similar study to this was performed where ribozymes specified to degrade dCK mRNA were used in leukaemic L1210 murine cells.[11] Transfection efficiency was 80%, while the reduction of dCK mRNA was about 40% and the activity was reduced by approximately 20%.[11] These cells did however not display any drug resistance to AraC compared to control cells [11] showing that a more pronounced downregulation of dCK activity as in this study was necessary to obtain an effect on nucleoside analogue activation. Despite the 70% downregulation at 24 hours after transfection, there were considerable amounts of dCK and dGK activity left in the cells. However, when measuring dCK and dGK activities in samples from leukemic patients with different degree of resistance to nucleoside analogues or in nucleoside analogue resistant cell lines they usually show some percentage of

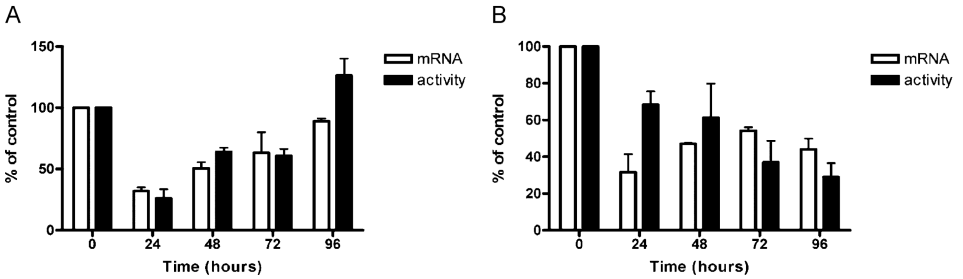

FIGURE 1 Means ± standard deviations of three or more independently performed experiments using dCK-siRNA (A) and dGK-siRNA (B) in CEM cells. Cells were transfected with 250 nM siRNA using electroporation. Cells were harvested at 24, 48, 72, and 96 hours and assayed for mRNA expression (white bars) and enzymatic activity (black bars) and compared to cells transfected with a negative control siRNA.

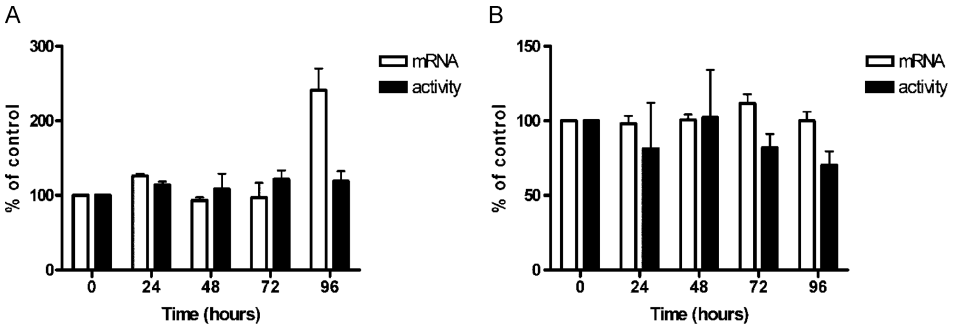

FIGURE 2 Means ± standard deviations of three or more independently performed experiments. dGK mRNA expression (white bars) and enzyme activity (black bars) were measured in dCK-siRNA transfected cells (A) and dCK mRNA expression (white bars) and enzyme activity (black bars) were investigated in dGK-siRNA transfected cells (B). This was done to determine any possible off-target effects of the siRNA binding to similar targets and to monitor compensatory mechanisms of one enzyme when the other enzyme had been downregulated.

dCK and/or dGK activities remaining in the cells resembling the levels seen here.[1,12] It may, therefore, not be necessary with a complete 100% downregulation of these two enzymes in order to study the effects of nucleoside analogue activation.

CEM cells with reduced activity of dCK or dGK were incubated with six different nucleoside analogues in order to see if and how their sensitivity toward these analogues changed. As control drugs 6-mercaptopurine (6-MP) and daunorubicin (Dnr) were used since they are independent of these enzymes for activity. IC50 values for the different drugs were compared

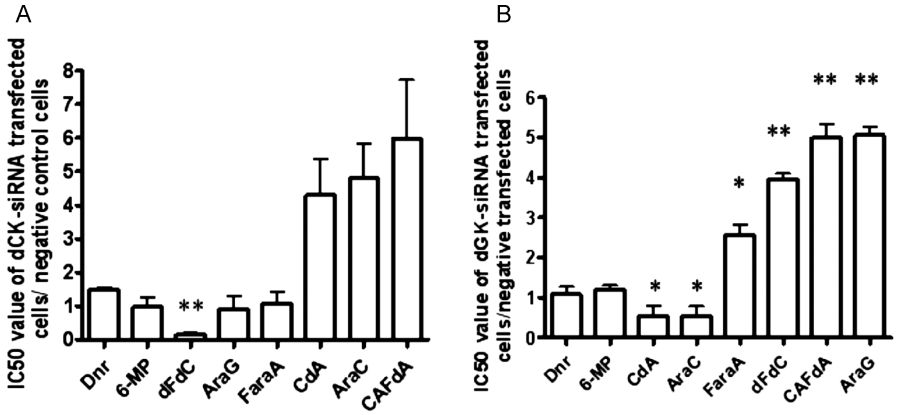

FIGURE 3 dCK-siRNA transfected cells with reduced dCK activity (A) or dGK-siRNA transfected cells with reduced dGK activity (B) were incubated with cytotoxic drugs and sensitivity (IC50 value) of the cells to the different compounds were compared with negative control transfected cells using MTT assay in three or more independently performed experiments. Daunorubicin (Dnr) and 6-mercaptopurine (6-MP) were used as a control drugs since their activities are not dependent of dCK or dGK. *-$p \geq 0.05$, **- $p \geq 0.01$.

between cells with low dCK or dGK activities and cells transfected with the negative control siRNA. As can be seen in Figures 3A and B, the ratio of IC50 values of 6-MP and Dnr were around one in cells with reduced dCK and dGK activities, indicating they were equally active despite downregulation of one of these two enzymes. Cells with reduced dCK activity showed unaltered sensitivity toward AraG and FaraA indicating they were efficiently phosphorylated by dGK since this enzyme was unaffected by the dCK downregulation. CdA, AraC and CAFdA were four to six fold less activated, while dFdC was 8-fold more activated in these cells. In cells with reduced dGK activity, CdA and AraC were significantly more activated indicating their dependency of dCK for activation, while FaraA, dFdC, CAFdA, and AraG were 2.5- to 5-fold less activated in these cells due to their dependence on dGK for activation. In these experiments it seems that CAFdA was equally dependent on both dCK and dGK for activation. However, other mechanisms than dCK and dGK activation may be of importance for activity of some of the nucleoside analogues but were not further investigated in this study.

Deoxycytidine kinase is reported to be the first and rate-limiting enzyme for the activation of several nucleoside analogues but many of the nucleoside analogues tested here, can be phosphorylated and activated to various extent by the mitochondrial dGK,[13–16] since AraG-resistant MOLT-4 cells deficient in dGK display cross-resistance to AraC, CdA, FaraA, and CAFdA.[13] It has previously been shown that AraG is incorporated into mitochondrial DNA only, while AraC is incorporated only into nuclear DNA suggesting different activating enzymes for the two drugs.[17] Our results are in agreement with these observations since the cytotoxic effect of AraG was unchanged or slightly increased in the dCK downregulated cells and significantly decreased in the dGK reduced cells, while the cytotoxicity of AraC was decreased almost 4-fold in dCK reduced cells and more efficiently activated in dGK downregulated cells.

Our data also show a rather strong dependence of CdA upon phosphorylation by dCK, indicating that dCK is the major activating enzyme of this drug, while dGK does not seem to contribute to its activation. In this study it also seems that FaraA is solely dependent on dGK for activation. A strong correlation between the IC_{50} value of FaraA and dCK activity has however been shown[18] but a mechanism other than dCK deficiency has been proposed for resistance to FaraA,[3] since dCK-deficient CEM cells may still be sensitive to the cytotoxicity of FaraA[19] as also seen here. The reason for FaraA-resistance has been shown to involve altered activity of ribonucleotide reductase (RR)[20] which is also true for dFdC where the RR activity was increased 2.4-fold in dFdC-resistant colon cancer cells.[21] In our siRNA transfected cells the R1, R2, and the alternative subunit p53R2 of the RR enzyme were more or less unchanged compared to control cells when looking at protein expression using western blot technique (not shown), which may be the reason for sustained cytotoxicity of FaraA in the cells with

reduced dCK activity. This enzyme reduces ribonucleotides to their corresponding deoxyribonucleotides leading to increased endogenous levels of dNTPs competing with the triphosphates of the active nucleoside analogues, and can thus be involved in resistance.

Resistance mechanisms toward dFdC have been shown to involve decreased dCK activity[22] but its cytotoxicity is less dependent on the activity of dCK than other drugs such as CdA, AraC and FaraA.[18] A dFdC-resistant murine leukaemic cell line showed cross-resistance to AraC and CdA but not to FaraA.[23] The current study shows that there may be additional mechanisms for activation of dFdC. We plan to investigate whether other metabolic pathways for dFdC activation or deactivation are present in these siRNA transfected cells using microarray analysis. This can possibly also rule out any off-target or cytotoxic effects of the siRNA or the method of transfection.

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