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The Subcellular Location of Nucleoside Analog Phosphorylation Is a Determinant of Synergistic Effects of Hydroxyurea

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The ribonucleotide reductase inhibitor hydroxyurea exhibits synergistic pharmacological activity with several nucleoside analogs used in antiviral and anticancer chemotherapy. We have used a cell model system where a deoxycytidine kinase (dCK)-deficient cell line was reconstituted with genetically engineered dCK targeted to the cytosol, the nucleus, or the mitochondria to investigate how the subcellular location of nucleoside analog phosphorylation affected the synergistic effects of a ribonucleotide reductase inhibitor. Hydroxyurea showed synergistic cytotoxicity with the nucleoside analogs 1-β-D-arabinofuranosylcytosine and 2-chloro-2′-deoxyadenosine when dCK was expressed in the cytosol or in the nucleus, but not when dCK was expressed in the mitochondria. These data indicate that the synergistic effect of ribonucleotide reductase inhibition is limited to nucleoside analogs phosphorylated in the cytosol or the cell nucleus.

Key Words: **ribonucleotide reductase; nucleoside analog; nucleoside kinase; chemotherapy; dNTP pools; nucleotide metabolism.**

Mammalian cells have two pathways for deoxyribonucleotide synthesis, the *de novo* and the salvage pathways (1). Ribonucleotide reductase is the key enzyme of the *de novo* pathway which constitutes the main supply of deoxyribonucleotides for nuclear DNA replication (2). The two subunits of ribonucleotide reductase are localized in the cytosol and the enzyme is active in the S-phase of the cell cycle. Recently, a novel gene product homologous to one of the ribonucleotide reductase subunits was identified, but its contribution to deoxyribonucleotide synthesis is not clear (3). In addition to the *de novo* deoxyribonucleotide synthesis, cells can salvage deoxyribonucleosides by phosphorylation to deoxyribonucleoside triphosphates catalyzed by nucleoside and nucleotide kinases. The rate-limiting step of the salvage pathway is catalyzed by the deoxyribonucleoside kinases: deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), and thymidine kinase 1 and 2 (TK1 and TK2) (4). TK1 is a cytosolic enzyme, dCK is located in the cytosol and/or nucleus, whereas both dGK and TK2 are mitochondrial enzymes (5–8). dCK, dGK and TK2 are constitutively expressed throughout the cell cycle whereas TK1 exhibits a strict S-specific expression pattern similar to ribonucleotide reductase (4).

The salvage pathway is important for the pharmacological activation of antiviral and anticancer nucleoside analogs. The nucleoside analog triphosphates compete with the deoxyribonucleotides as substrates of DNA polymerases and consequently interfere with cellular or viral DNA replication. The amount of nucleoside analogs incorporated into DNA is correlated with their cytotoxicity (9). One approach to enhance the efficiency of nucleoside analog chemotherapy is to combine nucleoside analogs with compounds that inhibit ribonucleotide reductase (10, 11). The inhibition of ribonucleotide reductase results in a decreased *de novo* synthesis of dNTPs that is favorable for the incorporation of nucleoside analogs into DNA. Furthermore, a decrease of the dNTP pool may also increase the phosphorylation of nucleoside analogs because several nucleoside kinases are feed-back inhibited by dNTPs. Ribonucleotide reductase inhibitors, such as hydroxyurea, have shown synergistic pharmacological effects with several different nucleoside analogs used in antiviral and anticancer chemotherapy (12–14). Recently, hydroxyurea was also shown to increase the cytotoxicity of ganciclovir in cells transfected with herpes simplex virus thymidine kinase (15).

Nucleoside analogs are phosphorylated in the nucleus, the cytosol or the mitochondria depending on

Abbreviations used: dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; TK1 and TK2, thymidine kinase 1 and 2; araC, 1-β-D-arabinofuranosylcytosine; CdA, 2-chloro-2′-deoxyadenosine; dFdC, 2′,2′-difluorodeoxycytidine; GFP, green fluorescent protein.

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which of the human nucleoside kinases that catalyze the phosphorylation. There is evidence that the mitochondrial dNTP pool is separated from the cytosolic/nuclear pool and that anti-metabolites have different effects on the pools in the different compartments (16–18). We have in the present study reconstituted dCK-deficient cells with dCK targeted to the cytosol, the cell nucleus or the mitochondria. By using this cell model we have investigated the synergistic effects of combining a nucleoside analog with hydroxyurea to further understand the subcellular compartmentalization of nucleoside analog phosphorylation *in vivo*.

MATERIALS AND METHODS

Culture and transfection of cell lines. The dCK-deficient CHO cell lines expressing dCK targeted to the nucleus, cytosol or mitochondria was created as described (18). Briefly, the dCK cDNA was cloned into the pEGFP-N1 plasmid vector to express the protein in fusion with the green fluorescent protein (GFP). The wild-type dCK-GFP fusion protein (nucDCK-GFP) efficiently translocates into the cell nucleus, whereas dCK with a mutated nuclear targeting signal is retained in the cytosol (cytDCK-GFP) (7). The N-terminal mitochondrial targeting sequence of cytochrome *c* oxidase was fused to the N-terminal of the cytosolic dCK-GFP fusion protein to target the dCK-GFP protein to the mitochondrial matrix (mitDCK-GFP). The cells were cultured in McCOY 5A modified medium supplemented with 10% (v/v) fetal calf serum (Gibco BRL), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were grown at 37°C in a humidified incubator with a gas phase of 5% CO_2.

Autoradiography. The cells were cultured on poly-L-lysine-coated chamber slides (Nunc, Inc.) for 24 h. Cells were labeled with [^{3}H]-1-β-D-arabinofuranosylcytosine (Moravek Biochem) for 6–20 h. The slides were rinsed with PBS, fixated 10 min in methanol:acetic acid (3:1), and washed three times with ice-cold 10% TCA, once with water and once with methanol. The slides were coated with Hypercoat photoemulsion (Amersham) and exposed 1–3 weeks at 4°C. The autoradiography films were developed using D-11 developer (Kodak).

Cell proliferation assays. 10^4 cells were plated in 96-well microtiter plates and indicated concentrations of araC (Sigma), CdA (Sigma), dFdC (Lilly Research Laboratories), and 0.25 mM hydroxyurea (Sigma) was added when indicated. Cell survival was assayed by the metrotetrazolium assay (Sigma) after 4 days of drug exposure. Each experiment was performed in triplicate.

RESULTS

A cell system with dCK-deficient CHO cell lines transfected with engineered dCK targeted to either the nucleus, the cytosol or the mitochondria was used in this study (18) (Fig. 1). These transfected cell lines allow studies of the pharmacological effects of nucleoside analog phosphorylation in the three different subcellular compartments. We used an autoradiography method to visualize the incorporation of tritiated nucleoside analogs into nuclear or mitochondrial DNA. As shown in Fig. 2, the cells expressing dCK in the cytosol or in the nucleus showed a distinct dark staining of the nucleus when incubated with ^{3}H-araC, indicating incorporation of the nucleoside analog into nuclear DNA. The cells expressing dCK in the mitochondria showed, however, a dotted autoradiography pattern distributed throughout the cell, indicating incorporation of ^{3}H-araC into mitochondrial DNA (18).

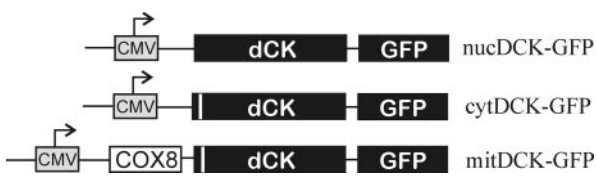

FIG. 1. Plasmids constructed to express dCK in the nucleus (nucDCK-GFP), cytosol (cytDCK-GFP), and mitochondria (mitDCK-GFP) (18). The nuclear signal in the N-terminal was mutated (white box) to express dCK in the cytosol. The mitochondrial targeting signal of cytochrome *c* oxidase subunit VIII (COX8) was cloned upstream of the cytosolic mutant to target dCK to the mitochondrial matrix. CMV, cytomegalovirus promoter.

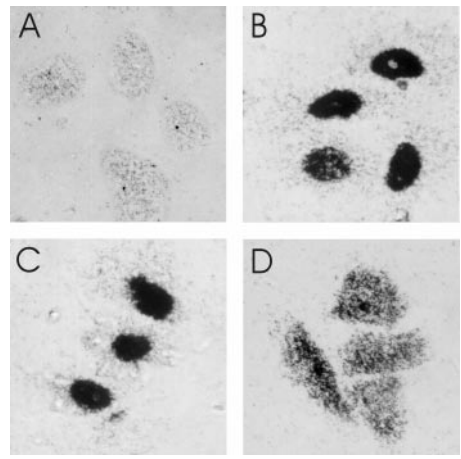

FIG. 2. ^{3}H-araC incorporation into nuclear and mitochondrial DNA visualized by autoradiography in dCK-deficient CHO cells (A) and cells transfected with nucDCK-GFP (B), cytDCK-GFP (C), and mitDCK-GFP (D).

The ribonucleotide reductase inhibitor hydroxyurea was used in combination with the dCK activated nucleoside analogs araC, CdA and dFdC. We first determined the sensitivity of the transfected and untransfected CHO cells to different concentration of hydroxyurea (Fig. 3). There was no difference in hydroxyurea sensitivity of the cells expressing dCK in the different subcellular locations. The lowest hydroxyurea concentration that did not affect cell survival under the assay conditions was 0.25 mM. We therefore decided to use this concentration in the subsequent studies of combinations of hydroxyurea and nucleoside analogs.

We determined the cell lines sensitivity to araC, CdA and dFdC in the presence and absence of 0.25 mM hydroxyurea (Fig. 4). The cells expressing dCK in the cytosol or nucleus incubated with araC or CdA showed approximately 10-fold lower IC_{50} in the presence of hydroxyurea, compared to the cells incubated with nucleoside analog alone. In contrast, there was no in-

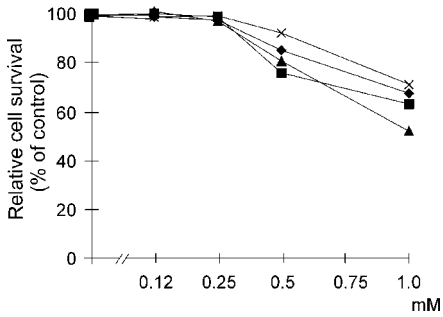

FIG. 3. Hydroxyurea sensitivity of dCK-deficient CHO cells (◆) and CHO cells transfected with dCK targeted to the nucleus (■), cytosol (▲), or mitochondria (×).

crease of araC or CdA sensitivity when hydroxyurea was added to the cells expressing dCK in the mitochondria. The sensitivity of dFdC was not affected by the addition of hydroxyurea for any of the cell lines expressing dCK.

DISCUSSION

We have shown in the present study that synergistic cytotoxicity of the ribonucleotide reductase inhibitor hydroxyurea and the nucleoside analogs araC or CdA occurred when the nucleoside analogs were phosphorylated in the cytosolic/nuclear compartment, but not when the nucleoside analogs were phosphorylated in the mitochondria. These findings support the notion that the cytosolic/nuclear dNTP pool is separated from the mitochondrial dNTP pool. Deoxyribonucleosides and nucleoside analogs phosphorylated in the cytosol or nucleus are incorporated into nuclear DNA. In contrast, when phosphorylated in the mitochondria, these compounds become trapped and are predominantly incorporated into mitochondrial DNA (16, 18, 19). Although studies suggest that deoxyribonucleotides and phosphorylated nucleoside analogs can be imported into the mitochondria from the cytosol (16, 20, 21), this transport system appears to be inefficient to equili-

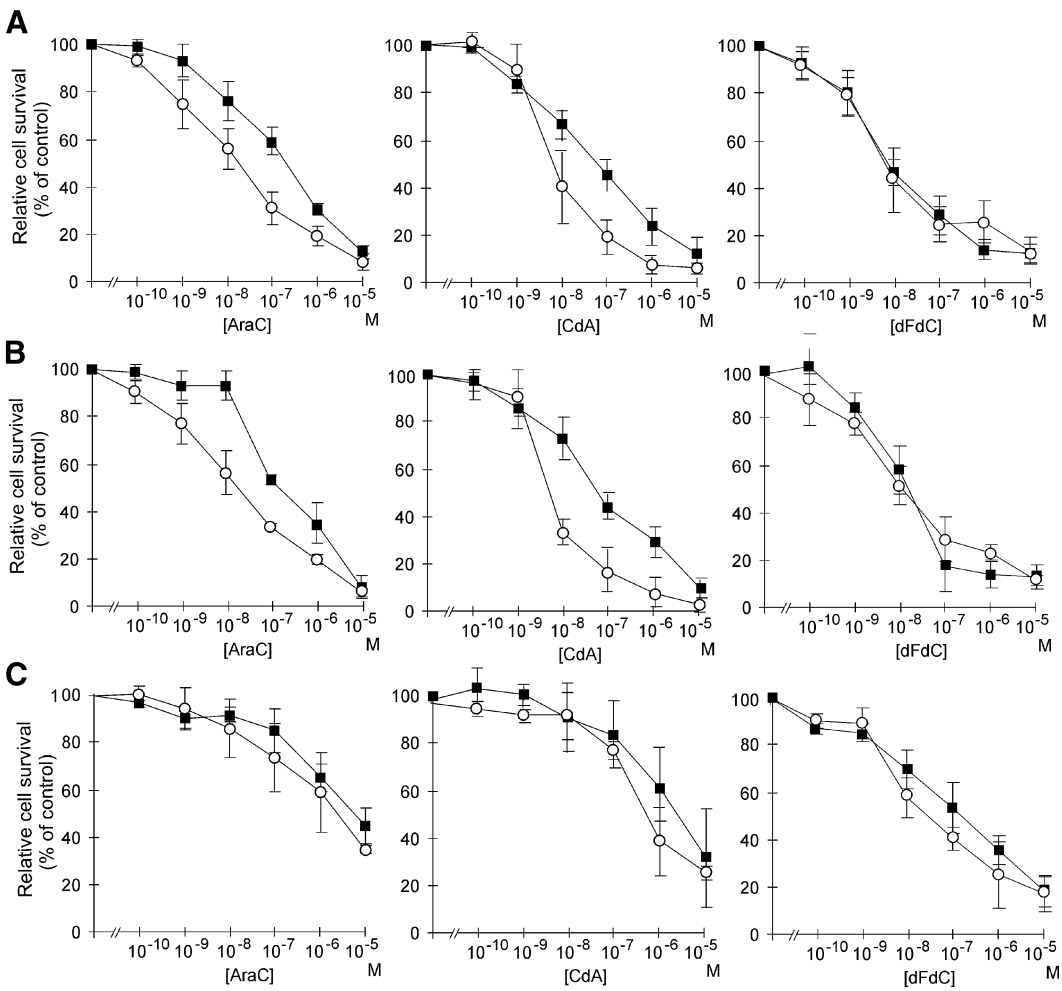

FIG. 4. Sensitivity of CHO cells expressing dCK in the nucleus (A), cytosol (B), or mitochondria (C) to nucleoside analogs in the absence (■) or presence (○) of 0.25 mM hydroxyurea. Mean values of three separate experiments. Error bars indicate standard deviation.

brate the two dNTP pools. Ribonucleotide reductase inhibition by hydroxyurea is likely to primarily affect the cytosolic/nuclear deoxyribonucleotide pool, since ribonucleotide reductase is a cytosolic enzyme. The decrease in the cytosolic/nuclear dNTP pool will cause an increase in sensitivity to the nucleoside analogs due to a change in the nucleoside analog triphosphate/dNTP ratio which favors incorporation of nucleoside analogs into nuclear DNA and/or cause an increase in nucleoside analog phosphorylation by decreasing the dNTP feedback inhibition of nucleoside kinases. Because the cytosolic/nuclear dNTP pool does not equilibrate with the mitochondrial dNTP pool, there was no cytotoxic synergy of hydroxyurea and the nucleoside analogs phosphorylated in the mitochondria.

In contrast to araC and CdA, the nucleoside analog dFdC did not exhibit synergistic cytotoxicity with hydroxyurea regardless the site of phosphorylation. Previous studies have shown that dFdC-diphosphate is a potent inhibitor of ribonucleotide reductase (22). It has also been shown that cells exposed to dFdC showed similar changes in dNTP pool levels as cells exposed to hydroxyurea (22, 23). Accordingly, it is likely that dFdC by itself inhibits ribonucleotide reductase and no further inhibition is achieved by hydroxyurea at the concentration used in these experiments.

In summary, our study demonstrates that it is important to consider the subcellular site of nucleoside analog phosphorylation when combining these compounds with other antimetabolites to achieve synergistic effect.

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