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Phosphorylation of Anticancer Nucleoside Analogs by Human Mitochondrial Deoxyguanosine Kinase

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ABSTRACT. The kinetic properties of recombinant human mitochondrial deoxyguanosine kinase (dGK, EC 2.7.1.113) for 2′-deoxyguanosine and the clinically important nucleoside analogs 2-chloro-2′-deoxyadenosine (CdA), 9-β -D-arabinofuranosylguanine (araG) and 2′,2′,-difluorodeoxyguanosine (dFdG) were determined. The Michaelis–Menten kinetic parameters, comparing ATP and UTP as phosphate donors, demonstrated a marked increase in phosphorylation efficiency ($V_{max}K_m$) with UTP in comparison with ATP for both CdA and araG. The difluoro analog dFdG was an efficient substrate for recombinant dGK with an apparent K_m of 16 μM with ATP as phosphate donor. We compared the kinetic properties of dGK with those of the related enzyme deoxycytidine kinase (dCK, EC 2.7.1.74). Although the purines 2′-deoxyguanosine (dGuo) and 2′-deoxyadenosine are substrates for both dGK and dCK, only CdA among the purine nucleoside analogs tested was an efficient substrate for both dCK and dGK. In competition with dGuo, the most efficient analog for phosphorylation by dGK was araG, as indicated by a lower K_i value than for CdA and dFdG. Of the purine analogs tested as substrates for dCK, only CdA could compete with 2′-deoxycytidine (dCyd). No inhibition of dCK-mediated dCyd phosphorylation was found by either araG or dFdG. In crude cell extract of HeLa and Capan 2 cells, the major CdA phosphorylation was contributed by dCK, while most araG phosphorylation was a result of dGK activity. Our study with pure recombinant enzymes confirms that dGK is mainly responsible for araG and dFdG phosphorylation, whereas dCK is the most important enzyme for activation of CdA and 2′,2′-difluorodeoxycytidine (dFdC). BIOCHEM PHARMACOL **56**;8:1035–1040, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. deoxyguanosine kinase; deoxycytidine kinase; nucleoside phosphorylation; nucleoside analogs; pyrimidine metabolism; purine metabolism

Nucleoside analogs are extensively used in antiviral and anticancer therapy. The pharmacological activities of nucleoside analogs are dependent on phosphorylation by cellular or viral enzymes. Phosphorylation of nucleoside analogs to the corresponding nucleoside monophosphates is the rate-limiting step for the pharmacological activation of most nucleoside analogs. There are four known deoxyribonucleoside kinases in mammalian cells: the S-phase-specific thymidine kinase 1 and the cell cycle constitutively expressed dCK§ (EC 2.7.1.74), dGK (EC 2.7.1.113) and thymidine kinase 2. We have recently cloned the cDNA that encodes human dGK [1]. The amino acid sequence as well as the substrate specificity of dGK have similarities with the previously well-characterized dCK. Both dGK and dCK phosphorylate the purine nucleosides deoxyguanosine and deoxyadenosine, while the pyrimidine nucleoside deoxycytidine is phosphorylated only by dCK. The purine nucleoside analogs CdA and araG are active against several types of lymphoid malignancies [2]. Both compounds are substrates for dCK, but are also reported to be efficiently phosphorylated by mitochondrial dGK *in vitro* [3]. However, the relative contribution of dGK and dCK to the phosphorylation of CdA and araG *in vivo* is not known. dFdC (gemcitabine) is a difluoro-substituted deoxycytidine analog that is active against solid malignant tumours [4]. This nucleoside analog is a substrate of both dCK and mitochondrial thymidine kinase 2 [5]. A recent report on dFdC's purine congener dFdG shows that this nucleoside analog is cytotoxic as well [6]. Studies using a dCK-deficient cell line and partially purified cellular deoxyribonucleoside kinases suggest that dGK is responsible for dFdG phosphorylation [6, 7].

We decided to use highly purified recombinant human dCK and dGK to determine the kinetic properties of the clinically important anticancer nucleoside analogs CdA, araG, dFdC and dFdG with the phosphate donors ATP and UTP. We have also determined how efficiently these analogs compete with the naturally occurring deoxyribonucleosides accepted by the enzymes, and studied the phosphorylation of CdA and araG in crude extracts of

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§ *Abbreviations:* araG, 9-β-D-arabinofuranosylguanine; CdA, 2-chloro-2′-deoxyadenosine; dAdo, 2′-deoxyadenosine; dCK, deoxycytidine kinase; dGuo, 2′-deoxyguanosine; dCyd, 2′-deoxycytidine; dFdC, 2′,2′,-difluorodeoxycytidine; dFdG, 2′,2′,-difluorodeoxyguanosine; and dGK, deoxyguanosine kinase.

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human cancer cell lines. These data are important to elucidate the mechanisms of tissue-selective cytotoxicity of nucleoside analogs and to develop combination therapy of different nucleoside analogs on a rational basis. In conclusion, we demonstrate that: i) both recombinant human dGK and dCK phosphorylate natural purines and the base-modified purine analog CdA, while purine analogs with sugar modifications, such as araG and dFdG, are substrates mainly for dGK; ii) CdA competes more efficiently with dCyd for dCK phosphorylation than with dGuo for dGK phosphorylation; and iii) UTP compared with ATP as phosphate donor for dGK increases the efficiency of nucleoside analog phosphorylation.

MATERIALS AND METHODS

Materials

2-chloro-2′-[8-^{3}H]-deoxyadenosine (4 Ci/mmol), 9-β-D-[^{3}H]arabino-furanosylguanine (6.5 Ci/mmol) and 2′-[8-^{3}H]-deoxyguanosine (5.5 Ci/mmol) were purchased from Moravek. Unlabeled CdA, araG, dGuo and dCyd were obtained from Sigma. All reagents were of highest purity available. dFdC and dFdG were gifts from Lilly Research Laboratories.

Enzyme Preparation and Purification

The human dGK cDNA open reading frame sequence was ligated into the pMAL-C2 plasmid (New England Biolabs) to express the enzyme as a fusion protein with the maltose-binding protein. The construct was transformed into the *E. Coli* TB1 host strain and transformants were selected. A single positive colony was inoculated in a large-scale expression culture (LB broth containing 100 μg/mL of ampicillin) at 37°. At OD_{600} ≈0.5, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce fusion protein expression. After 2 hr induction, the cells were harvested by centrifugation at 4000 *g* for 20 min. The bacterial pellet was lysed by freeze-thawing and sonication for 3 × 1 min on ice in column buffer (20 mM Tris, pH 7.6, 200 mM NaCl, 1 mM EDTA). The crude extract was cleared by centrifugation at 9000 *g* for 30 min. The supernatant was diluted 1:5 by column buffer and loaded onto the amylose resin column. The protein was eluted in the column buffer with 10 mM maltose added. The fusion protein was cleaved with factor Xa (New England Biolabs) for 6 hr at room temperature. The size and purity of the cleaved protein was determined by SDS/PAGE (PhastSystem, Pharmacia). The protein concentration was determined by the method of Bradford with BSA as standard.

Preparation of Cell Extracts

The human pancreatic adenocarcinoma cell line, Capan 2, and the cervix epithelium cancer cell line, HeLa, were obtained from the American Type Culture Collection. The Capan 2 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, respectively. The medium was supplemented with 10% foetal calf serum (New England Nuclear), 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Crude cell extracts were prepared from 2 × 10^6 exponentially growing cells. After washing with PBS, the cells were lysed in 150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 1% SDS and 0.5% deoxycholate. The cell lysate was transferred to an Eppendorf tube, vortexed and incubated on ice for 30 min. After centrifugation for 20 min at 4°, the supernatant was stored at −70°.

Enzyme Assay

dGK activity was measured by a radiochemical method as described [8]. ^{3}H-dGuo, ^{3}H-CdA or ^{3}H-araG was used as a substrate. Indicated concentrations of nucleosides, nucleoside analogs, enzymes and 1 mM ATP or UTP were added to a volume of 35 μL. At 10, 20 and 30 min incubation at 37°, 10 μL of the reaction mixtures were spotted on Whatman DE-81 filters. The phosphorylation activity in crude cell extract was measured by the above radiochemical assay with 30 μg of protein from the extract. The assays of crude extracts were repeated three times. Standard errors were <20%.

The phosphoryl transfer assay was performed with (γ-^{32}P)-ATP as described [9]. Ten μM dGuo, dCyd, dAdo, CdA, araG, dFdG or dFdC were added to 50 mM Tris, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 1 mM unlabeled ATP, 25 μCi of (γ-^{32}P)-ATP (3000 Ci/mmol, Amersham) and recombinant human dGK or dCK. The samples were incubated at 37° for 1 hr. Two μL of the reaction mixtures were spotted on polythyleneimine-cellulose sheets and separated in a solution of 99% (w/v) $C_4H_8O_2$, 25% (w/v) NH_3 and H_2O, (66: 33: 1) for 12 hr. The sheets were autoradiographed and the nucleoside monophosphate products were quantified with the Image Master system (Pharmacia). The experiment was repeated three times. Standard errors were <20%.

Substrate Competition Experiments

dFdC, dFdG, CdA and araG were tested for their capacity to compete with dGuo or dCyd phosphorylation by dCK or dGK using the phosphoryl transfer assay described above. The nucleoside analog monophosphates have different mobility on the TLC sheets as compared to the naturally occurring dNMPs. Each nucleoside analog was added to the reaction mixture at increasing concentrations (10–5000 μM), while dGuo or dCyd was added to their physiological concentration of ≈10 μM. The data were used to calculate K_i values for dFdC, dFdG, CdA and araG. The dFdC competition to dCyd with dCK could not be measured due to poor separation of their monophosphates on the chromatography sheets.

TABLE 1. The kinetic properties of recombinant human dGK

Substrate	Phosphate donor	K_m (μM)	V_{max} (nmol/mg/min)	V_{max}/K_m
dGuo	ATP	1.1	2.9	2.8
	UTP	0.6	57	95
CdA	ATP	45	128	2.9
	UTP	10	204	20
araG	ATP	4.7	7.5	1.6
	UTP	2.5	198	80
dFdG	ATP	16*	n.d.	n.d.
	UTP	n.d.	n.d.	n.d.

n.d. = Not determined.
*K_m value was determined by phosphoryltransfer assay.

RESULTS

Kinetic Properties of Recombinant Human dGK

The Michaelis–Menten kinetic constants of human recombinant dGK for dGuo, araG, CdA, dFdC and dFdG were determined (Table 1). Human recombinant dGK had a K_m of 1 μM and a V_{max} of 3 nmol/mg/min for dGuo with ATP as phosphate donor. Kinetic determinations of dGK purified from human and bovine tissues showed similar results, with a K_m of 4.7 and 7.6 μM for dGuo [3, 10]. UTP as phosphate donor decreased the K_m by 40% for dGuo and increased the V_{max} 19-fold as compared to ATP as phosphate donor. The efficiency of the reaction thereby increased 34-fold. A similar increase in efficiency was observed when we used UTP instead of ATP for the nucleoside analogs araG and CdA. The K_m for araG decreased less than two-fold, but since the V_{max} increased dramatically with UTP as phosphate donor, the increase in phosphorylation efficiency was almost 50-fold. For CdA the K_m decreased fourfold, and the phosphorylation efficiency increased sevenfold with UTP instead of ATP in the enzyme assays.

The kinetic properties of dFdC and dFdG were determined by a phosphoryl transfer assay with labeled ATP as described in Methods. The K_m of 16 μM for dFdG is in agreement with the K_m reported for partially purified dGK [7]. dFdC was not a substrate for dGK. The K_m for dFdC phosphorylation by dCK was determined as 3.9 μM. By comparing the substrate concentrations necessary to reach a plateau level of phosphorylation for dFdG and dGuo, we estimated the V_{max} for dFdG to be ≈2- to 3-fold higher than for dGuo. Because the V_{max} for dGuo was determined, by the radiochemical assay, as 2.9 nmol/mg/min, the estimated V_{max} for dFdG was 6–9 nmol/mg/min (data not shown). The V_{max} for dFdC was estimated by the same method as ≈100 nmol/mg/min. Michaeli–Menten kinetics were observed for all substrates assayed in this study within the tested concentration range.

Comparison of Substrate Specificity of dGK and dCK

To directly compare the efficiency of dGK and dCK for phosphorylation of nucleoside analogs, we used a phosphoryl transfer assay with dGuo, dCyd, dAdo, araG, CdA, dFdG and dFdC as substrates and ATP as phosphate donor (Table 2). dGuo was, as expected, efficiently phosphorylated by both recombinant dGK and dCK, while dCyd was only a substrate for dCK. The nucleoside analogs araG and dFdG were only efficiently phosphorylated by dGK, while dFdC was phosphorylated only by dCK. CdA had the unique feature among these nucleoside analogs of being efficiently phosphorylated by both dGK and dCK.

TABLE 2. The relative phosphorylation of nucleoside analogs by recombinant human dGK and dCK

Substrate (10 μM)	Recombinant human dGK	Recombinant human dCK
dGuo	100	116
dCyd	<1	100
dAdo	174	127
araG	76	<1
CdA	345	187
dFdG	46	<1
dFdC	<1	296

The value of substrate phosphorylation is given in relation to dGuo phosphorylation by dGK or dCyd by dCK.

We also determined the inhibitory concentrations (K_i) of araG, CdA and dFdG for dGuo phosphorylation by dGK and for dCyd phosphorylation by dCK (Table 3). araG was the most efficient competitor to dGuo for dGK with a K_i of 670 μM. CdA was the only purine nucleoside analog tested that competed with dCyd for phosphorylation by dCK within the tested concentration range. The K_i of CdA as an inhibitor of dCyd phosphorylation was 313 μM. CdA was a less efficient competitor to dGuo for dGK, with a sevenfold higher K_i value than for dCK.

Phosphorylation of araG and CdA in Crude Extracts of Cancer Cell Lines

In addition to the kinetic properties of dCK and dGK for nucleoside analogs, the level of enzyme expression in the target tissues is also an important determinant for the pharmacological effects of a nucleoside analog. We therefore decided to determine the phosphorylation of CdA and araG in crude cell extracts of the cervix epithelium cancer

TABLE 3. The kinetic constants of nucleoside analog competition with 10 μM dGuo or 10 μM dCyd for dGK or dCK

Kinetic constant	Value (μM)	Varied substrate	Enzyme
K_i(dGuo)	670	araG	dGK
K_i(dGuo)	2133	CdA	dGK
K_i(dGuo)	1729	dFdG	dGK
K_i(dCyd)	>7500	araG	dCK
K_i(dCyd)	313	CdA	dCK
K_i(dCyd)	>7500	dFdG	dCK

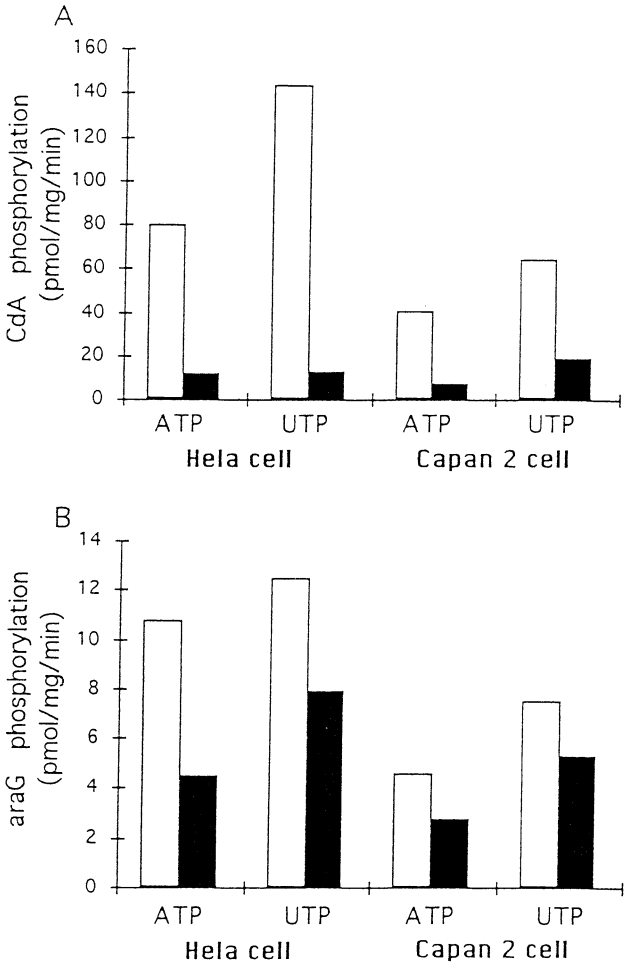

FIG. 1. Phosphorylation of CdA (A) and araG (B) in crude cell extracts of Hela and Capan 2 cells. White columns show the total substrate phosphorylation in the extract. Black columns show the phosphorylation contributed by dGK, after addition of 500 μM dCyd to inhibit dCK activity. One mM ATP or UTP was used as phosphate donor. The figure illustrates mean values of three independent experiments. Standard errors were <20%.

cell line HeLa and the pancreatic adenocarcinoma cell line Capan 2 (Fig. 1). Because CdA and araG are substrates of both dCK and dGK, the level of phosphorylation is likely to represent the total phosphorylation catalyzed by both enzymes. Because deoxycytidine is a substrate for dCK alone, addition of excess unlabeled deoxycytidine inhibits dCK-mediated nucleoside analog phosphorylation without affecting dGK activity [8]. The experiments showed that, in the investigated cell lines, CdA phosphorylation was efficiently inhibited by excess dCyd, and thus mediated by dCK, whereas more than 50% of the araG phosphorylation was catalyzed by dGK. Since the determination of kinetic parameters demonstrated a difference between ATP and UTP as phosphate donors for dGK, the dGK activity in cell extracts was measured with both ATP and UTP. There was, however, no significant difference in dGK activity determined with the different phosphate donors.

DISCUSSION

The synthesis of analogs of natural deoxyribonucleosides has been a successful strategy to find inhibitors of viral and cellular DNA replication. Because the nucleoside analogs must be phosphorylated by nucleoside and nucleotide kinases to their corresponding triphosphates for activity, a characterization of the enzymes involved in the phosphorylation will provide the basis for a rational use of these compounds. The cDNAs of all four known human deoxyribonucleoside kinases have now been cloned [1, 5, 11]. The present study was performed to characterize and compare the kinetic properties of dGK and dCK, and to study the overlapping substrate specificity for certain nucleoside analogs. dCK and dGK are closely related enzymes, but are located in different subcellular compartments. Biochemical data suggest that dGK is a mitochondrial enzyme [12]. The recent expression of dGK and dCK as fusion proteins with the green fluorescent protein (GFP) confirmed that dGK was targeted to the mitochondria. dCK was surprisingly found to be located in the cell nucleus [13]. However, nucleoside analogs phosphorylated by a mutated dCK protein located in the cytosol were as cytotoxic as when phosphorylated in the nucleus. This finding indicates free passage of nucleotides between the cytosolic and nuclear compartments. In contrast, the mitochondrial membrane is considered to be impermeable to hydrophilic molecules, and there is evidence for separate mitochondrial deoxyribonucleotide pools. Whether the cytotoxicity of a nucleoside analog is different when it is phosphorylated within the mitochondrial compartment as compared to the cytosolic/nuclear compartment is still to be elucidated. The data presented in this paper suggest that phosphorylation by mitochondrial dGK contributes to the cytotoxicity of araG, dFdG and CdA. The initial phosphorylation step of these compounds would, at least in part, occur in the mitochondrial matrix. The subcellular location of the enzymes responsible for the further metabolism of the nucleoside analogs and how the compounds are transported between the cytosol and the mitochondria are to a large extent unknown. Studies with dCK-deficient cell lines demonstrate resistance to CdA [1]. There is thus evidence that CdA toxicity is dependent on dCK activity in intact cells. Although dCK-deficient cells show decreased sensitivity to araG, there are studies that suggest phosphorylation of araG by biochemical pathways that are different from the 1-β-D-arabinofuranosylcytosine phosphorylating pathways [14].

The overlap in the substrate specificity of dCK and dGK for the purine nucleosides dGuo and dAdo did not apply to all of the purine nucleoside analogs investigated. Only CdA was an efficient substrate for both dGK and dCK, whereas araG and dFdG were very poor substrates for dCK, and dFdC was not phosphorylated by dGK. In this respect, dGK is strictly a purine kinase, while dCK can phosphorylate both purines and pyrimidines but does not accept modifications of the ribose sugar of purines. We confirmed the

different contributions of dGK and dCK to the phosphorylation of CdA and araG in crude cell extracts and thereby demonstrate the relevance of kinetic characterization of pure enzymes. We also demonstrated that recombinant dGK can efficiently use both ATP and UTP as phosphate donors both for natural substrates and nucleoside analogs. UTP improved the phosphorylation efficiency for all dGK substrates investigated. An increase in phosphorylation efficiency has previously been demonstrated for dCK but it is more pronounced for dGK [15, 16]. There was, however, no significant difference in enzyme activity in crude cell extracts when UTP instead of ATP was used as phosphate donor. Because a crude cell extract is a mixture of endogenous nucleotides from different cell compartments, it is possible that the *in vivo* kinetic parameters for dGK in the mitochondria are affected by the phosphate donors available. One could speculate that the presence of different phosphate donors might regulate the activity of dGK.

The cytotoxicity mediated by nucleoside analogs is caused by effects on nuclear and/or mitochondrial DNA synthesis. Mitochondrial toxicity has been reported for nucleoside analogs such as 2′,3′-dideoxycytidine [17]. However, this nucleoside analog is not a substrate for the mitochondrial nucleoside kinases [18], and there is no evidence that the nucleoside analogs phosphorylated by dGK increase mitochondrial toxicity. Rather, the mitochondrial toxicity is reported to be dependent on the affinity of the substrate for the γ-polymerase [19]. No other determinants of mitochondrial toxicity by nucleoside analogs have yet been described. The toxicity data do not exclude the possibility that a nucleoside analog is phosphorylated to its monophosphate in a specific cell compartment and is subsequently further metabolized to interfere with both nuclear and mitochondrial DNA replication. The metabolic pathways of nucleoside analogs so as to interfere with mitochondrial DNA have, however, not yet been elucidated.

Overexpression of human dCK cDNA in tumour cells was recently shown to increase 1-β-D-arabinofuranosylcytosine and CdA cytotoxicity [20]. Overexpression of dGK cDNA in tumour cells may be a new variant for a combined gene and chemotherapy treatment. The combined knowledge of kinetic parameters, substrate specificity, and tissue-specific expression of nucleoside kinases will be useful for the development of novel anticancer and antiviral nucleoside analogs as well as new modalities for treatment of these diseases.

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