

Antiviral Guanosine Analogs as Substrates for Deoxyguanosine Kinase: Implications for Chemotherapy

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A highly active form of human recombinant deoxyguanosine kinase (dGK) phosphorylated purine nucleoside analogs active against cytomegalovirus, hepatitis B virus, and human immunodeficiency virus, such as penciclovir, 2',3'-dideoxyguanosine and 3'-fluoro-2',3'-dideoxyguanosine. The antiherpesvirus drug ganciclovir, which is also used in gene therapy, was a substrate for dGK, but with low efficiency. ATP and UTP were both good phosphate donors, with apparent K_m values of 6 and 4 μ M and V_{max} values of 34 and 90 nmol of dGMP/mg of dGK/min, respectively. With a mixture of 5 mM ATP and 0.05 mM UTP, which represent physiologically relevant concentrations, the activities of dGK with ganciclovir and penciclovir was 1% and approximately 10%, respectively, of that with dGuo. The levels of dGK in different tissues were determined with a selective enzyme assay and the total activities per gram of tissues were similar in liver, brain, heart, and thymus extracts. The fact that the cellular dGK enzyme can phosphorylate antiviral guanosine analogs may help to explain the efficacies and side effects of several forms of chemotherapy.

Acyclovir is still the most-used antiherpesvirus drug, and the activity relies on its selective phosphorylation in infected cells, carried out by the virus-coded thymidine kinase (TK) (8, 11). Other acyclic purine analogs have been identified and used successfully as antiherpesvirus and antihepadnavirus agents, e.g., ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine], and penciclovir [9-(4-hydroxy-3-hydroxymethylbutyl-1-yl)guanine] (4, 6, 8–11, 30), as well as 2',3'-dideoxyguanosine (ddG), 3'-fluoro-2',3'-dideoxyguanosine (FLG) (1, 13, 17, 30), and lobucavir {(1R-1a,2b,3a)-9[2,3-bis(hydroxymethyl)-cyclobutyl]guanine} (29). The last three have also shown significant activity against human immunodeficiency virus (33).

Ganciclovir is very efficiently phosphorylated by herpesvirus TK, and transfer of the herpesvirus TK gene into tumor cells has led to the finding that the tumors become highly sensitive to ganciclovir. This is the basis for a large number of studies using herpesvirus TK in suicide gene therapy (7, 18). The fact that penciclovir is active against hepatitis B virus, which is the causative agent of both acute and chronic hepatitis, has posed a problem in that hepatitis viruses do not code for any protein known to be a nucleoside kinase (22, 30). Thus, the formation of phosphorylated penciclovir nucleotide has to be carried out by cellular enzymes, and a low but significant phosphorylation has been detected in uninfected cells (22, 23, 30).

Deoxyguanosine kinase (dGK) (nucleoside triphosphate:deoxyguanosine 5'-phosphotransferase, EC 2.7.1. 113) catalyzes the phosphorylation of purine deoxynucleosides and their analogs, using nucleoside triphosphate as phosphate donor (2). The cDNA for dGK has been cloned, and it codes for a 31-kDa protein with an N-terminal mitochondrial leader sequence (15, 32). The localization of dGK to mitochondria has been demonstrated in earlier biochemical studies and by recent immuno-

histological methods (16, 20, 31). A highly active form of recombinant dGK has been purified and shown to have a broad substrate specificity compared to earlier dGK preparations (24). In addition to dGuo, dAdo, and dIno several important analogs, e.g., arabinosyl guanine, 2-chlorodeoxyadenosine (CdA), 2-fluoro-arabinosyl adenosine, and 2',3'-dideoxyinosine were substrates for the enzyme. A low but significant activity with ganciclovir was also observed with the recombinant enzyme (24), similar to what was reported earlier with native dGK (25). This low activity may be involved in the toxicity observed with ganciclovir (26), although it is not clear how a mitochondrial enzyme can be responsible for the toxicity of nucleoside analogs, which supposedly target nuclear DNA synthesis (35).

The objective of the present study was to determine the substrate specificity of active recombinant dGK with different guanosine analogs. When UTP was used as a phosphate donor instead of ATP, the efficiency of nucleoside phosphorylation carried out by dGK was reported to be affected (34). This phosphate donor effect on the specificity was tested also with the highly active dGK. The levels of dGK in liver extracts and other tissue extracts were determined using a specific enzyme assay. The results presented here may change and extend our understanding of the biochemical basis for the efficacies and side effects of antiviral and gene therapy treatments based on guanosine analogs.

MATERIALS AND METHODS

Materials. The radiolabeled nucleotide [γ - 32 P]ATP (3000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. [8- 3 H]guanine-9- β -D-arabinofuranoside (6.5 Ci/mmol), 2'-[2,8- 3 H] deoxyguanosine (29.9 Ci/mmol), and [8- 3 H]ganciclovir (12.9 Ci/mmol) were from Moravек Biochemical Inc. Ganciclovir, penciclovir, and FLG were generously provided by N. G. Johansson, Medivir AB, Huddinge, Sweden. ddG was purchased from Calbiochem, and lobucavir was a gift from Bristol-Myers Squibb Pharmaceuticals, Wallingford, Conn.

Expression and purification. Recombinant dGK was expressed in pLys S BL 21(DE3) containing the dGK cDNA in the pET-9d vector and induced with IPTG (isopropyl- β -D-thiogalactopyranoside) as described earlier (24). Recombinant dGK was extracted and purified in the presence of 0.1% Triton X-100 and

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TABLE 1. Substrate specificity of dGK with nucleosides, using the phosphoryl transfer assay with 100 μM [$\gamma\text{-}^{32}\text{P}$]ATP as the phosphate donor

Substrate	Relative activity ^a with substrate at:	
	100 μM	500 μM
ddG	0.14	0.5
FLG	0.08	0.3
Acyclovir	<0.01	0.05
Ganciclovir	0.06	0.6
Penciclovir	0.5	1.2
Lobucavir	<0.01	<0.01

^a Activity values are relative to that with 10 μM dGuo. The values represent the means of at least two determinations, with the individual values varying less than 15% from the mean values.

0.1 mM ATP by metal affinity chromatography as described previously (24). The final preparation of pure dGK was concentrated by centrifugation in a Centriprep 10 tube and kept at -80°C until used. Preparation of cellular extracts from rapidly frozen bovine tissues (obtained from the local slaughterhouse) was done as described previously (31) with 0.5% Triton X-100 in the buffer to extract the dGK enzyme from the mitochondrial matrix (16).

Enzyme assays. dGK activity was determined using 50 μM [^3H]dGuo as a substrate as described previously (31, 32). The reaction solution contained 50 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 5 mM ATP, 0.5 mg of bovine serum albumin per ml, 1 mM dithiothreitol, 0.1% Triton X-100, and 0.05 to 0.1 μg of dGK in a total volume of 50 μl . The total amount of protein in the assay was 50 μg in the case of extracts from bovine tissues. The phosphoryl transfer assays were performed with 50 mM Tris-HCl (pH 7.6), 5 mM MgCl_2 , 100 mM KCl, 10 mM dithiothreitol, 0.5 mg of bovine serum albumin per ml, 0.05 μM [$\gamma\text{-}^{32}\text{P}$]ATP (10 mCi/ml), 100 μM ATP, 0.1 μg of dGK, and different concentrations of nucleosides in a total volume of 50 μl . The phosphorylated products were separated by thin-layer chromatography and quantified as described previously (31). Substrate kinetic parameters were determined using the Michaelis-Menten equation and nonlinear regression analysis with the Enzfitter program from Elsevier-Biosoft. The apparent K_m and V_{max} values are from one experiment which has been repeated at least twice with very similar results. The determination of the apparent K_i values was done with 55 μM [^3H]dGuo and 5 mM ATP as substrates and various concentrations of inhibitor, assuming that the inhibition was competitive.

RESULTS

Specificity of dGK with guanosine analogs. The capacity of recombinant dGK to phosphorylate several guanosine analogs was determined with a phosphoryl transfer assay in which the [^{32}P]ATP concentration was 0.1 mM and two different concentrations of the analogs were used (Table 1). In the assay with 100 μM ganciclovir and penciclovir as substrates, the activities compared to that with 10 μM dGuo were 6 and 50%, respectively (Table 1) and the activity with ddG corresponded to 14%, while the activity with FLG was 8% compared to that with dGuo (Table 1). No activity (less than 0.5% at 100 μM) was observed with the antiherpesvirus analog acyclovir [9-(2-hydroxy-ethoxymethyl)guanine] or with the cyclobutylguanosine analog lobucavir (Table 1).

ATP and UTP as phosphate donors for dGK. The apparent Michaelis-Menten kinetic constants for dGuo and CdA with recombinant dGK were determined using a radiochemical method with both ATP and UTP as phosphate donors (Table 2). dGK had an apparent K_m of 3 μM and a V_{max} of 30 nmol/mg/min with dGuo and ATP, similar to what was reported earlier (24). With UTP as the phosphate donor the apparent K_m was very similar but the V_{max} was threefold higher, and the efficiency of the reaction was thus increased. CdA was a good

TABLE 2. Kinetic constants for recombinant dGK with nucleoside substrates using different phosphate donors^a

Substrate	Phosphate donor	Apparent K_m (μM) ^b	Apparent V_{max} (nmol/mg/min) ^b	Efficiency (V_{max}/K_m)
dGuo	ATP	2.8 ± 0.5	30 ± 5.0	12
	UTP	3.7 ± 0.5	88 ± 1.5	23.7
CdA	ATP	62 ± 1.0	518 ± 1.5	8.3
	UTP	8 ± 0.5	83 ± 1.3	10.3

^a The values were determined by the radiochemical method using 5 mM ATP or 5 mM UTP as the phosphate donor.

^b Values and means and standard deviations.

substrate for dGK as demonstrated earlier (24, 31), with an apparent K_m of 62 μM and a V_{max} of 518 nmol/mg/ml with ATP as the donor. Both the K_m and V_{max} decreased sevenfold when UTP was the donor, and the efficiencies were similar with both donors (Table 2). The apparent kinetic constants for ATP with a fixed concentration of dGuo and an equimolar concentration of MgCl_2 were 5.8 ± 1.5 μM (K_m) and 34 ± 1.4 nmol/mg/min (V_{max}). With UTP as the donor, the K_m was 3.7 ± 1.9 μM and the V_{max} was 88.8 ± 2.8 nmol/mg/min. Thus, the efficiency was fourfold higher when UTP instead of ATP was used as the phosphate donor.

In an attempt to mimic the situation in vivo, we also carried out the experiments using different concentrations and combinations of donors and acceptors. The ATP and UTP concentrations that one could find in tissues are about 5 mM ATP and 0.05 mM UTP (27). A maximum concentration of 10 μM nucleoside has been measured in blood, and therefore this concentration was chosen. The results are presented in Table 3, and the activity with UTP was somewhat higher than that with ATP using dGuo, dAdo, or ganciclovir. The combination of ATP and UTP gave some further stimulation of the phosphorylation of dGuo but not of dAdo or ganciclovir (Table 3).

Inhibition of dGK by dGTP and dGMP. To clarify the regulation of dGK by deoxyguanosine nucleotides, we tested the effects of dGTP and dGMP as inhibitors. dGK was inhibited by addition of low concentrations of dGTP and dGMP, and the estimated apparent K_i values were 0.4 and 4 μM , respectively, using 5 mM ATP and 55 μM dGuo as substrates. The activity was also inhibited by higher concentrations of dATP, dAMP, and dIMP (the apparent K_i values were 41, 28, and 78 μM , respectively) using the same conditions as described above.

Level of dGK in bovine tissues. In order to determine the dGK expression in different tissues, the enzymes levels in extracts from bovine brain, heart, thymus, and liver were examined with a selective assay. The enzyme preparations were

TABLE 3. Effects of different concentrations of phosphate donors on the activity of dGK with various acceptors

³ H-labeled substrate (10 μM)	Activity (nmol/min/mg) ^a with:		
	5 mM ATP	0.05 mM UTP	5 mM ATP + 0.05 mM UTP
dGuo	55 ± 2	77 ± 8	88 ± 2
dAdo	7 ± 0.2	12 ± 2	11 ± 1
Ganciclovir	0.5 ± 0.2	1.1 ± 0.3	1 ± 0.2

^a Values are means and standard deviations from three independent determinations.

TABLE 4. Levels of dGK in extracts from bovine brain, thymus, heart, and liver^a

Tissue	Sp act (pmol/mg/min) ^b	Total dGK activity (nmol/min/g of tissue) ^b
Brain	39 ± 8	0.8 ± 0.2
Thymus	29 ± 6	1.1 ± 0.2
Heart	43 ± 9	1.4 ± 0.3
Liver	18 ± 4	1.9 ± 0.4

^a The activities were measured with 50 μM [³H]dGuo and in the presence of 500 μM dCyd to make the assay specific for dGK by blocking the dCyd kinase activity.

^b Values are means and standard deviations from at least three determinations.

made in the presence of the detergent Triton X-100, which is known to extract dGK from mitochondria (16). The highest specific activity was found in extracts from heart, with a specific activity of 43 pmol/mg/min, and the lowest (18 pmol/mg/min) was found in liver extracts (Table 4). However, when the total dGK activity was calculated based on the activity per gram of tissue, the levels in these tissues were relatively similar, with the highest total activity in liver extracts (Table 4).

DISCUSSION

The highly active recombinant dGK used in this study phosphorylates purine analogs like penciclovir, ganciclovir, ddG, and FLG to a significant extent. Minimal activity was observed with acyclovir, in accordance with earlier results (24, 31), and lobucavir (29) was not a substrate for dGK. The ganciclovir phosphorylation observed here with recombinant dGK is about 10-fold higher than that reported for the human cytomegalovirus (CMV) UL97 protein (28), which supposedly is the protein responsible for phosphorylation of ganciclovir *in vivo* in CMV-infected cells. These results suggest that dGK may be an important enzyme in the activation of ganciclovir and may in part be responsible for the anti-CMV activity of this drug.

The apparent kinetic parameters for dGK were determined with various concentrations of phosphate acceptors and donors. We observed that the efficiency with dGuo was two times higher with UTP compared to with ATP as the phosphate donor. The V_{max}/K_m ratio with CdA was similar with both donors. These results do not agree with a recent report demonstrating a much higher efficiency with UTP compared to ATP as a phosphate donor for dGK (34).

Most experiments in earlier studies are based on the concentrations of ATP and UTP observed in intact cells. At concentrations of ATP and UTP that may be more relevant in the mitochondria, i.e., 5 and 0.05 mM, respectively (27), dGK showed similar activities with the different substrates, with about twofold stimulation when UTP was used. Combining ATP and UTP gave results similar to those with UTP alone. Thus, the nature of the phosphate donor influences the specificity of dGK, as was shown previously for deoxycytidine kinase (14), but not to a very large extent.

Recombinant dGK is inhibited by different nucleotides. The most efficient inhibitors were dGTP and dGMP; other products, like dAMP and dIMP, also inhibited but at higher concentrations. These results show that recombinant dGK is efficiently inhibited by its end products, as described earlier for native dGK (12, 19). The very recent structural determination

of recombinant dGK in complex with ATP will lead to a better understanding of the substrate specificity and regulation of this enzyme (K. Johansson, S. Ramaswamy, C. Ljungcrantz, N. Knecht, J. Piskur, B. Munch-Petersen, S. Eriksson, and H. Eklund, submitted for publication).

The levels of dGK in different tissues have not been accurately determined. The activity of the enzyme was therefore tested, using [³H]dGuo as substrate in the presence of deoxycytidine (dCyd) at a concentration that blocks the activity of the competing enzyme deoxycytidine kinase (2, 24, 31). Due to the ethical and practical problems of obtaining tissues from human sources, we used bovine brain thymus, heart, and liver from the same animal. We expect that the results from the bovine samples are representative also for the situation in humans. Addition of detergents insured that dGK was extracted from the mitochondrial compartment, the highest specific activity was found in the extract from heart and the lowest was found in the extract from liver, but the overall variation in activity was only a factor of two. When the activity was calculated as the activity per gram of tissue, the liver contained the highest level of dGK and the brain contained the lowest, with the heart and thymus at intermediate levels. Still, the overall dGK contents per gram of tissue were similar, as expected with a constitutively expressed mitochondrial enzyme.

Assuming that the activity of recombinant dGK with penciclovir (approximately 10% of the activity of dGuo) is representative for the native enzyme, we estimate that in liver there is about 14 pmol of penciclovir phosphate formed per min per g of tissue, which is similar to that determined in uninfected hepatoma cells (23, 30). These results suggest that dGK is responsible for the phosphorylation of penciclovir in uninfected cells and, most likely, in hepatitis virus-infected cells or other cells infected with viruses not coding for deoxynucleoside kinase. Our results thus provide evidence for the activation of penciclovir by dGK in hepadnaviral infection, as recently suggested by Colledge et al. (6).

The role of mitochondrial dGK in activation of substrates acting in the cytosol or nuclei is not known. However, there are nucleotide transport systems in the mitochondrial membrane that could transport deoxynucleotides out from mitochondria (5). A cDNA coding for a cytosolic form of mouse dGK has been detected (21), and thus there may be at least in certain cells both a mitochondrial and a cytosolic form of dGK. In a recent immunohistological study with human 293 cells, dGK was found only in mitochondria and there was no evidence for a cytosolic form of the enzyme (16). A role for dGK in the mechanism of inherited purine nucleoside phosphorylase deficiency has been suggested based on recent results from a transgenic mouse model where the accumulation of high levels of dGTP in mitochondria was linked to extensive and selective apoptotic cell death of T lymphocytes (3). The availability of this type of animal model as well as the three-dimensional structure of dGK (Johansson et al., submitted) should lead to rapid progress in the understanding of the role of this enzyme in infected and uninfected cells.

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