Substrate Specificity of Mitochondrial 2'-Deoxyguanosine Kinase

EFFICIENT PHOSPHORYLATION OF 2-CHLORODEOXYADENOSINE*

Liyi Wang, Anna Karlsson‡, Elias S. J. Arner, and Staffan Eriksson

From the Department of Biochemistry 1, Medical Nobel Institute, Karolinska Institutet, Box 60 400, S-104 01 Stockholm, Sweden

Mitochondrial deoxyguanosine kinase (dGK) (EC 2.7.1.113) was purified to apparent homogeneity from bovine brain. The molecular mass of the native protein was 56 kDa, as judged by gel filtration, and one single band of 28 kDa was seen in sodium dodecyl sulfate-gel electrophoresis. 2'-Deoxyguanosine (dGuo) (Km, 7.6 µM), 2'-deoxyinosine, and 2'-deoxyadenosine (Km, 60 µM) were substrates for the enzyme as well as several dGuo analogs containing a lipophilic substituent at C-2'. Carboxyclic dGuo, 9-β-D-arabinofuranosylguanine, 9-β-β-arabinofuranosilyxanthine, and 9-β- β-arabinofuranosyladenine were substrates for the enzyme, whereas no 3'-modified dGuo analogs were effective. Interestingly, 2-chloro-2'-deoxyadenosine (CdA) was found to be an efficient substrate for dGK (Km, 85 µM). Since ara-G, 9-β-D-arabinofuranosylguanine, and 9-β-β-arabinofuranosyladenine were substrates for the enzyme, whereas no 3'-modified dGuo analogs were effective. Interestingly, 2-chloro-2'-deoxyadenosine (CdA) was found to be an efficient substrate for dGK (Km, 85 µM). The efficient CdA phosphorylation by mitochondrial dGK is a novel finding that may have far reaching implications for the clinical use of this potent cytostatic drug.

The use of nucleoside analogs as antiviral or anticancer drugs is extensive. A prerequisite for these drugs to achieve a therapeutic effect is their intracellular phosphorylation by nucleoside kinases. Knowledge of tissue distribution and substrate specificity of the phosphorylating enzymes is important for the successful use of these compounds. The mitochondrial purine deoxyribonucleoside kinase (dGK) has been purified from beef liver mitochondria by Park and Ives (1). They showed that dGK phosphorylates dGuo (Km, 4.7 µM) and dIno (Km, 21 µM). They also showed that ara-G and 8-aza-dGuo are inhibitors of dGuo phosphorylation.

2-Chloro-2'-deoxyadenosine (CdA) is a deoxyadenosine analog that has marked therapeutic efficiency in the treatment of leukemia (2, 3). It is generally believed that dCK is the sole CdA-phosphorylating enzyme and that dCK activity is a prerequisite for CdA cell toxicity (4). Human brain tissue lacks dCK, but it has been shown previously that extracts of brain, in spite of this, contain significant CdA phosphorylating activity. These observations suggest the presence of a second CdA-phosphorylating enzyme (5). In the present study we purified dGK from bovine brain to apparent homogeneity and identified dGK as the CdA-phosphorylating enzyme in the brain. We also explored dGK activity in extracts of isolated mitochondria and cytosol fractions from CEM cells. CdA phosphorylating activity, insensitive to inhibition by dCyd, was detected in the mitochondria. This finding is in agreement with our studies on the purified enzyme and shows that CdA is efficiently phosphorylated by dGK in mitochondria.

EXPERIMENTAL PROCEDURES

Materials

2'-[8-3H]Deoxyguanosine (9 Ci/mmol), 9-β-β-[8-3H]arabinofuranosylguanine (2 Ci/mmol), 2'-[2,8-3H]deoxyadenosine (34 Ci/mmol), and 2'-[8-3H]2-chlorodeoxyadenosine (20 Ci/mmol) were purchased from Moravek Biochemicals Inc. [Y-32P]ATP (5,000 mCi/mmol) was from Amersham Corp. DEAR-Sepharose (fast flow) and CH Sepharose 4B (CNBr activated) were obtained from Pharmacia LKB Biotechnology Inc. Isobutyril-1,4' dimethoxytrityl-2'-deoxyguanosine was obtained from Sigma, p-nitrophenylphosphorochloridate from Aldrich, hydroxyapatite from Clarkson Chemical Co., and unlabeled nucleotides and nucleosides were from Sigma. All reagents were of the highest purity available. All 2'-modified analogs of dGuo, dAdo, and dIno were synthesized by Prof. M. J. Robins, Brigham Young University, Provo, UT and kindly provided by Prof. J. Balzarini (Rega Institute for Medical Research, Leuven, Belgium). Purine arabinosides were kindly provided by Dr. G. Koszalka (Burroughs Welcome).

Enzyme Assays

Km and Vmax values were determined by the radiochemical method by Ives and Wang (6) using the substrates [8-3H]dGuo, [2,8-3H]dAdo, [8-3H]ara-G, or [8-3H]CdA. The reaction was performed as described previously (6). The assay was linear up to 45 min with a detection limit of 1 pmol/min/mg of protein. The phosphoryl transfer assay was performed with 100 µM [γ-32P]ATP (10 mCi/ml), 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 100 mM KCl, 10 mM DTT, 0.5 mg/ml bovine serum albumin, 600 ng of dGK, and 100 µM nucleoside in a total volume of 50 µl as described previously (7). Lactate dehydrogenase activity was measured spectrophotometrically at 340 nm temperature by determination of NADH oxidation during the initial linear decrease in absorbance at 340 nm upon addition of 10 µg of protein extract to a cuvette containing 60 µM NADH and 300 µM pyruvate in 50 mM Tris-HCl, pH 7.9.

22847
Preparation of dGlu-3′-(4-aminophenyl)phosphate-Sepharose—CH Sepharose 4B, with a 6-atom arm and activated by CNBr, was coupled to aminophenyl ester of dGlu-3′-(4-aminophenyl)phosphate, following the general procedure provided by Pharmacia. dGlu-3′-(4-aminophenyl)phosphate was synthesized from 2-aminoethyl-dGlu-3′-(4-aminophenyl)phosphate (Amerham, Arlington Heights, IL). The coupling was monitored by measuring the UV absorbance at 260 nm of the supernatant. The affinity column material contained 4.1 μmol of dGlu-3′-(4-aminophenyl)phosphate/μl of gel.

**Purification**—The temperature throughout the purification was 0-4°C. Bovine calf brain (1 kg) was homogenized in 4 liters of 0.3 M sucrose, 2 mM EDTA, 1 mM PMSF, 0.1 M benzamidine, 0.5% Triton X-100, 1 mM dithiothreitol, 0.5 M KC1 in buffer C. The column was washed with buffer C until no protein was detected. A linear gradient of 0-0.15 M potassium phosphate in buffer B was applied to a 0.5-ml hydroxylapatite column and subsequently eluted with 0.2 M potassium phosphate in buffer B. The purified enzyme was saved for further use. 60 ml of enzyme from the hydroxylapatite column with a single path monitor UV-1 detector (Pharmacia) was diluted with ice-cold water and applied to a hydroxylapatite column, equilibrated and eluted with 50 mM Tris-HCl, pH 7.6. A linear gradient of 0-0.3 M KC1 in buffer A was used to elute dGK activity. The fractions containing dGK activity were pooled and assayed for dGK activity. To concentrate the enzyme, the pooled fractions were applied to a 4-ml dGlu-Sepharose column equilibrated with buffer C. The column was washed with buffer C until no protein was detected in the eluate. Elution involved three steps: (i) 40 ml of 0.2 M KC1 in buffer C; (ii) 20 ml of 0.5 M KC1 in buffer C; and (iii) 20 ml of 1 mM dGlu in buffer C, containing 0.2 M KC1. Between each step, 2-ml fractions were collected and measured for protein content and assayed for dGK activity. To concentrate the enzyme, the pooled fractions from step iii were adjusted to pH 7.7 with 0.3 M NaOH and applied to a 0.5-ml hydroxylapatite column and subsequently eluted with 0.2 M potassium phosphate in buffer B. The purified enzyme was collected by SDS-gel electrophoresis (PhastSystem, Pharmacia). The buffer of the protein was changed to 50 mM Tris-HCl, pH 7.5, by Centricon centrifugation (Amicon) for enzyme activity measurements.

**Gel Filtration Chromatography**

Gel filtration chromatography was performed using fast protein liquid chromatography on a Superose Tm12 HR10/30 (10 x 300 mm) column with a single path monitor UV-1 detector (Pharmacia). About 45 μg of purified enzyme was injected to the column which was equilibrated and eluted with 5 mM MgCl2, 0.1 M NaCl, 1 mM DTT, and 1.0% Triton X-100, 50 mM Tris-HCl, pH 7.6, and 15% glycerol in 25 mM sodium acetate, pH 5.5 and applied to a 4-ml dGlu-Sepharose column equilibrated with buffer C. The column was washed with buffer C until no protein was detected in the eluate. Elution involved three steps: (i) 40 ml of 0.2 M KC1 in buffer C; (ii) 20 ml of 0.5 M KC1 in buffer C; and (iii) 20 ml of 1 mM dGlu in buffer C, containing 0.2 M KC1. Each fraction from step iii was adjusted to pH 7.7 with 0.3 M NaOH and applied to a 0.5-ml hydroxylapatite column and subsequently eluted with 0.2 M potassium phosphate in buffer B. The purified enzyme was collected by SDS-gel electrophoresis (PhastSystem, Pharmacia). The buffer of the protein was changed to 50 mM Tris-HCl, pH 7.5, by Centricon centrifugation (Amicon) for enzyme activity measurements.

**Substrate Specificity of Pure Bovine dGK**—In the DEAE chromatography of the bovine brain extract, dGlu phosphorylating activity was eluted at 0.15 M KC1. This activity coeluted with CTD phosphorylation but was separated from thymidine and dCD phosphorylating activity (Fig. 1). The pooled DEAE fractions of the dGlu phosphorylating activity were subsequently purified by hydroxylapatite chromatography and dGlu-Sepharose column chromatography as the final step. After the purification a single band of 28 kDa was detected on SDS-gel electrophoresis (Fig. 2). The yield from 1 kg of brain was 300 μg of protein, with a specific activity of 4.5 nmol/min/mg of protein. Gel filtration of the pure protein gave an estimated size of the native protein of 56 kDa (Fig. 3).

**Preparation of CEM Cytosolic and Mitochondrial Protein Extracts**

Mitochondria were isolated from 200 x 10^6 exponentially grown CEMwt cells, using the protocol described by Tapper et al. (9), with the following alterations. The supernatant (13 ml) from the centrifugation was resuspended in 7 ml of 50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, pH 8.1, and 0.25 M sucrose and were, after collection from the subsequent sucrose gradient, isolated by centrifugation at 1,500 X g for 15 min. Hereafter the mitochondrial pellet was resuspended in 1.5 ml of 10 mM MgCl2, 1 mM EDTA, pH 8.1, 7.5% glycerol, 1 mM DTT, 350 mM NaCl, 0.5 mM PMSF, 0.5% Triton X-100, and 10 mM Tris-HCl, pH 8.0. The mitochondria were then disrupted in a small glass homogenizer, and, after centrifugation at 130,000 X g for 60 min, the supernatant, containing extracted mitochondrial proteins, was kept at -70°C in 6 x 250-μl aliquots until analyzed.**

**RESULTS**

**dGK Activity in Extracts of Different Tissues and Cells**—

| Tissue/cell type | dGK activity* | No addition | +20 μM dCyd |
|------------------|---------------|-------------|-------------|
|                  | pmol/min/mg   |             |             |
| Human brain      | 58            | 57          |             |
| Bovine brain     | 54            | 54          |             |
| Human spleen     | 18            | 1.5         |             |
| Human skin       | 4.1           | 3.6         |             |
| PBMC + PHA*      | 89            | 6.1         |             |
| PBL              | 54            | 6.7         |             |
| CEM/wt           | 7.7           | 5.9         |             |
| CEM/dCK*         | 6.1           | 6.5         |             |

* dGlu (50 μM) was used as substrate.

* Peripheral blood mononuclear cells + phytohemagglutinin.
Substrate Specificity of Deoxyguanosine Kinase

Fig. 1. DEAE chromatography of bovine brain extract. The fractions were analyzed for dGuo (●) or CdA (○) activity (panel A) and dCyd (■) or thymidine (□) activity (panel B) as described under "Experimental Procedures." Protein was determined by absorbance at 280 nm.

Fig. 2. SDS-gel electrophoresis of the DEAE pool and the final fractions of the dGK purification. The size of the purified dGK was estimated to 28 kDa by the molecular mass markers.

Fig. 3. Molecular weight estimation of the purified dGK by gel filtration chromatography.

Table II

| Analog | Base | R1  | R2  | R3  |
|--------|------|-----|-----|-----|
| 2'-dGuo | Guanine      | H   | H   | O   |
| 2'-Cyclopropyl-2'-dGuo | Guanine      | H   | H   | O   |
| 2'-Methylene-2'-dGuo | Guanine      | H   | H   | O   |
| 2'-Azido-2'-dGuo | Guanine      | H   | H   | CH2|
| Carbocyclic 2'-dGuo | Guanine      | H   | H   | CH2|
| Ara-G  | Guanine      | O   | H   | O   |
| Guanosine | Guanine      | OH  | H   | O   |
| 2'-dIno | Hypoxanthine | H   | H   | O   |
| 2'-dIno | Hypoxanthine | H   | H   | O   |
| Inosine | Hypoxanthine | OH  | H   | O   |
| Ara-Hx | Hypoxanthine | O   | H   | O   |
| 2'-Cyclopropyl-2'-dIno | Hypoxanthine | O   | H   | O   |
| 2'-dAdo | Adenine      | H   | H   | O   |
| 2-Chloro-2'-dAdo | 2-Chloroadenine | H   | H   | O   |
| 7-Deaza-2'-dAdo | 7-Deazadenine | H   | H   | O   |
| Ara-A  | Adenine      | O   | H   | O   |

* Cyclopropyl.

with dGuo and dIno but not with dAdo, when this preparation was assayed directly (yielding a final potassium concentration of 40 mM in the assay). After changing the buffer to 50 mM Tris-HCl, pH 7.5, through Centricon centrifugation, the total activity increased, and dAdo could also be phosphorylated by the enzyme. For all further studies of the enzyme, it was kept in 50 mM Tris-HCl, pH 7.5.

The phosphoryl transferase reaction catalyzed by dGK was examined with several nucleoside analogs as substrates. The structures of the analogs phosphorylated by dGK are shown in Scheme 1 and Table II. The rate of phosphorylation of the nucleoside analogs was compared with dGuo as the substrate. The capacity of purified dCK to use the same compounds as the substrate were also investigated (Table III). No phosphorylation was found with 3'-modified purine 2'-deoxynucleoside analogs. 3'-Azido-2',3'-dideoxy-2,6-diaminopurine riboside, 2',3'-dideoxyguanosine, 3'-azido-2',3'-dideoxyadenosine, 3'-fluoro-2',3'-dideoxy-2,6-diaminopurine riboside, 3'-fluoro-2',3'-dideoxyadenosine, 3'-azido-2',3'-dideoxyinosine, 3'-fluoro-2',3'-dideoxyinosine, 3'-azido-2',3'-dideoxy-8-bromoadenosine, 3'-azido-2',3'-dideoxyadenosine, and 3'-fluoro-2',3'-dideoxyguanosine were not substrates for dGK, neither were the acyclic guanosine analogs acyclovir, ganciclovir, and buclovir. However, several 2'-modified 2'-dGuo analogs were efficient substrates, including the arabinosides of guanine (ara-G) and hypoxanthine (ara-Hx). The 6-substituted arabinosides, 6-methoxypurine arabinoside (ara-M), 6-methylaminopurine arabinoside (ara-MAP), and 6-dimethylaminopurine arabinoside (ara-DMAP) were not phosphorylated by dGK. The adenosine analogs 9-β-D-arabinofuranosyladenine (ara-A), 7-deaza-2'-dAdo, and CdA were also accepted as substrates for the enzyme. The most efficient substrate for dGK, at a 5 μM concentration, was carbocyclic 2'-dGuo, with a more than 6-fold higher relative phosphorylation rate as compared with 2'-dGuo. Both dGK and dCK phosphorylated...
substrates are 50 μM CdA, 50 μM CEM wt cells and 20 μM ara-G.

*The values in parentheses show the fold difference in enzyme activity between the cytosolic and mitochondrial fractions. Presented values are the means of three enzyme activity measurements using the cytosolic and mitochondrial fractions from one cell fractionation.

**The substrate phosphorylation is given in relation to dGuo phosphorylation by dGK or dCK.

**Substrate Specificity of Deoxyguanosine Kinase**

2'-Deoxyguanosine is known to be phosphorylated by two different 2'-deoxyribonucleoside kinases, dCK and dGK (11, 12). Assays on cell and tissue extracts, using 2'-dGuo as a substrate, will detect the activity of both enzymes. Inhibition of dCK by excess of dCyd will give a more specific and representative assay for dGK activity when using 2'-dGuo as a substrate in a crude extract. Using that approach, we could detect non-dCK-derived dGK activity in spleen, skin, resting and mitogen-stimulated lymphocytes, and in wild type and dCK-negative CEM cells (Table I). Interestingly, a 10-fold higher dGK activity, which was insensitive to dCyd inhibition, was detected in human and bovine brain tissue. This is in agreement with the data of Spasokoukotskaja (3) who could not detect any dCK enzyme in brain using Western blots. We purified dGK from bovine brain because of its high dGK activity and the lack of dCK activity. Our purification procedure yielded pure dGK, as judged by SDS-gel electrophoresis. The native dGK appeared to be a dimer with a molecular mass of 58 kDa, which is in accordance with earlier studies of bovine liver dGK (1). This is the first report on the purification and characterization of dGK from brain tissue.

dGK has previously been reported to phosphorylate dGuo and dlno (1). In earlier studies, dAdo was reported to be a substrate for dGK, but later this finding was explained by deamination of dAdo to dlno with subsequent phosphorylation of dlno to dIMP (11, 13). In this study we clearly show that dAdo is a substrate for purified dGK (Tables III and IV). To rule out any contaminating deaminase activity, the product of our enzyme assay was identified by high performance liquid chromatography to be dAMP, not dIMP (data not shown), which is also visualized by the autoradiogram of the

TABLE IV

| Substrate | dGuo | dCyd | dIno | dAdo | Ara-G | Ara-Hx | Carboxycyclic 2'-dGuo | 2'-Cyclopropyl-2'-dGuo | 2'-Azido-2'-dGuo | dAdo | CdA |
|-----------|------|------|------|------|-------|--------|----------------------|----------------------|------------------|------|-----|-------|
| Kₘ (μM)   | 100  | 5    | 100  | 5    | 100   | 5      | 100                  | 50                   | 50               | 100  | 50  | 30    |
| Vₘₐₓ (pmol/min/mg) | 5000 | 50   | 5000 | 50   | 5000  | 50     | 5000                 | 5000                 | 5000             | 5000 | 5000| 5000  |
| Efficiency | 592  | 0.01 | 592  | 0.01 | 592   | 0.01   | 592                  | 0.01                 | 592              | 0.01 | 592 | 0.01  |

*Not determined.*

**DISCUSSION**

2'-Deoxyguanosine is known to be phosphorylated by two different 2'-deoxyribonucleoside kinases, dCK and dGK (11, 12). Assays on cell and tissue extracts, using 2'-dGuo as a substrate, will detect the activity of both enzymes. Inhibition of dCK by excess of dCyd will give a more specific and representative assay for dGK activity when using 2'-dGuo as a substrate in a crude extract. Using that approach, we could detect non-dCK-derived dGK activity in spleen, skin, resting and mitogen-stimulated lymphocytes, and in wild type and dCK-negative CEM cells (Table I). Interestingly, a 10-fold higher dGK activity, which was insensitive to dCyd inhibition, was detected in human and bovine brain tissue. This is in agreement with the data of Spasokoukotskaja (3) who could not detect any dCK enzyme in brain using Western blots. We purified dGK from bovine brain because of its high dGK activity and the lack of dCK activity. Our purification procedure yielded pure dGK, as judged by SDS-gel electrophoresis. The native dGK appeared to be a dimer with a molecular mass of 58 kDa, which is in accordance with earlier studies of bovine liver dGK (1). This is the first report on the purification and characterization of dGK from brain tissue.

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phosphoryl transferase assay (Fig. 4). In contrast with the pure enzyme, the mitochondrial fraction showed deamination of dAdo. dGK has also been purified previously from pig skin (13), neonatal mouse skin (14), and human placenta (15). In all preparations of dGK so far reported the specific activity of this enzyme is quite low. If this is because of the physiological state of the enzyme or a result of the purification procedure is unclear.

Using the purified dGK, we studied the phosphorylation of several antiviral and cytotoxic purine nucleoside analogs (Tables II–V). Substitutions at the 3' position of purine 2'-deoxynucleosides, acyclic purine nucleoside derivatives, and 6-substitutions at the purine moiety other than oxygen or amino are not accepted by dGK. However, dGK accepted the purine bases guanine, hypoxanthine, and adenine when linked to 2'-deoxyribose, several 2'-substituted purine 2'-deoxyribose, and purine arabinosides. Interestingly, the 2-substituted dAdo analog, Cda, is an equally efficient substrate as dAdo for dGK phosphorylation (Table IV). This is a novel finding that may have far reaching implications for the application of this compound.

The only enzyme that is known to activate Cda so far is dCK, as shown by a markedly reduced Cda toxicity against dCK-deficient mutant cell lines and by blocking the cell toxicity of Cda by the addition of excess of dCyd (4). However, the present study shows clearly that a second enzyme, dGK, is able to phosphorylate Cda. In cells that lack dCK, Cda will thus be activated at a level corresponding to the cellular content of dGK. A lower Km for Cda phosphorylation by dCK (7) indicates that Cda will be phosphorylated more efficiently by this enzyme if both dCK and dGK are present in the cell. Since we showed that brain tissue contains no dCK activity but high dGK activity (Table I), this tissue may be of high value for Cda toxicity and antitumor studies with regard to diverse types of brain tumors or other tumors containing a high content of mitochondria. Thus, our findings provide a rational basis to consider brain tumors as a potential target for Cda chemotherapy.

Ara-G and ara-Hx were good substrates for dGK, whereas ara-A was less efficiently phosphorylated. Ara-G, an antileukemic compound that is also reported to act as an inhibitor of mitochondrial purine nucleoside phosphorylase (16), is selectively active in T-cell malignancies with low toxicity to other cells in the bone marrow (17). Accumulation of high ara-GTP levels in T cell malignancies compared with non-T cell leukemias has been reported by Shewach and Mitchell (18). They did not find any difference in ara-CTP accumulation in their study and suggested biochemical properties of T lymphoblasts to accumulate ara-GTP. T cells, but not B cells, also accumulate dGTP under purine nucleoside phosphorylase deficiency conditions (19). Since dCK is expressed in both T and B lymphocytes as well as in all lymphoid cells (5, 20), our findings may suggest that dGK could be a candidate enzyme to explain the differences of ara-G toxicity in these cells. The high efficiency of dGK for phosphorylation of ara-Hx could also account for drug-related toxicity in cells in which this metabolite is found. Ara-Hx is formed by deamination of ara-A and is also a metabolite of other biologically active purine arabinosides such as ara-DMAP and ara-M (21). Phosphorylation of ara-Hx forms ara-IMP, which can be converted to ara-AMP again. Ara-DMAP and ara-M show low toxicity in cell cultures but are neurotoxic in animals (21). Phosphorylation of ara-Hx by dCK has been suggested as a mechanism of ara-DMAP toxicity (22). However, our data strongly suggest dGK as the responsible enzyme to mediate the neurotoxicity of ara-Hx.

The mitochondrial location of dGK gives rise to some interesting questions. If nucleoside analogs must be activated by dGK to exert their effects on viral or nuclear DNA replication, they need to be transported as their phosphorylated derivatives from the mitochondria to the cytosol compartment. It is so far unclear if such a transport system exists. As a matter of fact, an accumulation of the corresponding 5'-triphosphate derivative of such nucleoside analogs may occur in the mitochondrial compartment. Starner and Cheng (23) have shown that the mitochondrial DNA polymerase γ has a broader substrate acceptance for modified nucleotide analogs than the nuclear DNA polymerases. This may lead to a toxicity of the test compound which is mediated through inhibition of mitochondrial DNA replication (24). Thus, in this regard the possibility of phosphorylation of nucleoside analogs by mitochondrial dGK should be kept in mind as a potential cause of side effects of certain drugs.

In conclusion we believe that dGK-catalyzed phosphorylation may play an important role in the evaluation and rational development of new antiviral and cytotoxic nucleoside analogs. The expression of dGK in normal and malignant cells and tissues should be explored more in terms of targeted chemotherapy.

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Substrate Specificity of Deoxyguanosine Kinase

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