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Phosphorylation of 9-β-D-arabinofuranosylguanine monophosphate by *Drosophila melanogaster* guanylate kinase

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Abstract

Nucleoside monophosphate kinases have an important role in the synthesis of nucleotides that are required for cellular metabolism. These enzymes are also important for the phosphorylation of nucleoside- and nucleotide analogs used in cancer and anti-viral therapy. We report the cDNA cloning and characterization of a 23 kDa guanylate kinase from *Drosophila melanogaster* (*Dm*-GUK). The predicted amino acid sequence was 58% identical to the human guanylate kinase and the enzyme was shown to phosphorylate GMP and dGMP with ATP as phosphate donor. The monophosphates of the deoxyguanosine analogs 2′,2′-difluorodeoxyguanosine (dFdG) and 9-β-D-arabinofuranosylguanine (araG) were also shown to be phosphorylated by the enzyme. We used the enzyme to reconstitute the complete in vitro three-step phosphorylation pathway for the conversion of dGuo and araG to the corresponding triphosphates.
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Keywords: Nucleoside kinase; Nucleoside analog; Purine; Anti-cancer therapy; Nucleoside phosphorylation; Suicide gene therapy

1. Introduction

Nucleoside analogs used in chemotherapy of cancer require phosphorylation inside the cells to become pharmacologically active [1]. The deoxyguanosine analog 9-β-D-arabinofuranosylguanine (araG) is in human cells initially converted to araG monophosphate by deoxycytidine kinase and deoxyguanosine kinase. Phosphorylation by guanylate kinase and nucleoside diphosphate kinase further convert araG to the pharmacologically active triphosphate form. Several suicide gene therapy strategies uses nucleoside kinases as the therapeutic gene to increase the conversion of cytotoxic nucleoside analog to the triphosphate form, and thereby, enhance their pharmacological effects [2]. We have previously shown that the expression of the deoxyribonucleoside kinase of *Drosophila melanogaster* (*Dm*-dNK) in cancer cell lines increases the cytotoxicity of several anti-cancer nucleoside analogs [3]. The *Dm*-dNK has broad substrate specificity and very high catalytic rate that makes it an attractive suicide gene candidate [3,4]. Although the naturally occurring deoxyribonucleosides are readily phosphorylated to the corresponding triphosphate, this may not be the case for nucleoside analogs. The first phosphorylation step catalyzed by nucleoside kinases appears for many nucleoside analogs to be the rate-limiting step in conversion to the triphosphate form. However, there is evidence that also the conversion to the diphosphate form by nucleoside monophosphate kinases and the conversion to the triphosphate form by nucleoside diphosphate kinases may be rate-limiting for certain nucleoside analogs [5,6]. For further development of nucleoside kinase-based gene therapy strategies, it may accordingly also be important to improve the phosphorylation of the nucleoside analog monophosphates and diphosphates. A combination of two or more nucleoside and nucleotide kinases activating a chemotherapeutic nucleoside analog may thus be a strategy to improve suicide gene therapy.

Thymidylate kinase and UMP–CMP kinase have among the nucleoside monophosphate kinases been carefully studied regarding their phosphorylation of nucleoside analog monophosphates [5,7,8]. The broad substrate specificity and high catalytic rate of the *Dm*-dNK made us

Abbreviations: *Dm*, *Drosophila melanogaster*; dNK, deoxyribonucleoside kinase; GUK, guanylate kinase; araG, 9-β-D-arabinofuranosylguanine; dFdG, 2′,2′-difluorodeoxyguanosine

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interested in investigating if the family of nucleoside monophosphate kinases from *D. melanogaster* had similar features. We have recently reported the cloning and characterization of the *D. melanogaster* UMP–CMP kinase [9]. However, this enzyme exhibited similar restricted substrate specificity as the human enzyme. In the present study, we have identified and cloned the cDNA of the *Drosophila* homologue of human guanylate kinase. We have studied the basic kinetic properties and substrate specificity of the enzyme. We also report for the first time an in vitro reconstitution of the three-step phosphorylation pathway converting deoxyguanosine and araG enzymatically to their triphosphate forms.

2. Materials and methods

2.1. Cloning and expression of Dm-GUK cDNA

We search the expressed sequence tag library of the GenBank database at the National Institute for Biotechnology Information with the Basic Local Alignment Search Tool [10] to identify novel *D. melanogaster* cDNA clones that encoded proteins similar to the human and bovine guanylate kinase [11–13]. The expressed sequence tag cDNA clones were obtained from Research Genetics (Huntsville, USA). The open reading frame of the cDNA sequence was amplified by the polymerase chain reaction using the primers 5′-ATGACCGCCGCCCCCGGGCCACGCCC and 3′-AATGTAGAATGATATTAACCTCA. The PCR product was cloned into the PCR T/TOPO TA cloning vector (Invitrogen). The DNA sequence of the plasmid was determined by DNA sequencing using an ABI310 automated DNA sequencer (Perkin Elmer Life Sciences).

The protein was expressed in *Escherichia coli* fused to an N-terminal polyhistidine tag encoded by the plasmid vector. The plasmid was transformed into the *E. coli* strain BL21(DE3)pLysS and cultured in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Protein expression was induced at $OD_{595} = 0.9$ with 1 mM isopropyl thio-β-D-galactoside for 3 h at 37 °C. The bacteria were harvested by centrifugation at 5000 × *g* for 10 min and resuspended in 50 mM NaH_2PO_4 pH 7.4 and 300 mM NaCl. The bacteria were lysed by the addition of 1 mg/ml lysozyme and by sonication. The protein extract was cleared by centrifugation at 12,000 × *g* for 20 min and loaded onto a Talon Metal affinity resin column (Clontech). The recombinant protein was eluted in 50 mM NaH_2PO_4 pH 7.4, 300 mM NaCl and 150 mM imidazole. The size and purity of the recombinant protein was determined by SDS–polyacrylamide (SDS–PAGE) gel electrophoresis (PhastSystem, Amersham Biotech). The protein concentration was determined with the Bradford Protein Assay (Bio-Rad). Bovine serum albumin was used as the concentration standard.

2.2. Enzyme assays

The enzymatic assays with radiolabeled ATP were performed in 10 μl containing 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 2.5 μCi/μl [γ-32P] ATP (3000 Ci/mmol, Amersham Biotech) and 1 mM nucleoside monophosphates (Sigma). Indicated amounts of enzymes were added and the reaction mixtures were incubated 30 min at 37 °C. The products were separated by thin-layer chromatography (TLC) on poly(ethyleneimine)-cellulose F sheets (Merck). The separation of the reaction products from ATP required a three-step TLC with different ammonium formate buffers. Chromatography was performed in 0.5, 1.2 and 1.8 M ammonium formate pH 6.5, that were allowed to rise to 3, 7 and 17 cm on the TLC sheets, respectively. The TLC sheets were analyzed by autoradiography using a phosphoimager (BAS 1000; Fuji Photo Film Co.). HPLC analysis of the reaction mixtures were performed by ion-pair reversed-phase chromatography on a 5 μm Hypersil ODS 250 mm × 4.6 mm column. The 1 ml/min mobile phase consisted of 0.1 M triethylamine phosphate buffer (pH 7.0) with a 95% methanol gradient (0–100%) The separated nucleotides were detected by absorbance at 254 nm. Mean values of three samples were calculated and a Michaelis–Menten function was fitted to the data points to calculate the kinetic parameters.

The coupled assay using multiple kinases were performed in 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 10 mM ATP, 5 mM DTT and 5 mM nucleosides. Creatin phosphate (2 mM) and 2.5 U creatine kinase (Sigma) were added to the reactions to regenerate the ATP consumed. Recombinant *Dm*-dNK was prepared as described [4] and NDPK was obtained from Sigma. The reaction mixtures were incubated 30 min at 37 °C and the reaction products analyzed by HPLC or TLC as described above.

3. Results

3.1. Identification and cDNA cloning of Dm-GUK

We decided to clone and recombinantly express the *Drosophila* homologue of the human guanylate kinase with the aim to determine the kinetic properties and substrate specificity of the enzyme. *D. melanogaster* expressed sequence tag cDNA clones with sequence similarity to the cDNA of human and bovine guanylate kinase [11–13] was identified in GenBank. The open-reading frame of one cDNA clone was subcloned and sequenced (clone ID: LD13007). The cDNA encoded a 204 amino acid residue open reading frame with a predicted molecular mass of 23 kDa. Alignment of the predicted amino acid sequence of the novel enzyme with different mammalian guanylate kinases showed that the *Drosophila* enzyme was ≈58% identical to the mammalian enzymes (Fig. 1). Based on the sequence similarity, the enzyme

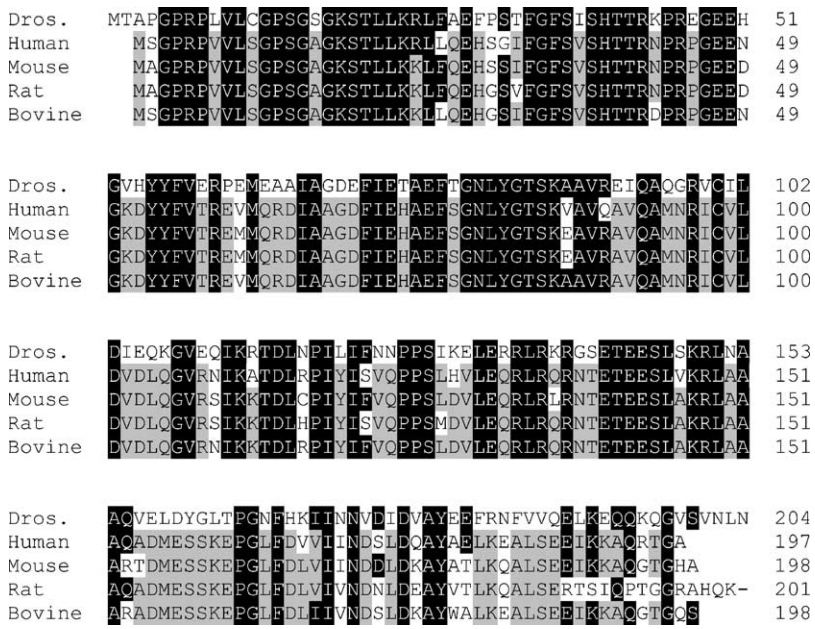

Fig. 1. Alignment of the guanylate kinase from *D. melanogaster*, human, mouse, rat and bovine origin. Black boxes indicate conserved amino acid residues compared to the *Drosophila* enzyme. Gray boxes indicate amino acid residues conserved in at least three of the enzymes.

was named *Dm*-GUK. Analysis of nucleotide sequences deposited in FlyBase [14] showed that *Dm*-GUK gene was located on chromosome 3L at 67E6-7 (FlyBase gene CG11811).

The *D. melanogaster* UMP–CMP kinase encodes a functional N-terminal mitochondrial import signal sequence [9]. Mitochondrial import signals are usually located in the N-terminal part of the protein and form an amphipathic α-helical structure that contains positively charged and hydrophobic amino acid residues. Although the N-terminal region of *Dm*-GUK contained several positively charged and hydrophobic amino acid residues, the presence of multiple proline residues strongly argue against the formation of an α-helix. Sequence analysis using the PSORT algorithm for prediction of subcellular protein localization was not able to identify features suggestive of a mitochondrial import signal sequence and the prediction suggests that the protein is located in the cytosol [15].

3.2. Expression and substrate specificity of Dm-GUK

The *Dm*-GUK was recombinantly expressed with an N-terminal poly-histidine tag and purified by one-step metal affinity chromatography (Fig. 2A). To verify the enzymatic activity and to determine the substrate specificity of the recombinant enzyme we tested the naturally occurring ribo- and deoxyribonucleoside monophosphates as substrates for the recombinant *Dm*-GUK in a phosphoryl transfer assay as described in Section 2. Several ribo- and deoxyribonucleoside monophosphates were tested at 1 mM concentration as substrates for the enzyme (Fig. 2C). The enzyme phosphorylated GMP and dGMP with similar relative efficiency using γ-^{32}P-ATP as phosphate donor. No phosphorylation of AMP, dAMP, CMP, dCMP, UMP, dUMP or dTMP were detected. The kinetic properties of *Dm*-GUK were determined using dGMP as substrate. Michaeli–Menten kinetics was found with an apparent K_m of 0.5 ± 0.1 mM and a V_{max} for the reaction of 1.0 ± 0.2 μmol/mg/min (Fig. 2B).

3.3. Phosphorylation of deoxyguanosine analogs by Dm-GUK

Deoxyguanosine nucleoside analogs are important chemotherapeutic agents and we decided to investigate if such analogs are substrates of the *Dm*-GUK. The deoxyguanosine analogs araG and dFdG are phosphorylated by the multisubstrate *Dm*-dNK and we assayed the coupled conversion of the nucleoside analogs to the diphosphate form catalyzed sequentially by *Dm*-dNK and *Dm*-GUK. Both araG and dFdG were phosphorylated to their corresponding diphosphates in this assay demonstrating that araGMP and dFdGMP are substrates for *Dm*-GUK (Fig. 3).

3.4. In vitro reconstitution of dGTP and araGTP synthesis

The entire pathway of dGTP synthesis from dGuo was reconstituted to investigate the role of *Dm*-GUK in the salvage pathway of dGTP formation. The *Dm*-dNK, *Dm*-GUK and NDPK were mixed in a single reaction with ATP as phosphate donor. The ADP generated in this reaction may lower the efficiency of conversation and we used creatine kinase/creatine phosphate as an ATP regenerating system. HPLC analysis demonstrated an almost complete

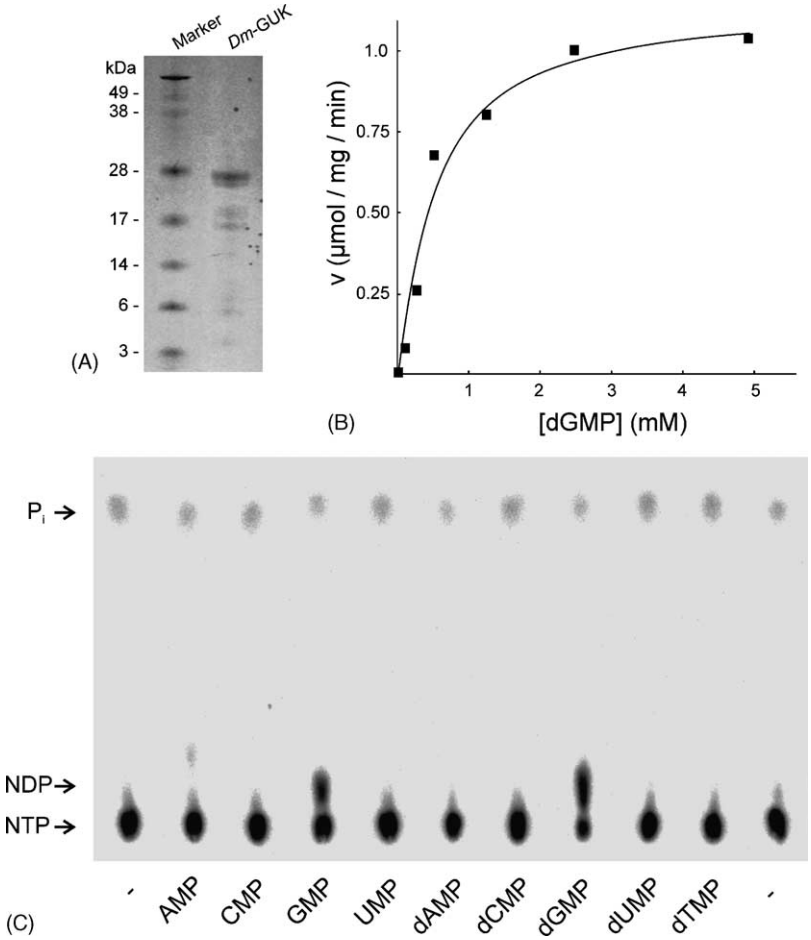

Fig. 2. Expression and enzymatic activity of recombinant *Dm*-GUK. (A) SDS–PAGE gel analysis of purified recombinant *Dm*-GUK. (B) Michaelis–Menten kinetic analysis of dGMP phosphorylation. (C) Substrate specificity for NMPs determined by TLC analysis. γ-^{32}P-ATP phosphotransferase assay using 1 mM nucleoside monophosphates as substrates. Control reactions (-) did not contain substrate. The arrows indicate the expected position of the nucleoside diphosphates (NDP) and triphosphates (NTP).

conversion of dGuo to dGTP in this reaction after 24 h of incubation (Fig. 4A). dGuo, dGMP and dGDP were present at <5% after the phosphorylation catalyzed by the enzymes. We used a similar assay to determine if araG could be converted to araGTP (Fig. 4B). Although araGTP was detected, the conversion was less efficient and araG, araGMP and araGDP were readily detected.

4. Discussion

The present study was initiated to study the nucleoside monophosphate kinase responsible for the second phosphorylation step of dGuo to dGTP in *D. melanogaster*. Previous studies of the multisubstrate *Dm*-dNK has demonstrated unique catalytic properties of this enzyme and we wanted to investigate if also other enzymes involved in the salvage of nucleosides in *Drosophila* had properties different from enzymes of other organisms. However, the analysis of *Dm*-GUK performed in this study demonstrated that the properties of the enzyme were similar to those reported for mammalian guanylate kinases. *Dm*-GUK only phosphorylated GMP and dGMP among the natural ribo- and deoxyribonucleoside monophosphates. The kinetic constants for *Dm*-GUK with dGMP as substrate were also found to be similar to those reported for the mammalian enzymes. The restricted substrate specificity for both *Dm*-GUK and the previously studied *D. melanogaster* UMP–CMP kinase suggest that the setup of nucleoside monophosphate kinases in *Drosophila* are similar to the enzymes present in mammalian cells. Although the insect cells contain only the single *Dm*-dNK multisubstrate deoxyribonucleoside kinase to catalyze the first step in the deoxyribonucleoside phosphorylation pathway, several different nucleoside monophosphate kinases are present in these cells to catalyze the further phosphorylation of the deoxyribonucleoside monophosphates.

Similar to other nucleoside monophosphate kinases, *Dm*-GUK has a K_m for its substrates that is much higher than the physiological concentration of the substrates in cells. To analyze the efficiency of the enzyme as part of the salvage pathway of dGuo to dGTP synthesis we reconstituted the entire pathway of dGuo phosphorylation to dGTP. The complete conversion of dGuo to dGTP in the

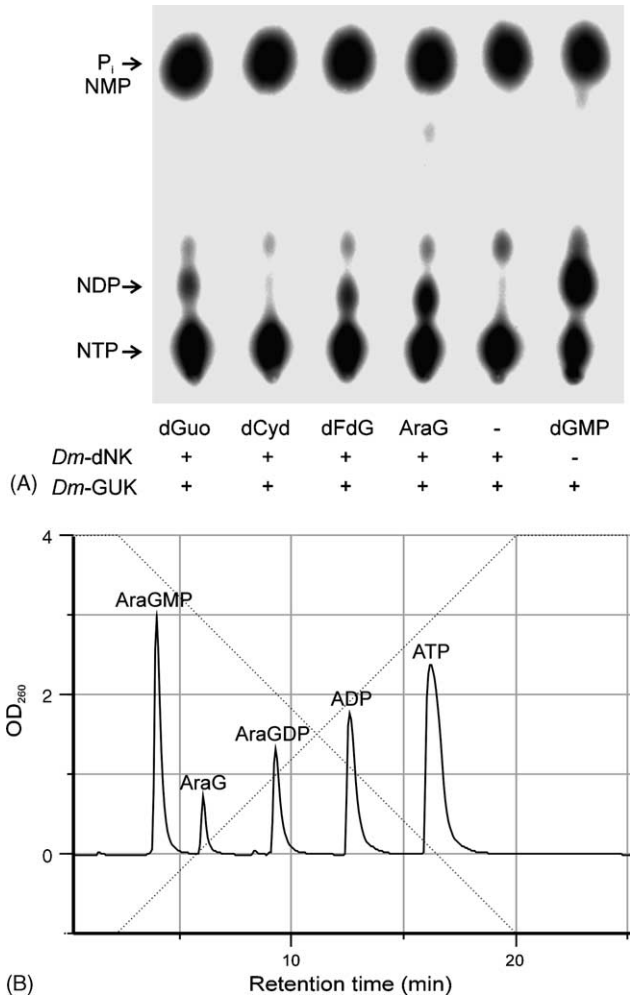

Fig. 3. Coupled phosphotransferase assay using *Dm*-dNK and *Dm*-GUK for phosphorylation of guanine nucleoside and nucleoside analogs to diphosphates. (A) TLC analysis of 1 mM dGuo, dCyd, dFdG and araG phosphorylation to the diphosphate form by 0.25 μg *Dm*-dNK and 1 μg *Dm*-GUK. (B) HPLC analysis of the araG phosphotransferase reaction using both kinases.

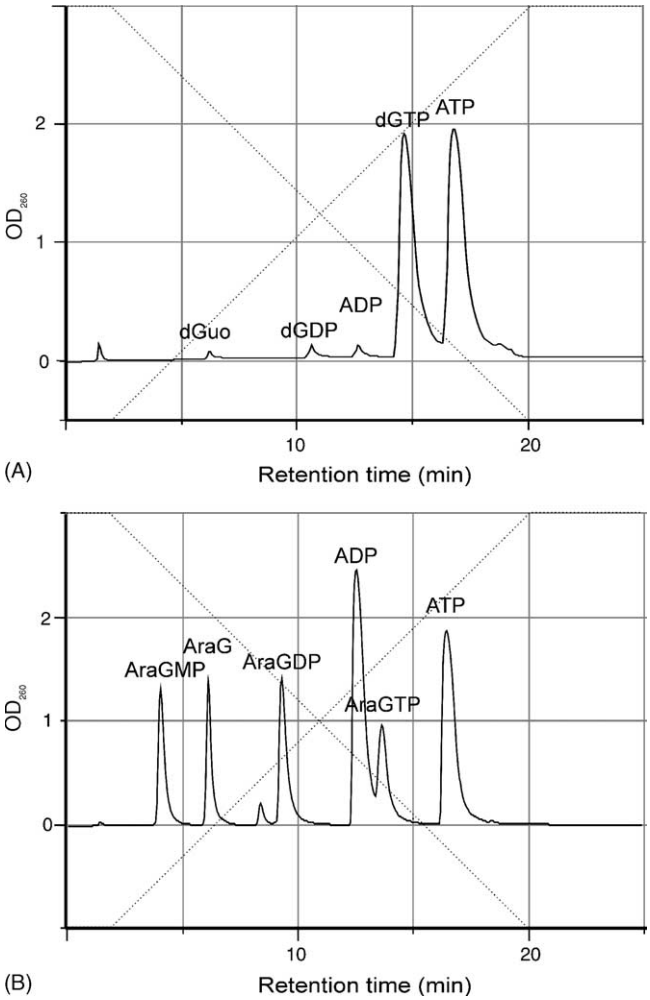

Fig. 4. Coupled phosphotransferase assay using *Dm*-dNK, *Dm*-GUK and NDPK for phosphorylation of dGuo (A) and araG (B) to nucleoside triphosphates.

experimental setup demonstrated that the kinetic properties of *Dm*-GUK did not prevent efficient phosphorylation to the triphosphate form. We found that the monophosphates of araG and dFdG can be efficiently phosphorylated by *Dm*-GUK. However, when araG was tested in the assay for conversion from nucleoside to nucleoside triphosphate, substantial amounts of araG, araGMP and araGDP were detected. These findings suggest that all of the three kinases used in the assay including *Dm*-GUK had problems to phosphorylate the nucleoside analog as efficiently as the natural deoxyribonucleoside. Nucleoside monophosphate kinases are not as thoroughly studied as the nucleoside kinases regarding their contribution to the phosphorylation of nucleoside analogs [16]. There are nucleoside monophosphate kinases that are known to be rate limiting in the activation of certain nucleoside analogs. Human thymidylate kinase is one example with a low efficiency of azidothymidine monophosphate phosphorylation that results in an accumulation of azidothymidine at the monophosphate level [5,17]. Our findings that also the conversion of the purine nucleoside analog araG to the triphosphate form may be limited by a nucleoside monophosphate kinase further emphasizes that also the kinases catalyzing the phosphorylation of nucleoside analog monophosphates and diphosphates should be investigated in the development of novel nucleoside analog-based therapies.

Acknowledgements

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