Phosphorylation of Pyrimidine Deoxynucleoside Analog Diphosphates

SELECTIVE PHOSPHORYLATION OF L-NUCLEOSIDE ANALOG DIPHOSPHATES BY 3-PHOSPHOGLYCERATE KINASE®

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D-Nucleoside analogs, which are in the natural configuration, as well as the L-nucleoside analogs, are clinically relevant antiviral and anticancer agents. Metabolism of L-nucleoside analog diphosphates to the triphosphates, however, remains unexplored. Studies with recombinant nm23-H1 and -H2 isoforms indicated that L-nucleoside analog diphosphates were not phosphorylated by their nucleoside diphosphate kinase (NDPK) activity. Therefore, roles of creatine kinase, 3-phosphoglycerate kinase, and pyruvate kinase were evaluated using preparations from commercial sources (NDPK) activity. Therefore, roles of creatine kinase, 3-phosphoglycerate kinase, and pyruvate kinase were evaluated using preparations from commercial sources and human HepG2 cells. Phosphorylation of L-OddC, L-SddC, L-Fd4C, L-FMAU, and L-ddC were compared with D-deoxynucleoside analogs, AraC, dFdC, and L-FMAU, and D-dideoxynucleoside analogs, ddC and d4T. Results based on preparations from HepG2 cells showed that L-nucleoside analog diphosphates were selectively phosphorylated by 3-phosphoglycerate kinase, whereas, D-deoxynucleoside analog diphosphates were phosphorylated by NDPK. Interestingly, ddCDP and d4TDP were substrates for creatine kinase, but not phosphorylated by NDPK. In conclusion, it is proposed that specificity of the phosphorylating enzymes toward the nucleoside analog diphosphates is dependent on the configuration of the analog (L or D) and the presence or absence of 3’-hydroxy group in the sugar moiety. The enzymatic process of phosphorylation of L- and D-nucleoside analog diphosphates is different in cells.

In the past decade the importance of L-nucleoside analogs in the inhibition of viral replication and treatment of cancer has been recognized. Controlling viral replication is also important for possibly preventing or delaying the onset of virus-associated cancers, especially in immunocompromised patients because of organ transplantation or acquired immunodeficiency syndrome (1, 2). Among the L-nucleoside analogs, L-SddC has been proven for the treatment of HIV and HBV (3–5); L-FMAU (1, 2) and L-Fd4C (6–8) are currently under phase I/II clinical trial as anti-HBV agents; and L-OddC (9–11) is in phase II clinical trials for the treatment of cancer. Among the D-nucleoside analogs, d4T and ddC are approved anti-HIV agents (12, 13), and dFdC (gancitabine) and AraC are anti-cancer agents (14).

Most nucleoside analogs require stepwise phosphorylation to the respective triphosphate metabolites to exert their pharmacological activity. L- and D-dCyd analogs are phosphorylated by cytoplasmic deoxycytidine kinase, and dThd analogs are phosphorylated by cytoplasmic thymidine kinase to the monophosphate metabolites. L-FMAU can be phosphorylated by both cytoplasmic deoxycytidine kinase and cytoplasmic thymidine kinase. dCd analog monophosphates are further phosphorylated by cytidine/uridine monophosphate kinase to the respective nucleoside diphosphate metabolites, whereas the dThd analogs are phosphorylated by thymidine monophosphate kinase (1, 2, 15, 16). Conversion of L-deoxynucleoside analog diphosphates to the pharmacologically active L-deoxynucleoside triphosphate metabolites remains largely unexplored; however, NDPK, which could phosphorylate naturally occurring nucleoside diphosphates, has been assumed to play a role (17–21). The last step of phosphorylation is of potential importance, because analogs like L-Fd4C, L-OddC, L-SddC, and ddC accumulate in the cells as diphosphate metabolites indicating inefficiency of the responsible enzyme (6, 22–24). However, L-FMAU is efficiently metabolized to L-FMAUTP (25).

Eight isoforms of NDPK have been isolated in humans, of which nm23-H1 and nm23-H2 have been shown to be cytoplasmic, and are capable of phosphorylating nucleoside diphosphates (26, 27). DR-nm23 (28), nm23-H4 (29) and nm23-H6 (30) are localized in the mitochondria, and nm23-H5 is testis-specific (31). Activities of nm23-H7 and nm23-H8 in terms of nucleoside diphosphate phosphorylation are not known (NCBI accession numbers Q9Y5B8 and XP_004705, respectively). NDPKs utilize ATP or other nucleoside triphosphates as a phosphate donor and transfer the phosphate residue onto nucleoside diphosphates via a phosphohistidine intermediate (ping-pong mechanism) (32, 33).

Other enzymes that are capable of phosphorylating nucleoside diphosphates are creatine kinase, 3-phosphoglycerate kinase, pyruvate kinase, phosphoenolpyruvate carboxykinase, and adenylsuccinate kinase (34–36). Based on the high rates of hydrolysis of the phosphate bonds of the donor compounds deoxycytidine; d4T, 2’,3’-dideoxy-2’,3’-dideoxythymidine; NDPK, nucleoside diphosphate kinase; DTT, dithiothreitol; HIV, human immunodeficiency virus; CK, creatine kinase; HBV, hepatitis B virus; dThd, thymidine; ddI, 2’,3’-dideoxyinosine; dCd, 2’,3’-deoxyctydine.

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such as creatine phosphate with $\Delta G$ equal to $-43$ kJ/mol, 1,3-biphosphoglycerate with $\Delta G$ equal to $-49$ kJ/mol, and phospho(en)pyruvate with $\Delta G$ equal to $-62$ kJ/mol (37), the enzymes, creatine kinase, 3-phosphoglycerate kinase, and pyruvate kinase, were also examined as potential enzymes for the phosphorylation of nucleoside analog diphosphates.

Creatine kinases are dimeric enzymes existing as three isoforms that are designated muscle type (MM), brain type (BB) and hybrid type (MB). These enzymes utilize creatine phosphate as a phosphate donor to convert ADP to ATP (38). The predominant role of creatine kinase is to generate creatine, which is used by myofibrils during muscular contraction. Pyruvate kinase is a tetramer that exists as three major isoforms named M1, M2, and L. The L-isoform is an allosteric enzyme requiring fructose 1,6-diphosphate as a co-factor and is known to be predominant in the liver (39, 40). Pyruvate kinase is a glycolytic enzyme that transfers a phosphate group onto ADP using phosphoenolpyruvate as a phosphate donor (37, 39).

For the purpose of this study, the diphosphates of the nucleoside analogs shown in Fig. 1 have been categorized into three groups: L-nucleoside analogs, L-ddC, L-OddC, L-SddC, L-Fd4C, and L-FMAU; D-nucleoside analogs with 3'-hydroxyl group, ddC, and d4T; and L-nucleoside analogs lacking the 3'-hydroxyl group, dCyd, AraC, dFdC (gemcitabine), and FMAU; and d-nucleoside analogs lacking the 3'-hydroxyl group, ddC, and d4T. This study compares the conversion of diphosphates of these analogs to triphosphates by different cellular enzymes. Since the role of L-nucleoside analogs has been implicated in the treatment of hepatitis B infections, which can also lead to progressive liver disease, studies have focused on phosphorylation of L-nucleoside analogs by enzymes isolated from HepG2 cells.

EXPERIMENTAL PROCEDURES

Synthesis of Nucleoside Analog Diphosphates—Monophosphate nucleoside analogs were synthesized according to the procedure published by Ruth and Cheng (44). Diphosphates of nucleoside analogs were synthesized enzymatically by the action of recombinant cytidine mono- or thymidine monophosphate kinase, using GTP as a phosphate donor. Nucleoside monophosphates and diphosphates were separated using DEAE Sephadex A-25 chromatography (Amersham, Uppsala, Sweden) eluted with step gradients between 0 and 200 mM KCl. GDP, generated subsequent to phosphate transfer, does not co-migrate with dCyd analog diphosphates during chromatographic purification (unlike ADP); therefore, GTP was used as a phosphate donor instead of ATP.

Cloning nm23-H1 and -H2 Proteins—Total RNA was extracted from KB cells (human epidermoid carcinoma cell line), using RNAzol (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. Aliquots of total RNA (20 µg) were reverse-transcribed in the presence of SuperScript RNase H-reverse transcriptase (Invitrogen, Rockville, MD). The reactions were incubated at 37 °C followed by incubation at 96 °C for 5 min to stop the reaction. cDNA was purified by passing through CHROMA SPIN-10 (CLONTECH, Palo Alto, CA) to remove unincorporated nucleotides and slig-dT primer. An aliquot of cDNA was amplified in the presence of DNA polymerase PWO (Roche Diagnostic Corp., Indianapolis, IN) and specific primers for nm23-H1 and nm23-H2. The sequence for sense and antisense primers for nm23-H1 were as follows: 5'-GAG GCA GCC ATG TTA GGC CAA CCA ACG GTC GTA TCC and 3'-GGG GCA GCC ATA TGG CCA ACT GTG. The PCR product was digested with the restriction enzyme NdeI and gel-purified using the Qiagen PCR purification kit (Qiagen Inc., Valencia, CA). The resulting DNA fragment was ligated to NdeI-digested pET28a bacterial expression vector. The sequences of nm23-H1 and -H2 were confirmed by DNA sequencing. The resulting pET28a-H1 and -H2 were N-terminally histidine fusion proteins.

Enzymatic Phosphorylation of Nucleoside Analog Diphosphates—All the buffers contained 50 mM Tris acetate (pH 7.5), 5 mM MgCl$_2$, 4 mM GTP, 5 mM NaF, and 5 mM DTT. NDPK activity was measured in the buffer containing 4 mM GTP as a phosphate donor. GDP was chosen as a phosphate donor based on its differential separation from dCyd monophosphate upon anion liquid chromatography (ALC). Creatine kinase activity was measured in the buffer containing 20 mM creatine phosphate as a phosphate donor. Pyruvate kinase activity was measured in a buffer containing 100 mM KCl with 2.5 mM phosphoenolpyruvate as a phosphate donor. For reactions including pyruvate kinase from HepG2 cells, 1 mM fructose 1,6-diphosphate was added as a co-factor. Phosphate activity was monitored by coupled reactions in a buffer containing 10 mM sodium phosphate, 4 mM NAD$^+$, and 4 mM N-glyceraldehyde 3-phosphate. 1,3-Biphosphoglycerate, which is the phosphate donor for the reaction, was generated 20 min prior to inclusion of 3-phosphoglycerate kinase, by addition of 8 units/ml glyceraldehyde-3-phosphate dehydrogenase. Indicated concentrations of nucleoside diphosphate analogs served as phosphate acceptors. Some of the studies included the commercial enzymes, creatine kinase (type I from rabbit muscle, Sigma Chemical Co., St. Louis, MO), pyruvate kinase (type II from rabbit muscle, Sigma), and 3-phosphoglycerate kinase (from yeast, Roche Molecular Biochemicals, Germany).

Lack of phosphorylation in the absence of the phosphate donor served as the positive control for the reactions. All samples were incubated at 37 °C, and speed on ice was sometimes used to stop the reaction. The buffer containing 10 mM sodium phosphate, 4 mM NAD$^+$, and 4 mM N-glyceraldehyde 3-phosphate dehydrogenase was prepared. NAD$^+$ was chosen as the phosphate donor for the reaction, was generated 20 min prior to inclusion of 3-phosphoglycerate kinase, by addition of 8 units/ml glyceraldehyde-3-phosphate dehydrogenase. Indicated concentrations of nucleoside diphosphate analogs served as phosphate acceptors. Some of the studies included the commercial enzymes, creatine kinase (type I from rabbit muscle, Sigma Chemical Co., St. Louis, MO), pyruvate kinase (type II from rabbit muscle, Sigma), and 3-phosphoglycerate kinase (from yeast, Roche Molecular Biochemicals, Germany). Lack of phosphorylation in the absence of the phosphate donor served as the positive control for the reactions. All samples were incubated at 37 °C, and speed on ice was used to stop the reaction. The buffer containing 10 mM sodium phosphate, 4 mM NAD$^+$, and 4 mM N-glyceraldehyde 3-phosphate dehydrogenase was prepared. NAD$^+$ was chosen as the phosphate donor for the reaction, was generated 20 min prior to inclusion of 3-phosphoglycerate kinase, by addition of 8 units/ml glyceraldehyde-3-phosphate dehydrogenase. Indicated concentrations of nucleoside diphosphate analogs served as phosphate acceptors. Some of the studies included the commercial enzymes, creatine kinase (type I from rabbit muscle, Sigma Chemical Co., St. Louis, MO), pyruvate kinase (type II from rabbit muscle, Sigma), and 3-phosphoglycerate kinase (from yeast, Roche Molecular Biochemicals, Germany).

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ADP generated by 3-phosphoglycerate kinase using 3-phosphoglycerate kinase as phosphate acceptor was evaluated by coupling to the glyceraldehyde-3-phosphate dehydrogenase system (modified from the procedure published previously (43)). The buffer contained 80 mM Tris acetate (pH 7.5), 8 mM MgCl₂, 1 mM ATP, 10 mM 3-phosphoglycerate, 0.15 mM NADH, 5 mM DTT, and 5 mM NaF. Oxidation of NADH by 1 unit of glyceraldehyde-3-phosphate dehydrogenase at 2-min intervals was monitored via spectrophotometer at 340 nm. Activity of each enzyme was measured by the conversion of ADP to ATP (or vice versa) in micromoles/min, which is proportional to oxidation or reduction of NADH or NAD (in micromoles) within a period of 1 min, using 6.22 × 10⁻⁵ cm⁻² as the molar extinction coefficient for NADH.

Western Blotting of nm23 Proteins—HepG2 fractions were pooled based on enzyme activity, and equal volumes were separated on 13% SDS-PAGE. Protein was transferred onto nitrocellulose membrane. The membrane was probe with polyclonal rabbit nm23-H1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); the membrane was then stripped (Tris-Cl, pH 6.8, 10% SDS, and 2-mercaptoethanol) and reprobed with monoclonal mouse nm23-H2 antibody (Seikagaku Corp., Tokyo, Japan) then stripped again and reprobed with monoclonal mouse nm23-H6 antibody (a generous gift from Dr. Miyuki Fukushima, Kyowa Hakko Kogyo Co. Ltd., Tokyo Research Laboratories, Japan). Protein bands were detected by chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

RESULTS

Phosphorylation of Nucleoside Analog Diphosphates by nm23-H1, -H2, and Some Commercial Cellular Enzymes—Immunofluorescence microscopy had established that the H1 and H2 isoforms of NDPK proteins are cytosolic (data not shown); therefore, these isoforms were cloned for further studies with nucleoside analog diphosphates. Table I represents phosphorylation of nucleoside analog diphosphates, normalized with respect to the phosphorylation of dCDP. Representative analogs from each category shown in Fig. 1 were used as phosphate acceptors. L-Nucleoside analogs, l-OddC, l-SddC, and l-Fd4C were not phosphorylated by both H1 and H2 enzymes. Moreover, ddCDP was also not a substrate for H1 and H2 enzymes. These results indicated that the configuration as well as the 3'-hydroxyl group are important for substrate specificity of nucleoside analogs toward these two NDPKs, which implied that other cellular enzymes might be responsible for phosphorylation of L-nucleoside diphosphates in the cytoplasm. Creatine kinase, 3-phosphoglycerate kinase, and pyruvate kinase are some cellular enzymes that are capable of phosphorylating nucleoside diphosphates in addition to ADP, which is their natural substrate. These enzymes, obtained from commercial sources, had been isolated from rabbit muscle or yeast. Interestingly, 3-phosphoglycerate kinase selectively phosphorylated L-nucleoside analog diphosphates (3- to 4-fold better), as compared with dCDP. L-Nucleoside analogs were also substrates for pyruvate kinase and, to a lesser extent, for creatine kinase. In addition, ddCDP was phosphorylated only by creatine kinase and pyruvate kinase, which had been isolated from rabbit muscle. Studies with these commercial enzymes indicated significance of configuration on phosphorylation by 3-phosphoglycerate kinase.

Purification of Enzymes from HepG2 Cells—As several L-nucleoside analogs are important for the treatment of hepatitis B infection, the metabolism of these analogs in liver cells is of potential interest. Therefore, HepG2 cells were selected for isolation of nucleoside diphosphate-metabolizing enzymes, using a Blue Sepharose CL6B affinity column.

The isolation profile of the enzymes that were identified is shown in Fig. 2. 3-Phosphoglycerate kinase was eluted in the wash buffer using its substrate 3-phosphoglycerate. Some activities of creatine kinase (CK-I) and NDPK (NDPK-I) were eluted in 20 mM KCl with a 0 to 5 mM ADP gradient. During previous attempts to isolate these enzymes from human fetal liver,² activities of pyruvate kinase, NDPK, and creatine kinase were not eluted with a buffer containing up to 0.5 mM KCl, however, they had co-eluted in a buffer containing KCl between 0.5 and 2 M. The elution procedure was therefore modified and the column was instead washed with a KCl gradient between 0.05 and 0.5 M to remove unwanted cellular proteins. This was followed by elution with 1 mM phosphoenolpyruvate and 1 mM fructose 1,6-diphosphate, which are substrates and co-factors for pyruvate kinase, respectively, in a buffer containing 0.5 mM KCl. Interestingly, some activities of NDPK (NDPK-II) and creatine kinase (CK-II) were also co-eluted with pyruvate kinase. Subsequent elution with 1 mM ADP in a buffer containing 0.5 mM KCl isolated another NDPK (NDPK-III) enzyme activity. Activity of the enzyme has been defined as the amount of enzyme required to phosphorylate 1 μmol of ADP to ATP in 1 min. The total activity (derived from Fig. 2), as well as the specific activity of each enzyme in the crude extract, and the blue Sepharose fractions, were evaluated using ADP or ATP as a substrate, and these values are shown in Table II. The blue Sepharose fractions were pooled on the basis of activity; fractions 8–11, 66–70, 75–79, 115–118, and 144–147 contain 3-phosphoglycerate kinase, CK-I, NDPK-I, NDPK-II (pyruvate kinase and CK-II), and NDPK-III, respectively. 3-Phosphoglycerate kinase was purified 920-fold, and NDPKs I and III were purified at least 690- and 320-fold, respectively. The apparent increase in the recovery of NDPK and creatine kinase activity subsequent to the purification procedure could have resulted from the removal of some contaminating proteins. Comparison of total enzyme activity in the crude extract and the blue Sepharose fractions showed that there was no significant loss of activity during the column chromatography procedure; therefore, values in Table II reflect the activity of each enzyme in HepG2 cells.

Identification of NDPK Isoforms in HepG2 Fractions—Three activity peaks of NDPK were separated using the elution procedure. The fractions could therefore contain different isoforms of the protein. Western blotting was carried out with nm23-H1 antibody (that was also slightly cross-reactive toward the H2 protein), nm23-H2 antibody, and nm23-H6 antibody. Fig. 3 is a

² P. Krishnan and Y. C. Cheng, unpublished observations.
Buffers used to elute the proteins from a blue Sepharose column are as L-nucleoside analogs, D-deoxynucleoside analogs, and D-dideoxynucleoside analogs.

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The analogs were phosphorylated by 3-phosphoglycerate kinase, NDPK enzymes were probably responsible for phosphorylation of these analogs in HepG2 cells. Different isoforms of NDPK were also found to have substrate preferences under the conditions used in this study. ddCDP was phosphorylated only by CK-I. d4TDP was a substrate for all the enzymes, however, consistent with the results for ddCDP, CK-I was probably responsible for its phosphorylation in these cells. L-Nucleoside analog diphosphates were phosphorylated by 3-phosphoglycerate kinase in the order as follows: L-FMAUDP > L-SddCDP > L-Fd4CDP > L-ddCDP > L-OddCDP. Importantly, l-nucleoside analogs were better or comparable to d-nucleoside analogs as substrates for 3-phosphoglycerate kinase. These results established that the specificity of the phosphorylating enzymes toward the nucleoside analogs is dependent on the configuration of the analog (l, or d) and the presence or absence of 3'-hydroxyl group.

**Inhibition of 3-Phosphoglycerate Kinase by L-Nucleoside Analog Diphosphates**—Several L-nucleoside analogs accumulate in the cells as diphosphates. Therefore, it is important to consider the impact of these diphosphates on nucleoside diphosphate-metabolizing enzymes, especially, 3-phosphoglycerate kinase. This was analyzed as the effect of nucleoside analog diphosphates (at a fixed concentration of 200 μM) on phosphorylation of ADP (used at 100 and 200 μM). The Kᵢ values were calculated from the amount of ATP formed in the presence of nucleoside analogs and are shown in Table IV. Although some of the d-nucleoside analogs such as dCDP, d4TDP, and d-FMAUDP had no impact on ATP formation at the tested concentrations, other nucleoside analog diphosphates were competitive inhibitors in the order dF4CDP >> AraCDP >> ddCDP. To allow for experimental variations, inhibition of ATP formation by less than 10% has been considered non-significant. L-Nucleoside analog diphosphates were very good competitive inhibitors of ADP phosphorylation in the order L-FMAUDP > L-Fd4CDP > L-SddCDP > L-ddCDP. The lack of inhibition by L-OddCDP could be due to the Kᵢ values being much greater than the Kᵢₘ for ADP. It is particularly interesting to note the marked difference between d-FMAUDP and d-FMAUDP, again indicating preference of l- over d-nucleoside analog diphosphates shown in Fig. 1, tested at a fixed concentration of 200 μM, were assessed as substrates with the fractions that were pooled on the basis of activity. The values shown in Table III represent the efficiency of phosphorylation of nucleoside analog diphosphates normalized with respect to the activity of enzyme required to phosphorylate ADP to ATP. Results showed that, although d-deoxynucleoside analog diphosphates were substrates for all the enzymes, except dF4CDP (gemcitabine), which was not phosphorylated by 3-phosphoglycerate kinase, NDPK enzymes were probably responsible for phosphorylation of these analogs in HepG2 cells. Different isoforms of NDPK were also found to have substrate preferences under the conditions used in this study. ddCDP was phosphorylated only by CK-I. d4TDP was a substrate for all the enzymes, however, consistent with the results for ddCDP, CK-I was probably responsible for its phosphorylation in these cells. L-Nucleoside analog diphosphates were phosphorylated by 3-phosphoglycerate kinase in the order as follows: L-FMAUDP > L-SddCDP > L-Fd4CDP > L-ddCDP > L-OddCDP. Importantly, l-nucleoside analogs were better or comparable to d-nucleoside analogs as substrates for 3-phosphoglycerate kinase. These results established that the specificity of the phosphorylating enzymes toward the nucleoside analogs is dependent on the configuration of the analog (l, or d) and the presence or absence of 3'-hydroxyl group.

**Phosphorylation of Nucleoside Analog Diphosphates by Enzymes Isolated from HepG2 Cells**—All the nucleoside analog diphosphates shown in Fig. 1, tested at a fixed concentration of 200 μM, were assessed as substrates with the fractions that were pooled on the basis of activity. The values shown in Table III represent the efficiency of phosphorylation of nucleoside analog diphosphates normalized with respect to the activity of enzyme required to phosphorylate ADP to ATP. Results showed that, although d-deoxynucleoside analog diphosphates were substrates for all the enzymes, except dF4CDP (gemcitabine), which was not phosphorylated by 3-phosphoglycerate kinase, NDPK enzymes were probably responsible for phosphorylation of these analogs in HepG2 cells. Different isoforms of NDPK were also found to have substrate preferences under the conditions used in this study. ddCDP was phosphorylated only by CK-I. d4TDP was a substrate for all the enzymes, however, consistent with the results for ddCDP, CK-I was probably responsible for its phosphorylation in these cells. L-Nucleoside analog diphosphates were phosphorylated by 3-phosphoglycerate kinase in the order as follows: L-FMAUDP > L-SddCDP > L-Fd4CDP > L-ddCDP > L-OddCDP. Importantly, l-nucleoside analogs were better or comparable to d-nucleoside analogs as substrates for 3-phosphoglycerate kinase. These results established that the specificity of the phosphorylating enzymes toward the nucleoside analogs is dependent on the configuration of the analog (l, or d) and the presence or absence of 3'-hydroxyl group.

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nucleoside analogs based on the conversion of their metabolites to triphosphates. These results indicate that most of the tested analogs had $K_v$ values comparable to the $K_m$ of ADP.

**DISCUSSION**

In addition to their roles in the treatment of HIV and cancer (L-OddC), L-nucleoside analogs are rapidly emerging as clinically relevant agents for the treatment of HBV (1, 2). To characterize nucleoside analogs based on the conversion of their diphosphate metabolites to triphosphates, these analogs were categorized as L-nucleoside analogs, d/ddC, L-OddC, L-SddC, L-Fd4C, and L-FMAU; d-nucleoside analogs with 3'-hydroxyl group, dCD4, AraC, dFdC (gemcitabine), and FMAU; and pyruvate kinase from rabbit muscle.

Kinases indicated selectivity toward L-nucleoside analogs, with dCDP and ddCDP using commercial 3-phosphoglycerate kinase. Isolation of these enzymes from a single source gives the table shows activity and the specific activity of the enzymes in the crude extract and the fractions from Blue Sepharose column chromatography, which were pooled on the basis of activity. One unit is defined as the amount of enzyme required to phosphorylate 1 μmol of ADP to ATP in 1 min.

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**FIG. 3. Western blot of NDPK proteins in HepG2 fractions.** Pooled fractions containing enzyme activities were probed with nm23-H1, nm23-H2, and nm23-H6 antibodies.

Since nucleoside analogs are phosphorylated in the cytosol, nm23-H1 and -H2 proteins (the cytosolic isoforms) were cloned. It was observed that these two proteins did not phosphorylate L-nucleoside analog diphosphates and could not be responsible for the conversion of diphosphate metabolites to triphosphates. In addition, ddCDP was also not a substrate for H1 and H2, indicating the importance of the 3'-hydroxyl group. It is likely that both ddCDP and ddADP (a metabolite of ddT) are also not phosphorylated by cytosolic H1 and H2 in cells. Initial comparison of several L-nucleoside analog diphosphates with dCDP and ddCDP using commercial 3-phosphoglycerate kinase indicated selectivity toward L-nucleoside analogs, whereas ddCDP was phosphorylated only by creatine kinase and pyruvate kinase from rabbit muscle.

Given the clinical relevance of L-nucleoside analogs as anti-HBV agents and their metabolism in human liver, HepG2 cells (human hepatoma cells) were selected for isolation of these kinases. Isolation of these enzymes from a single source gives an added advantage for direct comparison of the potential for phosphorylation of each nucleoside analog in the particular cell line or tissue. Using an affinity column (blue Sepharose CL6B) and the respective substrates in the elution buffer, 3-phosphoglycerate kinase, creatine kinase, and NDPK were eluted. Interestingly, despite the use of specific conditions and substrates for elution of pyruvate kinase, creatine kinase and NDPK (including H1 and H6 isoforms) also co-purified with pyruvate kinase. In addition, other NDPKs (including H1 and H2 isoforms) were eluted at higher salt concentrations in the presence of ADP. It is possible that specific protein-protein interactions may be responsible for the co-elution of creatine kinase and NDPK along with pyruvate kinase. This hypothesis is currently under investigation. The isoforms of creatine kinase and pyruvate kinase could not be differentiated in the enzyme fractions isolated from HepG2 cells. Pyruvate kinase was, however, more active in the presence than in the absence of fructose 1,6-diphosphate (data not shown), a co-factor for the L-isom, known to be a predominant form in liver (39).

Based on phosphorylation of the nucleoside diphosphate analogs by the enzymes isolated from HepG2 cells, it is concluded that all L-nucleoside analog diphosphates are likely to be phosphorylated by 3-phosphoglycerate kinase in cells. A recent report suggesting that NDPKs might not be the enzymes responsible for phosphorylating L-SddCDP (46) is also consistent with this conclusion. Phosphorylation by NDPKs seemed to require the presence of the 3'-hydroxyl group of d-deoxyribonucleoside analogs, whereas ddCDP was a substrate for creatine kinase. Consistent with the results for ddCDP, d4TDP was an excellent substrate for pyruvate kinase, although it could also be phosphorylated by NDPK. The possibility that ddCDP can be phosphorylated by pyruvate kinase in muscles (based on the results from rabbit muscle pyruvate kinase), due to differences in isoforms, is not ruled out, and is currently under investigation. The fact that d4TDP was also phosphorylated by NDPK (unlike other dideoxy analogs) supports published reports (20) that planarity and lack of steric hindrance in the sugar moiety probably afford it the required conformation for phosphate transfer by NDPK. Specificity of NDPK isoforms toward nucleoside analog diphosphates has not been evaluated.

Since some of the L-nucleoside analogs accumulated in the cells predominantly as diphosphate metabolites, their impact on 3-phosphoglycerate kinase was studied. It was observed that the $K_v$ values of most L-nucleoside analog diphosphates were comparable to the $K_m$ of ADP. This suggested that increased accumulation of L-nucleoside analog diphosphates over ADP levels in cells could have an impact on this enzyme. 3-Phosphoglycerate kinase is a glycolytic enzyme, and products of glycolysis are oxidized in citric acid cycle and allow transformation of carbohydrates to fat (47). The impact of inhibition of 3-phosphoglycerate kinase on the possible adverse effects of L-nucleoside analogs (48) is currently under investigation.
Nucleoside analog diphosphates were used as phosphate acceptors in reactions containing 3-phosphoglycerate kinase, CK-I, NDPK-I, pyruvate kinase (CK-II and NDPK-II), and NDPK-III. Efficiency of conversion to nucleoside analog triphosphates (mmol/min) has been normalized with respect to the activity of enzyme required to phosphorylate 1 μmol of ADP to ATP in 1 min. Values are mean ± S.D. from at least two independent experiments.

<table>
<thead>
<tr>
<th>Nucleoside diphosphate analog</th>
<th>Creatine kinase</th>
<th>Nucleoside diphosphate kinase</th>
<th>3-Phosphoglycerate kinase (× 10^-10)</th>
<th>Pyruvate kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>dCyd</td>
<td>8.4 ± 1.0</td>
<td>1.0 ± 0.7</td>
<td>10.0 ± 0.5</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>AraC</td>
<td>3.2 ± 0.8</td>
<td>1.6 ± 1.0</td>
<td>10.5 ± 0.5</td>
<td>18.1 ± 0.1</td>
</tr>
<tr>
<td>dFdC</td>
<td>35.0 ± 14.0</td>
<td>0.6 ± 0.2</td>
<td>83.5 ± 0.5</td>
<td>713.0 ± 157.0</td>
</tr>
<tr>
<td>dThd</td>
<td>2.3 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>16.5 ± 0.5</td>
<td>750.0 ± 50.0</td>
</tr>
<tr>
<td>dCD</td>
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<td></td>
<td>0.2 ± 0.03</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>dFT</td>
<td>16.0 ± 6.0</td>
<td>1.9 ± 1.3</td>
<td>0.9 ± 0.04</td>
<td>157.9 ± 9.3</td>
</tr>
<tr>
<td>d-dC</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>L-OddC</td>
<td>-</td>
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<tr>
<td>L-Fd4C</td>
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<tr>
<td>L-FMAU</td>
<td>-</td>
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<tr>
<td>L-FMAU</td>
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</table>

**TABLE IV**

*The values are representative results from single experiments due to difficulty in the synthesis of dFdCDP.*

**Hyphens represent inability to detect formation of nucleoside analog triphosphates by the respective enzyme.**

### REFERENCES


**Fig. 4. Schematic representation of enzymes phosphorylating nucleoside analog diphosphates in liver cells.** The model proposes structure-dependent phosphorylation of the analogs in cytosol of the cells. The figure is a modification of the model published by Bridges and Cheng (2).


Phosphorylation of Pyrimidine Deoxynucleoside Analog Diphosphates: SELECTIVE PHOSPHORYLATION OF L-NUCLEOSIDE ANALOG DIPHOSPHATES BY 3-PHOSPHOGLYCERATE KINASE
Preethi Krishnan, Qin Fu, Wing Lam, Jieh-Yuan Liou, Ginger Dutschman and Yung-Chi Cheng

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