Novel Role of 3-Phosphoglycerate Kinase, a Glycolytic Enzyme, in the Activation of L-Nucleoside Analogs, a New Class of Anticancer and Antiviral Agents*

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1-Nucleoside analogs are a new class of clinically active antiviral and anticancer agents. The phosphorylation of these analogs from diphosphate to triphosphate metabolites is crucial for their biological action. We studied the role of 3-phosphoglycerate kinase, a glycolytic enzyme, in the metabolism of L-nucleoside analogs, using small interfering RNAs to down-regulate the amount of this enzyme in HelaS3 and 2.2.15 cells, then shown as models for studying the impact of the enzyme on the anticancer and antihepatitis B virus activities of these analogs. Decrease in the expression of 3-phosphoglycerate kinase led to a corresponding decrease in the formation of the triphosphate metabolites of L-nucleoside analogs (but not D-nucleoside analogs), resulting in detrimental effects on their activity. The enzyme is important for generating as well as maintaining the steady state levels of L-nucleotides in the cells, thereby playing a key role in the activity of L-nucleoside analogs against human immunodeficiency virus, hepatitis B virus, and cancer. This study also indicates a structure-based distinction in the metabolism of L- and D-nucleoside analogs, disputing the classic notion that nucleoside diphosphates are responsible for the phosphorylation of all classes of nucleoside diphosphate kinases are responsible for the phosphorylation of all classes of nucleoside diphosphate kinases.

1-Nucleoside analogs are an emerging class of antiviral and anticancer agents that are being used clinically or are under advanced clinical trials (1, 2). Structurally, L-nucleoside analogs are mirror images of the natural D-nucleosides. Despite the unnatural L-configuration, these analogs are able to utilize the salvage pathway of deoxynucleoside metabolism to be phosphorylated to the active triphosphate form (3, 4). Similar to the D-nucleoside analog counterparts, L-nucleoside analog triphosphates exert their pharmacological activity through incorporation into cellular or viral DNA, leading to chain termination or inhibition of viral reverse transcriptase or DNA polymerase (5–10). In the past decade, the realization that L-nucleosides are capable of being activated in cells has led to the discovery of several antiviral pyrimidine L-deoxynucleoside analogs. Lamivudine (3TC) is currently used for the treatment of human immunodeficiency and hepatitis B viral infections (11–13). Clevudine (L-FMAU) and elvucitabine (L-Fd4C) are under phase II, and emtricitabine (FTC) is in phase III clinical trials as anti-HBV agents (14–19). L-Fd4C is also under phase II clinical trial for the treatment of HIV infection (17). Although several D-deoxynucleoside analogs such as gemcitabine and cytarabine are used as anticancer agents, troxacinabine (L-OddC) is the only L-nucleoside analog with anticancer properties. It is currently under phase II and III clinical trials for the treatment of pancreatic cancer and acute myelogenous leukemia, respectively (20–22).

The first two steps in the metabolism of L-nucleoside analogs seem to be similar to those of its D-nucleoside counterparts. The cytidine analogs, 3TC, L-OddC, L-Fd4C, and gemcitabine, are phosphorylated by cytoplasmic deoxycytidine kinase to the monophosphate metabolite, whereas L-FMAU can be phosphorylated by both deoxycytidine kinase and thymidine kinase (4, 10, 23–25). The cytidine analogs are then phosphorylated by cytidine/uridine monophosphate kinase to the respective diphosphates, and thymidine analogs are phosphorylated by thymidylate kinase (4, 26–28). The last step of pyrimidine nucleoside analog metabolism was assumed to be carried out by nucleoside diphosphate kinase (NDPK); however, its role in the phosphorylation of L-nucleoside analog diphosphates remained doubtful (29–31). We examined this further in detail by studying the phosphorylation of pyrimidine D-deoxy, D-dideoxy, and L-dideoxy nucleoside analog diphosphates by nucleoside metabolizing enzymes isolated from HepG2 cells (human hepatoma cell line). We showed that only L-nucleoside analogs containing 3'-hydroxyl groups are likely to be phosphorylated by NDPKs. The D-dideoxy nucleoside analogs are better substrates for creatine kinase, and L-nucleoside analogs are selectively phosphorylated by 3-phosphoglycerate kinase (PGK) (32).

The primary function of PGK, a 46-kDa glycolytic protein, is the utilization of 1,3-biphosphoglycerate as a phosphate donor to generate one molecule of ATP during glycolysis. The observation that L-nucleoside analog diphosphates are preferentially phosphorylated as compared with the D-nucleoside analog diphosphates, analyzed using recombinant human PGK and its active site mutants, was unexpected. The preference for L-nucleoside analog diphosphates was attributed to the higher $k_{cat}$ values for their phosphorylation to triphosphates, which is...
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A consequence of favorable orientations of the sugar and diphosphate moieties in the active site of the enzyme (33).

Although cell-free models support phosphorylation of L-nucleoside analogs by PGK, the model does not account for the possible regulation of activity or substrate specificity of PGK by other proteins. Moreover, enzymes other than NDPKs, PGK, creatine kinase, or pyruvate kinase may also be capable of phosphorylating L-nucleoside analog diphosphates in the cells. The formation of triphosphate metabolites is critical for the accuracy of further investigations. We carried out this study to assess the role of PGK in the metabolism of L-nucleoside analogs and to thereby analyze the physiological and clinical implications of the involvement of this enzyme in the activity of this class of drugs.

We have used siRNA technology to down-regulate PGK in cell lines relevant to the activity of the nucleoside analogs. The purpose is to evaluate the effect of down-regulation of PGK on the metabolism (specifically phosphorylation of diphosphates to triphosphates) and the subsequent effect on the pharmacological activity. The HelaS3 cell line (human cervical carcinoma) serves as a model for evaluating metabolism and cytotoxic activities of L-OddC and gemcitabine. 2.2.15 cell line (HBV-producing cells derived from HepG2 cells) has been used to evaluate the metabolism and anti-HBV activities of 3TC and L-FMAU.

EXPERIMENTAL PROCEDURES

Chemicals—[3H]3TC, [3H]-FMAU, [3H]-OddC, and [3H]gemcitabine were purchased from Moravek Chemicals (Brea, CA). 2–OH deprotected and annealed siRNA duplexes were synthesized by Dharmacon Research (Lafayette, CO). siRNAs with the following sequences were used: Seq 1, 5′-CCACGAGAUAAGCGGCUTATT, dTdTGACUCUG-UAAUUCGCGAGA-5′; Seq 2, 5′-UGGACCCAAGUCCGUAGUTATT, dTdTTACCUGGCUAGCCACUCAG-5′; Seq-con, 5′-CGAUUGGCUG-AACCGAGGUTATT, dTdTGACUACAGCGUCUCCA-5′.

siRNA Transfection—2 × 10⁴ HelaS3 cells grown in RPMI 1640 supplemented with 10% dialyzed FBS (dFBS), were plated into 6-well plates, 1 day before transfection (day 0). The next day (day 1), cationic lipid complexes were prepared by incubating 200 pmol/liter siRNA with 6 μl of Oligofectamine (Invitrogen) in 200 μl of OPTI-MEM (Invitrogen) for 15 min. The complexes were added to the cells to a final volume of 1 ml. After incubation for 5 h, 0.5 ml of RPMI supplemented with 30% dFBS was added to each well. The transfection was repeated on day 2. On day 3, cells from each well were replated in the absence of siRNA, based on the experimental requirements. (a) Cells were plated into 6-cm dishes for carrying out the metabolism experiments; (b) Cells were replated at 60% confluency into fresh 6-well plates for immunoblotting; (c) 2.5 × 10⁴ cells were plated in 8-chambered collagen coated culture slides (BD Labware) for immunofluorescence; (d) 1 × 10⁵ cells were plated into 6-well plates for clonogenic assays. The respective experiments were carried out 72 h after the first transfection on day 4. 2.2.15 cells grown in MEM-E supplemented with 10% dFBS were transfected similar to HelaS3 cells, on days 1, 3, and 4. Immunoblotting and metabolism of nucleoside analogs was carried out on day 6. Cells were plated and transfected into 2-chambered collagen coated slides at a similar schedule for immunofluorescence experiments.

Anti-PGK Antibody and Immunofluorescence—Antibody to human PGK was generated by subcutaneously injecting 200 μg of recombinant human PGK (produced and characterized in our laboratory previously as described in Ref. 33) mixed with equal volume of Freund’s complete adjuvant in New Zealand White rabbits. Booster shots with 100 μg of PGK and Freund’s incomplete adjuvant were administered twice, at 2-week intervals. Serum was collected 6 weeks after the first injection, and anti-PGK antibody was affinity-purified with N-hydroxysuccinimide-Sepharose beads conjugated to PGK according to the manufacturer’s protocol (Amersham Biosciences). The specificity of the antibody was evaluated by a competition experiment in which the antibody was allowed to bind to the recombinant human PGK prior to its use in immunoblots. This led to a dose-dependent decrease in the detection of PGK. In addition, nonspecific bands were not observed even at a 20-fold higher concentration of antibody in immunoblots of crude cell lysates (data not shown). The monospecific antibody was therefore suitable for detection of PGK in immunoblot and immunofluorescence experiments.

For immunofluorescence, cells were fixed and permeabilized using Fix and Perm kit according to the manufacturer’s protocol (Caltag Laboratories, Burlingame, CA). Cells were blocked with 10% FBS in permeabilization buffer, and all antibody dilutions were also prepared in similar buffers. Cells were incubated with anti-PGK for 1 h followed by anti-rabbit fluorescein isothiocyanate-conjugated antibody for another hour. Cells were washed with phosphate-buffered saline between treatments. Samples were fixed in Slow Fade light anti-fade kit (Molecular Probes, Eugene, OR). Confocal images were acquired with a Zeiss LSM510.

Metabolism of Nucleoside Analogos—The cells were treated with the respective compounds at the indicated concentrations and radiospecificity (24, 25). The cells were harvested in cold phosphate-buffered saline containing 20 μg/ml protease inhibitor (Sigma) and extracted with 15% trichloroacetic acid for 10 min on ice. The supernatant containing the nucleoside and its phosphorylated forms was extracted with a 45/55 ratio of trioctylamine and 1,1,2-trichlorotrifluoroethane (32, 33). The trichloroacetic acid insoluble pellet representing the nucleoside incorporated into the DNA was washed twice and resolubilized in Me2SO prior to evaluation on a Beckman LS5000TD scintillation counter. The nucleoside analog metabolites were analyzed by high pressure liquid chromatography (Shimadzu, Braintree, MA) connected to radiomatic detector (Flow Scintillation Analyzer, 150TR, Packard) using Partisil SAX column (Whatman, Clifton, NJ). All results are means and standard deviations from at least three independent transfections.

Clonogenic Assay—1 × 10⁴ cells were treated with L-OddC and gemcitabine for 24 h. 1 × 10⁸ cells were replated in 6-well plates in fresh medium. The colonies were counted 7 days later by staining the colonies with methylene blue. The results are means and standard deviations from three independent transfections.

Southern Analysis of HBV DNA—The cells were treated with 3TC and L-FMAU for 3 days. DNA was extracted using a DNeasy tissue kit (Qiagen, Valencia, CA) and digested with HindIII. HBV DNA was analyzed by Southern blots by hybridization of the blots with HBV DNA specific probes followed by autoradiography (34, 35). Inhibition of viral DNA in drug-treated cells as compared with untreated cells was assessed by analysis on Personal Densitometer SI (Molecular Dynamics) using the ImageQuant software. The results are means and standard deviations from three independent transfections.

RESULTS

Effect of Down-regulation of PGK on Anticancer Activity of L-Nucleoside Analogs—Two siRNAs, seq 1 and seq 2, have been used to down-regulate PGK in HelaS3 cells. Use of control siRNA, seq2-con, which has the inverse sequence of seq 2, helps to rule out the nonspecific effects of the siRNAs on PGK expression. PGK down-regulation by siRNA treatment was monitored by immunoblots using an anti-PGK antibody (Fig. 1A). Seq 1 and seq 2 decrease PGK expression by 2.5- and 6.6-fold, respectively, whereas seq2-con does not affect PGK content in the cells. To rule out differences due to transfection efficiency, the cells were subjected to immunofluorescence microscopy (Fig. 1B). The transfection efficiency of both seq 1 and seq 2 is around 90%, indicating that the down-regulation of PGK is a property of the siRNA itself. The intensity of fluorescein isothiocyanate-conjugated PGK visually correlates with the protein levels evaluated by immunoblotting.

Seq 2 was selected to study the effects of down-regulation of PGK on the metabolism of L-OddC and gemcitabine. Cells transfected with seq2-con siRNA served as the experimental control. The period of incubation was selected based on the linearity of uptake of the drugs with respect to time. The siRNA-treated cells were exposed to 1 μM (2.2 Ci/mmol) [3H]-L-OddC for 24 h. The formation of L-OddCTP is lower in seq 2-treated cells, leading to a specific 2.5-fold increase in L-OddCDP to L-OddCTP ratio (Fig. 2A and inset). The total amount of L-OddC metabolites in seq 2-transfected cells is lower than in the control cells. The amount of L-OddCTP incorporated into the DNA of cells treated with seq 2 is also significantly lower than in the control cells (Fig. 2B).

Cells were similarly transfected and exposed to 0.25 μM (0.9 Ci/mmol) [3H]gemcitabine for 30 min. The gemcitabine metab-
transfected cells (Fig. 2). This increase in LC50 is also compared with seq2-con transfected cells is 2-fold higher as compared with seq2-con transfected cells. After treatment with L-OddC, the colony-forming ability of seq 2 and seq2-con transfected cells were treated with 1 μM (2.2 Ci/μmol) [3H]3TC for 8 h. The amount of 3TCTP remains constant; however, the levels of 3TCTP in seq 2-treated cells are lower (Fig. 4A). This leads to a specific 4-fold increase in the 3TCDP to 3TCTP ratio in seq 2-transfected cells as compared with the control cells (Fig. 4A, inset). Due to differences in the formation of triphosphates, the cells transfected with seq 2 show a net decrease in the accumulation 3TC metabolites.

The metabolism of 1 μM (2.2 Ci/μmol) [3H]L-FMAU was evaluated by treating the siRNA-transfected cells for 2 h (Fig. 4B). There is an approximate 2-fold decrease in the formation of L-FMAUTP in seq 2-transfected cells, whereas the levels of other phosphorylated metabolites remain unaffected as compared with the control. As a consequence, there is a specific 2-fold increase in the diphosphate to triphosphate ratio, whereas the diphosphate to monophosphate ratio remains unchanged (Fig. 4B, inset). These data indicate that down-regulation of PGK correlates well with disturbances in the metabolism of 3TCDP and L-FMAUDP to the respective triphosphates.

To correlate the efficiency of generation of triphosphates with the pharmacological activities of 3TC and L-FMAU, their anti-HBV activities were evaluated in seq 2- and seq2-con-transfected cells. The cells were treated with drugs for 3 days, and HBV DNA content was then measured as an indication of anti-HBV activity (Fig. 4C). L-FMAU and 3TC were used at a concentration of 1 μM, similar to that used in the metabolism studies. In seq 2-transfected cells, inhibition of HBV DNA production by both analogs is 2-fold lower than in the control cells.

Role of PGK in Maintenance of Triphosphate Metabolite Levels—To evaluate the role of PGK in maintaining the levels of triphosphate metabolites, a model was designed to simulate phosphorylation and dephosphorylation of 1 μM (2.2 Ci/μmol) of [3H]3TC and [3H]L-FMAU. siRNA- and mock-transfected 2.2.15 cells were exposed to 3TC for 8 h followed by removal of the drug and replacement with fresh medium for different intervals of time (Fig. 5A). The content of the phosphorylated metabolites is an indication of the catabolism of nucleotides as a function of time. In seq 2-transfected cells, after 8 h (time 0) of incubation, there is an expected 4-fold increase in the 3TCDP to 3TCTP ratio as compared with the control cells. On the removal of 3TC, the ratios remain constant for around 8 h, but 3TCMP levels decrease gradually, and by 16 h, seq 2-treated cells only contain 3TCDP. In seq2-con-transfected cells, 3TCDP reduces gradually such that by 16 h, only 3TCDP and 3TCTP remain, but their levels and ratios remain constant at least up to 24 h after drug treatment. The dephosphorylation process of 3TCDP is slow, leading to its stability in the cells.

siRNA-transfected cells were treated with L-FMAU for 2 h (time 0) followed by replacement with fresh medium for the indicated periods of time (Fig. 5B). The triphosphate, diphosphate, and monophosphate metabolites of L-FMAU decrease...
progressively up to 8 h such that the diphosphate to triphosphate metabolite ratio remains constant. In seq 2-transfected cells, the amount of triphosphate metabolite diminishes rapidly after 16 h, leaving only the monophosphate metabolite at 24 h after drug treatment. In the control cells, the triphosphate is the major metabolite at 16 h, and the equilibrium between diphosphate and triphosphate metabolite is maintained even at 24 h after drug treatment.

These data indicate that the metabolites are catabolized stepwise to the respective nucleosides, which can freely diffuse out of the cells. Although the triphosphate metabolites are catabolized rapidly, they are rephosphorylated by PGK, indi-
cating the importance of the enzyme in the maintenance of steady state levels of triphosphate metabolite.

DISCUSSION

siRNA technology is useful for evaluating intracellular drug specificity and mechanism of action (36, 37). An added advantage to this technology is that the transient and specific targeting of the desired protein decreases the probability of selecting cells that can develop compensatory mechanisms for the protein function. Our strategy here was to down-regulate the expression of PGK to inhibit the phosphorylation of L-nucleoside analog diphosphate to triphosphate. Based on our previous results that showed preference of the enzyme for L- over D-nucleoside analogs, we also predicted that the metabolism of D-nucleoside analogs would be unaffected by changes in PGK content. The importance of PGK in L-nucleoside metabolism was tested in two representative cell lines, selected on the basis of their pharmacological activities: anticancer activity in HelaS3 and anti-HBV activity in 2.2.15. cells.FITC, fluorescein isothiocyanate.

Fig. 3. siRNA-mediated down-regulation of PGK in 2.2.15. cells. In A, the immunoblot shows the PGK (45 kDa) content in the cells on the 6th day, and actinin (100 kDa) has been used as an internal control. B, immunoblot indicating PGK content 3 days later (9th day). C, immunofluorescence of siRNA-mediated down-regulation of PGK in 2.2.15. cells. FITC, fluorescein isothiocyanate.

Fig. 4. Effect of PGK down-regulation on metabolism and activity L-nucleoside analogs in 2.2.15. cells. The graphs indicate the amounts of phosphorylated metabolites, and the inset shows the ratio of the indicated metabolites of 3TC (A) and l-FMAU (B). C, anti-HBV activity of 3TC and l-FMAU evaluated by quantification of partially double-stranded and double-stranded DNA normalized with respect to the integrated form of HBV DNA.
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The role of PGK in maintaining the levels of triphosphate metabolites was also examined. The dephosphorylation of nucleotides catalyzed by undefined enzymes and 5' nucleotidases results in the catabolism of nucleoside analog triphosphates to the respective nucleosides, which can diffuse out of the cells. It was therefore conceivable that PGK may affect the stability of triphosphate metabolites in the cells. Results indicated that in the cells with a decreased amount of PGK, removal of drug eventually leads to a rapid reduction in the amount of triphosphate metabolites, whereas in the control cells, the diphosphate to triphosphate ratio is retained, indicating constant regeneration of triphosphates by PGK. Since in cells lacking PGK, L-nucleoside triphosphates are catabolized but not regenerated, enzymes other than PGK may be involved in the reverse reaction. PGK is not only important for initial phosphorylation but also for the maintenance of steady state levels of L-nucleotides in the cells.

This report demonstrates that PGK phosphorylates L- but not D-nucleoside analog diphosphates in the cells. The siRNAs used in the study, however, did not knock out the expression of PGK, and generation of triphosphate metabolite was not completely inhibited. We can therefore conclude that although PGK plays a predominant role in the phosphorylation of anticancer and antiviral L-nucleoside analogs, we cannot rule out the possible role of other unknown enzymes in their metabolism to triphosphates. This study also emphasizes that contrary to the assumptions that NDPKs phosphorylate all nucleoside analog diphosphates, the structure of the analog is just another variable in determining the substrate specificity of the enzyme. Our earlier studies had in fact suggested that pyrimidine D-deoxynucleoside analog diphosphates are phosphorylated by creatine kinase (32). Given the diversity of nucleoside analogs currently used or under clinical trials for the treatment of cancer and viral infections, it is important to understand the structure-based distinction in the metabolism of these analogs. Clinically, PGK levels vary by tissue and possibly between patients. Since phosphorylation by PGK is a critical step in L-nucleoside uptake and metabolism, monitoring the PGK levels in the patients undergoing L-nucleoside therapy may prove to be important for predicting compliance to therapy. The clinical manifestations of involvement of PGK in the activation of anticancer and antiviral nucleoside analogs remain to be seen.

Fig. 5. Catabolism of L-nucleotides in 2.2.15. cells after removal of the drugs from the medium. The graph indicates the amounts of phosphorylated metabolites of 3TC (A) and L-FMAU (B) in the cells, as a function of time.

Experiments also included mock-transfected cells, and as expected, the metabolism and activity of nucleosides analogs in these cells were similar to those in seq2-con-transfected cells (data not shown). However, comparison of results obtained from seq 2- and seq2-con-transfected cells is more appropriate since the effects of siRNA duplexes on the activity of nucleosides analogs cannot be predicted.

Down-regulation of PGK in HelaS3 cells specifically affected formation of triphosphate metabolites, leading to increases in the diphosphate to triphosphate ratio of L-nucleoside analogs. In the case of L-OddC, the 2.5-fold decrease in the formation of L-OddCTP led to a decrease in the amount incorporated into the DNA. The lower expression of PGK correlated well with the subsequent decrease in the activity of L-OddC against the clonogenic activity of cells. The metabolism of gemcitabine, a d-deoxy nucleoside analog that is phosphorylated by NDPKs in cell-free models, was not affected by down-regulation of PGK; therefore, inhibition of the colony-forming ability of the cells remained unaffected.

In 2.2.15 cells, siRNA-induced decrease in PGK led to a specific increase in 3TC and in the L-FMAU diphosphate to triphosphate metabolites ratio. The change was due to a decrease in the formation of triphosphate metabolite; this was, however, not accompanied by a corresponding accumulation of diphosphate metabolites. One possible explanation is that the concentration of phosphorylated metabolites is in equilibrium, so the diphosphates are catabolized to the nucleosides, facilitating their diffusion out of the cells, thus preventing the accumulation of the diphosphate metabolites (38, 39). Therefore, when PGK was down-regulated, the total amount of 3TC/L-FMAU and its phosphorylated metabolites in the cells also decreased, indicating that the phosphorylation of diphosphate metabolites is the bottleneck for the total uptake of L-nucleoside analogs in the cells. These changes in metabolism consequently led to a decreased inhibition of HBV DNA production by 3TC and L-FMAU as well as L-Fd4C (data not shown). Metabolism of 3TC was also studied in the HelaS3 cell line transfected with siRNAs (data not shown). The amount of 3TC/CDP formation decreased 4- and 2-fold with seq 2 and seq 1, respectively, consistent with down-regulation of PGK in the HelaS3 cell line. This supports the important role of PGK in the phosphorylation of L- but not D-nucleoside analog diphosphates in cells.

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