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Metabolic Pathways for the Activation of the Antiviral Agent 2′,3′-Dideoxyguanosine in Human Lymphoid Cells

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SUMMARY

2′,3′-Dideoxyguanosine (ddGuo) is a selective inhibitor of the replication of human immunodeficiency virus *in vitro* and the most active antihepadnavirus nucleoside analog known *in vitro* and *in vivo*, in a Peking duck model. However, the exact route by which this and related guanosine analogs are anabolized to their putative active metabolites in target cells is controversial. The anabolic pathway for the activation of ddGuo was investigated with the use of mutant human lymphoid CCRF-CEM and WI-L2 cell lines deficient in known nucleoside kinases. Uptake of ddGuo by human lymphoid cells and subsequent conversion to mono-, di-, and triphosphorylated metabolites is dose dependent and occurs proportionately to the exogenous concentration of drug. Studies with kinase-deficient CCRF-CEM and WI-L2 mutants revealed that at least two different routes of metabolism are operating in these cells to initiate the phosphorylation of ddGuo to its active dideoxynucleotides, one being deoxycytidine (dCyd) kinase and the other a cytosolic-5′-nucleotidase acting in the anabolic direction as a phosphotransferase. The evidence for this included 1) a lower but significant accumulation of drug anabolites in dCyd kinase-deficient mutants, 2) a lack of cross-resistance of the kinase-deficient mutants to growth inhibition by ddGuo, compared with that by the related analogs dideoxycytidine and arabinosylcytosine, known substrates for dCyd kinase, and 3) identification of different phosphorylation activities for ddGuo in extracts of wild-type cells and kinase-deficient mutants. Knowledge of the enzyme systems involved in anabolism of ddGuo analogs should be important for both new drug design and optimal therapeutic application.

A number of 2′,3′-dideoxynucleosides have been shown to have anti-HIV activity *in vitro*, and 3′-azido-3′-thymidine (zidovudine) and ddIno (didanosine) are currently being used in the treatment of patients with the acquired immunodeficiency syndrome (1–3).

ddGuo is a purine dideoxynucleoside analog that exhibits selectivity similar to that of ddIno against HIV infectivity *in vitro* (4). ddGuo is of particular interest, however, because among the nucleosides it is the most active antihepdnavirus agent known both *in vitro* and *in vivo*, in a Peking duck model (5, 6). Moreover, studies (7–10) have demonstrated that the anti-HIV activity of ddGuo and the related purine analogs 2,6-diaminopurine riboside and ddIno is potentiated by combination with inhibitors of IMP dehydrogenase, such as ribavirin and tiazofurin. This potentiation by IMP dehydrogenase inhibitors also results in increased intracellular accumulation of the active 5′-triphosphate metabolites of these 2′,3′-dideoxynucleosides (8). Results such as these suggest the potential therapeutic use of ddGuo and related analogs as antiviral agents, either as single agents or in combinations with agents, such as ribavirin, that can enhance their antiviral activity.

In order to develop rational strategies for the therapeutic application of dideoxynucleosides, either alone or in combination, in the treatment of viral infections, it is important to have an understanding of the metabolic fates of these compounds. Previous reports have indicated that ddGuo is taken up and converted intracellularly to its mono-, di-, and triphosphorylated forms; however, the anabolic pathway involved in this activation remained unclear. In part, this is because human cells contain multiple enzyme systems that can promote purine dideoxynucleoside phosphorylation *in vitro*, including at least two nucleoside kinases, dCK and AK, and a cytosolic-5′-nucleotidase acting in the anabolic direction as a phosphotransferase (11–13). However, the role that these enzyme systems play in the phosphorylation of ddGuo and related guanine analogs in intact cells and *in vivo* has not been directly established.

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ABBREVIATIONS: HIV, human immunodeficiency virus; dCK, deoxycytidine kinase; AK, adenosine kinase; ddGuo, 2′,3′-dideoxyguanosine; ddIno, 2′,3′-dideoxyinosine; ddCyd, 2′,3′-dideoxycytidine; ara-C, arabinosylcytosine; ddGTP, dideoxyguanosine-5′-triphosphate; ddGDP, dideoxyguanosine-5′-diphosphate; ddGMP, dideoxyguanosine-5′-monophosphate; PNP, purine nucleoside phosphorylase; HPLC, high pressure liquid chromatography; dCyd, deoxycytidine; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HBV, hepatitis B virus; dGuo, deoxyguanosine; dAdo, deoxyadenosine.

The present study was undertaken to elucidate more definitively the anabolic pathways for the conversion of ddGuo to the nucleotides and to identify conditions that might promote the accumulation of these nucleotides in human lymphoid cells.

Materials and Methods

Chemicals. [^{3}H]ddGuo (44 Ci/mmol), labeled predominantly in the 2′- and 3′-positions of the dideoxyribose moiety, was obtained from Moravek Biochemicals (Brea, CA). The percentage of label in the 2′- and 3′-positions of the dideoxyribose moiety averaged 96–98%, with the remaining tritium being associated with the 8-position. The label was also analyzed by reverse phase HPLC and contained <1% crude labeled guanosine. Nucleosides and nucleotide analogs ddGuo, ddGTP, and ddGDP were purchased from Pharmacia (Piscataway, NJ) or obtained from the Pharmaceutical Resources Branch, National Cancer Institute, National Institutes of Health. All other nucleosides/nucleotides used were of the highest purity available and were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. CCRF-CEM, WI-L2, and MOLT-4 cells were grown as previously described (8, 14). The kinase-deficient mutants of the CEM and WI-L2 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated newborn calf serum (Sigma) and 2 mM L-glutamine, as previously described (14). Cells were verified to be in logarithmic growth at the time of use.

Growth inhibition determinations. Drug sensitivity studies were conducted in 25-cm^2 tissue culture flasks (Costar). Small volumes (10–200 μl) of inhibitory agent were pipetted into each flask, after which 5 ml of cells (1–2 × 10^5 cells/ml) in complete medium were added. After 48 hr, the number of untreated cells typically increased about 2–3-fold. The initial densities were subtracted from the final densities, and the number of cells in the flasks containing the nucleoside analogs was calculated as a percentage of the number of untreated cells in control flasks. The IC_{50} was determined from semilogarithmic graphs of the percentage of cell growth versus drug concentration.

Metabolism studies. Exponentially growing MOLT-4, CEM, and WI-L2 cells and the kinase-deficient mutants were incubated at 1 × 10^6 cells/ml with 10 μM radiolabeled ddGuo (5 μCi/ml). An incubation time of 6 hr was used for most of the studies to be described. At the end of the incubations, cells were centrifuged at 1500 rpm for 5 min, washed with 2 ml of ice-cold phosphate-buffered saline, and extracted with 250 μl of 70% cold methanol, 25 mM Tris·HCl, pH 7 (−20°), for about 20–30 min. The extracts was centrifuged at 12,000 × *g* for 30 sec, and the supernatant fraction was stored at −20° until analyzed by HPLC. Separation of drug metabolites by HPLC was carried out using a Partisil 10-SAX column (13). One-min fractions were collected, and radioactivity was determined. The intracellular concentrations of physiological purine nucleotides and drug metabolites were determined after separation on HPLC columns that had been prestandardized with known amounts of the nucleotide standards.

Enzyme assays. The assays for the phosphorylation of dCyd, ddGuo, and other nucleosides were carried out by using methodology similar to that previously described (15). For kinase activity measurements, dialyzed extracts (400–600 μg of protein) were incubated with 80 μM [^{3}H]dCyd (0.5 μCi) or 1 mM [^{3}H]ddGuo (0.5–1 μCi), in 10 mM $MgCl_2$, 10 mM ATP, 3 mM dithiothreitol, 15 mM pyruvate phospho(enol), 100 mM Tris·HCl (pH 7.4), with 8 units of pyruvate kinase. In the assay containing IMP as phosphate donor, the incubations were carried out with the same Tris·HCl buffer, 50 mM $MgCl_2$, 500 mM KCl, 5 mM IMP, and the appropriate nucleoside acceptor. The reactions (50 μl) were initiated by the addition of cell extract and were incubated for 30–60 min at 37°. The enzyme reactions were terminated by addition of 20 μl of the mixture to 80 μl of ice-cold water. A portion (50 μl) of this mixture was applied to a DE-81 disk (Whatman), which was then washed with water and dried. The filters were extracted with 1 ml of 0.1 N HCl, 0.2 M KCl, for 15 min, the eluate was collected in a scintillation vial, and radioactivity was determined by scintillation counting. Protein was quantitated in cell extracts by the procedure of Bradford (16), with bovine serum albumin as the standard.

Results

Accumulation of [^{3}H]ddGuo nucleotides in cells as a function of ddGuo concentrations in the medium. The metabolism of ddGuo in human lymphoid cells was examined over a range of concentrations. A representative HPLC analysis of extracts of wild-type human lymphoid WI-L2 cells incubated for 6 hr with 10 μM [2′,3′-^{3}H]ddGuo is shown in Fig. 1A. Radioactive peaks detected correspond to ddGDP, ddGTP, and the physiological purine ribonucleotides GTP, GDP, and GMP. This radioactivity in guanine nucleotides, we have previously shown (8), results from the cleavage of ddGuo by PNP and reutilization of the tritium-labeled guanine (present in this preparation at about 3–4%) via the HGPRT pathway. ddGMP coeluted in close proximity to the radioactive peak identified as GMP, and it was not possible to quantitate this metabolite under these conditions. In the presence of hypoxanthine in the medium at 10 times the concentration of ddGuo, there was a drastic reduction in the incorporation of radioactivity from ddGuo into the guanine nucleotides (Fig. 1B), presumably because of competition with the cleavage product guanine for the HGPRT pathway. Hypoxanthine, however, did not decrease the conversion of ddGuo to its nucleotides and, under these conditions, it was possible to demonstrate positively the presence of ddGMP (Fig. 1B). In addition, experiments using mutant CEM cells deficient in the activity for HGPRT and lacking the ability to incorporate label from ddGuo into the guanine nucleotides also confirmed the presence of ddGMP formation (data not shown). On the basis of these experiments, it was possible to determine that approximately 15–20% of the total labeled intracellular ddGuo nucleotides formed represented ddGMP. It is also noteworthy that, in the presence of hypoxanthine, there was a substantial radioactive peak (metabolite X) ahead of ddGMP, with a retention time of 10 min. The radioactivity incorporated into this peak represented well over 90% of the total radioactivity incorporated from ddGuo into the nucleotide fractions. Incubation of this radioactive peak with alkaline phosphatase did not result in the liberation of any nucleoside derivative, after subsequent analysis of the reaction products by reverse phase HPLC. However, treatment of [^{3}H]ddGuo-labeled cell extracts with alkaline phosphatase resulted in the disappearance of metabolite X when the extract was subjected to reverse phase HPLC. A majority of the radioactivity was not found to be associated with nucleosides. Although this needs further confirmation, it seems likely that metabolite X represents the dideoxyribose product formed during catabolism of ddGuo by PNP.

The effect of increasing ddGuo concentration on the accumulation of ddGDP and ddGTP is shown in Fig. 2. Both metabolites increased in proportion to the ddGuo concentration added, and no saturation of phosphorylation was evident up to 1 mM. Preincubation with or simultaneous addition of *p*-nitrobenzylmercaptopurine riboside, a potent inhibitor of nucleoside transport, had no detectable effect on the rate of phosphorylation of ddGuo in the cells (data not shown), indicating that the entry of this nucleoside analog is predominantly by a mechanism independent of the transport carrier utilized by physiological purine deoxynucleosides.

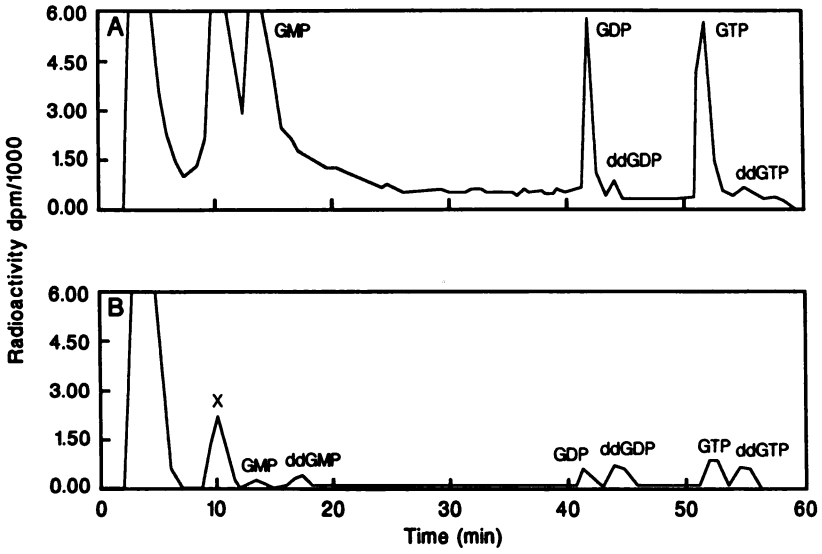

Fig. 1. Ion exchange (Partisil 10-SAX) HPLC elution profile of extracts from WI-L2 cells. Exponentially growing cells (approximately 10^7) were incubated for 6 hr with [2′,3′-^{3}H]ddGuo (10 μM), and the methanol-extracted cells were analyzed for nucleotide metabolites. Buffer A was 9 parts 0.05 mM ammonium phosphate buffer, pH 4.0, and 1 part 100% methanol; buffer B was 9 parts 0.7 mM ammonium phosphate, pH 4.6, and 1 part 100% ethanol. The elution program that was used was 20 min of buffer A, followed by a linear gradient to 100% of 700 mM ammonium phosphate with buffer B. A, Control; B, 100 μM hypoxanthine.

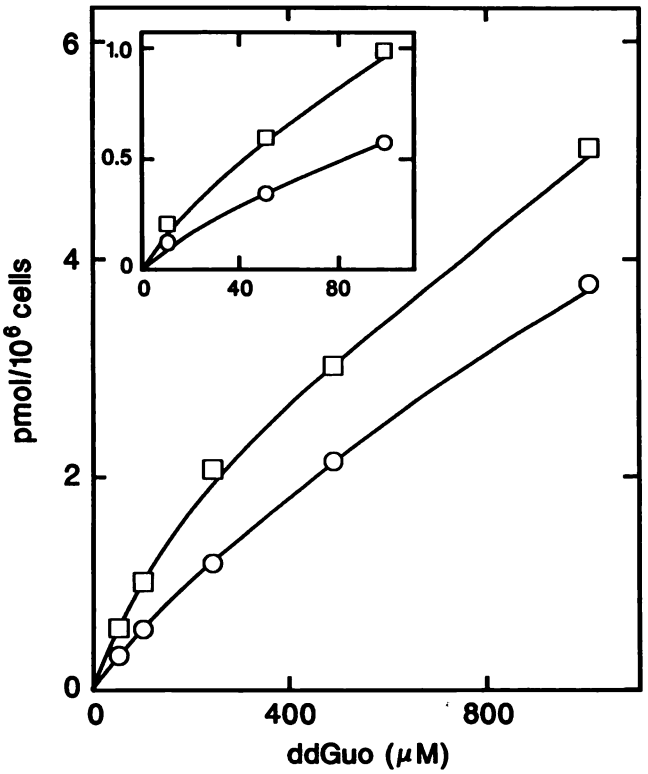

Fig. 2. Accumulation of ddGuo metabolites after incubation of MOLT-4 cells for 6 hr with different concentrations of ddGuo. □, ddGDP; ○, ddGTP. *Inset:* ddGuo, μM

Table 1 presents a summary of the level of ddGuo nucleotides achieved in three human lymphoid cell lines, i.e., the T cell lines MOLT-4 and CEM and the B cell line WI-L2, after incubation of the cells with a concentration of ddGuo sufficient to inhibit HIV replication (10 μM). Under these conditions (6-hr incubation), the cells accumulated ddGDP and ddGTP from ddGuo to levels of 200–500 pmol/10^9 cells. In all three cell lines, the analog diphosphate accumulated to a greater extent than the triphosphate (about 2–3-fold), suggesting that the phosphorylation of ddGDP to ddGTP is a rate-limiting step in the anabolism of this compound.

TABLE 1

Accumulation of ddGuo metabolites in human lymphoid cells

Exponentially growing cells (approximately 10^7 cells) were incubated with [2′,3′-^{3}H]ddGuo (10 μM 5 μCi/ml) at 37° for 6 hr. At the end of the incubation, methanolic extracts of the cells were prepared and intracellular metabolites were determined as described in Materials and Methods. The results are the mean of two separate experiments, which have been repeated.

Cell line	Nucleotide formed		Ratio, ddGDP/ddGTP
	ddGDP	ddGTP	
	pmol/10^9 cells		
MOLT-4	265	90	2.9
CEM	150	72	2.1
WI-L2	310	174	1.8

TABLE 2

Effect of 2′-deoxyribonucleosides on the cellular accumulation of ddGuo nucleotides in CEM and MOLT-4 cells

Exponentially growing cells (10^7) were incubated with [^{3}H]ddGuo (10 μM, 5 μCi/ml) at 37° for 5 hr, in the presence or absence of deoxynucleoside as indicated. After incubation, the dideoxynucleotides were quantitated as described in Materials and Methods. The results represent the mean of two separate experiments that have been repeated. In these experiments, the intracellular ddGDP and ddGTP concentrations after 6 hr were 361 and 280 pmol/10^9 in MOLT-4 and CEM cells, respectively.

Addition (100 μM)	ddGDP + ddGTP	
	CEM	MOLT-4
	% of control	
None	100	100
dCyd	55	69
dGuo	96	92
dAdo	188	203
dIno[a]	100	93

[a] dIno, 2′-deoxyinosine.

Effect of 2′-deoxynucleosides and the mutational loss of dCK and AK on phosphorylation of ddGuo. We reported previously (17) that dCK purified from human lymphoblastoid cells phosphorylates ddGuo, albeit at a rate only about 1% that of the physiological purine nucleosides dGuo and dAdo. In order to test whether ddGuo was phosphorylated by this enzyme in intact cells, we determined the ability of various 2′-deoxynucleosides, including dCyd, the preferred substrate for dCK, to compete with ddGuo for phosphorylation in CEM and MOLT-4 cells. Shown in Table 2 is the finding that the presence of an

excess of dCyd (100 μM) in the incubation medium decreased the formation of ddGuo nucleotides (ddGDP and ddGTP) in MOLT-4 and CEM cells by about 31 and 45%, respectively. In contrast, no other deoxynucleosides inhibited the anabolism of ddGuo.

To assist in a more definitive elucidation of the anabolic pathways followed by ddGuo, we determined whether mutants of CEM and WI-L2 that are severely deficient in dCK, AK, or both enzyme activities would exhibit altered drug activation. The results depicted in Table 3 show that the dCK-deficient mutants CEM/dCK$^-$ and WI-L2/dCK$^-$, when incubated with 10 μM ddGuo for 6 hr, were 20% and 40% less efficient in the synthesis of ddGDP and ddGTP than were the corresponding wild-type cells. AK$^-$ mutants of either CEM or WI-L2 cells showed no change in the profile of nucleotide analog accumulation, and the double-mutants (AK$^-$, dCK$^-$) of CEM and WI-L2 showed essentially the same profile of nucleotide analog formation as that seen with dCK$^-$ mutants. In marked contrast, and in agreement with others (18), the dCK-deficient mutants were virtually incapable of phosphorylating either ddCyd or its aranucleoside analog ara-C, both known substrates of dCK. Moreover, the ability of the dCK-deficient mutants to phosphorylate [^{3}H]ddGuo to the nucleotide could not be inhibited further by either dCyd or dGuo, each present at 100 μM. Surprisingly, addition of dAdo to the incubation stimulated (about 2-fold), rather than inhibited, the accumulation of ddGuo nucleotides from [^{3}H]ddGuo. We have no explanation for this effect, except to note that a similar result was obtained previously in studies we performed with ddIno and dideoxyadenosine in the CEM and MOLT-4 cells (11).

The lower levels of ddGuo anabolites in the kinase-deficient mutants could result from decreased activation or decreased stability of the phosphorylated analog. The extent of metabolic dephosphorylation of ddGuo metabolite was also examined in both cell types. As shown in Table 4, no significant differences in the stability of ddGuo nucleotides formed in the kinase-deficient WI-L2 cells and in wild-type cells were detected, indicating that decreased synthesis, rather than increased catabolism, was responsible for the lower levels of ddGuo anabolism observed.

Effects of ddGuo on growth of wild-type and kinase-deficient cells. We next compared the growth-inhibitory effects of ddGuo on wild-type cells and their kinase-deficient sublines. Table 5 shows a comparison of the effect of ddGuo with those of ddCyd and ara-C on the growth of the various human lymphoid cells. As can be seen, wild-type WI-L2 cells were about 3-fold more sensitive to ddGuo than were wild-type CEM cells (EC_{50} values of 150 and 560 μM, respectively). In contrast, both ddCyd and ara-C were more cytotoxic to CEM than to WI-L2 cells and inhibited the growth of these cells at lower concentration than did ddGuo (EC_{50} value of 2 and 3.6 μM, respectively). The growth-inhibitory effects of ddCyd and ara-C were decreased greatly in the dCK-deficient mutants of both CEM and WI-L2 cells; however, both kinase-deficient mutants showed a sensitivity to ddGuo similar to that of the wild-type counterpart. These data thus emphasize the marked differences that exist in the metabolism of ddGuo and ddCyd and indicate that dCK is not the only enzyme involved in the phosphorylation of ddGuo to the active intracellular anabolites in these human lymphoid cells.

TABLE 3

Accumulation of ddGuo nucleotides in wild-type and kinase-deficient CEM and WI-L2 cells

Exponentially growing cells (1 × 10^7) were incubated with [^{3}H]ddGuo (10 μM, 5 μCi/ml) for 6 hr at 37°. At the end of the incubation, extracts of the cells were prepared and analyzed by HPLC, as described. The results are the mean of two separate experiments.

Cell line	ddGDP	ddGTP	ddGDP	ddGTP
	pmol/10^9 cells	pmol/10^9 cells	% of control	% of control
CEM	124	101	100	100
CEM/dCK$^-$	107	83	86	82
CEM/AK$^-$	133	98	107	97
CEM/dCK$^-$,AK$^-$	96	76	77	75
WI-L2	290	163	100	100
WI-L2/dCK$^-$	189	98	65	60
WI-L2/AK$^-$	245	180	84	110
WI-L2/dCK$^-$,AK$^-$	194	114	66	69

TABLE 4

Stability of ddGuo nucleotides in wild-type and dCK-deficient human lymphoid cells

Half-lives were calculated from the intracellular ddGuo nucleotide concentrations after 4 and 8 hr of incubation of [^{3}H]ddGuo-prelabeled cells in drug-free medium.

Cell line	$t_{½}$	
	ddGDP	ddGTP
	hr	
CEM	4.3	5.2
CEM/dCK$^-$	3.8	5.9
WI-L2	6.6	7.3
WI-L2/dCK$^-$	7.8	5.6

TABLE 5

Comparative effects of nucleoside analogs on the growth of wild-type and kinase-deficient lymphoid cells

Values are the means from a single experiment performed in duplicate, which was repeated with similar results.

Cell type	EC_{50}*		
	ddGuo	ddCyd	Ara-C
	μM		
CEM			
Wild-type	560	2	0.016
dCK$^-$	720	380	12.5
WI-L2			
Wild-type	150	3.5	0.033
dCK$^-$	155	264	43

* Concentration of nucleoside analogs producing a 50% decrease in growth.

Phosphorylation of ddGuo in cell extracts. In light of the results presented thus far, we next examined the phosphorylating activity for ddGuo and dCyd in extracts of wild-type cells and kinase-deficient mutants. As expected, dCK-deficient CEM cells exhibited a very low capacity to phosphorylate dCyd and ddGuo when ATP was used as the phosphate donor (Table 6). Previous studies (13, 15) have shown that phosphorylation of various purine nucleosides can be catalyzed via a soluble 5′-nucleotidase. As shown in the results in Table 6, in the presence of IMP instead of ATP as a phosphate donor, ddGuo but not dCyd was phosphorylated to virtually the same extent in the kinase-deficient cell extracts and in the wild-type cell extracts. In order to determine how this reaction might function in the intact cells, the phosphorylation kinetics were estimated by using approximately physiological intracellular conditions. As shown in the results when ATP (3 mM) was added together with IMP, phosphorylation of ddGuo was stimulated about 2-fold. Addition of P_i (1 mM), a known inhibitor of 5′-nucleotidase, to the reaction containing IMP and ATP decreased ddGuo

TABLE 6

Nucleoside analog-phosphorylating activities in extracts of wild-type and dCK-deficient CEM cells

Extracts of wild-type and dCK$^-$ CEM cells were assayed for nucleoside kinase and phosphotransferase activity, using ATP and IMP as the phosphate donors, respectively, as described in Materials and Methods. The values are from one experiment that was repeated at least once with similar results. The enzyme assays were determined with 80 μM dCyd and 1 mM ddGuo. ATP and P_i were at 3 mM and 1 mM, respectively.

Substrate	Phosphorylating activity					
	Wild-type			dCK$^-$		
	ATP	IMP	IMP + ATP	ATP	IMP	IMP + ATP
	pmol/min/mg of protein					
dCyd	8.2	<0.01	6.7	0.12	<0.01	0.1
ddGuo	1.9	10.7	18.2	<0.01	11.8	20.4
ddGuo + P_i	1.5	3.3	13.6	<0.01	2.7	14.2

phosphorylation of ddGuo by about 40%, compared with that with ATP alone, and by about 70% when ATP was omitted. Overall, these data allow the conclusion that ddGuo may be activated to the monophosphate via two distinct metabolic pathways in human lymphoid cells.

Discussion

Although the therapeutic selectivity of ddGuo against the cytopathic effect of HIV *in vitro* is equivalent to that of ddIno, a compound currently in phase II/III trials, ddGuo has received relatively little attention as a potential anti-HIV drug. However, a feature that distinguishes ddGuo and related analogs, such as 2,6-diaminopurine riboside, is that it is the most potent of the dideoxynucleosides against hepadnavirus *in vitro* and also in suppressing *in vivo* replication of duck HBV in the Peking duck model (5, 6). Although the mechanism for this difference is unknown, these findings have raised the possibility that dideoxypurines of the guanosine family might, indeed, have some clinical utility as antiviral agents. Moreover, recent studies (7–10) have shown that the antiviral potency and anabolism of ddGuo and its 2,6-diamino derivative may be further enhanced by combination with inhibitors, such as ribavirin, of the enzyme IMP dehydrogenase of purine nucleotide synthesis.

The antiviral activity of ddGuo is thought to result from its conversion to the triphosphate analog ddGTP and subsequent inhibition of viral replication by the triphosphate analog (5). ddGTP is a potent inhibitor of reverse transcriptase, and its activity against HBV replication is likely due to its effect on the reverse transcription step known to occur in the HBV replication cycle (20). Previous studies (21) have shown that the anabolic phosphorylation of dideoxynucleosides is a major determinant of their antiviral effectiveness. The metabolic fate of ddGuo and related guanosine analogs has not been definitely elucidated. Previous studies by Busso *et al.* (22), using base-labeled [^{3}H]ddGuo, failed to detect any ddGuo metabolite formation from ddGuo in H-9 cells. However, the use of sugar-labeled ddGuo provides much greater sensitivity for detecting cellular ddGuo nucleotide formation. Our studies have demonstrated the cellular uptake and phosphorylation of ddGuo, in T lymphoid cells, to the mono-, di-, and triphosphate and have shown that the degree of analog nucleotide accumulation is directly related to the concentration of ddGuo to which the cells are exposed, up to at least 1 mM. The amount of accumulated ddGTP in human lymphoid CEM, MOLT-4, and WI-L2 cells incubated for 6 hr with a virally effective concentration

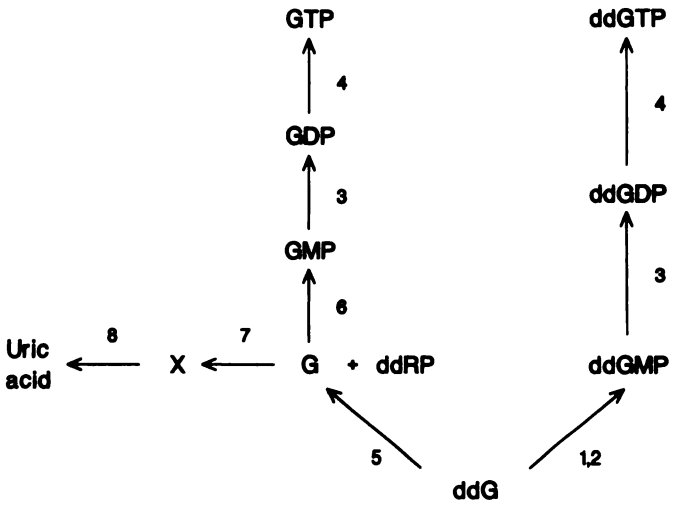

Fig. 3. Outline of metabolic pathways for ddGuo. *1*, dCK; *2*, phosphotransferase (5′-nucleotidase); *3*, nucleoside monophosphate kinase; *4*, nucleotide diphosphate kinase; *5*, PNP; *6*, HGPRT; *7*, guanine deaminase; *8*, xanthine oxidase; ddRP, dideoxyribose-1-phosphate.

of 10 μM ddGuo was approximately 90, 72, and 174 pmol/10^9 cells, respectively. Overall, this level of phosphorylated product formation was about 10-fold lower than that for ddIno or dideoxyadenosine in the same cell lines (11, 12). It is of interest that, in all three cell lines, the level of ddGDP accumulated to concentrations approximately 2–3-fold greater than that of ddGTP. This has recently been noted with another antiviral ddGuo analog, 3′-azido-2′,3′-dideoxyguanosine, but is not observed with either ddIno or the physiological guanosine nucleotides, which accumulate mainly as the triphosphates. These results indicate that the various anabolic steps that convert the ddGuo analogs to the triphosphate level do so in a fairly inefficient manner.

The specific enzyme involved in the phosphorylation of ddGuo in human lymphoid cells has not been determined previously. It has been demonstrated quite convincingly that dCK is primarily responsible for the initial step of phosphorylation of dGuo, dAdo, and a number of pharmacologically active analogs, in CEM and WI-L2 cells (14, 23). On the basis of the metabolic studies described here, utilizing kinase-deficient mutants of CEM and WI-L2 cells, it was possible to conclude that at least two different pathways for the anabolism of ddGuo to its corresponding metabolites are operative in these human lymphoid cells. Thus, moderate (20–40%) but not total inhibition of the cellular formation of ddGuo nucleotides was observed in the mutants deficient in dCK activity, under experimental conditions where ddCyd or ara-C, which are known substrates for this kinase, was inhibited by >95%. No detectable phosphorylation of ddGuo was detected with AK. In contrast, a cytosolic enzyme that transfers the phosphate group of a nucleoside 5′-monophosphate to a number of purine nucleosides (13, 15) did catalyze the phosphorylation of ddGuo. Incubation of extracts of wild-type or kinase-deficient CEM cells resulted in the formation of a product that was identified as ddGMP on the basis of its HPLC elution profile. The phosphotransferase reaction for ddGuo was mediated by a requirement for IMP or GMP, rather than ATP, as phosphate donor. This monophosphate specificity, in addition to a high salt requirement and ATP stimulation, is indicative of a reaction catalyzed via IMP-specific cytosolic 5′-nucleotidase, which

has been shown to act as a phosphotransferase for a number of purine nucleoside analogs, including ddIno (13), tiazofurin (15), acyclovir (24), and the carbocyclic 2',3'-didehydro-2',3'-dideoxyguanosine (25). The exact role of the nucleotidase in intracellular phosphorylation of ddGuo or other nucleoside analogs still remains to be clarified. The phosphotransferase reaction has an apparent K_m of 1–12 mM for ddGuo and other nucleosides, which is far above the active intracellular concentration of the analogs. However, the low affinity of the phosphotransferase for nucleoside substrates might be balanced by its relatively high abundance in the cells, and regulation by intracellular effectors may increase its nucleoside-phosphorylating capacity *in vivo*. In support of this hypothesis, Carson *et al.* (26) recently reported that a CEM mutant that overexpresses the IMP-specific cytoplasmic 5'-nucleotidase has significantly increased capacity to anabolize ddIno to its putative active antiviral species, dideoxyadenosine-5'-triphosphate.

In summary, the selective effect of ddGuo against HIV and hepadnaviruses, both *in vitro* and *in vivo* in an animal model system, may have important therapeutic implications. Its major liability appears to be its susceptibility to degradation by the ubiquitous PNP activity present in cells and its low specificity for anabolizing enzymes (Fig. 3). The availability of a compound that may be more actively anabolized to the analog nucleotides and that is not degraded by PNP could be of significant use as an antiviral agent.

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References

1. Yarchoan, R., J. M. Pluda, R. V. Thomas, H. Mitsuya, P. Brouwers, K. M. Wyvill, N. Hartman, D. G. Johns, and S. Broder. Long-term toxicity/activity profiles of 2',3'-dideoxyinosine in AIDS or AIDS-related complex. *Lancet* **336**:525–529 (1990).
2. Fishl, M. A., D. D. Richman, M. H. Grieco, *et al.* The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N. Engl. J. Med.* **317**:185–191 (1987).
3. Yarchoan, R., R. W. Klecker, K. J. Weinhold, *et al.* Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HILV-III/LAV replication, to patients with AIDS or AIDS-related complex. *Lancet* **1**:575–580 (1986).
4. Mitsuya, H., and S. Broder. Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc. Natl. Acad. Sci. USA* **83**:1911–1915 (1986).
5. Suzuki, S., B. Lee, W. Luo, D. Tovell, M. J. Robbins, and D. L. J. Tyrrell. Inhibition of duck hepatitis B virus replication by purine 2',3'-dideoxynucleosides. *Biochem. Biophys. Res. Commun.* **156**:1144–1151 (1988).
6. Lee, B., W. Luo, S. Suzuki, M. J. Robbins, and D. L. J. Terrell. *In vitro* and *in vivo* comparison of the abilities of purine and pyrimidine 2',3'-dideoxynucleosides to inhibit duck hepadnavirus. *Antimicrob. Agents Chemother.* **33**:336–339 (1989).
7. Baba, M., R. Pauwels, J. Balzarini, P. Herderwijn, E. DeClercq, and J. Desmyter. Ribavirin antagonizes inhibitory effects of pyrimidine 2',3'-dideoxynucleosides but enhances inhibitory effects of purine 2',3'-dideoxynucleosides on replication of human immunodeficiency virus *in vitro*. *Antimicrob. Agents Chemother.* **31**:1613–1617 (1987).
8. Ahluwalia, G., D. A. Cooney, L. L. Bondoc, Jr., M. J. Currens, H. Ford, D. G. Johns, H. Mitsuya, and A. Fridland. Inhibitors of IMP dehydrogenase stimulate the phosphorylation of the antiviral nucleoside 2',3'-dideoxyguanosine. *Biochem. Biophys. Res. Commun.* **171**:1297–1303 (1990).
9. Balzarini, J., P. Herderwijn, and E. DeClercq. Potentiating effect of ribavirin on the antiretrovirus activity of 3'-azido-2,6-diaminopurine-2',3'-dideoxyriboside *in vitro* and *in vivo*. *Antiviral Res.* **11**:161–172 (1989).
10. Hartman, N., G. S. Ahluwalia, D. A. Cooney, H. Mitsuya, S. Kageyama, A. Fridland, S. Broder, and D. G. Johns. Inhibitors of IMP dehydrogenase stimulate the phosphorylation of the anti-human immunodeficiency virus nucleosides 2',3'-dideoxyadenosine and 2',3'-dideoxyinosine. *Mol. Pharmacol.* **40**:118–124 (1991).
11. Ahluwalia, G., D. A. Cooney, H. Mitsuya, A. Fridland, K. P. Flora, Z. Hao, M. Dalal, S. Broder, and D. G. Johns. Initial studies on the cellular pharmacology of 2',3'-dideoxyinosine, an inhibitor of HIV infectivity. *Biochem. Pharmacol.* **36**:3797–3801 (1987).
12. Johnson, M. A., G. Ahluwalia, M. C. Connelly, D. A. Cooney, S. Broder, D. G. Johns, and A. Fridland. Metabolic pathways for the activation of the antiretroviral agent 2',3'-dideoxyadenosine in human lymphoid cells. *J. Biol. Chem.* **283**:15354–15357 (1988).
13. Johnson, M. A., and A. Fridland. Phosphorylation of 2',3'-dideoxyinosine by cytosolic 5'-nucleotidase of human lymphoid cells. *Mol. Pharmacol.* **36**:291–295 (1989).
14. Verhoef, V., J. C. Sarup, and A. Fridland. Identification of the mechanism of activation of 9-β-D-arabinofuranosyladenine in human lymphoid cells using mutants deficient in nucleoside kinases. *Cancer Res.* **41**:4478–4483 (1981).
15. Fridland, A., M. C. Connelly, and T. J. Robbins. Tiazofurin metabolism in human lymphoblastoid cells: evidence for phosphorylation by adenosine kinase and 5'-nucleotidase. *Cancer Res.* **46**:532–537 (1986).
16. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
17. Sarup, J. C., M. A. Johnson, V. Verhoef, and A. Fridland. Regulation of purine deoxynucleoside phosphorylation by deoxycytidine kinase from human leukemic blast cells. *Biochem. Pharmacol.* **38**:2601–2607 (1989).
18. Ullman, B., T. Coons, S. Rockwell, and K. McCartan. Genetic analysis of 2',3'-dideoxycytidine incorporation into cultured human T lymphoblasts. *J. Biol. Chem.* **263**:12391–12396 (1988).
19. Karlsson, A., P. Reichard, and F. Eckstein. The metabolism of 3'-azido-2',3'-dideoxyguanosine in CEM cells. *Biochem. Biophys. Res. Commun.* **166**:273–279 (1990).
20. Aoki-Sei, S., M. C. O'Brien, H. Ford, H. Fujii, D. A. Gilbert, D. A. Cooney, D. G. Johns, S. Broder, and H. Mitsuya. *In vitro* inhibition of hepatitis B virus replication by 2',3'-dideoxyguanosine, 2',3'-dideoxyinosine, and 3'-azido-2',3'-dideoxythymidine in 2.2.15 (PR) cells. *J. Infect. Dis.* **164**:843–851 (1991).
21. Hao, Z., D. A. Cooney, N. R. Hartman, C. F. Perno, A. Fridland, A. L. DeVico, M. G. Sarngadhavan, S. Broder, and D. G. Johns. Factors determining the activity of 2',3'-dideoxynucleosides in suppressing human immunodeficiency virus *in vitro*. *Mol. Pharmacol.* **34**:431–435 (1988).
22. Busso, M. E., L. Resnick, B. Yang, and A. M. Mian. Cellular pharmacology and anti-HIV activity of 2',3'-dideoxyguanoside. *AIDS Res. Hum. Retroviruses* **6**:1139–1146 (1990).
23. Hershfield, M. S., J. E. Fetter, C. W. Small, A. S. Baguara, S. R. Williams, B. Ullman, D. W. Martin, Jr., D. B. Wasson, and C. A. Carson. Effects of mutational loss of adenosine kinase and deoxycytidine kinase on deoxy-ATP accumulation and deoxyadenosine toxicity in cultured CEM human T-lymphoblastoid cells. *J. Biol. Chem.* **257**:6380–6386 (1982).
24. Keller, P. J., S. A. McKee, and J. A. Fyle. Cytoplasmic 5'-nucleotidase catalyzes acyclovir phosphorylation. *J. Biol. Chem.* **267**:8664–8667 (1985).
25. Bondoc, L. L., Jr., W. M. Shannon, J. A. Secrist III, E. Vince, and A. Fridland. Metabolism of the carboxylic nucleoside analogue carbovir, an inhibitor of HIV in human lymphoid cells. *Biochemistry* **29**:9839–9843 (1990).
26. Carson, D. A., C. J. Carrera, and D. B. Wasson. Deoxyadenosine-resistant human T lymphoblasts with elevated 5'-nucleotidase activity. *Biochim. Biophys. Acta* **1091**:22–28 (1991).

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