0026-895X/94/040777-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:777-782

Effects of IMP Dehydrogenase Inhibitors on the Phosphorylation of Ganciclovir in MOLT-4 Cells before and after Herpes Simplex Virus Thymidine Kinase Gene Transduction

RIAD AGBARIA, CRAIG A. MULLEN, NEIL R. HARTMAN, DAVID A. COONEY, ZHANG HAO, R. MICHAEL BLAESE, and DAVID G. JOHNS

Laboratory of Medicinal Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment (R.A., N.R.H., D.A.C., Z.H., D.G.J.), and Metabolism Branch, Division of Cancer Biology, Diagnosis, and Centers (C.A.M., R.M.B.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Received September 13, 1993; Accepted January 4, 1994

SUMMARY

We have undertaken to characterize the role of cytoplasmic 5′-nucleotidase (EC 3.1.3.5) in the phosphorylation of the anti-herpes simplex virus and anti-human cytomegalovirus agent ganciclovir (GCV) in MOLT-4 cells, a human T cell line adapted to grow in suspension culture. The rate of formation of GCV triphosphate was found to be approximately doubled by preincubation of nontransfected MOLT-4 cells with agents that cause the accumulation of IMP, such as ribavirin (20 μM) and mycophenolic acid (1 μM), and the reaction rate was found to be unaffected by high levels of thymidine (100 μM). With herpes simplex virus-1 thymidine kinase (HStk) gene-transduced MOLT-4 cells, the rate of GCV phosphorylation was approximately 40-fold faster than that in uninfected cells and, in marked contrast to uninfected cells, the reaction was significantly inhibited both by IMP dehydrogenase inhibitors and by thymidine. These latter effects appear to be the result of 1) the accumulation of high levels of dTTP in IMP dehydrogenase inhibitor-treated cells, with consequent feedback inhibition of HStk, and 2) direct competitive substrate inhibition by thymidine of the HStk-catalyzed phosphorylation of GCV. Thus, agents that enhance 5′-nucleotidase-catalyzed phosphorylation of GCV in uninfected cells do not play a similar role in HStk-transfected cells, a consequence of the quantitative predominance of the viral thymidine kinase-catalyzed reaction over that attributable to endogenous cytoplasmic 5′-nucleotidase.

The initial phosphorylation of the antiviral nucleoside analogues ACV [9-(2-hydroxyethoxymethyl)guanine] and GCV [9-((1,3-dihydroxy-2-propoxy)methyl)guanine] (Fig. 1) in HSV-infected cells is catalyzed by a virus-encoded thymidine kinase (1–5). Phosphorylation of these two agents is also detectable, although to a much lesser degree, in uninfected mammalian cells lacking the virus-encoded enzyme. In 1985, Keller *et al.* (6) reported that a highly purified cytoplasmic 5′-nucleotidase (EC 3.1.3.5), present in uninfected rat liver cells and acting as a phosphotransferase, could catalyze the phosphorylation of ACV, GCV, and other guanosine derivatives. The rate of phosphorylation of these nucleoside analogues by the 5′-nucleotidase was slow, compared with that seen with the natural acceptor substrate inosine (approximately 1–2%), but, in the case of ACV, appeared to be sufficient to account for the low level of ACV-phosphorylating activity present in uninfected mammalian cells. The phosphorylation of GCV by this 5′-nucleotidase appears not to have been further explored.

Our interest in the substrate specificity of cytosolic 5′-nucleotidase arose from the observation that this enzyme was in large part responsible for the initial 5′-phosphorylation of a number of purine dideoxynucleoside anti-human immunodeficiency virus compounds, including 2′,3′-dideoxyguanosine and 2′,3′-dideoxyinosine (7–9). Furthermore, because the preferred phosphate donor in phosphorylations catalyzed by this enzyme was IMP, exposure to agents that increased the intracellular levels of the latter nucleotide (e.g., the IMPD inhibitors ribavirin, tiazofurin, and mycophenolic acid) enhanced both the phosphorylation and the antiviral activity of these compounds. The increase in the rate of 5′-monophosphorylation consequent to IMPD inhibition varied considerably among these nucleosides, ranging from approximately 2-fold in the case of 2′,3′-dideoxyinosine (8, 9) to >20-fold in the case of the fluorinated analogue 2′-β-fluoro-2′,3′-dideoxyadenosine (10).

Because GCV, like ACV, is also subject to low but readily detectable 5′-nucleotidase-catalyzed phosphorylation in uninfected cells (6), we have undertaken studies to determine whether this reaction, like that observed for purine 2′,3′-dideoxynucleosides, is similarly enhanced by agents that increase intracellular IMP. In addition, in view of the reports of

ABBREVIATIONS: ACV, acyclovir; GCV, ganciclovir; IMPD, IMP dehydrogenase; HPLC, high performance liquid chromatography; HStk, herpes simplex thymidine kinase; HSV, herpes simplex virus; AZT, 3′-azido-3′-deoxythymidine.

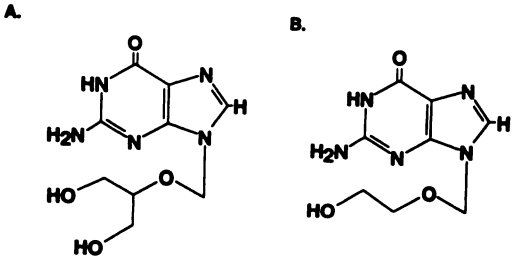

Fig. 1. Structures of ganciclovir (A) and acyclovir (B).

the cytostatic effects (and thus potential antitumor activity) of GCV in HSV-transfected cell lines *in vitro* (11–14) and *in vivo* (13, 15) and the current interest in GCV treatment of HSV-transfected brain tumors (16, 17), we have extended the scope of the study to include determination of the effects of IMPD inhibitors on GCV phosphorylation in human (MOLT-4) cells before and after transfection with the HSV-encoded thymidine kinase gene.

Materials and Methods

Chemicals. Ribavirin (NSC-163039) and mycophenolic acid (NSC-129185) were supplied by Dr. Karl Flora, Pharmaceutical Resources Branch, Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health. GCV was purchased from Syntex Laboratories, Inc. (Palo Alto, CA). [^{3}H]GCV (22 Ci/mmol; radiochemical purity, >99%), labeled in the 8-position of the purine base, was obtained from Moravek Biochemicals (Brea, CA). Other nucleoside standards were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells. Studies of the metabolism of GCV were carried out using exponentially growing MOLT-4 cells. Cells were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated (56° for 60 min) fetal bovine serum, 44 μg/ml gentamicin, and 4 mM L-glutamine, at 37° in a humidified atmosphere of 95% air/5% CO_2. For some experiments, proliferating MOLT-4 cells were transduced with the retroviral vector STK, containing the cDNA for the HStk gene promoted by the simian virus 40 promoter as well as the cDNA for the neomycin phosphotransferase (*Neo*^{R}) gene promoted by the retroviral long terminal repeat (15). Cells in logarithmic growth were exposed for 16 hr to the vector supernatants supplemented with protamine (5 μg/ml) (18). They were then selected for gene transduction by growth for 4 weeks in the presence of the neomycin analogue G418 (0.6 mg/ml; GIBCO, Grand Island, NY).

Metabolism studies. Ten-milliliter aliquots of a MOLT-4 cell suspension (2.5 × 10^6 cells/ml) were incubated with radiolabeled GCV (5 or 10 μM, 5 μCi/ml) in the presence or absence of IMPD inhibitors or other modulators. After a 5-hr incubation, the cells were recovered by centrifugation and the pellets were extracted with 0.4 ml of 60% methanol. Extracts were heated for 2 min at 95° and, after centrifugation, the supernatant was evaporated under vacuum and redissolved in 250 μl of water; a 200-μl aliquot of the reconstituted samples was subjected to chomatography on a Partisphere 5-SAX column (buffer A, 0.002 M ammonium phosphate, pH 3.5; buffer B, 0.7 M ammonium phosphate, pH 3.5). The following elution program was used: 0–10 min, linear gradient to 4% buffer B; 10–20 min, linear gradient to 25% buffer B; 20–45 min, linear gradient to 90% buffer B; 45–50 min, isocratic elution with 90% buffer B; 50–55 min, linear gradient to 100% buffer A; 55–70 min, reequilibration with buffer A before the next injection. The flow rate was 2 ml/min. One-minute fractions were collected and radioactivity was determined by liquid scintillation counting. Intracellular GTP and IMP pool sizes were determined from the areas under the elution peaks for these nucleotides, using a column precalibrated with IMP and GTP reference standards; these nucleotides eluted at 7 ± 1 and 39 ± 1 min, respectively.

Phosphorylation of tritiated thymidine in MOLT-4 cells and HStk-transfected MOLT-4 cells. Five-milliliter aliquots of suspensions of untransfected and HStk gene-transfected MOLT-4 cells (2.5 × 10^6 cells/ml) were incubated for 5 hr in the presence or absence of ribavirin (20 μM) or mycophenolic acid (1 μM). The cells were then pulse-labeled with [^{3}H]thymidine (2 μCi/ml; specific activity, 20 Ci/mmol) and the incubation was continued for another 15 min. Cells were harvested and extracted as described above. Cell extracts were then subjected to HPLC analysis of [^{3}H]thymidine nucleotides using a Partisil 10-SAX column and the following elution scheme: 0–5 min, isocratic elution with 100% buffer A (0.02 M ammonium phosphate, native pH); 5–20 min, linear gradient to 25% buffer B (0.7 M ammonium phosphate plus 10% methanol, native pH); 20–35 min, linear gradient to 100% buffer B; 35–40 min, linear gradient to 100% buffer A; 40–55 min, equilibration with buffer A. Eluted radiolabeled nucleotides were monitored with an in-line radioactivity flow detector. The times of elution of [^{3}H]thymidine, [^{3}H]dTMP, [^{3}H]dTDP, and [^{3}H]dTTP were 3, 10, 20, and 27 min, respectively.

Determination of deoxyribonucleoside triphosphate concentrations. Deoxyribonucleoside-5′-triphosphate concentrations in MOLT-4 cell extracts were determined by a modification of the method of Garrett and Santi (19), as described previously (8).

Results

Chromatographic separation of nucleotides arising from GCV. We initially determined whether, under the conditions of this study, the phosphorylated metabolites of GCV could be detected chomatographically in extracts of nontransfected MOLT-4 cells incubated with the radiolabeled drug. A typical HPLC tracing of the anabolites arising from GCV and separated by ion exchange chromatography is shown in Fig. 2; it can be seen that the principal radiolabeled metabolites eluted at 8, 24, and 42 min.

To aid in the identification of these nucleotides, a methanolic extract from MOLT-4 cells incubated for 5 hr with 5 μM [^{3}H] GCV was applied to a C-18 reverse phase column, which was eluted with 0.003 M NaCl. The GCV phosphates eluted at 2 min in this system; these were collected, lyophilized, and incubated with alkaline phosphatase (0.1 unit/ml, at pH 9) for 5 hr at 37°. The samples so treated were then chromatographed again on a C-18 column equilibrated and eluted isocratically with 0.1 M formic acid; the greater part of the radioactivity (>99%) was found to coelute with authentic GCV (data not shown).

Effects of IMPD inhibitors on phosphorylation of GCV in nontransfected MOLT-4 cells. The addition of ribavirin (20 μM) together with ^{3}H-labeled GCV resulted in an approximately 2-fold increase in the formation of the di- and triphosphates of the latter (Fig. 2); a lesser increase in accumulation (approximately 40%) was noted at the monophosphate level.

In an attempt to determine the level of ribavirin capable of exerting maximal stimulation of phosphorylation, a range of ribavirin concentrations from 5 to 20 μM was explored. As shown in Fig. 3, accumulation of GCV di- and triphosphates continued to increase up to the highest concentration studied (20 μM); ribavirin levels in excess of this amount exerted a cytostatic effect in MOLT-4 cells and were not further studied.

The ability of the IMPD inhibitor mycophenolic acid to stimulate phosphorylation was also determined. The potency of the latter compound was greater than that of ribavirin, with increases in GCV nucleotides at a concentration of 1 μM that were equivalent to those seen with 20 μM ribavirin (Fig. 4, *upper*, A-C).

Relative effectiveness of other modulators. In view of

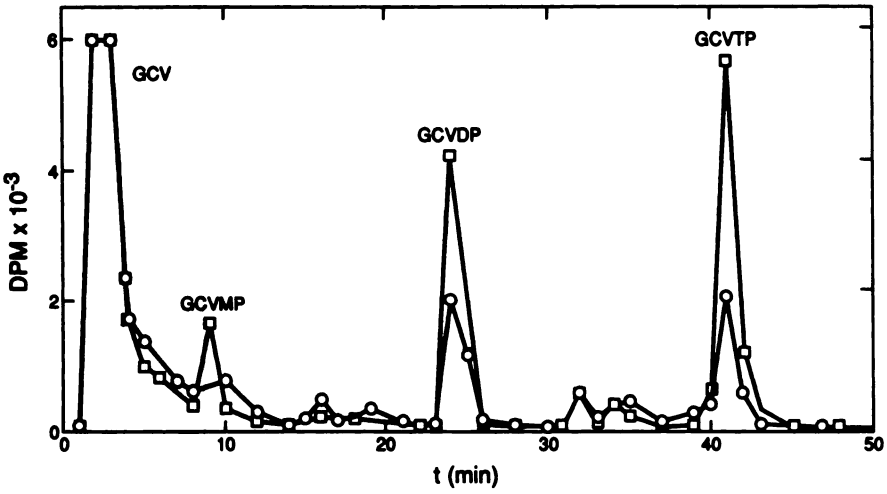

Fig. 2. Separation of ^{3}H-labeled metabolites arising from [8-^{3}H]GCV. Nontransfected MOLT-4 cells (2.5×10^6 cells/ml) were incubated with ^{3}H-labeled GCV (5 μM; 5 μCi/ml) for 5 hr. Methanolic cell extracts of 20×10^6 cells were subjected to ion exchange HPLC (Partisphere 5-SAX), using the elution program described in Materials and Methods. Representative chromatograms are shown (four experiments). ○, Control; □, plus ribavirin (20 μM). *GCVMP*, GCV monophosphate; *GCVDP*, GCV diphosphate; *GCVTP*, GCV triphosphate.

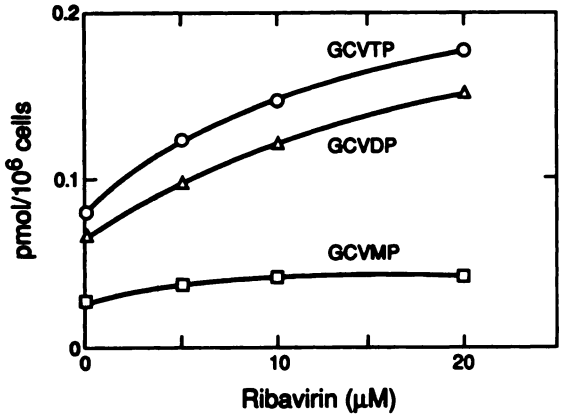

Fig. 3. Effect of ribavirin concentration on GCV nucleotide formation from [^{3}H]GCV. Nontransfected MOLT-4 cells in logarithmic growth (2.5×10^6 cells/ml at time 0) were incubated for 5 hr with 5 μM [^{3}H]GCV (5 μCi/ml) and varying concentrations of ribavirin. Methanolic cell extracts were analyzed as described in Materials and Methods. Each value represents the average of duplicate determinations, with the individual values obtained varying by <10%. *Ordinate*, intracellular GCV nucleotide concentration (pmol/10^6 cells). *GCVMP*, GCV monophosphate; *GCVDP*, GCV diphosphate; *GCVTP*, GCV triphosphate.

the marked enhancement of IMP pools and thus of GCV phosphorylation seen with ribavirin and mycophenolic acid, we felt it would be of interest to determine whether high concentrations of inosine (10 and 100 μM) in the medium might also be effective in increasing IMP and thus GCV phosphorylation. As shown in Table 1, however, inosine in the absence of ribavirin was only very slightly effective in increasing either IMP levels or GCV phosphate formation; this lack of enhancement of phosphorylation by inosine was previously observed in studies of the phosphorylation of 2′,3′-dideoxyadenosine and 2′,3′-dideoxyinosine (8).

The effects of the purine nucleosides guanosine and 2′-deoxyguanosine on the ribavirin stimulatory effect were next determined. As previously seen also with 2′,3′-dideoxyinosine (9), these nucleosides effectively reversed the ribavirin enhancement of GCV phosphorylation, most likely by restoring GTP to normal or supranormal levels (Table 1) and thus permitting resumption of preferential utilization of IMP in the adenylosuccinate synthetase/lyase-catalyzed synthesis of AMP (9), the first step of which is a GTP-requiring reaction.

Thymidine, even at high concentrations (10 and 100 μM), had no effect on GCV phosphorylation in this system (Table 1), providing additional evidence that autochthonous thymidine kinase does not play a detectable role in this reaction in nontransfected MOLT-4 cells.

Effects of IMPD inhibitors on phosphorylation of GCV in MOLT-4 cells after HStk gene transfection. As is well known, mammalian cells infected with HSV, or transfected with a HStk vector, elaborate a thymidine kinase enzyme that differs in many of its properties from the cytoplasmic thymidine kinase of uninfected cells (1, 2). Among these properties is the ability to monophosphorylate the guanosine analogues ACV and GCV (1–5), which is the rate-limiting reaction in the formation of the pharmacologically active ACV and GCV triphosphates. We thus determined whether agents that affected the phosphorylation of GCV by 5′-nucleotidase in nontransfected cells would exert any effect on the phosphorylation of this agent in MOLT-4 cells transfected with viral thymidine kinase. As shown in Fig. 4, *lower*, A-C, and Table 2, the rate of formation of GCV triphosphate was increased almost 40-fold in HStk-transfected MOLT-4 cells, compared with the rate seen in nontransfected cells. In marked contrast, however, to the situation in nontransfected cells, ribavirin and mycophenolic acid, rather than enhancing the phosphorylation of GCV, inhibited the formation of GCV phosphates to levels even below those seen in control cells. Guanosine and 2′-deoxyguanosine also lost their capacities to nullify the increased phosphorylation that is seen in nontransfected MOLT-4 cells incubated with ribavirin. Most importantly, thymidine, which was without effect on the GCV phosphorylation reaction in nontransfected cells, was a highly potent inhibitor of such phosphorylation in the HStk-transfected cells (Table 2).

Effects of IMPD inhibitors on dTTP pools and on [^{3}H] thymidine phosphorylation in HStk-transfected MOLT-4 cells. The inhibition of GCV phosphorylation by ribavirin and mycophenolic acid in HStk gene-transfected cells (Fig. 4, *lower*, A-C) can most readily be explained by the well known ability of these agents to increase dTTP levels (20), the latter nucleotide being a feedback inhibitor of both native thymidine kinase and HStk (21, 22). We thus determined the levels of dTTP after exposure of transfected cells to these compounds. Both ribavirin and mycophenolic acid were highly effective in increasing dTTP levels in these cells (Fig. 4, *lower*, D). The

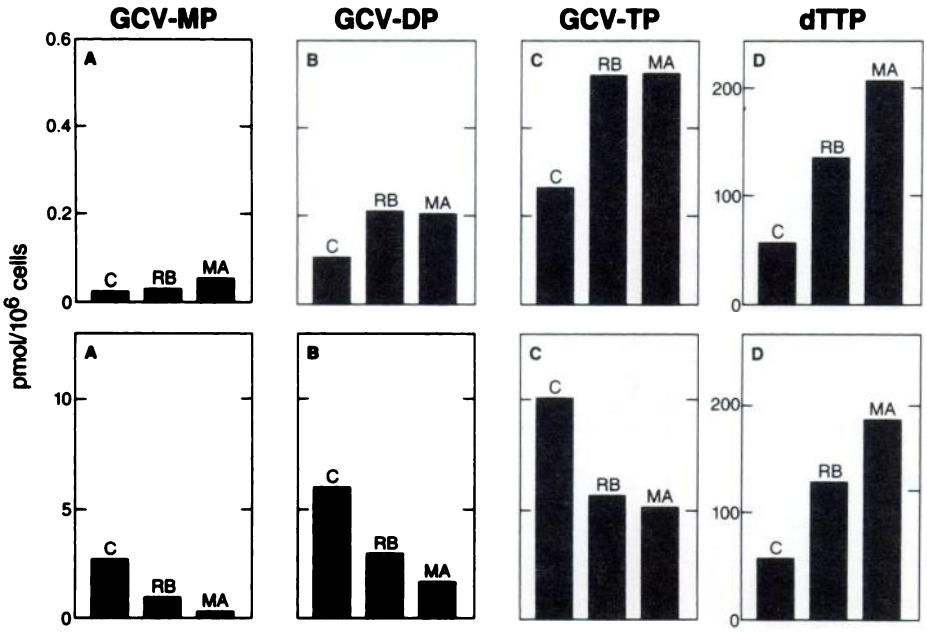

Fig. 4. Effects of ribavirin and mycophenolic acid on [^{3}H]GCV nucleotide formation from [^{3}H]GCV and on dTTP pools in nontransfected and HStk-transfected cells. MOLT-4 cells (2.5 × 10^6 cells/ml at time 0) were incubated for 5 hr with 10 μM [^{3}H]GCV (5 μCi/ml) in the absence (*C*, control) or presence of 20 μM ribavirin (*RB*) or 1 μM mycophenolic acid (*MA*). Each value represents the average of duplicate determinations, with the individual values obtained varying by <10%. Methanolic cell extracts were analyzed as described in Materials and Methods. A, [^{3}H]GCV monophosphate; B, [^{3}H]GCV diphosphate; C, [^{3}H]GCV triphosphate; D, dTTP. *Upper*, nontransfected cells; *lower*, HStk-transfected cells. *Ordinate*, intracellular nucleotide (GCV or dTTP) concentration (pmol/10^6 cells).

TABLE 1

Effect of selected nucleosides on the ribavirin enhancement of GCV phosphorylation in nontransfected cells

MOLT-4 cells (2.5 × 10^6 cells/ml) were incubated for 5 hr with 5 μM GCV (5 μCi/ml) in the presence of nucleosides and ribavirin (20 μM), as shown. Methanolic cell extracts were analyzed by ion exchange HPLC as described in Materials and Methods. Results are the average of two separate experiments, with the individual values varying by <10%. Values in parentheses are percentage of control (rate of GCV triphosphate formation in the absence of added nucleosides).

Addition	GCV triphosphate		IMP		GTP	
	Ribavirin omitted	+Ribavirin (20 μM)	Ribavirin omitted	+Ribavirin (20 μM)	Ribavirin omitted	+Ribavirin (20 μM)
	pmol/10^6 cells		*pmol/10^6 cells*		*pmol/10^6 cells*	
None	0.079 (100%)	0.188 (238%)	<10	267	520	120
Inosine, 100 μM	0.075 (95%)	0.214 (271%)	36	258	574	156
Guanosine, 10 μM	0.084 (106%)	0.098 (124%)	<10	<10	534	370
Deoxyguanosine, 10 μM	0.047 (59%)	0.062 (78%)	<10	<10	524	643
Thymidine, 10 μM	0.068 (86%)	0.173 (219%)	<10	376	496	145
Thymidine, 100 μM	0.075 (95%)	0.150 (190%)	<10	150	361	137

TABLE 2

Effect of selected nucleosides on the phosphorylation of GCV in HStk-transfected cells

HStk-transfected MOLT-4 cells (2 × 10^6 cells/ml) were incubated for 5 hr with 10 μM GCV (2 μCi/ml) in the presence of nucleosides and ribavirin (20 μM), as shown. Methanolic cell extracts were analyzed by ion exchange HPLC, as described in Materials and Methods. Results are the average of two separate experiments, with the individual values varying by <10%. Values in parentheses are percentage of control (rate of GCV triphosphate formation in the absence of added nucleosides).

Addition	GCV triphosphate		IMP		GTP	
	Ribavirin omitted	+Ribavirin (20 μM)	Ribavirin omitted	+Ribavirin (20 μM)	Ribavirin omitted	+Ribavirin (20 μM)
	pmol/10^6 cells		*pmol/10^6 cells*		*pmol/10^6 cells*	
None	10.2 (100%)	5.6 (55%)	40	221	600	118
Guanosine, 10 μM	11.2 (110%)	17.2 (169%)	18	19	800	1284
Deoxyguanosine, 10 μM	11.2 (110%)	9.3 (91%)	13	12	960	1378
Inosine, 10 μM	12.2 (120%)	6.4 (63%)	21	186	709	139
Thymidine, 10 μM	1.4 (14%)	2.7 (26%)	<10	196	546	186

data for a range of concentrations of ribavirin are shown in Fig. 5; Fig. 5 also illustrates the substantial depression of dGTP produced in these cells as a consequence of inhibition of IMPD by ribavirin.

As shown previously by Drach *et al.* (23, 24), in uninfected cells treated with ribavirin the ribavirin-induced increase in dTTP pools leads to decreased radiolabeling of dTTP and other thymidine nucleotides after pulse-labeling with [^{3}H]thymidine, both because of the feedback inhibition of the enzyme by elevated dTTP and because of the isotope dilution effect resulting from the increase in total dTTP. To determine whether a similar effect was demonstrable with HStk-transfected cells, we pulse-labeled HStk-transfected MOLT-4 cells with [^{3}H]thymidine and determined the formation of tritiated thymidine nucleotides with HPLC. As shown in Fig. 6, *lower*, decreased radiolabeling was seen, with mycophenolic acid (1 μM) being slightly more potent in this respect than ribavirin (20 μM).

Effect of dTTP on GCV phosphorylation by lysates from HStk-transfected MOLT-4 cells. To confirm directly the inhibitory action of increased dTTP on GCV phosphoryl-

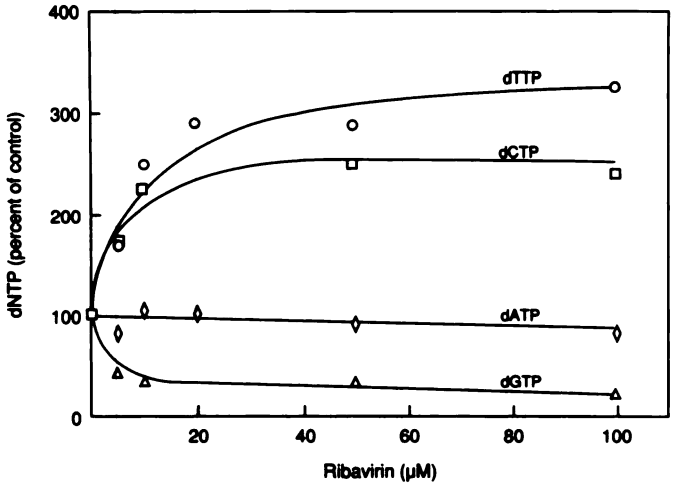

Fig. 5. Effect of ribavirin on deoxynucleoside-5′-triphosphate concentrations in HStk-transfected MOLT-4 cells. Transfected MOLT-4 cells (2.5×10^7 cells, at a density of 2.5×10^6 cells/ml) were incubated at 37° for 5 hr with varying concentrations of ribavirin. Deoxyribonucleotide concentrations were determined as described in Materials and Methods. Each value represents the average of duplicate determinations, with the individual values varying by <10%. Control values (no ribavirin) were as follows: dCTP, 18.3; dTTP, 43.3; dATP, 34.8; and dGTP, 18.2 pmol/10^6 cells.

ation, we then determined the effect of the addition of exogenous dTTP on the formation of GCV monophosphate by lysates of HStk-transfected MOLT-4 cells; as shown in Table 3, dTTP was an effective inhibitor of GCV monophosphate formation by cell lysates, at levels equivalent to those formed in whole cells after ribavirin or mycophenolic acid treatment (i.e., 50–250 μM).

Discussion

Keller *et al.* (6) reported that, in uninfected cells from rat liver, much of the phosphorylating activity for the similar compounds GCV and ACV in the cytosolic fraction could be accounted for by the 5′-nucleotidase operative in the experiments described here. The present observations, however, support the contentions that 1) the process responsible for the monophosphorylation of GCV in HStk-transfected T cells differs substantially from that seen in nontransfected cells and 2) 5′-nucleotidase plays a quantitatively minor role in this process in transfected cells, with the HStk playing the major role. Supporting this interpretation are the observations that GCV phosphorylation in HStk-transfected cells is inhibited rather than stimulated by IMPD inhibitors and that thymidine, which had no effect on GCV phosphorylation in nontransfected cells, is a potent inhibitor of this reaction in HStk-transfected cells.

The inhibition by thymidine of GCV phosphorylation in HStk gene-transfected cells can be readily explained as a consequence of substrate inhibition, i.e., competition between GCV and thymidine for phosphorylation by the same HSV vector-encoded thymidine kinase. The fact that the thymidine inhibition effect is not seen in nontransfected cells (Table 1) is a reflection of the inability of autochthonous thymidine kinase to utilize GCV as a substrate. The mechanism of inhibition of GCV phosphorylation by IMPD inhibitors appears, however, to be more complex than the mechanism of inhibition by thymidine. It has long been known that both ribavirin and mycophenolic acid bring about significant increases in dTTP pools (20) and also that dTTP is a feedback inhibitor of both native thymidine kinase and HStk (21, 22). This increased dTTP (see Figs. 4, *lower*, D, and 5) could therefore account for the decrease in GCV phosphorylation. To test this point directly, we examined the ability of dTTP to inhibit the formation of GCV monophosphate by lysates of HStk-transfected MOLT-4 cells, and we found significant inhibition at dTTP levels equimolar to those produced in ribavirin- or mycophenolic acid-treated cells (Table 3). The basis for the increase in dTTP after treatment of cells with IMPD inhibitors is still, however, not fully established. Lowe *et al.* (20) postulated that the drop in GTP levels consequent to IMPD inhibition (see Tables 1 and 2) results in a compensatory increase in phosphoribosylpyrophosphate synthesis and that, as a consequence of the latter, the synthesis of pyrimidine ribonucleotides (including UTP) is greatly increased, an effect we have also observed in the present study (data not shown). The increased uridine ribonucleotide

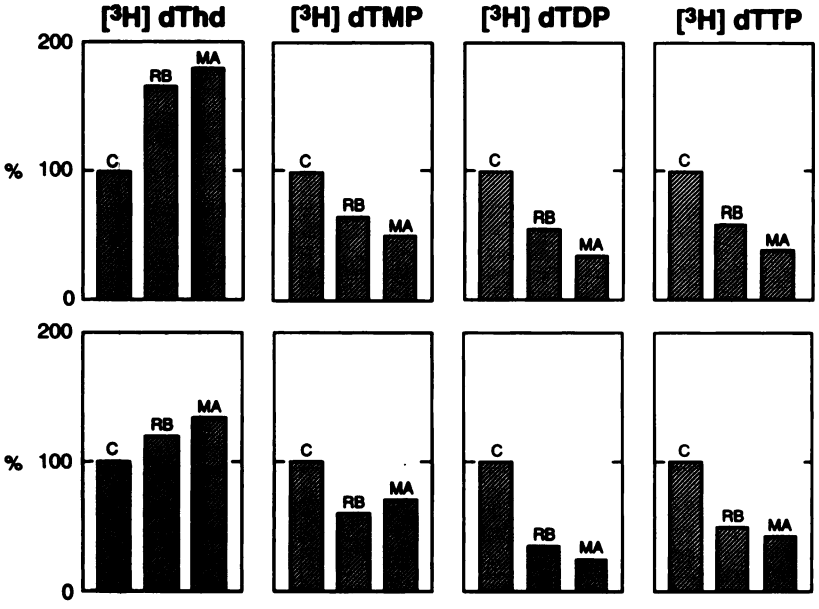

Fig. 6. Effects of IMPD inhibitors on phosphorylation of tritiated thymidine by MOLT-4 cells and HStk-transfected MOLT-4 cells. Untransfected and HStk gene-transfected MOLT-4 cells (2.5×10^6 cells/ml) were pulse-labeled with [^{3}H]thymidine (2 μCi/ml), as described in Materials and Methods, and ^{3}H-labeled thymidine nucleotides were separated by HPLC. *Upper*, untransfected MOLT-4 cells; *lower*, HStk gene-transfected MOLT-4 cells. *C*, control (no inhibitor); *RB*, plus ribavirin (20 μM); *MA*, plus mycophenolic acid (1 μM). Control (100%) values for [^{3}H]thymidine (*[^{3}H]dThd*), [^{3}H]dTMP, [^{3}H]dTDP, and [^{3}H]dTTP radioactivity were as follows: untransfected cells: 5122, 1168, 1586, and 3931 dpm/10^6 cells, respectively; HStk gene-transfected cells, 6576, 594, 2217, and 4970 dpm/10^6 cells, respectively.

TABLE 3

Inhibition by dTTP of the rate of phosphorylation of GCV by lysates of HStk-transfected MOLT-4 cells

Exponentially growing HStk-transfected MOLT-4 cells were collected by centrifugation for 10 min at 1000 × g (4°), resuspended in 0.05 M Tris buffer with 2 mM dithiothreitol, pH 7.8, and ruptured by sonication. The reaction mixture consisted of 5 mM ATP, 5 mM $MgCl_2$, 10 μM [^{3}H]GCV (220 μCi/ml), inhibitor (dTTP) at the concentration indicated, and buffered cell lysate (40 μg of protein), in a total volume of 45 μl. GCV-monophosphate formation was allowed to proceed for 60 min at 37° and the reaction was terminated by heating at 95° for 2 min. Product formation was determined by ion exchange HPLC as described previously (26), with an elution time for GCV monophosphate of 16.6 min. Values shown are means ± standard errors from three separate determinations.

dTTP concentration	Rate of GCV monophosphate formation	Inhibition
μM	pmol/mg of protein/min	%
0	2.73 ± 0.18	
50	2.12 ± 0.05	22
100	1.02 ± 0.04	63
250	0.96 ± 0.03	65

levels then lead, by well established biosynthetic pathways, to increased formation of uridine deoxyribonucleotides, including dUMP, which in turn is the precursor (via thymidylate synthetase) of increased dTMP and thus of increased dTTP.

Irrespective of the mechanism, the resulting inhibition of GCV phosphorylation in HStk-transfected cells by the IMPD inhibitors ribavirin and mycophenolic acid is analogous to the reversal of the phosphorylation and antiviral activity of AZT by ribavirin (25), which is similarly attributable to dTTP feedback inhibition of the thymidine kinase-catalyzed phosphorylation of AZT, although in this case it is the endogenous thymidine kinase of the cell, rather than a virus-encoded enzyme, that is operative. In the case of AZT and ribavirin, this partial antagonism would appear to be a contraindication to the use of combinations of these two agents in antiviral therapy (25). The situation is less clear, however, with respect to guanosine analogues such as GCV and ACV; ribavirin and other IMPD inhibitors, in addition to increasing intracellular dTTP, effectively deplete GTP and dGTP levels (see Tables 1 and 2 and Fig. 5), thus lessening the ability of these physiological nucleotides to compete with GCV triphosphate or ACV triphosphate inhibition of HSV replication or of HStk-transfected neoplastic cell replication. The net therapeutic result thus cannot be predicted on biochemical grounds alone.

Acknowledgments

The authors would like to thank Sandra Taubenkibel for excellent secretarial assistance.

References

1. Elion, G. B., P. A. Furman, F. A. Fyfe, P. De Miranda, L. Beauchamp, and H. J. Schaeffer. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci. USA* **74**:5716–5720 (1977).
2. Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J. Biol. Chem.* **253**:8721–8727 (1978).
3. Field, A. K., M. E. Davies, C. DeWitt, H. C. Perry, R. Liou, J. Germershausen, J. D. Karkas, W. T. Ashton, D. B. R. Johnston, and R. L. Tolman. 9-[[2-Hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine: a selective inhibitor of herpes group virus replication. *Proc. Natl. Acad. Sci. USA* **80**:4139–4143 (1983).
4. Smith, K. O., K. S. Galloway, W. L. Kennell, K. K. Ogilvie, and B. K. Radatus. A new nucleoside analog, 9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]guanine, highly active *in vitro* against herpes simplex virus types 1 and 2. *Antimicrob. Agents Chemother.* **22**:55–61 (1982).
5. Smee, D. F., R. Boehme, M. Chernow, B. P. Binho, and T. R. Matthews. Intracellular metabolism and enzymatic phosphorylation of 9-(1,3-dihydroxy-2-propoxymethyl)guanine and acyclovir in herpes simplex virus-infected and uninfected cells. *Biochem. Pharmacol.* **34**:1049–1056 (1985).
6. Keller, P. M., S. A. McKee, and J. A. Fyfe. Cytoplasmic 5′-nucleotidase catalyzes acyclovir phosphorylation. *J. Biol. Chem.* **260**:8664–8667 (1985).
7. 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).
8. Hartman, N. R., 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).
9. Balzarini, J., C.-K. Lee, P. Herdewijn, and E. De Clercq. Mechanism of the potentiating effect of ribavirin on the activity of 2′,3′-dideoxyinosine against human immunodeficiency virus. *J. Biol. Chem.* **266**:21509–21514 (1991).
10. Johns, D. G., G. S. Ahluwalia, D. A. Cooney, H. Mitsuya, and J. S. Driscoll. Enhanced stimulation by ribavirin of the 5′-phosphorylation and anti-human immunodeficiency virus activity of purine 2′-β-fluoro-2′,3′-dideoxynucleosides. *Mol. Pharmacol.* **44**:519–523 (1993).
11. Balzarini, J., E. De Clercq, D. Ayusawa, and T. Seno. Murine mammary FM3A carcinoma cells transformed with the herpes simplex virus type 1 thymidine kinase gene are highly sensitive to the growth-inhibitory properties of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine and related compounds. *FEBS Lett.* **185**:95–100 (1985).
12. Oliver, S., G. Bubley, and C. Crumpacker. Inhibition of HSV-transformed murine cells by nucleoside analogs, 2′-NDG and 2′-nor-cGMP: mechanisms of inhibition and reversal by exogenous nucleosides. *Virology* **145**:84–93 (1985).
13. Moolten, F. L. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res.* **46**:5276–5281 (1986).
14. Balzarini, J., C. Bohman, and E. De Clercq. Differential mechanism of cytostatic effect of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, 9-(1,3-dihydroxy-2-propoxymethyl)guanine, and other antiherpetic drugs on tumor cells transfected by the thymidine kinase gene of herpes simplex virus type 1 or type 2. *J. Biol. Chem.* **268**:6332–6337 (1993).
15. Moolten, F. L., and J. M. Wells. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J. Natl. Cancer Inst.* **82**:297–300 (1990).
16. Culver, K. W., Z. Ram, S. Walbridge, H. Ishii, E. H. Oldfield, and R. M. Blaese. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science (Washington D. C.)* **256**:1550–1552 (1992).
17. Ram, Z., K. W. Culver, S. Walbridge, R. M. Blaese, and E. H. Oldfield. *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res.* **53**:83–88 (1993).
18. Cornetta, K., and W. F. Anderson. Protamine sulfate as an effective alternative to Polybrene in retroviral-mediated gene-transfer: implications for gene therapy. *J. Virol. Methods* **23**:187–194 (1989).
19. Garrett, C., and D. V. Santi. A rapid and sensitive high pressure liquid chromatography assay for deoxyribonucleoside triphosphates in cell extracts. *Anal. Biochem.* **99**:268–273 (1979).
20. Lowe, J. K., L. Brox, and J. F. Henderson. Consequences of inhibition of guanine nucleotide synthesis by mycophenolic acid and virazole. *Cancer Res.* **37**:736–743 (1977).
21. Breitman, T. R. The feedback inhibition of thymidine kinase. *Biochim. Biophys. Acta* **67**:153–155 (1963).
22. Cheng, Y.-C. Deoxythymidine kinase induced in HeLa TK$^-$ cells by herpes simplex virus type I and type II: substrate specificity and kinetic behavior. *Biochim. Biophys. Acta* **452**:370–381 (1976).
23. Drach, J. C., M. A. Thomas, J. W. Barnett, S. H. Smith, and C. Shipman, Jr. Tritiated thymidine incorporation does not measure DNA synthesis in ribavirin-treated human cells. *Science (Washington D. C.)* **212**:549–551 (1981).
24. Drach, J. C., J. W. Barnett, M. A. Thomas, S. H. Smith, and C. Shipman, Jr. Inhibition of viral and cellular DNA synthesis by ribavirin, in *Ribavirin: A Broad Spectrum Antiviral Agent* (R. A. Smith and W. Kirkpatrick, eds.). Academic Press, New York, 119–128 (1980).
25. Vogt, M. W., K. L. Hartshorn, P. A. Furman, T.-C. Chou, J. A. Fyfe, L. A. Coleman, C. Crumpacker, R. T. Schooley, and M. S. Hirsch. Ribavirin antagonizes the effect of azidothymidine on HIV replication. *Science (Washington D. C.)* **235**:1376–1379 (1987).
26. Ford, H., Jr., D. A. Cooney, G. S. Ahluwalia, Z. Hao, M. E. Rommel, L. Hicks, K. A. Dobyns, J. E. Tomaszewski, and D. G. Johns. Cellular pharmacology of cyclopentenylcytosine in MOLT-4 lymphoblasts. *Cancer Res.* **51**:3733–3740 (1991).

Send reprint requests to: Riad Agbaria, Building 37, Room 5B22, National Cancer Institute, NIH, Bethesda, MD 20892.