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Demonstration of Viral Thymidine Kinase Inhibitor and Its Effect on Deoxynucleotide Metabolism in Cells Infected with Herpes Simplex Virus

LOUISE M. NUTTER,[1] SUSAN P. GRILL,[1] GINGER E. DUTSCHMAN,[1] RAM A. SHARMA,[2] MIROSLAV BOBEK,[2] AND YUNG-CHI CHENG[1]*

Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514,[1] and Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14263[2]

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The thymidine analog 5'-ethynylthymidine was a potent inhibitor of herpes simplex virus type 1 (strain KOS)-induced thymidine kinase with a K_i value of 0.09 μM. 5'-Ethynylthymidine was less inhibitory against herpes simplex virus type 2 (strain 333)-induced thymidine kinase with a K_i of 0.38 μM and showed no inhibition against human cytosolic thymidine kinase under the conditions tested. The compound was effective against the altered thymidine kinase induced by acyclovir- and bromovinyldeoxyuridine-resistant virus variants. At 100 μM 5'-ethynylthymidine, the cellular pool size of dTTP in herpes simplex virus type 1-infected cells was 5% that of infected cells receiving no drug treatment, while there was no significant effect on the pool sizes of dATP, dGTP, and dCTP. There was a positive correlation between dTTP pools and the intracellular thymidine kinase activity of herpes simplex virus type 1-infected cells. When tested alone, 5'-ethynylthymidine exhibited no antiviral activity, but it antagonized the antiviral efficacy of five compounds which require viral thymidine kinase for their action.

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) and varicella-zoster virus induce unique virus-specified thymidine kinases (dThd kinase) in infected cells (2, 8, 18, 20). In the past, major efforts were made to develop antiviral compounds that would serve as "selective alternative substrates" (3) for virus-specified dThd kinase. While the activity of viral dThd kinase does not appear to be critical for virus replication in cell culture systems (19), studies have suggested that it is important for virus pathogenicity and reactivation of latent virus from neural cells (9, 14, 30). In this report, we describe the effects of 5'-ethynylthymidine (5'-Et-dThd) (Fig. 1) as a selective inhibitor for HSV dThd kinase and its impact on deoxynucleotide metabolism in virus-infected cells.

MATERIALS AND METHODS

Materials. All chemicals used were reagent grade or better. ^{3}H-labeled deoxynucleoside 5'-triphosphates (dNTPs) were purchased from ICN Radiochemicals, Irvine, Calif. dThd, dNTPs, and calf thymus DNA were purchased from Sigma Chemical Co., St. Louis, Mo. DNA polymerase I (endonuclease-free) and hydroxyurea were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. RPMI 1640 medium, fetal bovine serum, and kanamycin were purchased from Hazleton Research Products, Inc., Denver, Pa. 5'-Et-dThd was synthesized by published procedures (29). Acyclovir (ACV) was a gift from Burroughs Wellcome Co., Research Triangle Park, N.C., and 9-(1,3-dihydroxy-2-propoxy-methyl)guanine (DHPG) was from Syntex Co. 5'-Amino-dThd (5'-NH_2-dThd) was provided by William Prusoff, Yale University, New Haven, Conn. Fluoroiodo-arabinosyl-cytosine (FIAC) and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) were gifts from J. J. Fox (Sloan-Kettering Cancer Institute) and G. D. Searle & Co., respectively.

* Corresponding author.

Cells. The cells used in this study were HeLa S3, HeLa BU (dThd kinase deficient), Vero, and KB6B (13). The cells were maintained at 37°C in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg of kanamycin per ml. All cultures were found to be mycoplasma-free by the Bethesda Research Laboratories Mycotect (24) and 4,6-diamidino-2-phenylindole fluorescence (28) techniques.

Virus stocks. The viruses used in this study were HSV-1 (strains KOS, SC16, B3, and Tr7) and HSV-2 (strain 333). The HSV SC16 parental strain and dThd kinase mutants B3 and Tr7, which were isolated in the presence of BVDU at 10 μg/ml and ACV at 1 μg/ml, respectively (9, 15), were gifts from G. Darby and H. Field (Cambridge University, Cambridge, United Kingdom). HSV-1 or HSV-2 was added at a low multiplicity of infection (0.01 PFU per cell) to confluent Vero monolayers. After a 1-h adsorption period, 30 ml of RPMI 1640 supplemented with 2% fetal bovine serum was added. The cells were then incubated at 37°C for 24 h followed by incubation at 34°C for 48 h (HSV-1) or 24 h (HSV-2). At the end of the incubation period the cells were suspended and frozen at −70°C. Before use the virus stocks were sonicated with two 10-s bursts on a Branson Sonifier with a cup horn adapter to retain sterility. The suspension was centrifuged for 10 min at 1,200 × *g* to remove the remaining cells. The medium containing virus was then aliquoted into sterile tubes and stored at −70°C.

Cell and virus growth inhibition. Cell growth inhibition and the virus yield assay were performed as previously described (4) with the exceptions that all studies were done with 5% dialyzed fetal bovine serum; the multiplicity of infection was 3 PFU per cell, and Vero cells were used instead of CV-1 cells for titration in the virus yield assays.

dNTP pool assay. dNTP pool measurements were done as previously described (31). 5'-Et-dThd alone had no effect on the dNTP pool assay.

Enzyme purification, enzyme assays, IC_{50}, and K_i determinations. The cellular cytosolic dThd kinase used in studies to

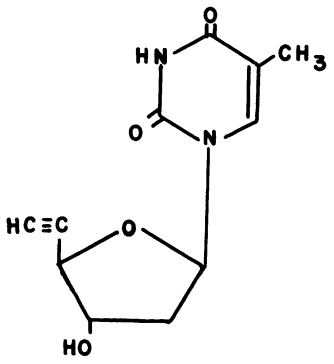

FIG. 1. Structure of 5'-Et-dThd.

determine the K_i values was isolated from KB6B cells. The HSV-1 and HSV-2 dThd kinases were extracted from dThd kinase-deficient HeLa BU cells infected with HSV-1 (strain KOS) and HSV-2 (strain 333), respectively. All these dThd kinases were purified by affinity column chromatography procedures as described previously (22). The dThd kinase assay was the same as that described previously (5). Incubations were conducted at 37°C for 60 min, and the K_i and inhibitor concentration for 50% inhibition of enzyme activity (IC_{50}) values were determined as described previously (6).

The thymidylate kinase and synthase assays were performed as described previously (7, 12, 27). dThd phosphorylase activity was determined by monitoring conversion of [^{14}C]dThd to [^{14}C]thymine by high-performance liquid chromatography. Briefly, after a 30-min incubation of enzyme extract with radiolabeled dThd at 37°C, the reaction was stopped by the addition of 25% trichloroacetic acid. After neutralization and centrifugation, the supernatants were loaded onto an Alltech Lichrosorb RP-18 column, and the column was eluted at a rate of 1 ml/min with 5 mM KH_2PO_4 (pH 3.5)–10% methanol. Fractions of 1 ml were collected, and radioactivity associated with dThd and thymine (as previously determined by retention times of authentic markers measured by their A_{265}) was measured by scintillation counting. For evaluation of whether 5'-Et-dThd could serve as a substrate for dThd phosphorylase, reactions were done as described above, except that 5'-Et-dThd was used instead of [^{14}C]dThd; metabolites were then analyzed by high-performance liquid chromatography as described above.

Intracellular dThd kinase activity. HeLa S3 or HeLa BU cells (1.5×10^6/ml) were mock infected or infected with HSV-1 or HSV-2 at 3 PFU per cell and incubated at 37°C for a 1-h adsorption period. The medium was then removed, and the cells were washed with growth medium and resuspended at a cell density of 5×10^5/ml in RPMI 1640 medium supplemented with kanamycin (100 μg/ml) and 5% dialyzed fetal bovine serum. At 9 h post-HSV infection, [^{14}C]dThd (0.16 μCi/ml; 1 μM dThd) and different amounts of 5'-Et-dThd were added to the medium depending on experimental conditions. At the end of the labeling period (1 h), the cells were pelleted by centrifugation, washed with phosphate-buffered-saline, and resuspended in a small volume of phosphate-buffered saline. Samples were then pipetted directly onto DE81 disks which were immediately washed in 100% ethanol. The DE81 disks were washed two additional times in 100% ethanol, and then radioactivity associated with them was measured by scintillation counting in 5 ml of aqueous counting fluid. In this manner, total intracellular dThd kinase activity was determined from the sum of radioactive dThd nucleotides plus the dTMP associated with DNA both precipitated directly on the DE81 disks.

RESULTS

Activity of 5'-Et-dThd against viral and cellular dThd kinase activities in vitro and in cell culture. HSV-1- and HSV-2-induced dThd kinases that are different from those of their host cellular counterparts (2) were examined for their sensitivity to 5'-Et-dThd. The activity of 5'-Et-dThd against purified HSV-1 (KOS), HSV-2 (333), and human cytosolic dThd kinases at a concentration of 100 μM [^{14}C]dThd in the assay mixture is shown in Fig. 2A. At 10 μM, 5'-Et-dThd inhibited 50 and 25%, respectively, of HSV-1 and HSV-2 dThd kinase activities. Furthermore, 5'-Et-dThd was a potent inhibitor of HSV-1 (strain KOS) dThd kinase-associated

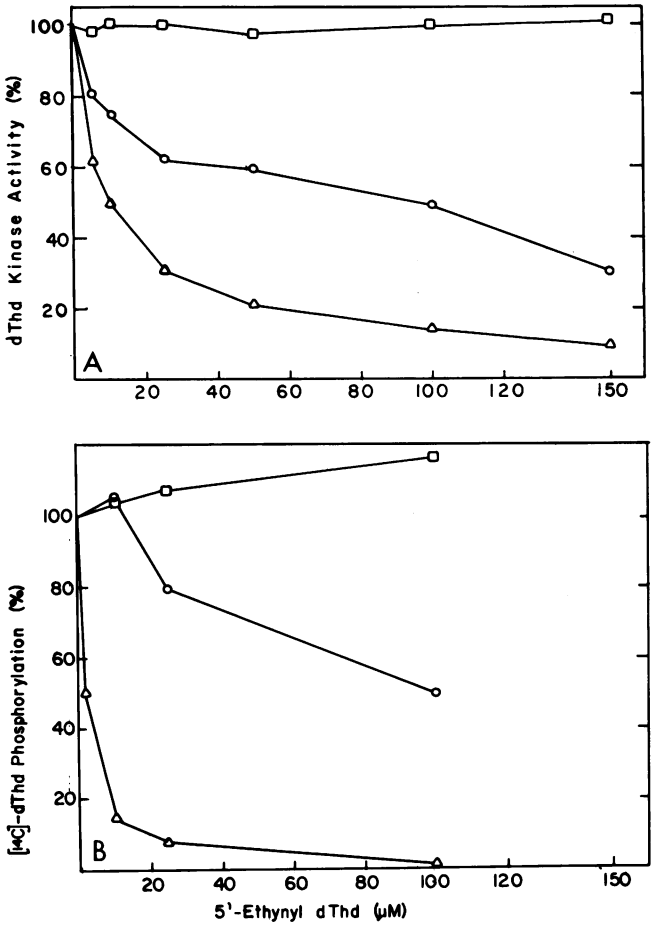

FIG. 2. Effect of 5'-Et-dThd on in vitro dThd kinase activity and intracellular dThd phosphorylation. (A) Affinity column-purified human cytosolic (□), HSV-1 (KOS) (△), and HSV-2 (333) (○) dThd kinases were examined for their sensitivity to 5'-Et-dThd as described in Materials and Methods. The 100% dThd kinase activity values for human cytosolic, HSV-1, and HSV-2 enzymes were 9.4×10^{-3}, 23×10^{-3}, and 21×10^{-3} units of enzyme, respectively. (B) The effect of 5'-Et-dThd on intracellular dThd phosphorylation was measured in HSV-1 (KOS) (△)-, HSV-2 (333) (○)-, or mock (□)-infected cells. HeLa BU cells were the host cells for HSV-1 and HSV-2 9-h infections, and HeLa S3 was the host for the mock infection. The 100% dThd phosphorylation values for HeLa BU cells infected with HSV-1 and HSV-2 and mock-infected HeLa S3 cells were 33,000, 20,000 and 10,667 cpm/10^6 cells, respectively. For details see the text.

dTMP kinase activity with IC_{50} values of 0.3 and 0.8 μM, respectively, when dTMP substrate concentrations of 25 and 100 μM were used in the assay (Table 1). No demonstrable effects on purified human cytosolic dThd kinase were observed when concentrations of 5′-Et-dThd as high as 150 μM were used under identical assay conditions (Fig. 2A). Results from kinetic studies showed that 5′-Et-dThd was a competitive inhibitor with respect to dThd, and K_i values were estimated to be 0.09 ± 0.02 and 0.38 ± 0.18 μM for HSV-1 (strain KOS) and HSV-2 (strain 333) dThd kinase, respectively (Table 1). 5′-Et-dThd did not serve as a substrate for viral dThd kinase as determined by the phosphate transfer assay with dThd kinase from HSV-1 (KOS)-infected HeLa BU cells as an enzyme source and 2 mM ATP (11).

The effects of 5′-Et-dThd on intracellular dThd kinase activities of HSV-1- and HSV-2-infected HeLa BU cells and on mock-infected HeLa S3 cells at 9 h postinfection were examined (Fig. 2B). At 100 μM, 5′-Et-dThd inhibited 97% of HSV-1 dThd kinase-mediated [^{14}C]dThd phosphorylation, but only 50% of that mediated by HSV-2 dThd kinase in HSV-2-infected HeLa BU cells. Thus, differences in the potency of 5′-Et-dThd against HSV-1- and HSV-2-induced dThd kinases in vitro and in cell culture are clearly demonstrated. The accumulation of intracellular phosphorylated [^{14}C]dThd metabolites was increased slightly in mock-infected HeLa S3 cells treated with increasing amounts of 5′-Et-dThd (Fig. 2B).

The role of host dThd kinase in virus-infected cells was explored by comparing the effect of 5′-Et-dThd (25 μM) on intracellular dThd phosphorylation in HeLa S3 and HeLa BU cells at 9 h postinfection by the procedures described above. After a 1-h labeling period in the presence of drug, 12.5 and 80% of the untreated control dThd-phosphorylating activities were present in HeLa BU cells infected with HSV-1 and HSV-2, respectively (Fig. 3A and B). When HeLa S3 cells were used as the host cells for infection, these values were increased to 25 and 88%, respectively (Fig. 3C and D). Thus, the host dThd kinase activity in virus-infected cells was still functional at 9 h postinfection.

Activity of 5′-Et-dThd against dThd kinase activities induced by different types and strains of HSV. Virus mutants which induce viral dThd kinases that exhibit altered substrate specificities (dThd kinase^{A}) and are resistant to selective antiviral agents have been isolated (21). The effect of 5′-Et-dThd on the in vitro and intracellular dThd kinase activities of two such dThd kinase variants, Tr7 and B3 (ACV and BVDU resistant, respectively) was examined, together with their parental HSV-1 strain (SC16). The results are shown in Table 1. The dThd kinase activities of these dThd kinase mutants, whose induced dThd kinase levels are similar to those of the parent SC16 strain (21), were sensitive to 5′-Et-dThd, with IC_{50} values of less than 10 μM both in vitro and in cell culture.

TABLE 1. Effect of 5′-Et-dThd on viral dThd kinase, viral dThd kinase-associated dTMP kinase, and host dTMP kinase activities[a]

Virus type and strain	IC_{50} of dThd kinase (μM)		K_i[b]	IC_{50} of virus-associated dTMP kinase (μM)	
	Intracellular[c]	In vitro[d]		25 μM[e]	100 μM[e]
HSV-1					
KOS	3	6	0.09 ± 0.02 (49)	0.3	0.8
SC16	5	5			
Tr7	2	3			
B3	6	7			
HSV-2 (333)	100	46	0.38 ± 0.18 (23)	60.0[f]	

[a] All studies were performed with HeLa BU cells.
[b] K_i determinations were performed with affinity column-purified dThd kinase; numbers in parentheses reflect number of determinations for K_i ± standard deviation.
[c] Intracellular [^{14}C]dThd phosphorylation.
[d] IC_{50} determinations were performed with enzyme extracts of cells infected with designated virus; dThd concentration was 100 μM in the assay.
[e] dTMP substrate concentrations used in assay; crude extracts were used for the assay.
[f] The activity of HSV-2 (333)-infected HeLa BU cells reflects host dTMP kinase activity.

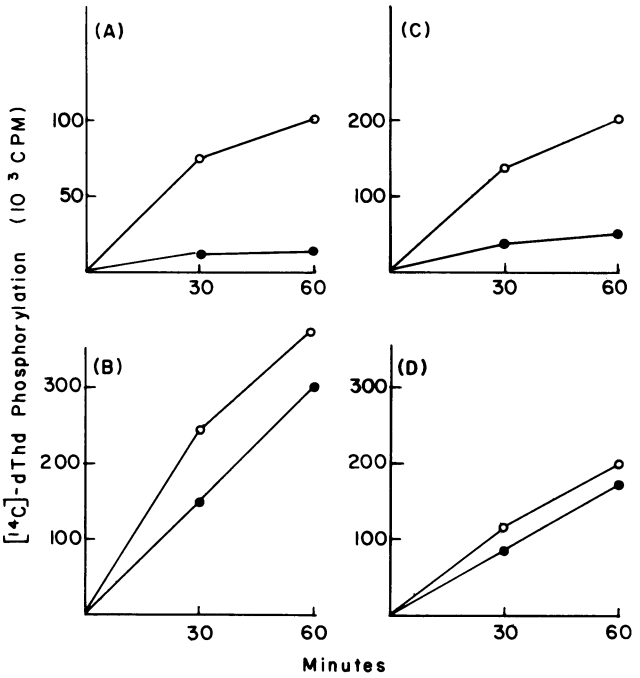

FIG. 3. Role of host dThd kinase in HSV-1 (KOS)- and HSV-2 (333)-infected cells. Intracellular dThd phosphorylation was evaluated in HeLa BU cells infected for 9 h with HSV-1 (A) or HSV-2 (B) and in HeLa S3 cells infected with HSV-1 (C) or HSV-2 (D) in the absence (○) or presence (●) of 25 μM 5′-Et-dThd. For details, see the text.

Interaction of 5′-Et-dThd with other dThd-metabolizing enzymes. The effect of 5′-Et-dThd against human dTMP synthase was examined. At 100 μM 5′-Et-dThd, no effect on dTMP synthase from HSV-1-infected HeLa BU cells was observed when 25 μM dUMP substrate and 150 μM 5,10-methylene-tetrahydrofolate were used in the enzyme assay. Furthermore, 5′-Et-dThd was neither a substrate nor an inhibitor of dThd phosphorylase. Specifically, no thymine product was detected with 735 μM 5′-Et-dThd substrate with 0.26 or 21.5 U of dThd phosphorylase from HSV-1 (strain KOS)-infected cells or human liver, respectively. Similarly, 150 μM 5′-Et-dThd did not inhibit the phosphorylase reaction with 600 μM dThd as the substrate for the same units of activity described above. In terms of the interaction of 5′-Et-dThd with purified human chronic lymphocytic leukemia dTMP kinase, the IC_{50} was 37.5 μM when 25 μM dTMP was used in the enzyme assay (data not shown).

Effect of 5′-Et-dThd on dNTP pools in HSV-1-infected cells. In view of the potent inhibition of 5′-Et-dThd on HSV-1 dThd kinase and its associated dTMP kinase, we examined

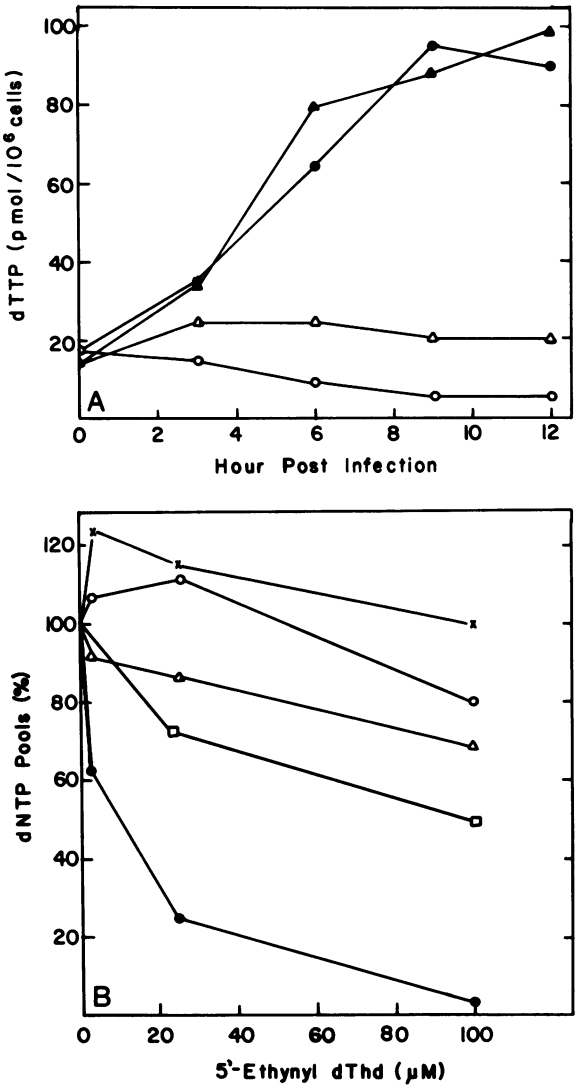

FIG. 4. Effect of 5'-Et-dThd on dNTP pools in HSV-1-infected cells. (A) dTTP pools were measured in acid-soluble extracts from HSV-1-infected HeLa BU cells (●, ○) and HSV-1-infected HeLa S3 cells (▲, △) in the absence (solid symbols) or presence (open symbols) of 100 μM 5'-Et-dThd. (B) The effect of 5'-Et-dThd on dCTP (○), dGTP (△), dATP (×), and dTTP (●) pools in HeLa BU cells infected for 9 h with HSV-1 (KOS) and on dTTP pools in HeLa BU cells infected with a dThd kinase-dTMP kinase-deficient HSV-1 strain (MDK) (□) was examined. The 100% values for dCTP, dGTP, dATP, and dTTP pools in HSV-1 (KOS)-infected HeLa BU cells were 64, 65, 75, and 159 μM, respectively, and the dTTP pool of MDK-infected HeLa BU cells was 70 μM.

the effect of 5'-Et-dThd on dNTP pools in HSV-1-infected cells. At 12 h postinfection, the dTTP pool of HeLa BU cells treated with 100 μM 5'-Et-dThd was reduced by 95% as compared with that of untreated infection control cells (Fig. 4A). When HeLa S3 cells were used as the host cell for infection, the dTTP pool was reduced by 80% as compared with that of untreated infected control cells (Fig. 4A). This difference could be related to the activity of host dThd kinase in HeLa S3 cells. When other dNTP pools in the virus-infected cells were examined, no significant effect of 5'-Et-dThd on dATP, dCTP, and dGTP pools in 9-h HSV-1 (KOS)-infected HeLa BU cells was observed (Fig. 4B). Thus, the possibility that ribonucleotide reductase is inhibited by 5'-Et-dThd is not likely.

The decrease of dTTP pools in virus-infected cells caused by 5'-Et-dThd could be due to inhibition of viral dThd kinase or its intrinsic dTMP kinase. To explore whether inhibition of this intrinsic dTMP kinase activity (which constitutes 10% of the total dTMP kinase activity in 9-h-infected cells) is sufficient to explain these results, we examined the impact of 5'-Et-dThd on dTTP pools of HSV-1 (strain MDK)-infected HeLa BU cells; the MDK strain of HSV-1 is unable to induce viral dThd kinase or its intrinsic dTMP kinase. At 100 μM 5'-Et-dThd, there was only 45% inhibition of dTTP pools (Fig. 4B). This suggested that the activity of host dTMP kinase in infected cells, even though partly inhibited by 5'-Et-dThd, is sufficient enough to maintain partially the dTTP pool, and the inhibition of the viral dThd kinase-associated dTMP kinase is not a major factor in decreasing dTTP pools in 5'-Et-dThd-treated cells.

Correlation of viral dThd kinase inhibition and dTTP pools. The impact of viral dThd kinase inhibition on dTTP pools in infected cells is demonstrated in Fig. 4 and suggests that viral dThd kinase activity is important for maintenance of this pool. We investigated this further by examining the correlation of viral dThd kinase activity with dTTP pool levels in HSV-1 (strain KOS)-infected cells (Fig. 5). In this study, dThd kinase activity and dTTP pool levels were measured at 9 h postinfection of HeLa S3 or HeLa BU cells with HSV-1 (strain KOS) in the presence of different concentrations of 5'-Et-dThd. When the results were plotted, a positive correlation was found between dThd kinase activity and dTTP levels in the infected cells. When the host dThd

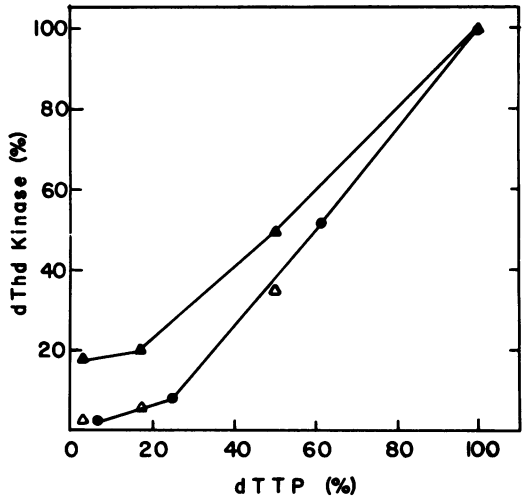

FIG. 5. Correlation of intracellular dThd kinase activity with dTTP pools. Intracellular dThd kinase activity and dTTP pools were measured in HeLa S3 (▲) and HeLa BU (●) cells infected with HSV-1 (KOS) for 9 h in the presence of different concentrations of 5'-Et-dThd. The contribution of host dThd kinase activity (15%) was subtracted from total dThd kinase activity, and the resulting values were plotted versus the dTTP pool (△). All values are depicted as the percentage of the control dThd kinase activity and dTTP pool measurements in the absence of 5'-Et-dThd (100%). The concentrations of 5'-Et-dThd used for studies with HSV-1-infected HeLa S3 cells were 0, 2.5, 25, and 100 μM, and those for HSV-1-infected HeLa BU cells were 0, 2, 10, and 100 μM. The 100% values for the dTTP pools in HSV-1-infected HeLa S3 and HeLa BU cells were 56 and 40 μM, respectively. The 100% intracellular dThd kinase activity values for HSV-1- and HSV-2-infected HeLa BU cells were 90,000 and 50,000 cpm/10^6 cells, respectively.

kinase activity contributed by HeLa S3 cells was subtracted from the total dThd kinase activity and replotted against the dTTP level, the result was superimposable on that obtained from dThd kinase-deficient infected cells (Fig. 5). These results suggest that viral dThd kinase activity is of major importance in dTTP pool maintenance in HSV-1-infected cells. The presence of a significant dTTP pool in the dThd kinase strain (MDK) of HSV-1-infected cells (Fig. 4) was not taken into consideration for this conclusion since this strain could have more than one mutation in addition to not being able to induce viral dThd kinase in infected cells.

Effects of 5'-Et-dThd on antiviral activity of several agents. By itself, 5'-Et-dThd had no effect on virus yield (Table 2) and on virus DNA synthesis as determined from isopycnic centrifugation studies in which $^{32}PO_4$-labeled virus DNA was separated from host DNA (L. M. Nutter, S. P. Grill, and Y.-C. Cheng, unpublished observation). Nucleoside analogs have been developed based on the "selective alternate substrate" strategy (3) and depend on viral dThd kinase for their antiviral activity. We examined the effect of 5'-Et-dThd on the antiviral efficacy of five such analogs, ACV, DHPG, FIAC, BVDU, and 5'-NH_2-dThd. The effect of 5'-Et-dThd on the activity of the five agents was determined by the virus yield assay with HSV-1 (strain KOS)-infected HeLa BU cells. At 100 μM, 5'-Et-dThd reversed the antiviral activity of ACV (15 μM), DHPG (2 μM), FIAC (1 μM), BVDU (1 μM), and 5'-NH_2-dThd (500 μM) 35-, 100-, 98-, 15-, and 250-fold, respectively (Table 2). Results from the present study suggest that viral dThd kinase is important for dTTP pool maintenance in infected cells (Fig. 4 and 5). Therefore, we evaluated the combined effects of inhibition of viral dThd kinase and ribonucleotide reductase activities on virus replication. The results of this study are depicted in Table 2 and show that there was no significant effect of 5'-Et-dThd on the antiviral activity of hydroxyurea.

TABLE 2. Effect of 5'-Et-dThd on activity of antiviral agents[a]

Drug (μM)	% PFU with a 5'-Et-dThd concn (μM) of:			
	0	2.5	25	100
None	100	97	96	99
ACV (15)	2	9	45	70
FIAC (1)	0.6		26	59
DHPG (2)	0.1	0.2	4	10
BVDU (1)	0.9		9.5	13
5'-NH_2-dThd (500)	0.2	0.3	3	50
Hydroxyurea (1 mM)	15	19	14	10

[a] The antiviral activities of several agents in the presence and absence of 5'-Et-dThd were determined by the virus yield assay with HSV-1 (KOS)-infected cells.

DISCUSSION

We demonstrated that 5'-Et-dThd is a selective inhibitor of HSV-induced dThd kinase and has no effect on human cytosolic dThd kinase. 5'-Et-dThd is more potent against HSV-1 than HSV-2 dThd kinase in vitro and in cell culture. The differential activity of 5'-Et-dThd against type 1 and type 2 dThd kinases appears to be due to a decreased affinity of the compound for type 2 dThd kinase as reflected by their K_i values (HSV-1 K_i, 0.09 μM; and HSV-2 K_i, 0.38 μM; Table 1).

It is possible that some metabolism of 5'-Et-dThd occurs inside cells. The increase of dThd phosphorylation in uninfected cells treated with 5'-Et-dThd (Fig. 2) may be due to the catabolism of 5'-Et-dThd by degradative enzymes (e.g., dThd phosphorylase). In this manner, 5'-Et-dThd could protect dThd nucleotides from these degradative activities. Alternatively, it cannot be ruled out that a decreased efflux of dThd from uninfected cells occurred in the presence of 5'-Et-dThd. Our results suggest that 5'-Et-dThd is not metabolized by dThd phosphorylase. However, possible effects of this compound on dThd nucleotide efflux or intracellular phosphatases were not ruled out. 5'-Et-dThd could not be phosphorylated by viral dThd kinase. Thus, possible mutagenic effects caused by metabolism and subsequent incorporation of 5'-Et-dThd into cellular DNA are not anticipated. Furthermore, 5'-Et-dThd (100 μM) had no effect on host (HeLa BU) DNA synthesis as measured by $^{32}PO_4$ incorporation studies, nor did it inhibit HeLa BU or HeLa S3 cell growth.

The amount of phosphorylated dThd nucleotides in HSV-1-infected HeLa S3 cells was higher than that in HeLa BU cells, owing to the fact that host and viral dThd kinase can utilize dThd as a substrate. The dThd phosphorylation in virus-harboring, resting-nondividing cells with diminished host dThd kinase activities would, therefore, have increased susceptibility to 5'-Et-dThd relative to that in a dividing cell. Thus, utility for such a compound against the viral dThd kinase activity in a neural cell can be envisaged, which could be critical for the viral reactivation process.

Virus variants which induce dThd kinase activities with altered substrate specificities (dThd kinase^A) and are resistant to antiviral agents such as ACV have been isolated in the laboratory (21) and, recently, from clinic samples (23, 25). We examined whether 5'-Et-dThd could inhibit the dThd kinase induced by the virus variants Tr7 and B3 (ACV and BVDU resistant, respectively) and found that 5'-Et-dThd was an efficient inhibitor of these dThd kinase^A activities in vitro and in cell culture (Table 1). Thus, a potential use of 5'-Et-dThd for inhibition of viral dThd kinase activity, whether of a wild-type or altered substrate phenotype, is suggested.

Treatment of HSV-1-infected HeLa BU cells with 5'-Et-dThd (100 μM) resulted in a 95% decrease of the dTTP pool, whereas dCTP, dATP, and dGTP pools were not significantly altered (Fig. 4). The effect of 5'-Et-dThd on dTTP pool levels was more pronounced in HeLa BU than in HeLa S3 cells which is consistent with the pattern observed for intracellular dThd phosphorylation (Fig. 3), suggesting a role of host dThd kinase in dThd phosphorylation in virus-infected cells. When the relationship of intracellular dThd kinase activity and dTTP pools of infected cells was examined, a good correlation was found (Fig. 5). The differences in dTTP pool levels of HeLa S3 and HeLa BU cells were correlated with their dThd kinase activities. Since no effects of 5'-Et-dThd were found on the de novo synthesis of dTTP (i.e., dTMP synthase and ribonucleotide reductase), whereas significant inhibition of viral dThd kinase was observed, it is suggested that viral dThd kinase plays a major role in the maintenance of dTTP pools in virus-infected cells. Previously, we showed that approximately 30% of the dTTP pool was derived from host DNA breakdown and that the remainder was from de novo sources in HSV-2-infected cells (26). A role for dThd kinase in the formation of dTTP originating from host DNA breakdown was postulated. The results of the present study with HSV-1 suggest that viral dThd kinase, possessing dThd kinase and dTMP kinase activities, is very important in dTTP pool regulation, regardless of the dTTP origin (e.g., de novo or host DNA breakdown). Viral dThd kinase but not its associated dTMP kinase activity is most important in this dTTP pool maintenance.

However, it is not known whether dThd nucleotide derived from de novo synthesis and host DNA is excreted from the cell and phosphorylated after reentry or whether thymidine so derived is phosphorylated directly inside the cell, or both.

It should be pointed out that 5'-Et-dThd may have other effects intracellularly, which could also result in decreased dTTP pools (e.g., dTMP synthase, nucleotide phosphatase, nucleoside diphosphate kinase). We do not favor such a possibility. This is based on the following observations. We did not observe any effects of 5'-Et-dThd on dTMP synthase activity in vitro, and effects of this compound on phosphatases and nucleoside diphosphate kinase activities are unlikely since results from this study show that other dNTP pools in HSV-1-infected cells were not significantly affected by 5'-Et-dThd.

The remainder of the dTTP pool (approximately 5 μM) present in HSV-1-infected HeLa BU cells treated with 100 μM 5'-Et-dThd could be derived from host dTMP kinase activity. This level of dTTP is apparently able to sustain HSV-1 DNA synthesis, with no adverse effects on virus replication (Table 2). Thus, extensive depletion of dTTP pools, below the dTTP K_m for virus polymerase (ca. 0.45 μM [17]) is probably required before antiviral effects are seen.

Alone, 5'-Et-dThd exhibited no antiviral activity in HSV-1-infected HeLa BU cells, which is in accordance with the observation that viral dThd kinase is not critical for replication of HSV in cell culture systems. 5'-Et-dThd reversed the antiviral efficacy of ACV, DHPG, FIAC, BVDU, and 5'-NH_2-dThd, all of which require viral dThd kinase for their activity. It was anticipated that the diminished dTTP pools in HSV-1-infected cells caused by 5'-Et-dThd would augment the antiviral action of the phosphorylated forms of 5'-NH_2-dThd and BVDU, the latter which could compete with dTTP for virus polymerase; this was not the case. Thus, appreciable levels of phosphorylated 5'-NH_2-dThd or BVDU derivatives apparently did not form in HSV-1-infected cells in the presence of 5'-Et-dThd. The antiviral actions of FIAC and the guanosine analogs ACV and DHPG, whose phosphorylated derivatives compete against dCTP and dGTP, respectively, for HSV polymerase (1, 10, 16), were reversed by 5'-Et-dThd. Since 5'-Et-dThd significantly decreases the dTTP pools of infected cells, this compound could be very beneficial in combination with viral dThd kinase-independent agents that must compete with dTTP for virus polymerase.

In summary, we showed that 5'-Et-dThd is a potent inhibitor of HSV dThd kinase. Mutagenic effects of 5'-Et-dThd owing to its incorporation into host DNA are not anticipated, nor does 5'-Et-dThd have significant cytotoxicity in uninfected human cells. A critical role for viral dThd kinase in dTTP pool maintenance in virus-infected cells was postulated.

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