L-Thymidine Is Phosphorylated by Herpes Simplex Virus Type 1 Thymidine Kinase and Inhibits Viral Growth

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We have demonstrated that herpes simplex 1 (HSV1) thymidine kinase (TK) shows no stereospecificity for D- and L-β-nucleosides. In vitro, L enantiomers are not recognized by human TK, but function as specific substrates for the viral enzyme in the order: L-thymidine (L-T) ≫ 2′-deoxy-L-guanosine (L-dG) > 2′-deoxy-L-uridine (L-dU) > 2′-deoxy-L-cytidine (L-dC) > 2′-deoxy-L-adenosine (L-dA). HSV1 TK phosphorylates both thymidine enantiomers to their corresponding monophosphates with identical efficiency and the K_i of L-T (2 μM) is almost identical to the K_m for the natural substrate D-T (2.8 μM). The L enantiomer reduces the incorporation of exogenous [^{3}H]T into cellular DNA in HeLa TK$^-$/HSV1 TK$^+$ but not in wild-type HeLa cells, without affecting RNA, protein synthesis, cell growth, and viability. L-T markedly reduces HSV1 multiplication in HeLa cells. Our observations could lead to the development of a novel class of antiviral drugs characterized by low toxicity.

Introduction

Viruses, contrary to bacteria and parasites, meet their metabolic needs by using both their host's and their own enzymes. The fact that the viral enzymes are often quite similar to their cellular equivalents is one of the reasons that makes the development of effective, nontoxic drugs against viral infections a rather challenging task.

One way to overcome this problem has been to identify, within viral enzymes, those which have properties not shared by the host counterparts. These enzymes could then be specific targets for chemicals which impair the viral replication without harming the infected organism.

In the case of herpes simplex virus this strategy has lead to the development of several nucleoside analogues, such as idoxouridine,[1] (bromovinyl)deoxyuridine (BVDU),[2] and acyclovir,[3] whose remarkable antiviral activity is ultimately due to a specific interaction of their 5′-triphosphates with the viral DNA polymerase.[4,5] Another herpes enzyme which is the target of the same type of investigation is thymidine kinase (TK), because, contrary to the cellular enzyme, it phosphorylates also several pyrimidine and purine nucleotide analogues. In fact, all the previously mentioned antiherpetic agents depend on this enzyme for the first two steps of their conversion into the active triphosphate form and therefore act as substrate inhibitors of the viral TK.

More recently several potent nonsubstrate inhibitors of this enzyme have been found.[6-11] Those described by some of us in vivo significantly diminished the frequency of reactivation of HSV1 from explant cultures of latently infected murine trigeminal ganglia,[12] suggesting that viral TK is required for viral reactivation.

However the therapeutic potential of most of the above compounds, in particular of those acting as substrate for the viral TK, is often hampered by variable degrees of cytotoxicity to host cells.

From the point of view of their chemical structure most inhibitors of thymidine kinase and of HSV replication/

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reactivation can be grouped into two main categories. One includes analogues of deoxyuridine, with modifications at the 5-position of the pyrimidine ring and/or the 5'-OH group of the sugar mojety. The second includes guanines and 2'-deoxyguanosines carrying different substituents on the amino group or guanine derivatives with an acyclic side chain at the 9-position.

Less attention has been given to alternative substrates where the 2'-deoxy-D-ribosyl group is replaced by other sugar moieties. Recently, α,β-D-xylo- and -lyxofuranosyl nucleosides have been evaluated for their activity against RNA and DNA viruses, including HSV1 and -2, with interesting results.[13] In fact, it appears that some viral enzymes can process analogues of their natural substrates with an inverted configuration at a chiral center close to the reactive site. However, to the best of our knowledge, no investigation has been done on the antiviral activity of the enantiomers of the natural nucleosides, which have an inverted configuration at all chiral centers. If cellular and viral enzymes greatly differ in their stereoselectivity for the substrate, a better therapeutic index could be foreseen for nucleoside analogues where effective modifications of the base moiety are associated with the presence of a sugar of unnatural configuration, namely 2'-deoxy-L-ribose.

As a preliminary test of this hypothesis we have studied the interaction of HSV-1 and -2 thymidine kinases with L-thymidine and other enantiomers of natural 2'-deoxy-D-nucleosides.

In the past, a few studies have been reported on the biological activity of L-nucleosides and nucleotides—like interactions with bacterial polynucleotide phosphorylases and nucleolytic enzymes.[14,15] The metabolism of pyrimidine L-nucleosides in mice has been the subject of a very detailed investigation showing that they are not, or very poorly, metabolized.[16]

Nowadays, 2'-deoxy-L-nucleosides are also taken into consideration[17-20] as building units of nuclease-resistant antisense[21] oligonucleotides, potentially useful for therapeutic purposes.

Results

Chemistry. The five 2'-deoxy-L-nucleosides, whose activity has been tested in the present study, are all known compounds, and the strategy we have followed for their preparation is summarized in the Scheme I. A convenient synthesis of the pyrimidine derivatives L-dU (**2**), L-T (**3**), and L-dC (**4**) has been reported by Holy[22] starting from the common intermediate 3',5'-di-*O*-benzoyl-2'-deoxy-L-uridine (**1**), which was prepared on a large scale from L-arabinose, following a five-step procedure.[22]

Accordingly, compounds **2** and **3** were prepared as described,[22] while compound **4** was obtained in two steps from **1**, applying the chemistry developed more recently by Sung[23] for the natural D enantiomer.

Instead, the method reported in literature[24] for the synthesis of the purine nucleosides L-dA **6** and L-dG **8** is rather unsatisfactory, since it involves many steps and affords the final products in few percent yield.

Thus, we investigated the possibility of using compound **1** as the common chiral pool also for the synthesis of **6** and **8**. In fact, as shown in the scheme, the acid-catalyzed transglycosylation reaction[25] between **1** and N^6-benzoyladenine or N^2-palmitoylguanine in the presence of a silylating agent proceeded with good chemical yield, giving a mixture of two (**5a,b**) and four (**7a–d**) isomers, respectively. Chromatography allowed the separation of **5a** and **7a**, which were identified by comparison (TLC and ^{1}H NMR) with the corresponding D enantiomers, prepared from the natural nucleosides by established procedures. Acid-catalyzed "equilibration" of the recovered **5b** and **7b–d**, under the same conditions used for the transglycosylation reaction, afforded a further amount of **5a** and **7a**. 3',5'-Di-*O*-benzoyl-2'-deoxy-L-N^6-benzoyladenosine (**5a**) and 3',5'-di-*O*-benzoyl-2'-deoxy-L-N^2-palmitoylguanosine (**7a**) were obtained in 35% and 24% overall yield, respectively, and converted into 2'-deoxy-L-adenosine (**6**) and 2'-deoxy-L-guanosine (**8**), by removal of the protective groups under basic conditions.

The purity of the 2'-deoxy-L-nucleosides **2–4**, **6**, and **8**, whose ^{1}H NMR spectra were identical with those of the corresponding D enantiomers, was checked by elemental analysis and optical rotatory power.

Biology. L-Deoxynucleosides Selectively Inhibit D-Thymidine Phosphorylation by HSV 1 Thymidine Kinase. We have screened five L-β-nucleosides, namely L-T, L-dC, L-dA, L-dG, and L-dU for their capacity to inhibit the phosphorylation of [^{3}H]-D-T catalyzed by the human and the HSV1 TKs. All five compounds were found inactive against the human enzyme up to 1 mM concentration. On the contrary they exerted differential inhibitory capabilities against the viral enzyme in the following order: L-T > L-dG > L-dU > L-dC > L-dA. Inhibition curves shown in Figure 1A indicate that the ID_{50} values for HSV1 TK are 2, 300, 400, 1000, and more than 1000 μM for L-T, L-dG, L-dU, L-dC, and D-dA, respectively. Figure 1B shows that human TK is completely resistant to L-T up to 1 mM, the highest tested concentration.

L-Thymidine Is a Competitive Inhibitor of HSV1 TK. The Lineweaver–Burk plot of inhibition by L-T of

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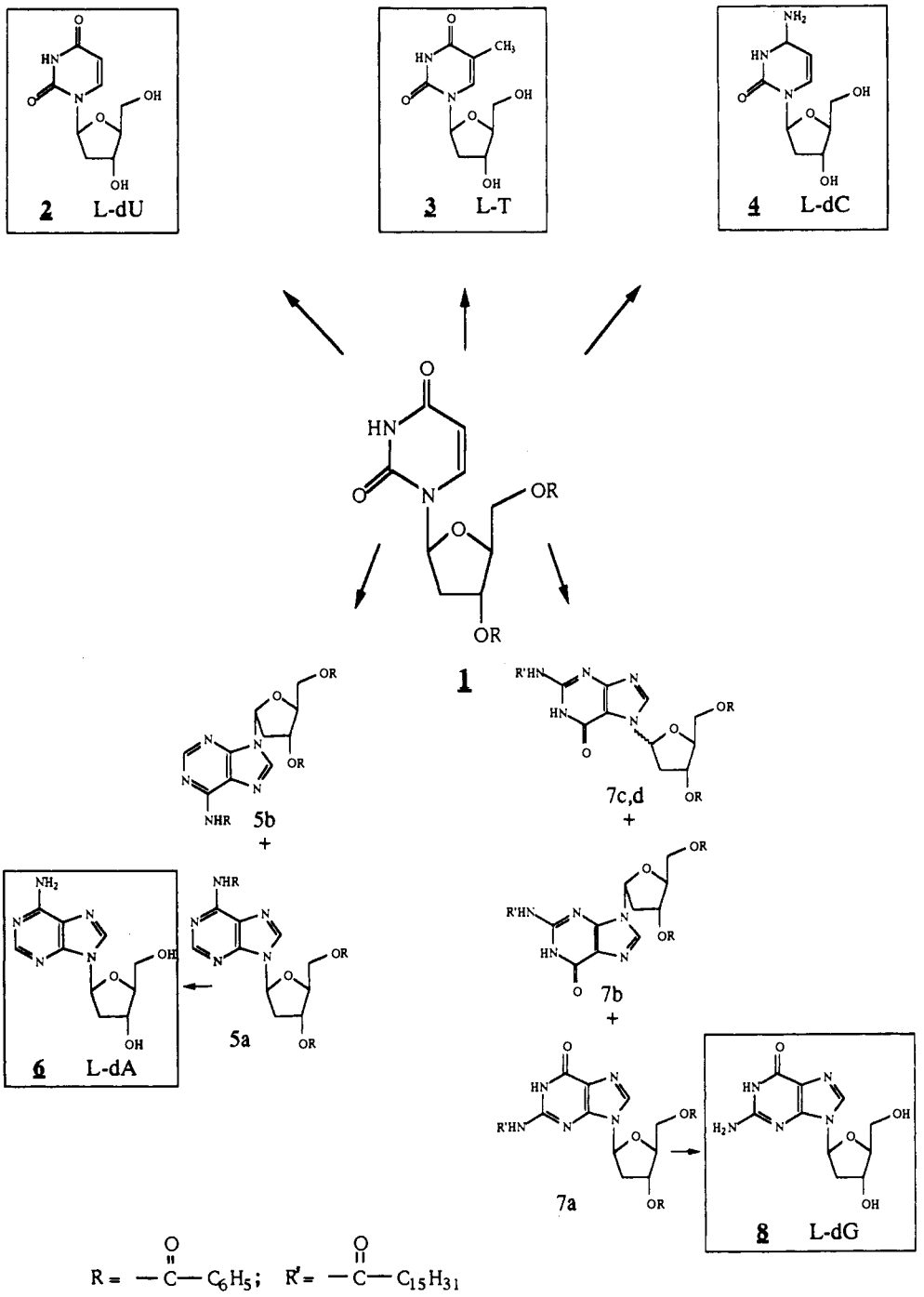
Scheme I



D-T phosphorylation by HSV1 TK, shown in Figure 2, is consistent with a competitive mechanism. In fact at each concentration of L-T, a concentration of D-T substrate can be found that prevents inhibition, K_m for D-T and K_i of L-T were found to be 2.8 and 2 μM, respectively. These data strongly support the hypothesis that L-T binds to the active site of the enzyme with an efficiency close to that of the natural substrate D-T.

L-Thymidine Is Phosphorylated by HSV1 TK as Well as Its Natural Counterpart D-Thymidine. To understand whether L-T is a competing substrate or a nonsubstrate inhibitor of the viral enzyme, we incubated HSV1 TK with D- and L-T in the presence of [γ-^{32}P]ATP under conditions that allow the formation of the 5'-monophosphate of the natural substrate. Resolution of reaction products was performed by HPLC as described in the Experimental Section. Figure 3 shows the chromatograms of the reactions with L-T (panel A) and D-T (panel B) as substrates. Two identical peaks of radioactivity in the position of the marker TMP can be observed, suggesting that the two enantiomers are indifferently recognized as substrate by the viral enzyme. In parallel HPLC experiments where no nucleoside substrate was used, the radioactivity was confined to the ATP position (data no shown).

It is worth mentioning here that our chromatographic conditions do not allow the detection of, both with D and L enantiomers of thymidine, the reaction product (TDP) of the thymidylate kinase activity associated with HSV1 TK. Preliminary results indicate that L-T is phosphorylated to the diphosphate form by viral TK with an

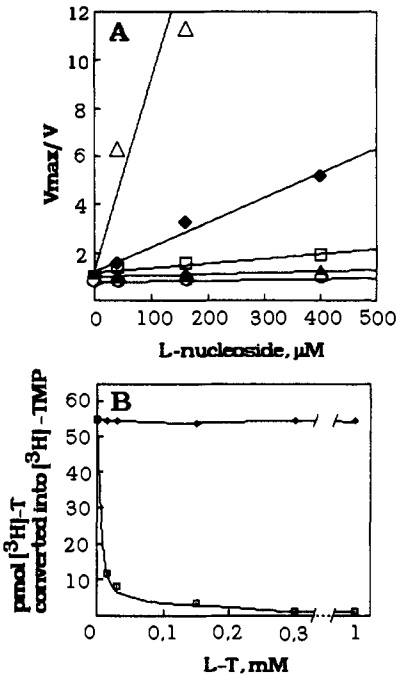


Figure 1. Panel A: Effect of increasing amounts of (△) L-T, (◆) L-dG, (□) L-dU, (▲) L-dC, and (○) L-dA on the phosphorylation of the natural substrate D-T (1.13 μM) by HSV1 thymidine kinase. Lines passing beyond the last point shown represent lines to the next data point, which is not shown. Panel B: Effect of increasing amounts of L-T on the phosphorylation of the natural substrate D-T by human (◆) and HSV1 (□) TKs.

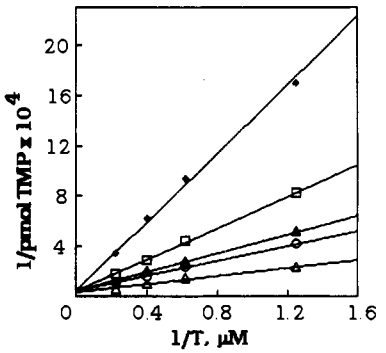


Figure 2. Lineweaver–Burk plot of the effect of L-T on the activity of HSV1 TK in the presence of increasing concentrations of the natural substrate [^{3}H]T. The enzyme was assayed as described in Materials and Methods with variation of the concentration of L-T: (△) 0 μM, (○) 2 μM, (▲) 5 μM, (□) 10 μM, and (◆) 15 μM.

efficiency comparable with that observed for the D enantiomer.

L-Thymidine Selectively Inhibits [^{3}H]Thymidine Incorporation in HSV1-TK Transformed HeLa Cells without Affecting Cellular Growth and Viability. In order to verify the ability of L-T to compete with the natural substrate in vivo, we used three lines of HeLa cells: wild type, its TK$^-$ mutant, and the same made TK$^+$ by transfection with the HSV1 TK gene (HeLa 5a). L-T, like BVDU used in parallel experiments (data not shown), inhibits the utilization of [^{3}H]T only in the cell line which depends on viral TK for T incorporation (compare Figure 4A with Figure 4C), while no effect was detected on normal and TK$^-$ cell lines (Figure 4B).

RNA and protein synthesis were not affected by L-T up to 300 μM, the highest concentration tested, in all tested cell lines (data not shown). Contrary to the reported

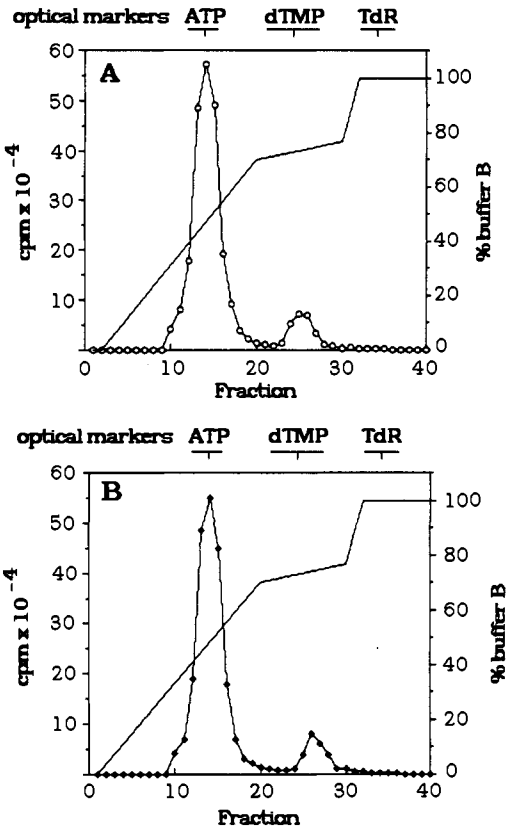


Figure 3. HPLC analysis of the products of incubation of HSV1 TK with [γ-^{32}P]ATP and either L-T (panel A) or D-T (panel B). The TK reaction and the HPLC analysis were performed as described in Materials and Methods.

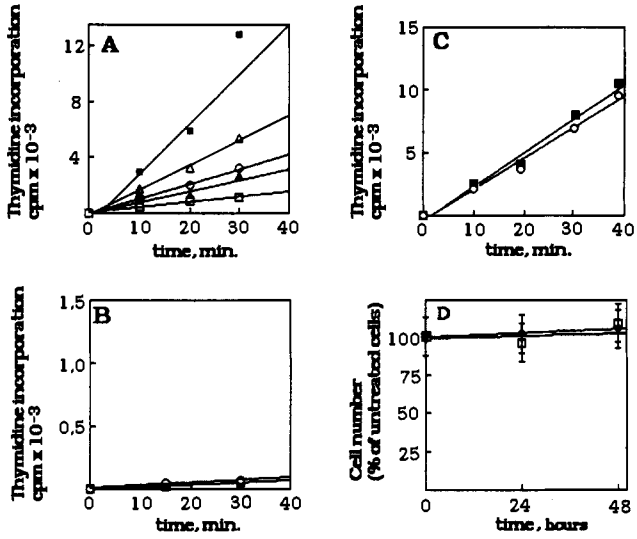


Figure 4. Effect of L-T on the incorporation of [^{3}H]-D-T and on cell growth. Incorporation of [^{3}H]-D-T into DNA of subconfluent HeLa 5a (HeLa TK$^-$/HSV TK$^+$) (panel A), HeLa TK$^-$ (panel B), and HeLa wild type (panel C) was assayed as described in the Experimental Section in the absence of L-T (■) and in the presence of (△) 35 μM L-T, (○) 70 μM L-T, (◆) 100 μM L-T, and (□) 200 μM L-T. The effect of L-T (250 μM) on cell growth (panel D) was measured in HeLa wild type (□) and in HeLa TK$^-$/HSV TK$^+$ (●).

cytotoxicity of BVDU and related inhibitors of viral TKs, L-T had no effect on the growth of HeLa wild type and HeLa TK$^-$/HSV1 TK$^+$ cells exposed for 48 h to 250 μM L-T (Figure 4D) either as a single addition or replaced every 12 h. At each time point no difference was observed

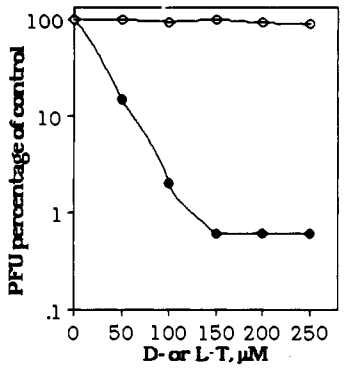


Figure 5. Effect of different concentrations of D-T and L-T on virus growth. The compounds were added to HeLa TK⁻ cells at 1 h post-infection, and the virus was harvested and titrated 24 h post-infection as described in the Experimental Section: (○) D-T, (●) L-T.

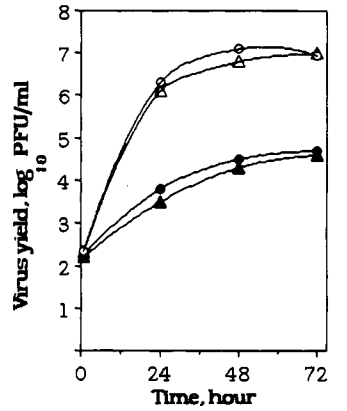


Figure 6. Effect of D-T and L-T on the multiplication of HSV1 (KOS strain) in HeLa S3 and HeLa BU cells. The compounds were added immediately after virus adsorption at 90 μM. Virus yield was determined by plaque formation in Vero cell cultures from HSV1 infected HeLa S3 cells (△, ▲) and HSV1 infected HeLa BU cells (○, ●), in presence of D-T (open symbols) or L-T (solid symbols).

in the cell viability determined as colony forming ability (data not shown).

L-Thymidine Inhibits HSV1 Growth in HeLa Cells. The concentration-dependent inhibition of viral multiplication by L-T is shown in Figure 5. The different activity of the two enantiomers on HSV1 replication in HeLa TK⁻ cells is clearly demonstrated by the reduction of more than 2 order of magnitude in the PFU taking place only in the case of L-T. The inhibitory effect of L-T on virus multiplication was tested in HeLa S3 and HeLa BU cells by measuring the viral yield at increasing times of exposure to the compound. L-T added at 90 μM immediately after virus adsorption, significantly reduced virus yield as compared to D-T (Figure 6).

Discussion

The aim of the present study was to verify whether Herpes simplex 1 thymidine kinase would bind and process L-thymidine, the enantiomer of its natural substrate and whether this analogue had an inhibitory activity toward the proliferation of the virus in dividing cell cultures. Indeed, the results show that (a) the affinity of HSV1 TK for L-T is very close to that for the natural substrate, whose K_m (2.8 μM) is comparable with the K_i (2 μM) of the analogue; (b) the two enantiomers are phosphorylated at the same extent, as indicated by HPLC analysis of the reaction products; (c) on the contrary, no inhibitory activity of L-T is detected against the human enzyme up to 1 mM, the higher tested concentration; (d) in vivo L-T selectively inhibits viral proliferation without any detectable toxic effect on noninfected cells.

Hence, the overall configuration of the sugar ring has practically no influence on the recognition by the viral enzyme, whereas it strongly affects the recognition by its human counterpart. More evidence along this line comes from the experiments with wild type and TK⁻/HSV1 TK⁺ HeLa cells: L-T inhibits short-term incorporation of exogenous [³H]T into cellular DNA, without affecting total DNA synthesis, only in the cell line where the incorporation depends on the activity of the viral enzyme. The selective behavior of L-thymidine is further substantiated by the absence of any effect upon either cellular RNA and protein synthesis or upon the growth and viability of HeLa TK⁻/HSV1 TK⁺ cells exposed to a 250 μM L-T for 48 h. These last results prove that the activity of L-T occurs primarily through its phosphorylation by viral TK. They also suggest that the de novo synthesis overcomes the blockage of D-TTP synthesis via the salvage pathway under the condition of the cell culture. In addition, the phosphorylated form(s) of L-T have no detrimental effect on the human cell viability.

A reduction of 2 orders of magnitude was instead found on the virus multiplication in infected cell culture in the presence of 100 μM L-T. It is worth mentioning that Cheng and co-workers[7] failed to observe a reduction of the virus yield with 5′-ethynylthymidine, a potent nonsubstrate inhibitor of HSV1 TK at concentrations reducing the TTP pools to 5%. At present, we do not know if the decrease of the viral yield caused by L-T is due only to its activity toward HSV1 TK or to other mechanisms, like inhibition of the viral DNA polymerase. This and related aspects are currently under investigation.

After the completion of the first part of the present work,[26] we became aware of the results obtained with the carbocyclic analogues of 5-iodo- and 5-(2-bromovinyl)-2′-deoxyuridine.[27] Both enantiomeric forms of these compounds are potent inhibitors of HSV1 replication and have remarkably similar affinity for the viral TK. At odds with the behavior of L-thymidine, which is a purely competitive inhibitor of the enzyme, in the carbocyclic series the compounds whose overall configuration is opposite to that of the natural substrate show linear mixed-type inhibition.

Our results and those of Balzarini et al.[27] prove that HSV1 TK is very tolerant to the overall configuration of either natural or alternative substrate molecules, in contrast to the general behavior of enzymes. Instead, the lack of antiherpetic activity of the α anomers of L-thymidine[28] and of 5-substituted 2′-deoxy-D-uridines[2] could indicate that a key factor of the activity of this enzyme is

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the relative configuration of the 1′ and 4′ carbons of the sugar ring of the substrate.

From a therapeutic point of view, the most interesting result of the present study is that the marked inhibitory activity of L-T toward HSV1 TK and virus proliferation is not accompanied by toxic effects on the host enzymes and cells (either HeLa or HeLa TK⁻/HSV1 TK⁺) most probably ascribable to the absolute stereospecificity of cellular enzymes. On the contrary, the very potent antiherpetic agents D-idoxouridine and D-BVDU, substrate inhibitors of the viral TK, have some affinity for cytosolic and/or mitochondrial TK.[29] This leads to adverse effects on highly proliferating bone marrow and lymphoid tissues. Also N^2-phenyldeoxyguanosine, an excellent inhibitor of HSV1 TK developed by some of us, displays some toxicity for the mammalian host cell,[8] most likely because it partially inhibits cellular DNA polymerases.

Thus, we feel that L-T, an efficient nontoxic inhibitor of this enzyme, represents a prototype molecule for the development of novel more potent and safer drugs for the therapy of recurrent herpes infections.

Experimental Section

Chemicals. Unless otherwise stated, commercial reagents and solvents were used as received. For TLC MERCK 5719 silica gel 60 precoated plates were used. Flash and short-column chromatography were done with MERCK 9385 silica gel 60 (230–400 mesh). Medium-pressure chromatography was performed with a Jobin Yvon Cromatospac using MERCK 7736 silica gel 60 H. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. ^{1}H NMR spectra were recorded with a Varian VXR-200 spectrometer.

[^{3}H]Thymidine ([^{3}H]T, 20 Ci/mmol, [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]uridine ([^{3}H]U, 27 Ci/mmol, [^{3}H]dTTP (30 Ci/mmol), and [^{3}H]-L-leucine ([^{3}H]Leu, 59 Ci/mmol) were obtained from Amersham. High-pressure liquid chromatography (HPLC) reagents were purchased from BDH. T and TMP were from Boehringer. HPLC reverse-phase C_{18} BioSil ODS-5S column was from Bio-Rad.

2′-Deoxy-L-uridine (2). *1* (18 g)[22] was deprotected as described[22] and afforded 8.1 g (86%) of 2′-deoxy-L-uridine: $[\alpha]^{26}_D$ −30.0° (*c* 2.1, H_2O) lit.[20] $[\alpha]^{26}_D$ −29.8° (*c* 1.5, H_2O). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

L-Thymidine (3). *2* (4.5 g) was converted into the title compound according to the reported procedure.[22] The crude material was purified by flash chromatography (eluent CH_2Cl_2/MeOH 85:15) and crystallized from ethanol to give 2.3 g of *3* (49%), $[\alpha]^{20}_D$ −19.9° (*c* 1.15, H_2O) lit.[30] $[\alpha]^{20}_D$ −20.3° (*c* 0.19, H_2O). Anal. ($C_{10}H_{14}N_2O_5$) C, H, N.

2′-Deoxy-L-cytidine 4. *1* (5 g) was treated as described,[23] giving 1.64 g (62.8%) of crystallized (MeOH) *4*: $[\alpha]^{23}_D$ −57.2° (*c* 2, H_2O) lit.[31] for the D enantiomer $[\alpha]^{23}_D$ +57.6° (*c* 2, H_2O). Anal. ($C_9H_{13}N_3O_4$) C, H, N.

Transglycosylation Procedure. To 3′,5′-di-*O*-benzoyl-2′-deoxy-L-uridine (*1*) (4.4 g, 10 mM) dissolved in anhydrous 1,2-dichloroethane (100 mL) *N,O*-bis(trimethylsilyl)acetamide (BSA) (7.2 mL, 31 mM) was added followed by 20 mM of N^6-benzoyladenine or N^2-palmitoylguanine. The resulting mixture was refluxed for 30 min under careful exclusion of moisture and cooled to room temperature, and freshly distilled (trimethylsilyl)-methyl trifluoromethanesulfonate (TMS-triflate) (2.6 mL, 13 mM) was added. The reaction mixture was then refluxed for 4 h in an atmosphere of dry argon, cooled to room temperature, diluted with CH_2Cl_2 (250 mL), and vigorously shaken with 5% sodium bicarbonate (60 mL). After standing, the solid precipitated was filtered, the organic layer was separated, washed with water (100 mL), and dried with sodium sulfate. The solvents were evaporated under reduced pressure leaving a brown gummy residue which was dissolved in CH_2Cl_2 (50 mL) and filtered through a small pad of silica gel. Evaporation of the solvent gave the crude mixtures of isomers as a yellow brittle foam, from which the N-9-β isomers were separated by chromatography (see below). A further yield of these compounds was obtained by treating the "unwanted" isomer(s) with TMS-triflate in the presence of BSA and the appropriate protected base in the same ratios and conditions detailed above.

2′-Deoxy-L-adenosine 6. The crude material from the transglycosylation reaction was purified by short-column chromatography (eluent CH_2Cl_2/MeOH, 97:3), giving 4.7 g of a mixture of N-9-α (**5b**) and N-9-β (**5a**) anomers of 3′,5′-di-*O*-benzoyl-2′-deoxy-L-N^6-benzoyladenosine. α derivative: ^{1}H NMR ($CDCl_3$) δ 6.67 (dd, 1′), 5.73 (m, 3′); R_f = 0.27 [eluent CH_2Cl_2 (86)/dioxane (7)/CH_3CN (7)]. β derivative: ^{1}H NMR ($CDCl_3$) δ 6.58 (dd, 1′), 5.85 (m, 3′); R_f = 0.37 (eluent as above). Medium-pressure chromatography of the isomer mixture [eluent CH_2Cl_2 (87)/dioxane (6)/CH_3CN (7)] afforded 1.4 g of pure **5a** and 2.7 g of **5b**. A second crop (0.6 g) of **5a** was obtained from anomerization (see above) of the recovered α isomer. Overall yield was 35.5%.

5a (1.7 g) was dissolved in 30 mL of pyridine and treated with 60 mL of 28% aqueous ammonia; after standing at 50 °C for 24 h the reaction mixture was evaporated under reduced pressure and pyridine was completely removed by repeated coevaporations with water. Water (40 mL) was added to the residue, the mixture was extracted with ether (3 × 20 mL), the clear solution was concentrated to a small volume, the pH was adjusted at 5.5-6.0 with acetic acid. After further extractions with ether the solution was evaporated to dryness, and the white residue was crystallized from water to give needles which were filtered, washed several times with acetone, and dried under vacuum: final yield of **6**, 0.52 g (69%), $[\alpha]^{23}_D$ +23.1 (*c* 1, H_2O) lit.[24] $[\alpha]^{23}_D$ +23.2° (*c* 1, H_2O). Anal. ($C_{10}H_{13}N_5O_3 \cdot H_2O$) C, H, N.

2′-Deoxy-L-guanosine 8. The crude material from the transglycosylation reaction was purified by short-column chromatography [eluent CH_2Cl_2 (100–97%)/MeOH (0–3%)] to give 0.9 g of N-7-α/β anomers **7c,d** and 4.8 g of N-9-α/β anomers **7b,a**, from which 1.3 g of pure 3′,5′-di-*O*-benzoyl-2′-deoxy-β-L-N^2-palmitoylguanosine (**7a**) was obtained by medium-pressure chromatography [eluent CH_2Cl_2 (86)/dioxane (7)/CH_3CN (7)/MeH (1)]. Treatment of the recovered N-7-α/β and N-9-α isomers mixture (3.4 g) with TMS-triflate, as above described, afforded, after workup and chromatography, 0.42 g of **7a**. Overall yield was 24%. TLC [eluent CH_2Cl_2 (86)/dioxane (7)/CH_3CN (7)/MeOH (2)] and relevant ^{1}H NMR ($CDCl_3$) data of the four isomers were as follows: (N-7-α/β) R_f 0.58, δ 6.8 (dd) and 6.73 (dd) (1′ protons), 5.72 (m) and 5.68 (m) (3′ protons); (N-9-α) R_f 0.42, δ 6.26 (dd, H-1′), 5.67 (m, H-3′); (N-9-β) R_f 0.36, δ 6.3 (t, H-1′), 5.83 (m, H-3′). **7a** (1.5 g) was deprotected as described above for the adenosine derivative; the final product was crystallized from water and, after filtration, washed several times with absolute ethanol and dried under vacuum. A total of 0.42 g (76%) of 2′-deoxy-L-guanosine (*8*) was obtained: $[\alpha]^{26}_D$ +20.1° (*c* 1.1, DMF) lit.[24] $[\alpha]^{26}_D$ +20.5° (*c* 1, DMF). Anal. ($C_{10}H_{13}N_5O_4 \cdot H_2O$) C, H, N.

Cells and Viruses. The cells used in this study were HeLa S3, HeLa BU (thymidine kinase deficient, TK⁻), HeLa 5a (HeLa TK⁻ transformed to the TK⁺ phenotype with a functional copy of the HSV1 TK gene), and Vero. The cells were maintained at 37 °C in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum (FCS). KOS strain of HSV1 was used in all the experiments.

Cell Growth and Viability. Cells were seeded 24 hours before L-T addition in 50 × 10 mm Falcon dishes, with 4 mL of medium at a density of 10‰ cells/mL. At each time point, cells were collected by trypsinization before counting. To test the colony forming ability, cells were seeded at a density of 500 cells/dish. After 8 days, cell colonies were stained with Coomassie Blue R-250 and counted.

Virus Growth Inhibition. Confluent HeLa S3 and HeLa BU cells in 25-cm^2 flasks were used as host cells for virus infection. After 1-h adsorption period of virus at 2 PFU per cell, the monolayers were rinsed twice with phosphate-buffered saline.

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Growth medium (5 mL) containing various concentration of drugs was added. The cells were then incubated at 37 °C for different times. At the end of the incubation period the cells were suspended and aliquoted into sterile plastic tubes and kept frozen at −70 °C until titration.

Virus Plaque Titration. The titer of the samples was determined by plaque titration method.[32] Tissue culture dishes (35 × 10 mm, Falcon) were seeded with Vero cells and used for assays when they were approximately 75% monolayer. Volumes (0.2 mL) of logarithmic dilutions of each sample were inoculated onto each of two tissue culture dishes and adsorbed for 1 h with intermittent shaking. The inoculum was removed, and 2 mL of DMEM containing 1% FCS and 0.3% of human immune serum globulin was added. After a 48-h incubation period at 37 °C in a 5% CO_2 atmosphere, the overlay medium was removed, and the cell sheets were fixed with methanol and stained with Giemsa. The number of plaques was determined with a Nikon profile projector which magnified the dishes 10×. The duplicates were averaged, and the number of PFU was calculated. The infectious titer was expressed in PFU/mL.[31]

Thymidine Kinase Assay. The host- and virus-specific enzymes were assayed as follows: when [^{3}H]T was used, 0.1–0.4 milliunits of enzyme were incubated at 37 °C for 30 min in 25 μL of a mixture containing 30 mM HEPES-K^+, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol (DTT), and 1.12 μM [^{3}H]T (1500 cpm/pmol). The reaction was terminated by spotting 20 μL of the incubation mixture on a 25-mm DEAE paper disk (DE-81 paper; Whatman). The disk was washed twice in an excess of 1 mM ammonium formate pH 5.6 in order to remove unconverted nucleoside and then once in distilled water and finally in ethanol. Radioactive TMP was estimated by scintillation counting in Omnifluor (NEN).

When [γ-^{32}P]ATP was used in TK assay, enzyme was incubated for 30 min in the same mixture except for 10 μM T or L-T and 100 μM [γ-^{32}P]ATP (1500 cpm/pmol).

One unit of TK is defined as the amount of enzyme which converts, under the above assay conditions, 1 nmol of T into TMP per minute.

Enzymes. Both the host- and virus-specific TKs were purified through affinity chromatography using CH Sepharose 4B column coupled with thymidine 3′-(*p*-aminophenyl phosphate) as described in Focher et al.[33] The specific activity of the host and viral enzymes was approximately 500 and 600 nmol/min per mg, respectively; the latter values corresponded to a purification of approximately 600-fold for each enzyme.

High-Pressure Liquid Chromatography. The reverse-phase method employing the Bio-Rad 100 MAPS preparative system was used in order to separate nucleosides from nucleotides. A 0.4- × 15-cm reverse-phase C_{18} BioSil ODS-5S column was used at room temperature in the following conditions: injection volume, 20 μL of reaction mixture made 100 μM each in ATP, TMP, and T before injection; detection, UV 260 nm; eluents, buffer A (20 mM KH_2PO_4, pH 5.6), buffer B (20 mM KH_2PO_4, pH 5.6, 60% methanol). Gradient conditions were as follows: 0 min, 100% buffer A; 20 min, 30% buffer A/70% buffer B; 30 min, 23% buffer A/77% buffer B; 32 min, 100% buffer B. The flow rate was 0.5 mL/min. A total of 40 fractions were collected and counted in a Kontron betamatic IV scintillation counter.

In Vivo Incorporation of [^{3}H]Thymidine, [^{3}H]Uridine, and [^{3}H]Leucine in HeLa Cells. HeLa cells were grown in DMEM with fetal calf serum at 37 °C in suspension in flasks to a density of 10^6 cells/mL. They were resuspended in DMEM without calf serum and incubated for 30 min at 37 °C, and then either [^{3}H]T (25 Ci/mmol), [^{3}H]U (27 Ci/mmol), or [^{3}H]L (59 Ci/mmol) was added to a concentration of 33 μCi/mL, 35 μCi/mL, or 78 μCi/mL, respectively; incubation was then continued. At 10, 20, and 40 min, 0.08-mL samples of culture were spotted on 25-mm GF/C (Whatman) filters. Trichloroacetic acid insoluble material was determined as previously described.[34] Incorporation of labeled T was inhibited more than 90% by the inclusion of 1.5 μg/mL aphidicolin during the labeling period.

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