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5-Iodo-2′-deoxy-L-uridine and (*E*)-5-(2-Bromovinyl)-2′-deoxy-L-uridine: Selective Phosphorylation by Herpes Simplex Virus Type 1 Thymidine Kinase, Antiherpetic Activity, and Cytotoxicity Studies

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SUMMARY

5-Iodo-2′-deoxy-L-uridine (L-IdU) and (*E*)-5-(2-bromovinyl)-2′-deoxy-L-uridine (L-BVdU) have been prepared and found to inhibit herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) with activities comparable to those of their analogs with the natural D-sugar configuration. The mechanism of inhibition is purely competitive for L-IdU (K_i = 0.24 μM) and mixed-type for L-BVdU (K_i = 0.13 μM). High performance liquid chromatographic analysis of the reaction products demonstrated that the viral enzyme phosphorylates both L-enantiomers to their corresponding monophosphates with efficiency comparable to that for D-enantiomers. Neither L-enantiomer inhibits the human cytosolic TK. In contrast to their D-enantiomers, L-IdU and L-BVdU have no effect on human thymidylate synthase, either in HeLa cells or in TK-deficient HeLa cells transformed with the HSV-1 TK gene. Both L-enantiomers (i) have no effect on HeLa cell growth, (ii) are 1000-fold less cytotoxic toward TK-deficient HeLa cells transformed with the HSV-1 TK gene than are their D-enantiomers, (iii) in contrast to their D-enantiomers, are fully resistant to hydrolysis by nucleoside phosphorylase, and, (iv) in spite of their much lower cytotoxicity, most probably due to the very low affinity of L-BVdU monophosphate and L-IdU monophosphate for thymidylate synthase, are only 1 or 2 orders of magnitude less potent than their D-enantiomers in inhibiting viral growth, with potency comparable to that of acyclovir.

Virus-encoded enzymes are essential for either viral infection or reactivation and often present biochemical properties different from those of their cellular counterparts. For instance, whereas human cytosolic TK is dThd specific, HSV-1 TK phosphorylates also dCyd and a variety of pyrimidine and purine nucleoside analogs, which represent most of the antiherpetic drugs clinically used or under investigation. Selectivity of action has been pursued mainly by using analogs that differ from the natural D-nucleosides in the chemical structure of the base and/or the sugar but not in the absolute configuration. We have recently found that L-dThd is a good substrate for HSV-1 TK but is not recognized by the human cytosolic enzyme and reduces virus proliferation in HeLa cells with little or no toxic effect on host cells (1). The possibility that L-nucleoside analogs might be more selective antiviral agents than the corresponding D-enantiomers has been confirmed for some derivatives of dCyd (2). For example, D-ddCyd and L-ddCyd have comparable antiviral activities against human immunodeficiency virus type 1, but L-ddCyd does not inhibit host mitochondrial DNA synthesis and shows lower cytotoxicity (2). These findings led us to synthesize and study the antiherpetic activity of dThd analogs in which a modified base moiety is combined with a sugar of unnatural configuration, namely 2′-deoxy-L-ribose.

D-IdU is an effective antiviral agent, but its therapeutic

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ABBREVIATIONS: TK, thymidine kinase; HSV-1, herpes simplex virus type 1; HeLa TK$^-$/HSV-1 TK$^+$ cells, thymidine kinase-deficient HeLa cells transformed with the herpes simplex virus type 1 thymidine kinase gene; TK$^-$, thymidine kinase-deficient; dThd, thymidine; dCyd, deoxycytidine; ddCyd, dideoxycytidine; TS, thymidylate synthase; L-IdU, 5-iodo-2′-deoxy-L-uridine; L-BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxy-L-uridine; D-IdU, 5-iodo-2′-deoxy-D-uridine; D-BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxy-D-uridine; CFA, colony-forming ability; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HPLC, high performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IdUMP, 5-iodo-2′-deoxyuridine monophosphate; BVdUMP, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine monophosphate.

value is severely limited by the fact that it is also a good substrate for human TK. D-BVdU, whose potency against HSV-1 proliferation in cell culture and *in vivo* is remarkable, is therapeutically useful, although potential drawbacks, such as good affinity for the mitochondrial TK (3), inhibition of TS by its monophosphate (4), and degradation by pyrimidine phosphorylase (5), limit its use. This last problem has already been addressed using the carbocyclic analogs carba-BVdU and carba-IdU, where the sugar moiety is substituted with a cyclopentane ring. Unluckily, the carba derivatives, which are resistant to degradation and in cell cultures have antiviral activities comparable to those of the parent compounds, perform very poorly *in vivo* (6). Thus, we have synthesized L-IdU and L-BVdU and studied (i) their affinity for human, cytosolic, and HSV-1 TKs, (ii) their susceptibility to be phosphorylated by the aforementioned TKs and to be hydrolyzed by human nucleoside phosphorylase, (iii) their effect on human TS *in vivo*, (iv) their ability to inhibit HSV-1 proliferation in cell cultures, and (v) their cytotoxicity for the uninfected host cells.

Materials and Methods

Chemicals

Commercial reagents and solvents were used unless otherwise stated. Flash column chromatography was performed with Merck 9385 silica gel 60 (230–400 mesh). Optical rotations were measured with a Perkin Elmer 141 polarimeter. ^{1}H NMR spectra were recorded with a Varian VXR 200-MHz spectrometer. CD spectra were recorded with a Jasco 500A spectropolarimeter equipped with a DP100 data processor. [^{3}H]dThd (20 Ci/mmol), [^{3}H]dCyd (18–30 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. D-BVdU and acyclovir were supplied by Sigma. Antiviral drugs were dissolved in dimethylsulfoxide and diluted in complete medium just before the treatment.

Synthesis of L-IdU

L-IdU was obtained in two steps from 3′,5′-di-*O*-benzoyl-2′-deoxy-L-uridine, starting from L-(+)-arabinose, as described for the corresponding D-enantiomer (7). The pure compound was characterized by ^{1}H NMR spectroscopy and CD spectroscopy.

Synthesis of L-BVdU

L-BVdU was synthesized in three steps as described for the corresponding D-ribose analog (8), starting from L-IdU. The final product was purified by silica gel flash chromatography (eluent, CH_2Cl_2/CH_3OH, 88:12), and the solvent was coevaporated with absolute ethanol several times. The product was then characterized by ^{1}H NMR spectroscopy and CD spectroscopy [$[\alpha]^{25}_D = -7.8$ (concentration, 1.41 g/100 ml, in CH_3OH)].

Enzymes

Both the host- and virus-specific TKs were purified by affinity chromatography using a CH Sepharose 4B column coupled with dThd-3′-(*p*-aminophenyl phosphate), as already described (9). The specific activities of the host and viral enzymes were approximately 500 and 600 nmol/min/mg, respectively; the latter values correspond to a purification of approximately 600-fold for each enzyme. One unit of TK is defined as the amount of enzyme that converts, under the aforementioned assay conditions, 1 nmol of dThd to TMP per minute.

Cells and Viruses

HeLa S3 cells (a human epithelial cell line derived from a cervix carcinoma), HeLa BU cells (TK$^-$), and HeLa TK$^-$/HSV-1 TK$^+$ cells were obtained from Prof. G. Della Valle, Department of Genetics and Microbiology A. Buzzati Traverso, University of Pavia (Pavia, Italy), U-373 MG cells (a glioblastoma astrocytoma line) from the American Type Culture Collection, and MRC-5 cells (an embryonic human lung fibroblast line) from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia. The cell lines were tested for *Mycoplasma* contamination with the Hoechst 33258 staining method and were found to be negative. The cells were grown in DMEM with 10% fetal calf serum (NBL Laboratories); HeLa TK$^-$ medium was supplemented with bromodeoxyuridine (25 μg/ml), whereas MRC-5 medium was supplemented with 1 mM sodium pyruvate. Media were invariably supplemented with 2 mM L-glutamine and penicillin-streptomycin (GIBCO). HSV-1 (strain HF) was obtained from the American Type Culture Collection.

TK Assay

HSV-1 TK was assayed as follows. Enzyme was incubated at 37° for 30 min in 25 μl of a mixture containing 30 mM K$^+$-HEPES, pH 7.5, 6 mM $MgCl_2$, 6 mM ATP, 0.5 mM dithiothreitol, and 0.15 μM (K_m concentration) [^{3}H]dThd (2200 cpm/pmol). Human cytosolic TK was assayed under the same reaction conditions, except for the use of 1.12 μM (K_m concentration) [^{3}H]dThd (2200 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 with an excess of 1 mM ammonium formate, pH 5.6, to remove unconverted nucleoside, once with distilled water, and finally with ethanol. Filters were then placed in 20-ml scintillation vials, and it was confirmed that the filters were flat on the bottom of the vials. One milliliter of 1 M KCl/0.1 M HCl solution was added and radioactivity was extracted by shaking for 20–30 min. To each vial 4 ml of EcoLume (ICN) were added, and the radioactivity was counted in a β counter.

When [γ-^{32}P]ATP was used in TK assays, enzyme was incubated in the mixture described above, containing 1 mM [γ-^{32}P]ATP (125 cpm/pmol), 2 mM $MgCl_2$, and 40 μM levels of the test compound (dThd, D-BVdU, L-BVdU, D-IdU, or L-IdU). After 1 hr at 37°, 1.5 μl of 0.5 M EDTA were added to each sample to stop the reaction. To each sample were then added 2 μl of a solution containing TMP, dThd, D-IdU, and D-BVdU (10 mM each) as markers at 260 nm. Samples were heated for 5 min at 80° and centrifuged at 10,000 rpm for 10 min. Twenty microliters of the supernatant were injected for HPLC.

Nucleoside and Nucleotide Separation by Reverse Phase HPLC

The reverse phase method, employing the Bio-Rad 100 MAPS preparative system, was used to separate nucleosides from nucleotides. A 0.4- × 15-cm, reverse phase, C_{18} BioSil ODS-5S column was used at room temperature under the following conditions: injection volume, 20 μl; detection, absorbance at 260 nm; buffer A, 20 mM KH_2PO_4, pH 5.6; buffer B, 20 mM KH_2PO_4, pH 5.6/60% methanol; gradient conditions, 0–5 min, 0% buffer B; 5–15 min, 0–50% buffer B; 15–16 min, 50–100% buffer B; 16–28 min, 100% buffer B; flow rate, 0.5 ml/min. Fifty-six fractions (fraction volume, 250 μl) were collected and counted in a β counter.

In Vivo Incorporation of [^{3}H]dThd

HeLa TK$^-$/HSV-1 TK$^+$ cells were grown in DMEM with fetal calf serum, at 37° 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°, and then [^{3}H]dThd (25 Ci/mmol) was added to a concentration of 33 μCi/ml; incubation was then continued. At 10, 20, and 40 min, 0.08-ml culture samples were spotted on 25-mm GF/C filters (Whatman). Trichloroacetic acid-insoluble material was determined as described previously (10). Incorporation of [^{3}H]dThd was >90% inhibited by the inclusion of 1.5 μg/ml aphidicolin during the labeling period.

Cell Growth and Viability

To test the CFA, cells were seeded in 50- × 10-mm Falcon dishes, 24 hr before L-IdU or L-BVdU addition, in 4 ml of medium at a density of 500 cells/dish. After 8 days, cell colonies were stained with Comassie blue R-250 and counted.

Cytotoxic Effects

Serial 2-fold dilutions of test compound in 100 μl of medium were prepared in quadruplicate in flat-bottomed, 96-well, microtiter plates, and an equal volume of cell suspension (2×10^5 cells/ml) was added. After 72 hr of incubation at 37° in 5% CO_2, the plates were stained in an MTT assay. The absorbance was measured with a microplate reader (Bio-Rad model 3550), with a test wavelength of 750 nm and a reference wavelength of 690 nm (11). Cytotoxicity was estimated as cellular growth inhibition, and maximum tolerated dose represents the concentration required to induce a minimal cytostatic effect. The data represent average values from two or three separate experiments.

Determination of Tritium Release from [^{3}H]dCyd for Assay of TS

The activity of TS in HeLa and HeLa TK$^-$/HSV-1 TK$^+$ cells was measured by estimation of tritium release from [^{3}H]deoxyuridylate (formed in the cells from [^{3}H]dCyd) in the reaction catalyzed by TS. This method, described by Balzarini *et al.* (12), was modified as follows. Cells, growing in DMEM (HeLa TK$^-$/HSV-1 TK$^+$ cells) or minimal essential medium (HeLa cells) with 10% fetal calf serum, were collected by centrifugation at 200 × *g* for 10 min and resuspended in fresh medium without fetal calf serum, at the concentration of 1.6×10^6/ml. Aliquots of 100 μl were incubated at 37° in the presence of 2.3 μM [^{3}H]dCyd (1.3 μCi) and an appropriate amount of compound to be tested. At the end of the reaction time (30 min), 75 μl of the reaction mixture were withdrawn and mixed with 250 μl of an ice-cold suspension of carbon black (160 mg/ml) in 5% trichloroacetic acid. After centrifugation at 1000 × *g* for 20 min, 100-μl samples of the supernatants were mixed with 1 ml of EcoLume (ICN) and analyzed for radioactivity in a β counter.

Nucleoside Degradation by Intact Human Blood Platelets and HPLC Analysis of the Reaction Products

The phosphorolysis of D-BVdU, L-BVdU, D-IdU, and L-IdU by intact human blood platelets was measured by HPLC. The nucleoside degradation assay and HPLC analysis were performed as described by Desgranges *et al.* (5). Briefly, 3×10^8 platelets/ml were incubated at 37° in 350 μl of a mixture containing 10 mM Tris·HCl, pH 7.5, 1 mM sodium phosphate, pH 7.5, 0.15 M NaCl, 1 mM EDTA, and 100 μM D- or L-nucleoside. At 0, 20, and 40 min, 100-μl aliquots were removed, rapidly cooled in ice, and centrifuged for 10 min at 3000 × *g* at 4°. Twenty microliters of the supernatant were analyzed by HPLC.

Nucleosides were separated from their corresponding bases by HPLC on a reverse phase, C_{18} BioSil ODS-5S column eluted with a linear gradient according to the method described by Desgranges *et al.* (5), with some modifications. The conditions were as follows: injection volume, 20 μl; detection, absorbance at 285 nm for IdU and 292 nm for BVdU; buffer A, 10 mM KH_2PO_4, pH 4.6; buffer B, 10 mM KH_2PO_4, pH 4.6/60% methanol; gradient conditions, 0–5 min, 0% buffer B; 5–35 min, 0–100% buffer B; flow rate, 0.5 ml/min.

Antiviral Activity

Reduction of cytopathic effects. Serial 2-fold dilutions of test compound in medium were prepared in flat-bottomed, 96-well, microtiter plates, and an equal volume of cell suspension (2×10^5 cells/ml, infected with virus at a multiplicity of infection of 0.05) was added. After an incubation period of 72 hr at 37° in CO_2, antiviral activity measurements were based on microscopic evaluation of HSV-1-induced cytopathic effect. In all experiments each drug concentration was assayed in triplicate and averaged data were plotted as dose-effect curves, from which the IC_{50}, i.e., the concentration required to reduce the virus-induced cytopathic effect by 50%, was extrapolated. The data represent average values from two or three separate experiments. Finally, the culture plates were frozen and stored at −70° for additional experiments.

Reduction of infectious virus production. To determine the infectious virus amount, the cell culture plates were treated with three cycles of freezing/thawing at −70°, triplicate well contents were centrifuged at 4000 rpm for 10 min at 4°, and the viral titer was determined as follows. A series of semilogaritmic dilutions were obtained from supernatants in a 96-well microtiter plate, cells were added, and the plate was incubated for 72 hr at 37° under 5% CO_2. The infected wells were recorded after a microscopic evaluation of HSV-1-induced cytopathic effect, and the virus titer was calculated using the Reed-Muench method. The virus titer was estimated by infection of Vero cells. From the reduction of infectious virus production at three or more different drug concentrations, the IC_{50}, i.e., the drug concentration required to reduce the virus yield progeny by 50%, was extrapolated.

Results

Selective inhibition by L-IdU and L-BVdU of D-dThd phosphorylation by HSV-1 TK. We have studied L-IdU and L-BVdU, as well as their D-enantiomers, for their ability to inhibit the phosphorylation of D-[^{3}H]dThd catalyzed by human and HSV-1 TKs. The L-nucleosides exerted an inhibitory action similar to that of their D-counterparts against the viral enzyme, while being inactive against the human TK (Table 1). Thus, L-IdU, in contrast to D-IdU, which also inhibits the human TK (selectivity index, 50), is a selective inhibitor of the viral enzyme (selectivity index, >2000). L-IdU (Fig. 1A), like L-dThd (1), behaved as a competitive inhibitor of HSV-1 TK (K_i = 0.24 μM). L-BVdU (Fig. 1B) revealed a linear mixed-type inhibition of D-dThd phosphorylation (K_i = 0.13 μM, as calculated from a Dixon plot of slope versus inhibitor concentration); its behavior resembled that of the L-enantiomers of carba-BVdU (13).

Phosphorylation of L-IdU and L-BVdU by HSV-1 TK. To understand whether L-IdU and L-BVdU are substrate inhibitors of the viral enzyme, like their D-counterparts, or pure nonsubstrate inhibitors, we incubated HSV-1 TK with either L- or D-enantiomers in the presence of [γ-^{32}P]ATP (Fig. 2). Resolution of reaction products was performed by HPLC, as described in Materials and Methods. Fig. 2 shows the comparison of the chromatograms of the products of the reactions in which L- or D-IdU, L- or D-BVdU, or D-dThd was tested as substrate. For each enantiomer a peak of radioactivity appears between the position of ATP and that of the corresponding nucleoside analog, where the nucleoside

TABLE 1

Effect of dThd analogs on D-[^{3}H]dThd phosphorylation by HSV-1 and HeLa TKs

Compound	IC_{50}		Selectivity Index, HeLa TK/HSV-1 TK
	HSV-1 TK	HeLa TK	
	μM		
D-IdU	0.12	6.05	50
L-IdU	0.35	>2000[a]	>5000
D-BVdU	0.07	>600[a]	>8000
L-BVdU	0.26	>600[a]	>2000
L-dThd	0.15	>1000[a]	>6000

[a] Highest tested concentration.

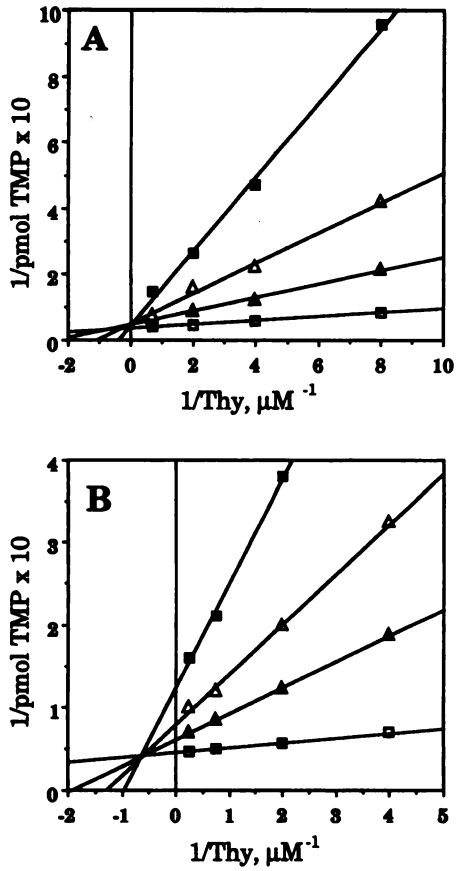

Fig. 1. Lineweaver-Burk plot of the effect of L-IdU (A) and L-BVdU (B) on the activity of HSV-1 TK in the presence of increasing concentrations of the substrate [^{3}H]dThd (*Thy*). L-IdU and L-BVdU concentrations were 0 μM (□), 0.5 μM (▲), 1 μM (△), and 2 μM (■).

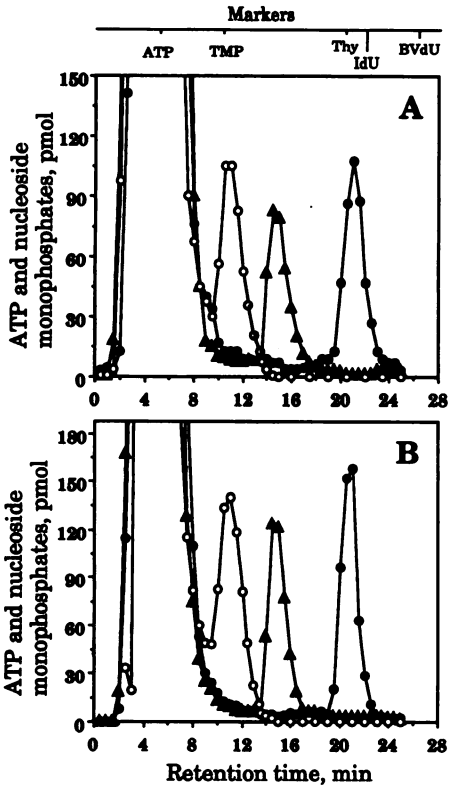

Fig. 2. HPLC elution profiles of the products of reactions of HSV-1 TK with [γ-^{32}P]ATP and the D-enantiomers (A) and L-enantiomers (B) of IdU (▲), BVdU (●), and dThd (*Thy*) (○). The profiles of the three analyses for each enantiomer are overlaid.

monophosphates are eluted, in a sequence that mirrors that of the markers D-dThd, D-IdU, and D-BVdU.

Under our chromatographic conditions we can quantitate only the radioactivity of the nucleoside monophosphates, because the labeled nucleoside diphosphates eventually formed by the thymidylate kinase activity associated with the HSV-1 TK are not completely separated from the labeled ATP. Therefore, we have no information regarding whether L-IdUMP and L-BVdUMP are further converted to diphosphates by the viral thymidylate kinase activity, as happens to the nucleoside monophosphates of their corresponding D-enantiomers. Direct studies with radiolabeled compounds will resolve this issue. However, the observed inability of L-TMP to compete with the phosphorylation of D-TMP by HSV-1 TK (14) suggests that L-IdUMP and L-BVdUMP might not be transformed to diphosphates, at least by the viral enzyme.

Inhibition of [^{3}H]dThd incorporation by L-IdU and L-BVdU in HeLa TK$^-$/HSV-1 TK$^+$ cells. To verify the ability of L-IdU and L-BVdU to compete with the natural substrate in intact cells, HeLa and HeLa TK$^-$/HSV-1 TK$^+$ cells were incubated in the presence of [^{3}H]dThd and increasing concentrations of L-enantiomers (and of D-enantiomers, as controls). D-IdU affected [^{3}H]dThd incorporation in HeLa cells, in agreement with its affinity for the cytosolic TK (Table 1), whereas both BVdU enantiomers and L-IdU did not affect [^{3}H]dThd incorporation (data not shown), confirming their lack of affinity for the cellular cytosolic TK (IC_{50} of >600 μM for BVdU enantiomers and >2000 μM for L-IdU) (Table 1). In contrast, both enantiomers of IdU (Fig. 3A) and BVdU (Fig. 3B) strongly inhibited [^{3}H]dThd incorporation in HeLa TK$^-$/HSV-1 TK$^+$ cells, as expected from the affinity of these compounds for the viral TK (Table 1).

Effects of L-IdU and L-BVdU on HeLa cell growth. We have also evaluated the effect of D- and L-enantiomers of IdU and BVdU on cellular growth and viability of three different HeLa cell lines, i.e., HeLa, HeLa TK$^-$, and HeLa TK$^-$/HSV-1 TK$^+$ (Fig. 4). Cell viability was determined as CFA after 8 days of exposure to the drug. The effect of equal concentrations of D-dThd is always reported for comparison.

Both enantiomers of BVDU, like D-dThd, had no or little effect on the CFA of HeLa cells (Fig. 4, A and D) or HeLa TK$^-$ cells (Fig. 4, B and E), even at the highest concentration tested (100 μM). This is clearly linked to their lack of affinity for the cellular cytosolic TK. In contrast, with the HeLa TK$^-$/HSV-1 TK$^+$ cells a marked difference in cytotoxicity was seen between BVDU enantiomers (Fig. 4, C and F). Despite the fact that the two enantiomers are equally well recognized as substrates by the viral TK, D-BVDU completely abolished the CFA of HeLa TK$^-$/HSV TK$^+$ cells at concentrations (0.01 μM) 1000 times lower, compared with L-BVDU (10 μM). We speculate that the lower cytotoxic effect of the L-enantiomer results at least in part from the absence of inhibitory effects of L-BVdUMP on cellular TS (see next paragraph).

Regarding the IdU enantiomers, a marked difference was observed between the effects of D- and L-IdU in both HeLa

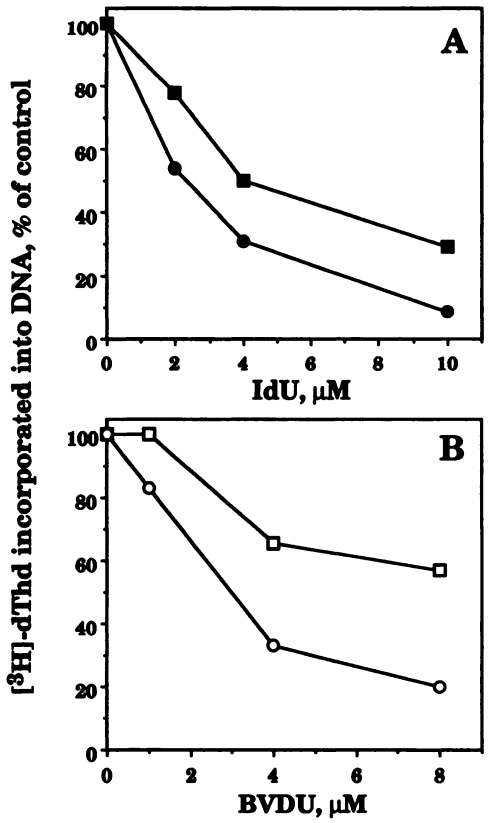

Fig. 3. Inhibition of [^{3}H]dThd incorporation in HeLa TK$^-$/HSV-1 TK$^+$ cells by D-IdU (●) and L-IdU (■) (A) or D-BVdU (○) and L-BVdU (□) (B). The decrease in [^{3}H]dThd incorporation reflects the inhibition of [^{3}H]dThd phosphorylation due to competition with IdU and BVdU; 100% corresponds to 0.03–0.04 μCi of [^{3}H]dTMP incorporated. Each *point* is the average of three determinations.

cells (Fig. 4A) and HeLa TK$^-$/HSV-1 TK$^+$ cells (Fig. 4C). At concentrations of D-IdU that almost completely abolished the CFA in both cell lines (10 μM), the L-enantiomers only had an effect comparable to that of D-dThd. In HeLa cells, the very low cytotoxicity of L-IdU is certainly linked to the lack of affinity for the cytosolic TK, whereas in the HeLa TK$^-$/HSV-1 TK$^+$ cells it may again be due to the lack of inhibitory activity of L-IdUMP on cellular TS (see next paragraph).

Effects of D- and L-enantiomers of IdU and BVdU on TS activity *in vivo*. It is known that several 5-substituted dUMP analogs, such as 5-fluoro-dUMP and 5-BVdUMP, are potent inhibitors of cellular TS (4). This inhibition depletes the dTTP pool in infected cells, directly affecting DNA synthesis and contributing to the cytotoxicity of these compounds. Therefore, we have investigated and compared the effects of both L- and D-enantiomers of IdU and BVdU on TS activity in HeLa cells and HeLa TK$^-$/HSV-1 TK$^+$ cells. TS represents the *de novo* pathway of dTMP synthesis, because it catalyzes the reductive methylation of dUMP to dTMP, in which the hydrogen atom on C-5 of the uracil ring is replaced by a methyl group. Thus, the activity of TS in intact cells can be measured by estimating the tritium release from [5-^{3}H]dUMP formed upon administration of [5-^{3}H]deoxyuridine or [5-^{3}H]dCyd, as described by Balzarini *et al.* (12). In fact, both labeled nucleosides are converted to [5-^{3}H]dUMP, [5-^{3}H]deoxyuridine directly by phosphorylation and [5-^{3}H]dCyd either via deamination to [5-^{3}H]deoxyuridine and phosphorylation to [5-^{3}H]dUMP or via phosphorylation to [5-^{3}H]dCMP and deamination to [5-^{3}H]dUMP. The results shown in Fig. 5 were obtained by administering to growing cells [5-^{3}H]dCyd, because [5-^{3}H]deoxyuridine is not commercially available.

In HeLa cells D-IdU is phosphorylated by cellular TK and inhibited the tritium release at micromolar concentrations (Fig. 5A). In contrast, D-BVdU is not a substrate for cellular TK and exerted an inhibitory effect at much higher concentrations (Fig. 5C), in agreement with the observation of Balzarini *et al.* (12). In the case of both L-enantiomers, which we have just described not to be substrates for the cellular TK, we observed no inhibition of the release of tritium when the compounds were assayed at up to 200 μM (Fig. 5, A and C). Therefore, the L-enantiomers are inactive.

In HeLa TK$^-$/HSV-1 TK$^+$ cells, where both D-IdU and D-BVdU are phosphorylated by viral TK, these compounds inhibited tritium release from [5-^{3}H]dUMP (Fig. 5, B and D). The corresponding L-enantiomers were again totally inactive (also when assayed up to 200 μM) (data not shown). The overall results suggest that TS is a stereospecific enzyme.

Resistance of L-IdU and L-BVdU to phosphorolysis by intact human blood platelets. Human blood platelets contain dThd phosphorylase as the sole pyrimidine nucleoside phosphorylase and have been used to monitor the intracellular degradation of dThd and of various 5-substituted 2′-deoxyuridines (5). In this system it was found that D-BVdU, D-IdU, and other 5-substituted 2′-deoxyuridines are effective substrates of dThd phosphorylase, which cleaves the uracil ring from the sugar moiety (5), thus limiting their potential therapeutic activity. To compare the susceptibility of D- and L-enantiomers to dThd phosphorylase, we incubated human blood platelets with 0.1 mM D- or L-enantiomers, as described in Materials and Methods. Aliquots of the incubation mixture were removed at 0, 20, and 60 min and analyzed by HPLC. Under these conditions D-BVdU and D-IdU were catabolized to their corresponding bases by approximately 94% and 80%, respectively, in 60 min, whereas L-BVdU and L-IdU were found to be fully resistant to degradation. Fig. 6 reports, for comparison, the HPLC elution profiles of D-BVdU after 20-min (Fig. 6A) and 60-min (Fig. 6B) incubations and of L-BVdU after a 60-min incubation (Fig. 6C). Because L-dThd also was completely resistant to phosphorolysis by human dThd phosphorylase,[1] the human dThd phosphorylase, like TK (1) and TS, appears to be a stereospecific enzyme.

Comparison between the antiviral activities of D- and L-enantiomers of IdU and BVdU in cell cultures. The anti-HSV-1 activity was tested in HeLa cells for IdU enantiomers and in four different cell lines for BVdU enantiomers. After 72 hr of continuous treatment in every cell line, no microscopically detectable alteration of normal cell morphology or proliferation was observed at the maximum concentrations tested for L-BVdU and D-BVdU (400 and 800 μM, respectively). In HeLa cells a maximum tolerated dose of >300 μM for L-IdU and of approximately 20 μM for D-IdU was observed. The antiviral activity of L-IdU and L-BVdU was evaluated both as their inhibition of virus-induced cytopathic effect in cell lines infected with HSV-1 (Table 2A) and as their ability to reduce viral yield (Table 2B). L-IdU was only 6–30 times less potent than D-IdU and L-BVdU was 30–200 times

[1] S. Spadari, F. Focher, and A. Verri, unpublished observations.

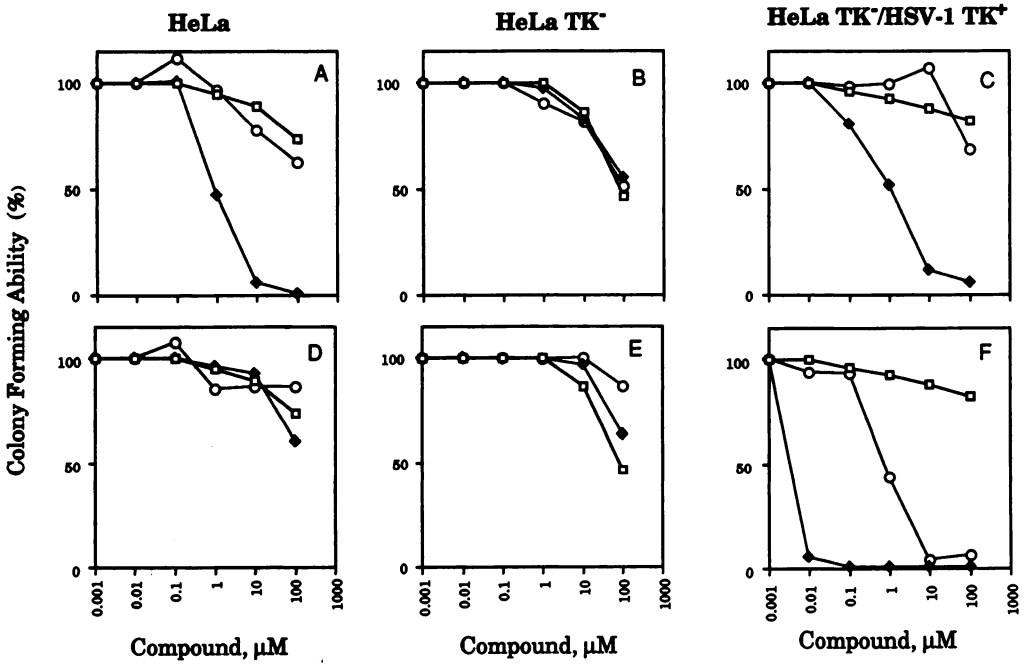

Fig. 4. CFA of HeLa (A and D), HeLa TK$^-$ (B and E), and HeLa TK$^-$/HSV-1 TK$^+$ (C and F) cells in the presence of increasing concentrations of IdU (*upper*) and BVdU (*lower*) enantiomers. ◆, D-Enantiomers; ○, L-enantiomers; □, D-dThd. Cell colonies were counted after 8 days of exposure.

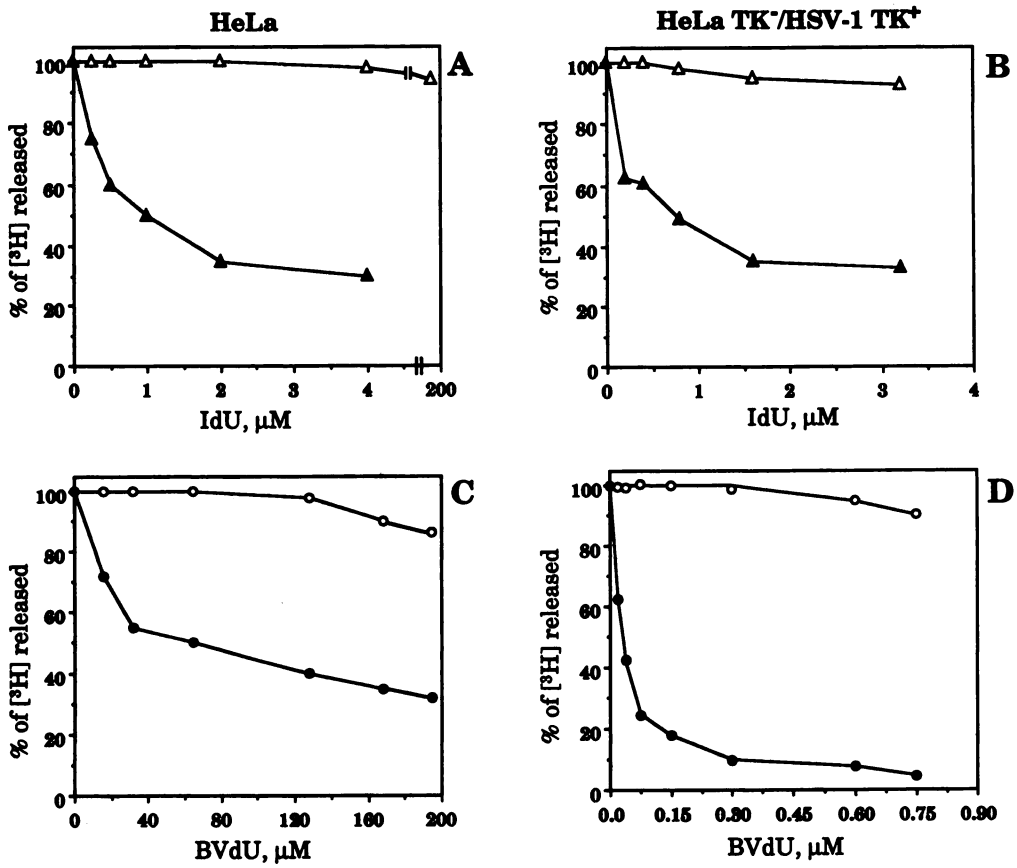

Fig. 5. Effect of D-IdU (▲), L-IdU (△), D-BVdU (●), and L-BVdU (○) on tritium release from [5-^{3}H]deoxyuridylate by TS in cells exposed to [5-^{3}H]dCyd. A and C, HeLa cells; B and D, HeLa TK$^-$/HSV-1 TK$^+$ cells. The 100% value corresponds to 0.02 or 0.04 μCi of tritium released in HeLa or HeLa TK$^-$/HSV-1 TK$^+$ cells, respectively. Each *point* is the average of three determinations.

less potent than D-BVdU in inhibiting the cytopathic effect of the virus or viral growth. The possibility that the observed anti-HSV-1 activity of L-BVdU is due to contamination (0.6–1.5%) by the D-enantiomer is unlikely, for the following reasons. Because L-BVdU is obtained from L-IdU in a two-reaction process that does not involve the chiral center of the

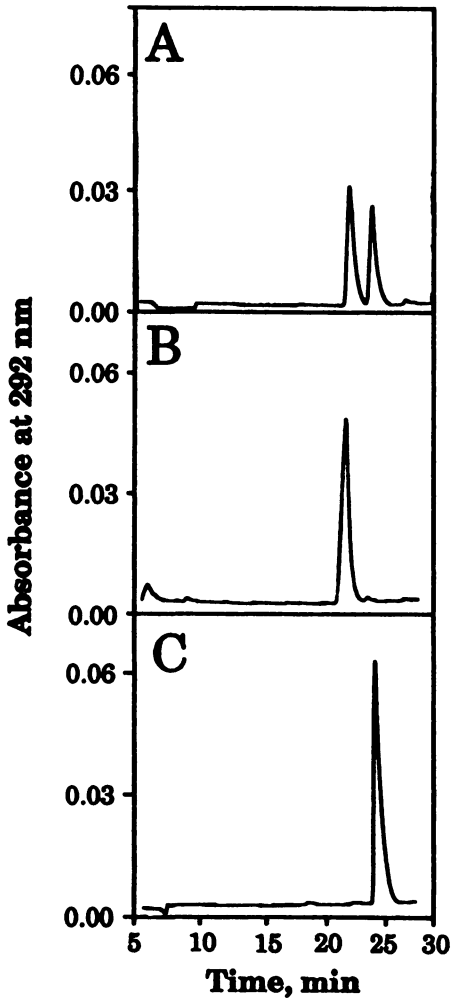

Fig. 6. HPLC elution profiles for D-BVdU after 20-min (A) and 60-min (B) incubations and for L-BVdU after a 60-min incubation (C) with intact human blood platelets. BVdU retention time, 24.5 min; (*E*)-5-(2-bromovinyl)-2′-uracil retention time, 23.5 min.

TABLE 2

Antiherpetic activity of IdU and BVdU enantiomers

Cells	IC_{50}				
	D-IdU	L-IdU	D-BVdU	L-BVdU	Acyclovir
			μM		
A. Inhibition of virus-induced cytopathic effect					
HeLa	1.7	51.7	0.07	5.1	30.5
HeLa TK^-	ND[a]	ND	0.2	23.3	22
U-373 MG	ND	ND	0.1	6.2	6.3
MRC-5	ND	ND	0.55	89.4	5.9
B. Reduction of viral yield					
HeLa	0.5	2.8	<0.001	0.2	0.1
HeLa TK^-	ND	ND	<0.03	0.9	4.2
U-373 MG	ND	ND	<0.01	1	0.8
MRC-5	ND	ND	<0.05	3.2	2.2

[a] ND, not determined.

molecule, its enantiomeric purity must be at least equal to that of the starting L-IdU. For the latter compound, the results of the assay with purified HeLa TK (Table 1) show the maximum possible contamination of the "L-enantiomer preparation" by the D-enantiomer to be <0.3% (IC_{50} D-IdU/IC_{50} L-IDU). Thus, the antiviral activity of the L-enantiomer preparations can be largely, if not exclusively, ascribed to the L-enantimers in their own right. This is in line with the anti-HSV-1 activity (IC_{50} = 25 μM) of L-dThd (1), whose enantiomer, natural D-dThd, is obviously inactive. Under the present experimental conditions, the potency of L-BVdU, with the exception of that in the infected MRC-5 cells, was comparable to that of acyclovir, which was used as a reference drug.

Discussion

The present results demonstrate that L-IdU and L-BVdU exert inhibitory actions similar to those of the D-enantiomers against HSV-1 TK at micromolar concentrations, while being inactive against human TK. The kinetics of their interactions with the viral TK are different; D- and L-IdU are purely competitive inhibitors, whereas L-BVdU shows linear mixed-type inhibition. Thus, the inversion of configuration affects the enzyme behavior towards BVdU enantiomers in the same way as it does with the carbocyclic derivatives carba-BVdU, carba-IdU, and carba-dThd (13). In contrast, it has no influence on the mechanism of inhibition displayed by IdU enantiomers, as already found for L-dThd (1) and for the enantiomers of carba-deoxyguanosine (15). The HPLC analysis of the reaction products shows that L-IdU and L-BVdU are converted to the corresponding monophosphates by HSV-1 TK with an efficiency comparable to that of the D-enantiomers. Thus, the absolute configuration of these analogs does not influence their processing by the viral enzyme. In contrast, the cytosolic TK is very sensitive to the stereochemical features of alternative substrates, as proved by the finding that inversion of configuration totally abolishes the sizable affinity of IdU for the human enzyme. As a result, L-IdU, in contrast to D-IdU, has no toxicity toward HeLa cells. Not all cytosolic kinases, however, accept as putative substrates only nucleoside analogs having the natural D-configuration. Recently, several analogs of the pyrimidine nucleosides have been tested as potential antiviral agents against human immunodeficiency virus and hepatitis B virus. Because these viruses do not possess their own pyrimidine kinases, modified nucleosides can be converted into the active phosphorylated derivatives only when accepted as substrates by the host kinases. When both enantiomeric forms were studied, it was always found that, among effective dThd analogs, only those having the absolute configuration corresponding to that of the natural D-substrate were active (2, 16), whereas with dCyd analogs both enantiomers may inhibit viral proliferation (2, 17–19). In some cases, the isomer with the unnatural L-configuration was significantly more potent than its natural counterpart (20). Thus, it can be concluded that, among human nucleoside kinases, TK is far more stereoselective than dCyd kinase.

The present work also shows that TS, the target enzyme for the cytostatic action of D-BVdU (12), is highly sensitive to the configuration of putative substrates. Thus, in HeLa TK^-/HSV-1 TK^+ cells (where D- and L-BVdUMP are formed with comparable efficiencies), L-BVdU is approximately 1000-fold less cytotoxic than its corresponding D-enantiomer, probably as a result of the very low affinity of L-BVdUMP for TS. However, an additional reason could derive from the 20-fold

lower affinity of L-BVdU, compared with D-BVdU, for the mitochondrial TK.[2]

Furthermore, we have also shown that the inversion of configuration of the sugar moiety strongly affects the affinity of the nucleoside for dThd phosphorylase, which appears unable to release the corresponding base from L-IdU, L-BVdU, and L-dThd. This fact favors the anabolic conversion of L-enantiomers to their monophosphate forms by viral TK.

On the basis of (i) selective phosphorylation by HSV-1 TK, (ii) complete inactivity toward cytosolic TK, TS, and dThd phosphorylase, (iii) very low affinity for mitochondrial TK, and (iv) very low cytotoxicity, L-IdU and L-BVdU appear very promising selective antiherpetic agents. However, their inhibitory potency against HSV-1 proliferation in infected cells is reduced by approximately 1 (L-IdU) or 2 (L-BVdU) orders of magnitude, compared with the D-enantiomers. This behavior is similar to that of the carba-IdU and carba-BVdU enantiomeric pairs (13). Most antiherpetic nucleoside analogs act, as their triphosphate forms, by interfering with viral DNA polymerase (13). Therefore, the lower potency of the L-enantiomers, compared with the D-enantiomers, could be due to a decreased efficiency of (i) cellular uptake, (ii) the anabolic pathway from the starting nucleoside to the 5′-triphosphate form, or (iii) the interaction of the L-triphosphate with the viral DNA polymerase. These aspects will be investigated when radiolabeled L-IdU and L-BVdU and their triphosphate forms are available. However, the very low affinity of L-IdUMP and L-BVdUMP for TS in intact cells (as described here), which is one possible reason for the low cytoxicity of L-IdU and L-BVdU, could explain their lower antiviral potency, compared with their D-enantiomers. In fact, TS, which is strongly inhibited by D-BVdUMP and D-IdUMP, is considered an important additional target for the antiviral activity of D-BVdU and D-IdU, due to the specific cell killing of newly HSV-1-infected and (now) HSV-1 TK-expressing cells (4, 12).

Altogether, the present findings show that inversion of configuration enhances the selectivity of the antiherpetic agents IdU and BVdU, although with the penalty of reduced antiviral potency. Nevertheless, L-BVdU is as active as acyclovir in reducing the virus yield and the virus-induced pathogenicity and deserves further evaluation as a potentially useful therapeutic agent for the treatment of acyclovir-resistant HSV-1 infections.

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