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Carbocyclic 5-Iodo-2′-deoxyuridine (C-IDU) and Carbocyclic (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine (C-BVDU) as Unique Examples of Chiral Molecules where the Two Enantiomeric Forms Are Biologically Active: Interaction of the (+)- and (−)-Enantiomers of C-IDU and C-BVDU with the Thymidine Kinase of Herpes Simplex Virus Type 1

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SUMMARY

The (+)- and (−)-enantiomers of the carbocyclic analogues of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (C-BVDU) and 5-iodo-2′-deoxyuridine (C-IDU) were synthesized by separate routes. Both the (+)- and (−)-enantiomers of C-BVDU and C-IDU were markedly inhibitory to herpes simplex virus type 1 (HSV-1) replication. (+)-C-BVDU and (+)-C-IDU were as inhibitory to HSV-1 as the racemic (±)-C-BVDU and (±)-C-IDU, respectively, whereas the (−)-enantiomers were only 10-fold less active. Also, the (+)- and (−)-enantiomers of C-BVDU were equally inhibitory to the growth of murine mammary carcinoma cells transformed by the HSV-1 or HSV-2 thymidine kinase (TK) gene (designated FM3A TK^-/HSV-1 TK^+ and FM3A TK^-/HSV-2 TK^+). The (+)- and (−)-enantiomers of C-BVDU and the (+)- and (−)-enantiomers of C-IDU had a remarkably similar affinity for HSV-1 TK [K_i, 0.09 and 0.19 μM for (+)-C-BVDU and (+)-C-IDU and 0.16 and 0.19 μM for (−)-C-BVDU and (−)-C-IDU, respectively]. The inhibition of HSV-1 TK by BVDU, IDU, (+)-C-BVDU, and (+)-C-IDU was purely competitive with regard to the natural substrate (thymidine), whereas (−)-C-BVDU, (−)-C-IDU, (±)-C-BVDU, and (±)C-IDU showed a linear mixed-type inhibition of HSV-1 TK. C-BVDU and C-IDU are examples of chiral molecules of which both isomeric forms are markedly active at both the cellular and enzymatic level.

The antiviral and cytostatic activity of nucleoside analogues is often limited because of their rapid degradation by catabolic enzymes. In an attempt to avoid the rapid degradation of pyrimidine nucleoside analogues by pyrimidine nucleoside phosphorylases (i.e., uridine and dThd phosphorylase), carbocyclic analogues of the antiviral (IDU, BVDU, and IVDU) and cytostatic (5-fluoro-2′-deoxyuridine and 5-nitro-2′-deoxyuridine) nucleosides were synthesized (1–4). In these carbocyclic nucleoside analogues, the sugar moiety is replaced by a cyclopentyl ring (Fig. 1). C-BVDU and C-IVDU are completely resistant to phosphorolytic cleavage by dThd phosphorylase and uridine phosphorylase (5). Furthermore, C-BVDU and C-IVDU, like their parent compounds, are highly potent and selective inhibitors of HSV-1 replication in cell culture (6). The selectivity of BVDU, IVDU, C-BVDU, and C-IVDU as anti-HSV-1 agents depends to a large extent on their phosphorylation by the virus-encoded TK (6–8). Consequently, BVDU and IVDU are much more inhibitory to the growth of murine mammary carcinoma (FM3A) cells that have been transformed by the viral TK genes (FM3A TK^-/HSV-1 TK^+ and FM3A TK^-/HSV-2 TK^+) than to the wild type (FM3A/0) cells (9–11). For example, BVDU is inhibitory to the growth of FM3A TK^-/HSV-1 TK^+ cells at a 2000-fold lower concentration than that required to inhibit FM3A/0 cell growth. Evidently, BVDU

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ABBREVIATIONS: dThd, thymidine; IDU, 5-iodo-2′-deoxyuridine; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; C-dGuo, carbocyclic analogue of deoxyguanosine; C-dThd, carbocyclic analogue of thymidine; C-IDU, carbocyclic analogue of 5-iodo-2′-deoxyuridine; C-BVDU, carbocyclic analogue of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; C-IVDU, carbocyclic analogue of (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; TK, thymidine kinase; HSV-1, herpes simplex virus type 1; C-Ado, carbocyclic analogue of adenosine; C-AMP, carbocyclic analogue of AMP; HSV-2, herpes simplex virus type 2; C-dDAPR, carbocyclic analogue of 2,6-diaminopurine 2′-deoxyriboside; $CCID_{50}$, 50% cell culture-infective dose; MIC, minimum inhibitory concentration.

must be phosphorylated by the virus-encoded TK to exert its cytostatic effect on FM3A TK⁻/HSV-1 TK⁺ or FM3A TK⁻/HSV-2 TK⁺ cells. Also, C-BVDU is much more inhibitory to the growth of FM3A TK⁻/HSV-1 TK⁺ cells than wild type FM3A/0 cells.

Carbocyclic nucleoside analogues are chiral molecules, the synthesis of which normally results in the formation of a racemic mixture containing both the (−)- and (+)-optical enantiomers. As a rule, only one of the enantiomeric forms of chiral molecules has biological activity. Among the (+)- and (−)-enantiomers of aristeromycin [(±)-C-Ado] following selective enzymatic conversion of (−)-C-AMP to (−)-C-Ado by 5′-ribonucleotide phosphohydrolase and conversion of the remaining (+)-C-AMP to (+)-C-Ado by alkaline phosphatase (12), only the (−)-form, the configuration of which corresponds to natural adenosine, showed significant cytostatic and antiviral activity. The (+)-enantiomer was totally inactive. Also, Secrist and coworkers (13) separated the (+)- and (−)-enantiomers of (±)-C-dDAPR by specific deamination of D-(+)-C-dDAPR to the corresponding D-(+)-C-dGuo derivative. Again, poor if any antiviral activity was noted for the L-(−)-enantiomer.

We have now synthesized the (+)-enantiomers of C-BVDU and C-IDU, the isomers mimicking the absolute configuration of the natural D-nucleosides, and the corresponding (−)-enantiomers, by totally independent routes (14, 15). The four enantiomers, as well as the racemic mixtures, were evaluated for their anti-HSV-1 and anti-HSV-2 activity, their affinity for HSV-1 TK, and their cytostatic activity against FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells. Both the (+)- and (−)-enantiomer forms of C-BVDU inhibited the replication of HSV-1 and the growth of FM3A cells transformed by the HSV-1 or HSV-2 TK gene. The (+)- and (−)-enantiomers of C-BVDU and C-IDU were also markedly inhibitory to HSV-1 TK; this inhibition was strictly competitive for the (+)-enantiomers but of the mixed type for the (−)-enantiomers. These carbocyclic pyrimidine nucleoside analogues are examples of chiral molecules of which both enantiomeric forms show biological activity.

Materials and Methods

Cells and viruses. FM3A cells (subclone F28-7), originally established from a spontaneous mammary carcinoma in a C3H/He mouse (16) and designated FM3A/0, were cultured as published earlier (17). FM3A TK⁻/HSV-1 TK⁺ and FM3A TK⁻/HSV-2 TK⁺ cells, which lack host cell TK activity but express the HSV-1 or HSV-2 TK activity, respectively, were derived from the TK-deficient FM3A TK⁻ cell line, as previously described (18–22). The cells transformed by the HSV-1 TK or HSV-2 TK gene were cultured in the same culture medium as the FM3A/0 cells.

Primary rabbit kidney cells were grown in Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% fetal calf serum (Flow Laboratories), 2 mM L-glutamine (Flow Laboratories), and 0.075% $NaHCO_3$.

The origin of the virus strains HSV-1 (KOS) and HSV-2 (G) has been described previously (23).

Compounds. The racemic mixtures (±)-C-BVDU and (±)-C-IDU were synthesized according to previously published procedures (2). The synthesis of the optically pure (+)- and (−)-enantiomers of C-BVDU and C-IDU will be described elsewhere (14, 15). Physical constants of the enantiomers are as follows. (+)-C-BVDU {(+)-1-[(1*R*,3*S*,4*R*)-3-hydroxy-4-(hydroxymethyl)-cyclopentyl]-5-[(*E*)-2-bromovinyl]-1*H*,3*H*-pyrimidine-2,4-dione}: m.p., 184–185°, decomposition; $[\alpha]_D^{20}$, +4.9° (concentration, 1.58, CH_3OH). (−)-C-BVDU: m.p., 184–185°, decomposition; $[\alpha]_D^{20}$, α −4.9° (concentration, 1.73, CH_3OH); ^1H NMR (CD_3OD) δ, 1.60–2.31 (overlapping multiplets, 5 H, 2 × CH_2, C*H*CH_2OH), 3.67 (m, 2 H, CH_2OH), 4.15 (m, 1 H, C*H*OH), 5.08 (m, 1 H, C*H*N), 6.79 (d, J = 13.6 Hz, 1 H, vinylic H), 7.34 (d, J = 13.6 Hz, 1 H, vinylic H), 7.75 (s, 1 H, H-6); ^{13}C NMR (CD_3OD) δ, 33.55 (C-5′), 40.43 (C-2′), 50.20 (C-4′), 56.62 (C-1′), 64.24 (C-6′), 73.79 (C-3′), 109.17 (C-α), 112.48 (C-5), 130.14 (C-β), 141.82 (C-6), 151.84 (C-4), 163.66 (C-2); UV (H_2O) λ_{max}, 254 nm, ϵ = 14,500; analysis, ($C_{12}H_{16}BrN_2O_4$) C, H, N. (+)-C-IDU {(+)-1-[(1*R*,3*S*,4*R*)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-5-iodo-1*H*,3*H*-pyrimidine-2,4-dione}: m.p. 265–266°; $[\alpha]_D^{20}$, + 6.9° (concentration, 2.75, CH_3OH). (−)-C-IDU: m.p., 165–166°; $[\alpha]_D^{20}$, − 7.0° (concentration, 4.56, CH_3OH); ^1H NMR (dimethyl sulfoxide-d_6) δ, 1.09–2.26 (overlapping multiplets, 5 H, 2 × CH_2, C*H*CH_2OH), 3.40 (m, 2 H, CH_2OH), 4.00 (m, 1 H, C*H*OH), 4.43–5.05 (overlapping multiplets, 3 H, CHN, 2 × OH), 8.13 (s, 1 H, H-6), 11.2 (s, 1 H, NH); UV (H_2O) λ_{max}, 242 nm, ϵ = 11,500; analysis, ($C_{10}H_{13}IN_2O_4$) C, H, N. The physical data obtained for (+)- and (−)-C-BVDU and (+)- and (−)-C-IDU indicated an optical purity of up to 100% for both enantiomers. Enantiomeric purity was also checked by high performance liquid chromatography, using the chiral column Nucleosil Chiral 2 (Macherey-Nagel) (14). Because further recrystallization did not change optical rotations, we concluded that both compounds were enantiomerically pure. BVDU was synthesized according to a modification of the method described by Jones *et al.* (24). IDU was obtained from Sigma (St. Louis, MO). [*methyl*-^{3}H]dThd (specific radioactivity, 40 Ci/mmol) was obtained from Amersham International Limited.

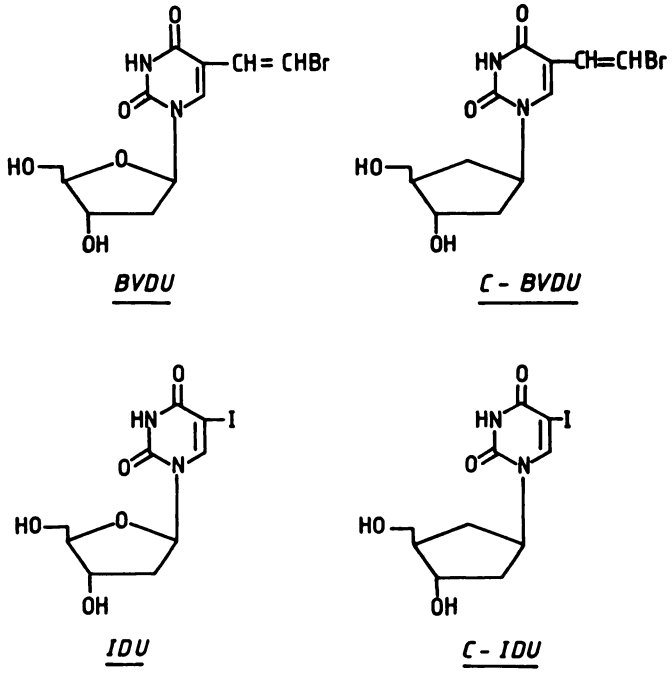

Fig. 1. Structural formulae of BVDU, C-BVDU [(+)-, (−)-, or (±)-C-BVDU], IDU, and C-IDU [(+)-, (−)-, or (±)-C-IDU].

Inhibition of cell proliferation. The methods for evaluating the cytostatic effects of the compounds on FM3A cells have been described previously (9, 17). Briefly, 5 × 10^4 cells were suspended in growth medium and added to microplate wells in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 48 hr at 37° in a humidified CO_2-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter.

Preparation of HSV-1 TK. HeLa TK⁻ cells (lacking cytosol TK) were seeded in 75-cm² culture bottles in Eagle's minimum essential medium supplemented with 10% (v/v) inactivated fetal calf serum (GIBCO Bio-Cult, Glasgow, Scotland), 2 mM L-glutamine (Flow Lab-

oratories), and 0.075% (w/v) $NaHCO_3$. When 90% confluent, the cell monolayers were infected with HSV-1 (KOS) at a multiplicity of infection of 10 $CCID_{50}$/culture. After 1-hr virus adsorption, the cells were further incubated in cell culture medium until viral cytopathogenicity reached 50–75%. The cell cultures were then washed four times with 50 mM Tris·HCl, pH 8.0, containing 0.9% NaCl, and were frozen at −70°. After thawing, the cells were treated with 0.1 M Tris·HCl, pH 8.0, containing 20 mM β-mercaptoethanol. Following sonication, the cell homogenate was cleared by centrifugation for 60 min at 105,000 × *g*. The 30–70% $(NH_4)_2SO_4$ precipitate of the cell homogenate was resuspended in Tris·HCl, pH 8.0, containing 20 mM β-mercaptoethanol, and was dialyzed overnight against 100 volumes of the same buffer supplemented with glycerol (final concentration of glycerol, 10%).

Enzyme (TK) assay and determination of inhibition constants (K_i). The standard assay mixture contained 5 mM ATP, 5 mM $MgCl_2$, 9 mM KF, 5 mM phosphoenolpyruvate, 5 μg of pyruvate kinase, 10 mM β-mercaptoethanol, varying concentrations of the radiolabeled substrate (0.2, 0.4, 0.6, 0.8, and 1.0 μM), and 10 μl of enzyme extract, in a total volume of 90 μl of 50 mM Tris·HCl, pH 8.0. The assay mixture was incubated at 37° for 15 min and the reaction was terminated by spotting 50 μl onto DE81 discs. The discs were immersed in 95% ethanol, washed three times in 95% ethanol, dried at 60° for 20 min, and analyzed for radioactivity by liquid scintillation counting in a toluene-based scintillant.

The K_i values of the test compounds against HSV-1 TK were calculated from the Lineweaver-Burk diagrams, using the following formula: $K_i = [I]/\left(\frac{K_m'}{K_m} - 1\right)$. Estimations of the kinetic behavior of the test compounds were based on the Dixon (slope versus $[I]$ plots) and slope versus $1/[S]$ plots.

Antiviral activity. The antiviral test procedures were based on an inhibition of virus-induced cytopathogenicity in primary rabbit kidney cell cultures. Briefly, confluent cell cultures in microtiter trays were inoculated with 100 $CCID_{50}$ of virus [HSV-1 (strain KOS), HSV-2 (strain G), or TK^- HSV-1 (strain VMW 1837)], 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After 1 hr of virus adsorption, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Viral cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

Results

Antiviral activity of the (+)- and (−)-enantiomers of C-BVDU and C-IDU. BVDU, IDU, the (+)- and (−)-enantiomers of C-BVDU and C-IDU, and the racemic mixtures (±)-C-BVDU and (±)C-IDU were evaluated for their activity against HSV-1 (KOS), HSV-2 (G), and the TK-deficient HSV-1 strain VMW 1837. BVDU and (±)-C-BVDU strongly inhibited HSV-1 replication (MIC, 0.01 and 0.02 μg/ml, respectively) (Table 1). Also, both the (+)- and (−)-enantiomers of C-BVDU were markedly inhibitory to HSV-1 replication, (−)-C-BVDU being 14-fold less effective than (+)-C-BVDU. BVDU and its carbocyclic congeners were 150- to 300-fold less inhibitory to HSV-2 replication than HSV-1 (Table 1). Again, BVDU was most effective (MIC, 2 μg/ml), followed by (+)-C-BVDU and (±)-C-BVDU, whereas (−)-C-BVDU was only weakly active (MIC, 20 μg/ml). None of the BVDU analogues showed activity against the TK^- HSV-1 strain VMW 1837, pointing to the virus-induced TK as a pivotal enzyme for the activation of the compounds.

IDU, like BVDU, proved to be active against HSV-1 replication, and so did C-IDU. The (+)-enantiomer had an activity similar to that of (±)-C-IDU, whereas the (−)-enantiomer was about 10-fold less effective. The carbocyclic IDU congeners were 40- to 200-fold less active than IDU against HSV-2, whereas none of the compounds proved to be inhibitory to TK^- HSV-1. Thus, IDU and its carbocyclic congeners showed an activity spectrum similar to that of BVDU and its carbocyclic analogues (Table 1).

TABLE 1
Antiherpetic activity of BVDU, IVDU, and their carbocyclic analogues
The data represent average values for three or four separate experiments.

Compound	MIC[a]		
	HSV-1 (KOS)	HSV-2 (G)	TK^- HSV-1 (VMW 1837)
	μg/ml		
BVDU	0.01	2	≥400
(+)-C-BVDU	0.05	7	>400
(−)-C-BVDU	0.7	20	>400
(±)-C-BVDU	0.02	7	>400
IDU	0.1	0.4	≥400
(+)-C-IDU	0.07	10	>400
(−)-C-IDU	1.0	70	>400
(±)-C-IDU	0.1	20	>400

[a] Concentration required to reduce virus-induced cytopathogenicity by 50%.

Cytostatic activity of the (+)- and (−)-enantiomers of C-BVDU and C-IDU. BVDU, IDU, and their carbocyclic (+)- and (−)-enantiomers and (±)-racemic mixtures were evaluated for their cytostatic effects on murine mammary carcinoma FM3A/O, FM3A TK^-/HSV-1 TK^+, and FM3A TK^-/HSV-2 TK^+ cells.

BVDU was much more cytostatic to the growth of FM3A TK^-/HSV-1 TK^+ and FM3A TK^-/HSV-2 TK^+ cells than its carbocyclic congeners (Fig. 2). The 50% inhibitory dose of BVDU for FM3A TK^-/HSV-1 TK^+ and FM3A TK^-/HSV-2 TK^+ cell proliferation was 35,000- to 300,000-fold lower than those recorded for its carbocyclic derivatives. However, no marked differences in cytostatic activity were noted for the (+)- and (−)-enantiomers of C-BVDU and their racemic mixtures, whether evaluated with FM3A TK^-/HSV-1 TK^+ or FM3A TK^-/HSV-2 TK^+ cells. The C-BVDU derivatives almost completely inhibited FM3A TK^-/HSV-2 TK^+ cell proliferation at the highest concentration tested (500 μg/ml) but only partially inhibited FM3A TK^-/HSV-1 TK^+ cell proliferation under these conditions (Fig. 2).

IDU and the carbocyclic IDU enantiomers were not markedly cytostatic to FM3A TK^- cells transformed by the HSV-1 or HSV-2 TK genes (data not shown).

Kinetics of the phosphorylation of the (+)- and (−)-enantiomers of C-BVDU and C-IDU. BVDU, the (+)- and (−)-enantiomers of C-BVDU, and the (±)-racemic mixture of C-BVDU were evaluated for the kinetics of their interaction with the HSV-1 encoded TK. Both BVDU and (±)-C-BVDU are potent inhibitors of HSV-1 TK (Figs. 3 and 4). Their K_i values are 0.039 and 0.220 μM and K_i/K_m ratios are 0.14 and 0.78, respectively (Table 2). These K_i values, which were calculated from the formula given in Materials and Methods, corresponded very closely to the K_i values obtained from a replot of the Lineweaver-Burk data (Fig. 3) as slope versus $[I]$ (Dixon plot). A slope versus $1/[S]$ plot derived from the Dixon ($1/V$ versus $[I]$) plot showed a linear correlation between slope and $1/[S]$ and resulted in a straight line through the origin (indicative of a competitive inhibition) for BVDU and a straight line intersecting the x-axis (indicative of noncompetitive or linear mixed-type inhibition) for (±)-C-BVDU (Fig. 4).

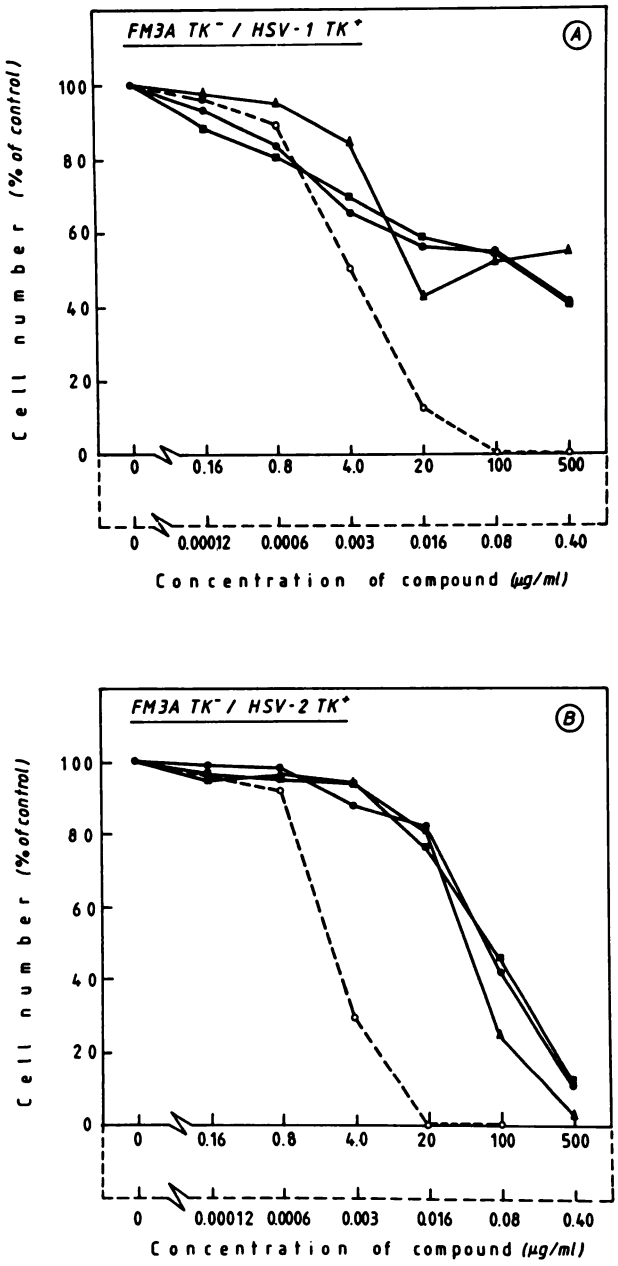

Fig. 2. Inhibition of the growth of FM3A TK⁻/HSV-1 TK⁺ (A) and FM3A TK⁻/HSV-2 TK⁺ cells (B) by BVDU (○), (+)-C-BVDU (■), (−)-C-BVDU (▲), and (±)-C-BVDU (●). The compound concentrations indicated on the *x*-axis refer to BVDU (– – –) on the one hand and (+)-C-BVDU, (−)-C-BVDU, and (±)-C-BVDU (——) on the other hand.

When the (+)- and (−)-enantiomers of C-BVDU were separately evaluated for their interaction with HSV-1 TK, both optical isomers proved to be markedly inhibitory to [*methyl*-^{3}H]dThd phosphorylation. In fact, both (+)- and (−)-enantiomers of C-BVDU were slightly more inhibitory to HSV-1 TK than the racemic mixture (±)-C-BVDU (Table 2). However, the kinetics of interaction of (+)-C-BVDU and (−)-C-BVDU with HSV-1 TK were clearly different. Like BVDU, (+)-C-BVDU was a potent competitive inhibitor of HSV-1 TK (Table 2), as is evident from the Lineweaver-Burk plots (Fig. 3) and Dixon replots (data not shown). Indeed, with increasing concentrations of BVDU and (+)-C-BVDU, the apparent K_m of the substrate [*methyl*-^{3}H]dThd increased proportionally, while the V_{max} remained unchanged (Fig. 3, *left*). Also, the slope versus 1/[*S*] replot resulted in a straight line going through the origin (Fig. 4, *left*). In contrast, (−)-C-BVDU and, to a lesser extent, (±)-C-BVDU proved to be linear mixed-type inhibitors of HSV-1 TK. This is clearly shown by the Lineweaver-Burk plots, in which the lines intersect left of the *y*-axis (Fig. 3, *right*) and by the Dixon slope versus 1/[*S*] replots (Fig. 4, *right*). As shown in Fig. 4 (*right*), the straight lines fitting the experimental data did not go through the origin. The K_i values of (−)-C-BVDU and (±)-C-BVDU listed in Table 2 were calculated from the slope versus [*I*] plots (Fig. 3, *inserts* in the *right*).

IDU, the (+)- and (−)-enantiomers of C-IDU, and the (±)-racemic mixture of C-IDU were also investigated for the kinetics of their interaction with HSV-1 TK. The K_i and K_i/K_m values are listed in Table 2. IDU and (+)-C-IDU were potent inhibitors of HSV-1 TK. They were about 2-fold less effective than BVDU and (+)-C-BVDU, respectively. Both (−)-C-IDU and (±)-C-IDU were about as inhibitory to HSV-1 TK as their carbocyclic BVDU counterparts (Table 2). The kinetic behavior of the IDU derivatives was essentially the same as that found for the BVDU derivatives. Thus, IDU and (+)-C-IDU were competitive inhibitors of HSV-1 TK, whereas (−)-C-IDU and (±)-C-IDU showed linear mixed-type inhibition of HSV-1 TK (Table 2). This kinetic behavior was evident from Lineweaver-Burk plots, Dixon (slope versus [*I*]) plots, and slope versus [1/*S*] replots thereof (data not shown).

Discussion

BVDU, IDU, and their carbocyclic derivatives (±)-C-BVDU and (±)-C-IDU require phosphorylation by the HSV-1- and HSV-2-encoded TK to exert their antiherpetic activity. A surprising finding, however, was that both enantiomers of C-BVDU and C-IDU proved to be inhibitory to the replication of HSV-1. These data suggested that both (+)- and (−)-enantiomers of C-BVDU and C-IDU might act as substrates for HSV-1 TK. That both optical isomers of chiral molecules would be biologically active is unexpected and such behavior has only exceptionally been demonstrated with the (+)- and (−)-enantiomers of carbocyclic purine nucleoside analogues. For example, we previously established that the (−)-enantiomer of aristeromycin [(−)-C-Ado] has significant cytostatic and virustatic activity, whereas the (+)-enantiomer is totally inactive (12). Secrist *et al.* (13) reported that the (+)-enantiomer of C-dDAPR is highly susceptible to enzymatic deamination to C-dGuo, whereas the (−)-enantiomer of C-dDAPR remains largely unaffected; accordingly, (−)-C-dGuo is much less active as an antiviral agent than (+)-C-dGuo. However, L-(+)-C-dGuo was found to have a modest antiviral activity not attributable to a minor impurity of D-(−)-C-dGuo, suggesting that L-(+)-C-dGuo was endowed with (poor) antiviral activity in its own right. The biological and kinetic behavior of the (+)- and (−)-enantiomers of C-IDU and C-BVDU may be extended to other carbocyclic pyrimidine nucleoside analogues. Indeed, we have recently examined the (+)- and (−)-enantiomers of C-dThd (obtained from J. Beres, Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, Hungary) and found both optical enantiomers to be markedly inhibitory to HSV-1 TK but not cytosol TK (derived from human lymphocyte MT-4 cells). Again, the (+)-enantiomer of C-dThd was a competitive inhibitor of HSV-1 TK, whereas the (−)-enan-

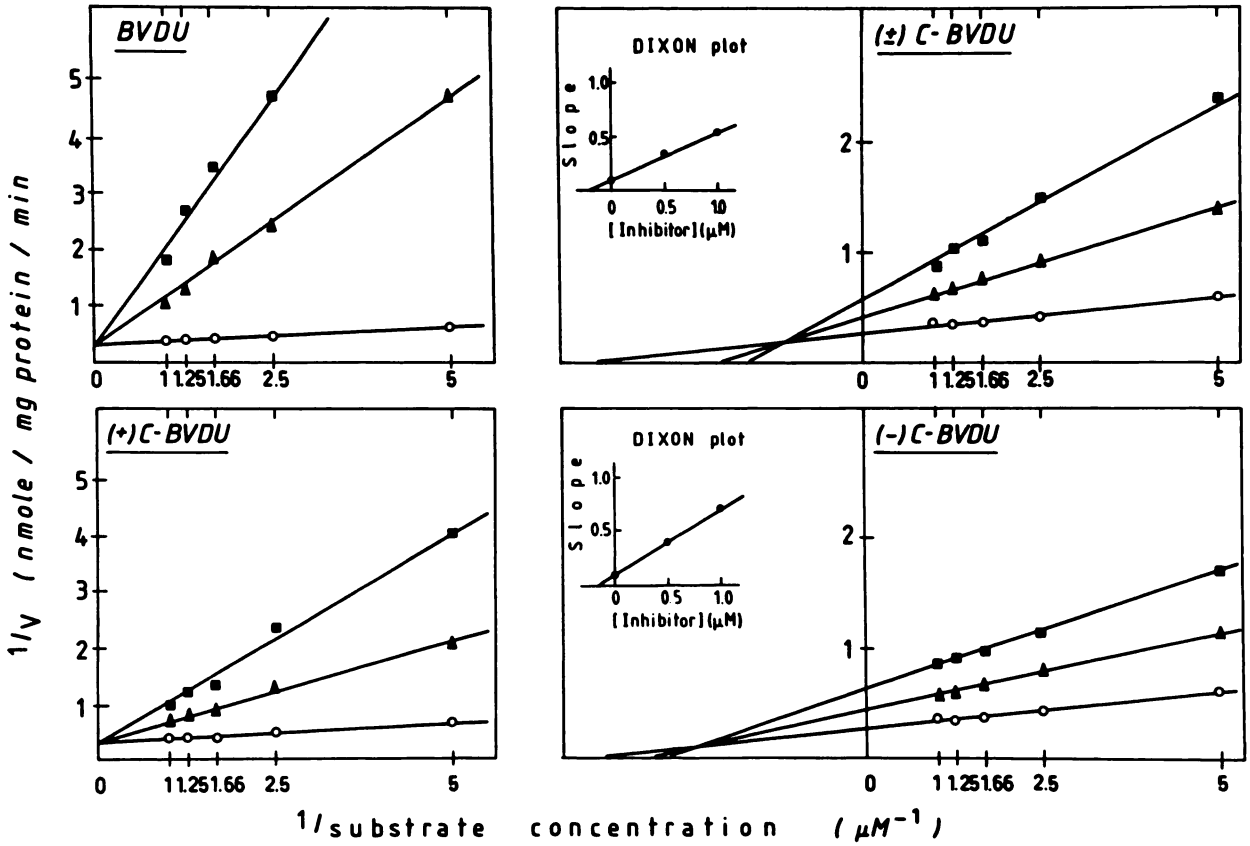

Fig. 3. Lineweaver-Burk ($1/v$ versus $1/[S]$) and Dixon (slope versus $[I]$) plots for HSV-1 TK activity (with [*methyl*-^{3}H]dThd as the substrate) in the presence of BVDU, (+)-C-BVDU, (−)-C-BVDU, and (±)-C-BVDU. Concentrations of the test compounds were 0 (○), 0.5 (▲), and 1.0 μM (■).

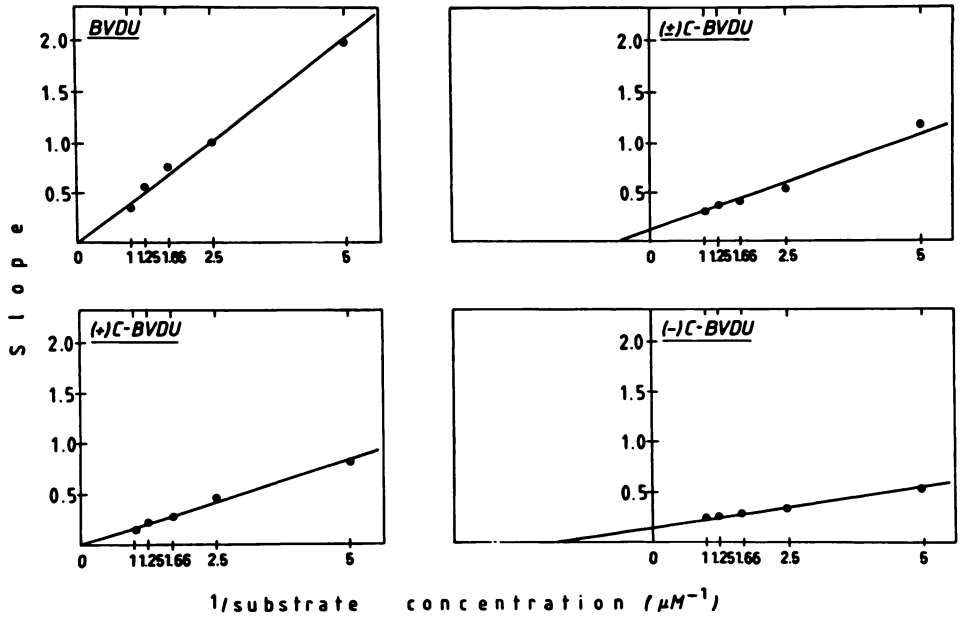

Fig. 4. Slope versus $1/[S]$ plots for HSV-1 TK activity (with [*methyl*-^{3}H]dThd as the radiolabeled substrate) in the presence of BVDU, (+)-C-BVDU, (−)-C-BVDU, and (±)-C-BVDU.

tiomer of C-dThd afforded a linear mixed-type inhibition of HSV-1 TK (data not shown). Moreover, both enantiomers were considerably less effective inhibitors for cytosol TK, with (−)-C-dThd being almost inert. Thus, in our studies, we could unequivocally demonstrate that the optical enantiomers of at least three different carbocyclic pyrimidine nucleoside analogues behaved in a similar way regarding their kinetic properties against HSV-1 TK. Due to the lack of radiolabeled enantiomers of C-BVDU, C-IDU, and C-dThd, we were unable to determine the exact substrate affinities of these compounds by virtue of their K_m values. However, because the carbocyclic nucleoside analogues must be phosphorylated to exert their antiherpetic activity, one would guess that the (−)-enantiomers as well as the (+)-enantiomers must be substrates for the HSV-1-encoded TK. Therefore, one may speculate that the lesser antiviral activity of the (−)-enantiomers compared with the

TABLE 2

Inhibition of HSV-1-encoded TK by BVDU, IDU and their carbocyclic analogues

K_m values for [*methyl*-^{3}H]dThd in the individual experiments ranged from 0.198 to 0.340 μM. The average K_m values obtained in the BVDU and IDU experiments were 0.281 and 0.286 μM, respectively. Data represent mean values for four to six separate experiments ± SD. K_i values for BVDU, (+)-C-BVDU, IDU, and (+)-C-IDU were calculated from the Lineweaver-Burk plots. The K_i values for (−)-C-BVDU, (±)-C-BVDU, (−)-C-IDU, and (±)-C-IDU were derived from Dixon (slope versus [*I*] replots.

Compound	K_i	K_i/K_m	Type of inhibition
	μM		
BVDU	0.039 ± 0.004	0.14	Competitive
(+)-C-BVDU	0.096 ± 0.010	0.34	Competitive
(−)-C-BVDU	0.160 ± 0.03	0.57	Linear mixed-type
(±)-C-BVDU	0.220 ± 0.095	0.78	Linear mixed-type
IDU	0.092 ± 0.004	0.32	Competitive
(+)-C-IDU	0.190 ± 0.011	0.66	Competitive
(−)-C-IDU	0.192 ± 0.109	0.67	Linear mixed-type
(±)-C-IDU	0.208 ± 0.077	0.73	Linear mixed-type

(+)-enantiomers of C-BVDU and C-IDU may be due to a lower V_{max} value of the (−)-enantiomers for HSV-1 TK. Direct studies with radiolabeled compound will resolve this issue. In any event, the unexpected finding that both (+)- and (−)-enantiomers of several carbocyclic pyrimidine nucleoside analogues are recognized by HSV-1 TK may be related to the unusual substrate affinities of the herpetic TK.

In the present study we have demonstrated that both (+)- and (−)-enantiomers of C-BVDU and C-IDU are (i) active against HSV-1 and, to a lesser extent, HSV-2, (ii) highly dependent on phosphorylation by the HSV-1-specified TK to exert their antiviral properties, (iii) equally cytostatic against tumor cells expressing HSV-1 or HSV-2 TK activity, and (iv) strongly inhibitory to the isolated HSV-1 TK (in experiments where [*methyl*-^{3}H]dThd served as the radiolabeled substrate). Furthermore, enzyme kinetics of the (+)-enantiomers were clearly different from those of the (−)-enantiomers. Whereas (+)-C-BVDU, like BVDU, competitively inhibited dThd phosphorylation by HSV-1 TK, (−)-C-BVDU showed a linear mixed-type inhibition. This differential behavior was ascertained by plotting the kinetic data as Lineweaver-Burk plots, Dixon (slope versus [*I*]) plots, and slope versus 1/[*S*] plots. All three plots were in agreement with regard to the purely competitive mode of action of (+)-C-BVDU and linear mixed-type inhibition of (−)-C-BVDU. The same conclusion was reached for the enantiomers of C-IDU (Table 2) and C-dThd (data not shown) and may well extend to the enantiomers of other carbocyclic pyrimidine nucleoside analogues.

At first glance, it seems contradictory that, on the one hand, C-BVDU has an affinity for HSV-1 TK and an inhibitory effect on HSV-1 replication (Table 1) similar to those of BVDU but, on the other hand, C-BVDU is much less inhibitory than BVDU to the proliferation of FM3A TK$^-$/HSV-1 TK$^+$ cells (Fig. 2). This apparent paradox can be explained by the differences in the mechanism of antiviral and cytostatic action of the compounds. The target for the antiviral action of both BVDU and C-BVDU is the viral DNA polymerase, and both BVDU 5′-triphosphate and C-BVDU 5′-triphosphate have been shown to be potent inhibitors of the DNA polymerization reaction (25, 26). In contrast, the cytostatic activity of 5-substituted pyrimidine 2′-deoxynucleoside analogues is mediated by an inhibitory effect on thymidylate synthase as the target enzyme (27), which implies that to exert their antiviral effect the compounds have to be converted intracellularly to the 5′-triphosphate stage, whereas for their cytostatic effect they ought to be phosphorylated to the 5′-monophosphate only (28). Thus, the pronounced inhibitory effects of BVDU on the growth of FM3A TK$^-$/HSV-1 TK$^+$ and FM3A TK$^-$/HSV-2 TK$^+$ cells can be attributed to the inhibition of thymidylate synthase by BVDU 5′-monophosphate (11, 29). The carbocyclic derivatives of 5-substituted dUMP analogues (i.e., 5-nitro-dUMP) are much less inhibitory to thymidylate synthase than the parent deoxyribosyl compounds (4). This may then explain why the carbocyclic deoxyuridine derivatives, despite their intracellular phosphorylation to the 5′-monophosphate by HSV TK, are much less cytostatic for the FM3A TK$^-$/HSV TK$^+$ cells than the parent compounds.

Our findings on the marked biological activity of both (+)- and (−)-enantiomers of carbocyclic pyrimidine nucleoside analogues have both a fundamental and a practical impact. The fact that both (+)- and (−)- optical enantiomers of carbocyclic IDU and BVDU are recognized to a similar extent by the HSV-1 TK clearly points to the unique and unusual characteristics of the viral TK relative to other enzymes (i.e., cytosol TK). The herpetic TK clearly shows a much lower substrate specificity for nucleoside analogues than its cytosolic counterpart. Our data also imply that a stereospecific synthesis of a chemically pure enantiomer of the carbocyclic pyrimidine nucleoside analogue does not necessarily improve its biological activity. However, such stereospecific synthesis may well be recommended in those cases where one of the enantiomers is endowed with a higher cytotoxic potential than the other. Separate synthesis of the enantiomers should then significantly increase the selectivity index (therapeutic index) of the compound. A case in point is (+)-C-dThd, which was markedly more inhibitory than (−)-C-dThd to the growth of FM3A cells, whereas both enantiomers were equally inhibitory to HSV-1 TK (data not shown).

In conclusion, this is the first report addressing the biological and biochemical effects of the pure (+)- and (−)-enantiomeric forms of C-BVDU and C-IDU. In contrast to optical isomers of chiral molecules in general, both the (+)- and (−)-enantiomeric forms of C-BVDU and C-IDU showed a marked inhibitory effect on HSV replication and a similar affinity for the viral TK. Carbocyclic pyrimidine nucleoside analogues such as C-BVDU and C-IDU can be considered as examples of chiral molecules of which the two isomeric forms exhibit a similar affinity at the enzymatic (i.e., HSV TK) level.

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