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SELECTIVE ANTIHERPETIC ACTIVITY OF CARBOCYCLIC ANALOGUES OF (E)-5-(2-HALOGENOVINYL)-2'-DEOXYURIDINES : DEPENDENCE ON SPECIFIC PHOSPHORYLATION BY VIRAL THYMIDINE KINASE

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SUMMARY The carbocyclic analogues of (E)-5-(2-bromovinyl)-2'-deoxyuridine (C-BVDU) and (E)-5-(2-iodovinyl)-2'-deoxyuridine (C-IVDU), in which the sugar moiety is replaced by a cyclopentane ring, are as efficient substrates for the herpes simplex type 1 (HSV-1)-encoded thymidine kinase (TK) as their parent compounds (BVDU and IVDU). This conclusion is based on the binding affinities (Ki) of BVDU, IVDU, C-BVDU and C-IVDU to the HSV-1 TK and on the phosphorylation rates (Km, V_{max}) of (^{125}I)IVDU and (^{125}I)C-IVDU by the enzyme. The specific phosphorylation of C-BVDU and C-IVDU by the viral TK may explain why these compounds are highly selective inhibitors of HSV-1 replication.

BVDU and IVDU are among the most potent and most selective inhibitors of HSV-1 and VZV replication that have been described to date[1]: i.e., they are 5-fold more potent against HSV-1 and 1000-fold more potent against VZV than the recently licensed antiviral drug, acyclovir.[2,3] The selectivity of BVDU and IVDU is attested by their low cytotoxicity : they do not interfere with normal host cell functions unless their concentrations are raised to 5,000-50,000 times the minimal antiviral concentration.[2,3]

An untoward feature of BVDU and IVDU is that they are efficient substrates for pyrimidine nucleoside phosphorylases, i.e. dThd phosphorylase[4] and Urd phosphorylase, which cleave BVDU and IVDU at their N-glycosidic linkage, thereby releasing the free pyrimidine bases. As a consequence, BVDU is rapidly cleared from the plasma, within 2-3 hours after intraperitoneal administration to rats.[5]

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Abbreviations : BVDU, (E)-5-(2-bromovinyl)-2'-deoxyuridine; IVDU, (E)-5-(2-iodovinyl)-2'-deoxyuridine; C-BVDU, carbocyclic BVDU; C-IVDU, carbocyclic IVDU; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus; TK (deoxy)thymidine kinase; PBS, phosphate-buffered saline.

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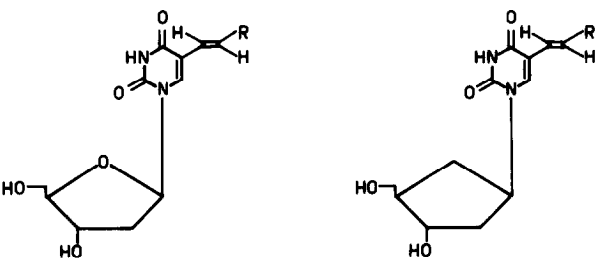

Fig. 1. Structural formulae of BVDU, IVDU, C-BVDU and C-IVDU.

To circumvent the problem of phosphorolytic cleavage of BVDU and IVDU, we have recently synthesized[6] the carbocyclic analogues, C-BVDU and C-IVDU, in which the sugar moiety is replaced by a cyclopentane ring (Fig. 1). C-BVDU and C-IVDU are completely resistant to phosphorolytic cleavage by dThd phosphorylase and Urd phosphorylase.[7] Furthermore, C-BVDU and C-IVDU are, like their parent compounds BVDU and IVDU, highly potent and selective inhibitors of HSV-1 replication in cell culture.

The selectivity of BVDU and IVDU as antiherpes agents depends to a large extent on a specific phosphorylation by the virus-encoded (deoxy)thymidine kinase (TK). BVDU and IVDU, as well as several other selective antiherpes agents, have a much greater affinity for HSV-1 TK than for cellular (cytosol) TK;[8,9] and this explains why BVDU and IVDU are preferentially phosphorylated by HSV-1-infected cells.[10]

In the present study we addressed the question whether the selective antiherpetic action of C-BVDU and C-IVDU may also be explained by a specific interaction with the viral TK. Such an interaction would have implications from a fundamental molecular-biological viewpoint, because it would mean that a nucleoside analogue containing a cyclopentane ring instead of the usual pentose moiety could act as substrate in an enzymatic reaction that would allow it to be metabolized by the cell.

MATERIALS AND METHODS

Compounds. BVDU and IVDU were synthesized by R. Busson and H. Vanderhaeghe (Rega Institute, Katholieke Universiteit Leuven, Belgium), following a method similar to that of Jones et al.[11] C-BVDU and C-IVDU were synthesized as described by Herdewijn et al.[6] [Methyl-^{3}H]dThd (specific radioactivity : 52 Ci/mmol) and [2-^{14}C]dThd (specific radioactivity : 50-60 mCi/mmol) were obtained from Amersham International Limited (Amersham, England).

Synthesis of [^{125}I]IVDU. [^{125}I]NaI (Amersham) (2.5 mCi in 25 µl of 0.05 N NaOH) was added to a solution of 0.5 mg IVDU and 2 µmol HCl in 0.15

ml ethanol. The mixture was heated in a sealed tube at 130°C for 15 min. After cooling, [^{125}I]IVDU was isolated from other reaction products by HPLC on a short-alkyl reversed phase (C_2) column (250 mm x 10 mm i.d.). The HPLC apparatus was equipped with both a U.V. detector (Waters Associates, model 440) connected to a chart recorder and a 2" [^{125}I]NaI radiation detector coupled to a multichannel analyzer (Canberra series 40). The column was eluted in an isocratic fashion at 3 ml/min with methanol-water (5:95). The eluate fraction containing [^{125}I]IVDU (eluting time : 20-26 min) was collected, and the solvent was removed by lyophilization. The residue was taken up in PBS to yield 2 mCi [^{125}I]IVDU with a specific radioactivity of 1.89 Ci/mmol.

Synthesis of [^{125}I]C-IVDU. [^{125}I]NaI (Amersham) (1.5 mCi in 15 µl of 0.05 N NaOH) was added to a solution of 0.2 mg C-IVDU and 1 µmol HCl in 60 µl ethanol. The mixture was heated in a sealed tube at 130°C for 15 min. After cooling, [^{125}I]C-IVDU was isolated from other reaction products by HPLC on a short-alkyl RP2 column (250 mm x 10 mm i.d.). The HPLC apparatus was equipped as described above. The column was eluted in an isocratic fashion at 3 ml/min with dioxane-water (20:80). The eluate fraction containing [^{125}I]C-IVDU (eluting time : 8.5-11 min) was collected and evaporated to dryness at reduced pressure (0.1 mm Hg) at 10°C. The residue was taken up in PBS to yield 1.25 mCi [^{125}I]C-IVDU with a specific radioactivity of 2.35 Ci/mmol.

Antiviral assays. The procedure for measuring inhibition of virus-induced cytopathogenicity (in primary rabbit kidney cells) as well as the sources of the virus strains have been described previously.[2]

Preparation of HSV-1 TK. Primary rabbit kidney cells were seeded in 75 cm^2 tissue culture flasks (Sterilin, Teddington, Middlesex, England) 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 Laboratories, Irvine, Scotland) and 0.075 % (w/v) $NaHCO_3$. When 90 % confluent, the cell monolayers were infected with HSV-1 (strain KOS) at a multiplicity of $10^{6.5}$ $CCID_{50}$ (cell culture 50 % infective doses)/ml/culture. After 1-hour virus adsorption, the cells were further incubated in cell culture medium for 14 hours. At that time viral cytopathogenicity reached 50-75 %. The cell cultures were then washed 4 times with 50 mM Tris-HCl, pH 8.0, containing 0.9 % NaCl and frozen at -20°C. After thawing, the cells were treated with 0.1 M Tris-HCl, pH 8.0, containing 20 mM β-mercaptoethanol. Following sonication (2 times, 10 sec), the cell homogenate was cleared by centrifugation for 30 min at 70,000 g and the supernatant was stored at -20°C until used as source of HSV-1 TK.

Determination of inhibition constants (Ki). Ki values of BVDU, IVDU, C-BVDU and C-IVDU for HSV-1 TK were determined as described by Cheng and Ostrander.[12] In these experiments [2-^{14}C]dThd served as the radiolabelled substrate.

Determination of phosphorylation rates. Km and V_{max} values were determined for HSV-1 TK with [methyl-^{3}H]dThd, [^{125}I]IVDU or [^{125}I]C-IVDU as the radiolabelled substrate. The standard assay mixture contained 5 mM ATP, 5 mM $MgCl_2.6\ H_2O$, 9 mM KF, 5 mM phosphoenolpyruvate, 5 µg pyruvate kinase, 10 mM β-mercaptoethanol, varying concentrations (100, 50, 25, 12.5 and 6.25 µM) of the radiolabelled substrate (100 µM corresponding to 0.2 µCi for [methyl-^{3}H]dThd, 0.34 µCi for [^{125}I]IVDU and 0.67 µCi for [^{125}I]-C-IVDU, respectively) and 10 µl enzyme extract in a total volume of 40 µl 50 mM Tris-HCl, pH 8.0. The assay mixture was incubated at 37°C for 15 min. and the reaction was terminated by the addition of 75 µl ice-cold 50 mM Tris-HCl, pH 8.0. After boiling for 90 sec., the assay mixture was applied onto DE81 discs and washed successively with 1 mM NH_4OOCH, pH 8.2, ethanol and ether. The discs were then evaluated for radioactivity in a toluene-based scintillant.

RESULTS

The carbocyclic analogues C-BVDU and C-IVDU were almost as active as their riboside counterparts BVDU and IVDU in inhibiting the replication of HSV-1 in primary rabbit kidney cells (Table I). Like their parent compounds, C-BVDU and C-IVDU were only inhibitory to HSV-2 at a concentration 100- to 300-fold higher than that required to inhibit HSV-1 replication. C-BVDU and C-IVDU were totally inactive against a TK-deficient (TK^-) variant of HSV-1, and also inactive against vaccinia, a virus that is known to code for a TK with a substrate specificity different from that induced by HSV-1. The latter findings suggested that C-BVDU and C-IVDU owed their selective activity against HSV-1 to a specific interaction with and phosphorylation by the HSV-1-encoded TK.

That C-BVDU and C-IVDU specifically interfered with HSV-1 TK was ascertained by measuring the cell-free enzyme kinetics with varying concentrations of $(2\text{-}^{14}C)$dThd as substrate and varying concentrations of C-BVDU, C-IVDU, BVDU or IVDU as inhibitor. As shown in Fig. 2, C-BVDU and C-IVDU proved equally effective in inhibiting HSV-1 TK activity as their parent compounds BVDU and IVDU, and all four nucleoside analogues behaved as competitive inhibitors with respect to $(2\text{-}^{14}C)$dThd phosphorylation. The Km of HSV-1 TK for $(2\text{-}^{14}C)$dThd in these assays was 6.25 µM and the Ki/Km ratios were as follows : 1.52 for BVDU, 0.91 for C-BVDU, 1.03 for IVDU and 0.64 for C-IVDU. On the assumption that Ki/Km ratios reflect binding affinity, these data suggested that C-BVDU and C-IVDU had at least as great an affinity for HSV-1 TK as their riboside counterparts BVDU and IVDU.

The rates of phosphorylation by HSV-1 TK were then measured with (^{125}I)C-IVDU or (^{125}I)IVDU as radiolabelled substrate. As demonstrated by the respective Km and V_{max} values (Table II), (^{125}I)C-IVDU was as efficient, if not more efficient, as substrate for HSV-1 TK than either (^{125}I)-

Table I. Antiviral activity of the carbocyclic analogues of BVDU and IVDU in primary rabbit kidney cell cultures

Compound	MIC_{50}* (µM)			
	HSV-1	HSV-2	TK^- HSV-1	Vaccinia virus
C-BVDU	0.15	45	> 1200	900
C-IVDU	0.21	26	> 1050	780
BVDU	0.03	6	300	21
IVDU	0.026	5.3	263	18

*Minimum inhibitory concentration required to reduce virus-induced cytopathogenicity by 50 % : average values for three HSV-1 strains (KOS, F, McIntyre), two HSV-2 strains (Lyons, G) and one TK^- HSV-1 strain (B2006).

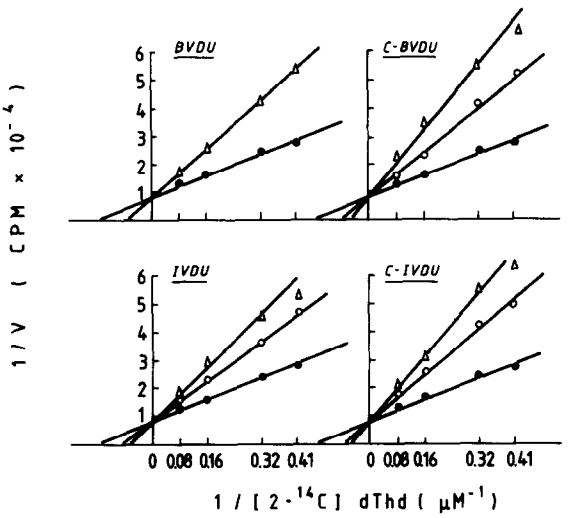

Fig. 2. Lineweaver-Burk plots for HSV-1 TK activity (with (2-^{14}C)dThd as substrate) in the presence of BVDU, C-BVDU, IVDU or C-IVDU at a final concentration of 0 μM (●), 5.55 μM (○) or 11.1 μM (Δ).

IVDU or (methyl-^{3}H)dThd, and its V_{max} was even 2-fold higher than that for (methyl-^{3}H)dThd.

Additional experiments have indicated that (5-^{125}I)C-IVDU is not a substrate for cellular TK (data not shown). While most of the radioactivity recovered from HSV-1-infected cells exposed for varying times (i.e. 6, 12 or 24 hours) to (5-^{125}I)C-IVDU was identified as (5-^{125}I)C-IVDUMP, not even a trace of (5-^{125}I)C-IVDUMP could be detected in uninfected cells exposed to (5-^{125}I)C-IVDU. From these cells only intact (5-^{125}I)C-IVDU could be recovered, suggesting that it was not phosphorylated by host cell TK.

DISCUSSION

Our findings indicate that the carbocyclic analogues of BVDU and IVDU are recognized as substrate by the HSV-1 encoded TK, and that, consequently, C-BVDU and C-IVDU may be metabolized (i.e. phosphorylated) in HSV-1

Table II. Phosphorylation rates of dThd, IVDU and C-IVDU by HSV-1 TK

Compound	Parameter	
	Km (μM)	V_{max} (nmol/mg protein/hour)
(Methyl-^{3}H)dThd	4.84	142
(5-^{125}I)IVDU	5.58	178
(5-^{125}I)C-IVDU	3.15	298

infected cells. Uninfected cells would be unable to phosphorylate these carbocyclic analogues.

Carbocyclic analogues of 5-substituted 2'-deoxyuridines such as 5-fluoro- and 5-iodo-2'-deoxyuridine have been the subject of previous studies.[13,14] The carbocyclic analogue of 5-fluoro-2'-deoxyuridine was inactive against HSV-1, but the carbocyclic 5-iodo-2'-deoxyuridine proved as active against HSV-1 as 5-iodo-dUrd itself. Carbocyclic 5-iodo-dUrd did not show any activity against TK^- HSV-1, and while this finding[14] suggested that carbocyclic uracil nucleosides must be activated by the virus-induced TK to be effective against HSV-1, no direct evidence was offered for this activation process. The present study demonstrates that carbocyclic 2'-deoxyuridines, i.e. C-BVDU and C-IVDU, are indeed phosphorylated by the HSV-1 TK, and this virus-specific mechanism of activation may be the prime reason for the high degree of antiviral selectivity of C-BVDU and C-IVDU.

Previous studies have demonstrated that the high affinity of BVDU for the HSV-1 induced TK is not substantially altered upon substitution of the 2-deoxyribofuranosyl moiety of BVDU by β-D-arabinofuranosyl, 2-deoxy-2-fluoro-β-D-arabinofuranosyl, 2,3-dideoxy-3-chlororibofuranosyl or 2,3-dideoxy-3-aminoribofuranosyl.[9,15] As shown here, BVDU analogues in which the 2-deoxyribofuranosyl moiety is replaced by a non-sugar, i.e. a cycloalkane (cyclopentane), are also able to act as substrate of HSV-1 TK.

This, in turn, indicates that such cyclopentyl pyrimidine derivatives can be metabolized by the HSV-1-infected cell. It would now seem imperative to follow the fate of these derivatives within the virus-infected cell, to examine whether they are phosphorylated to the 5'-triphosphate stage, and, if so, to determine whether they are incorporated into viral DNA or cellular DNA, or both.

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