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Role of the Incorporation of (*E*)-5-(2-Iodovinyl)-2′-deoxyuridine and Its Carbocyclic Analogue into DNA of Herpes Simplex Virus Type 1-Infected Cells in the Antiviral Effects of These Compounds

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SUMMARY

The carbocyclic analogue of (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (C-IVDU) is, like its parent compound (*E*)-5-(2-iodovinyl)-2′-deoxyuridine (IVDU), a potent and selective inhibitor of herpes simplex virus type 1 (HSV-1). There is a close correlation between the inhibition of viral DNA synthesis and the antiviral activity of both IVDU and C-IVDU. IVDU and C-IVDU inhibit viral DNA synthesis at 0.2 and 0.5 μM, respectively, and interfere with cellular DNA synthesis at concentrations that are 10- to 40-fold in excess of their antivirally effective doses. At concentrations affording a similar antiviral effect, C-[^{125}I]IVDU is incorporated into viral and cellular DNA of HSV-1-infected Vero cells to a 7- to 10-fold lesser extent than IVDU. [^{125}I]IVDU but not C-[^{125}I]IVDU leads to breakage of both DNA strands when incorporated into HSV-1 DNA.

BVDU and IVDU are potent and highly selective inhibitors of the replication of several herpes viruses in both cell cultures and animal models (1–4). The selectivity of these compounds as inhibitors of HSV-1 replication primarily depends upon a preferential activation (phosphorylation) by the HSV-1-encoded TK (5). BVDU and IVDU are also excellent substrates for pyrimidine nucleoside phosphorylases, which cleave the *N*-glycoside linkage between the pyrimidine ring and the sugar moiety (6, 7). Consequently, this rapid degradation may affect the therapeutic efficacy of BVDU and IVDU and, therefore, attempts have been made to prevent the phosphorolytic cleavage of BVDU and IVDU.

In this perspective, C-BVDU and C-IVDU (Fig. 1) were synthesized (8). In these nucleoside analogues, the sugar moiety is replaced by a cyclopentane ring. As expected, these carbocyclic derivatives of BVDU and IVDU did not act as substrates for pyrimidine nucleoside phosphorylases (8, 9). In cell culture, C-BVDU and C-IVDU proved to be almost as potent and at least as selective as BVDU and IVDU against HSV-1 replication (8). We also established that C-IVDU is effectively metabolized by HSV-1-infected cells and converted to its 5′-triphosphate form (10). C-IVDU is not phosphorylated to an appreciable extent by either mock-infected cells or cells infected with a TK$^-$ mutant of HSV-1 (10). These data point to the pivotal role of HSV-1 TK in the activation (phosphorylation) of the drug. Recently, we demonstrated that C-IVDU is incorporated into both viral and cellular DNA of HSV-1-infected Vero cells (10). This finding represents the first example of the incorporation of a cyclopentyl pyrimidine into DNA. What has remained unclear, however, is to what extent the incorporation of C-IVDU (or C-BVDU) into viral DNA contributes to their antiviral activity. Nor has it been resolved whether the incorporation of the carbocyclic nucleoside analogue affects the integrity of viral (and cellular) DNA.

In this study, we have investigated the relationship between the incorporation of C-IVDU into viral DNA and its antiherpetic activity, the effect of C-IVDU on viral and cellular DNA synthesis, and the effect of incorporation of C-IVDU on the integrity of HSV-1 DNA. From our data it will become clear that C-IVDU achieves its antiviral activity through a different mechanism than IVDU.

Materials and Methods

Compounds. IVDU was synthesized by R. Busson and H. Vanderhaeghe (Rega Institute for Medical Research, Katholieke Universiteit

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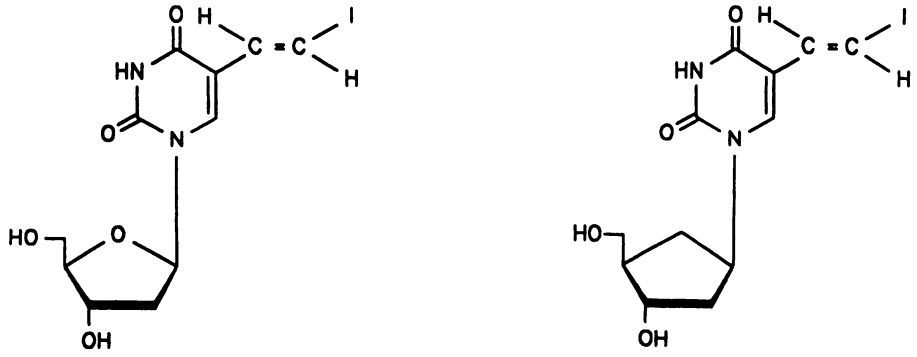
ABBREVIATIONS: BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; IVDU, (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; HSV-1, herpes simplex virus type 1; TK, thymidine kinase; C-BVDU, carbocyclic analogue of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; C-IVDU, carbocyclic analogue of (*E*)-5-(2-iodovinyl)-2′-deoxyuridine; EMEM, Eagle's minimum essential medium; PFU, plaque-forming unit; dThd, thymidine.



(E)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU)

Carbocyclic(E)-5-(2-iodovinyl)-2'-deoxyuridine (C-IVDU)

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

Leuven, Belgium), following a procedure described by Jones *et al.* (11). C-IVDU was synthesized as described by Herdewijn *et al.* (8). [*methyl*-^{3}H]dThd (specific radioactivity, 52 Ci/mmol) was obtained from Amersham International Limited. [^{125}I]IVDU and C-[^{125}I]IVDU were synthesized according to a previously described procedure (10).

Cells. Vero cells were grown in EMEM (Flow Laboratories) supplemented with 10% fetal calf serum (Flow Laboratories), 2 mM L-glutamine (Flow Laboratories), and 0.075% $NaHCO_3$.

Virus. The origin of HSV-1 (strain KOS) has been described previously (4).

Antiviral activity of IVDU and C-IVDU. Vero cells were seeded in 60-mm Petri dishes (Falcon, Becton Dickinson, Orangeburg, NY) at 5×10^5 cells/Petri dish and cultured in supplemented EMEM. Twenty-four hours later, the cell cultures were infected with 0.5 ml of HSV-1 (KOS) at 1 PFU/cell. After a 1-hr adsorption period, the virus was removed and 2 ml of supplemented EMEM, containing 0, 0.5, 1, 5, 10, or 50 μM IVDU or C-IVDU, were added to the cell cultures. The infected cell cultures were incubated for an additional 24 hr at 37° in a humidified CO_2-controlled atmosphere. Then, the cultures were frozen and stored at −70°. To determine the virus amount present in the culture fluids, the cell cultures were thawed and centrifuged at 10,000 × *g* for 10 min at 4°, and the virus titer in the supernatants was determined as follows. A series of decimal dilutions (1:10, 1:100, 1:1000, etc.) were prepared from the supernatants. Then, confluent Vero cell cultures (in 60-mm Petri dishes) were infected with 0.5 ml of the appropriate dilutions of the virus suspension. After a 1-hr adsorption, residual virus was removed and cells were further incubated at 37°. When viral plaques became visible, a second 1.2% agarose overlay containing 10% neutral red was added. Then, the number of plaques were recorded and the amount of virus in the cell culture supernatants was expressed as PFU/ml.

Cesium chloride equilibrium gradient analysis. In a first set of experiments, confluent Vero cells (in 60-mm Petri dishes) were infected with HSV-1 (KOS) at a multiplicity of 0.1 PFU/cell. After a 1-hr incubation at 37°, residual virus was removed and the cells were further incubated upon addition of IVDU at 0, 0.2, 0.5, or 2 μM or C-IVDU at 0.5, 5, or 20 μM, in the presence of [^{32}P]orthophosphate (at 25 μCi/infected cell culture), for 24 hr. Then, the medium was removed and the cells were treated with 200 μl of lysis buffer containing 0.2% sodium dodecyl sulfate, 0.5% *N*-laurylsarcosylate, 1 mM EDTA, 0.1 M NaCl, 10 mM Tris·HCl, pH 7.4, and 0.2 mg of Pronase (Calbiochem-Behring, La Jolla, CA). The lysates were layered onto 8 ml of a CsCl solution (1.71 g/ml) in distilled water and centrifuged for 64 hr at 40,000 rpm in a TFT 65.13 rotor (Kontron, Beun de Ronde, Amsterdam, The Netherlands). Seven-drop fractions were collected from the bottom of the tubes, and 70 μl of each fraction were spotted on Whatman GF/C paper disks, which were washed twice with 5% trichloroacetic acid and once with ethanol. The radioactivity of the samples was determined in a liquid scintillation counter. The density of each fifth fraction was calculated from the refraction indexes measured with a Zeiss refractometer.

In a second set of experiments, confluent Vero cell cultures were infected as described above and incubated with [^{125}I]IVDU (at 0.2, 0.5, or 2 μM) or C-[^{125}I]IVDU (at 0.5, 5, or 20 μM) for 24 hr. The radioactivity input per culture was 6 and 24 μCi for [^{125}I]IVDU and C-[^{125}I]IVDU, respectively. Cell cultures were treated as described above, viral and cellular DNA were separated by CsCl equilibrium gradient centrifugation, and the radioactivity of the samples was determined in a Hewlett Packard γ-counter.

Linear sucrose gradient analysis. HSV-1 (KOS) DNA was separated from cellular DNA by CsCl equilibrium gradient centrifugation and desalted by Sephadex gel filtration (Gilson-Analis, Gent, Belgium). Then, 200 μl of the DNA solution were placed on top of a 4.8-ml linear gradient [10% to 30% (w/w)] of ultrapure sucrose (Schwarz/Mann, Becton Dickinson) in water. The sucrose gradients were centrifuged at 100,000 × *g* for 2.5 hr in a SW 56.1 titanium rotor (MSE Scientific Instruments) at room temperature. Then, five-drop fractions were collected from the top of the tubes (upon pumping up of the sucrose gradients with a 50% (w/v) sucrose solution). The density of each third fraction was determined with a Zeiss refractometer. Sixty microliters of each fraction were precipitated on Whatman GF/C paper discs with 5% trichloroacetic acid, and the discs were dried with ethanol. Radioactivity of the samples was determined in a Hewlett Packard γ-counter (^{125}I-labeled samples) or a liquid scintillation counter (^{3}H-labeled samples).

Results

Antiherpetic activity of IVDU and C-IVDU in Vero cells. Vero cells were infected with HSV-1 (KOS) at 1 PFU/cell and incubated with varying concentrations of IVDU and C-IVDU for 24 hr. Virus yield was determined based on the number of PFU present in the cell culture supernatants. IVDU and C-IVDU afforded 75%, 99%, and 99.9% reduction in HSV-1 yield at concentrations of 0.2 and 0.5 μM, 1 and 4 μM, and 2.5 and 6 μM, respectively (Fig. 2). At 10 μM, IVDU and C-IVDU reduced the HSV-1 titer by 5 and 4 orders of magnitude, respectively. Thus, over a broad range of concentrations, IVDU brought about a 10-fold greater decrease in virus titer than C-IVDU.

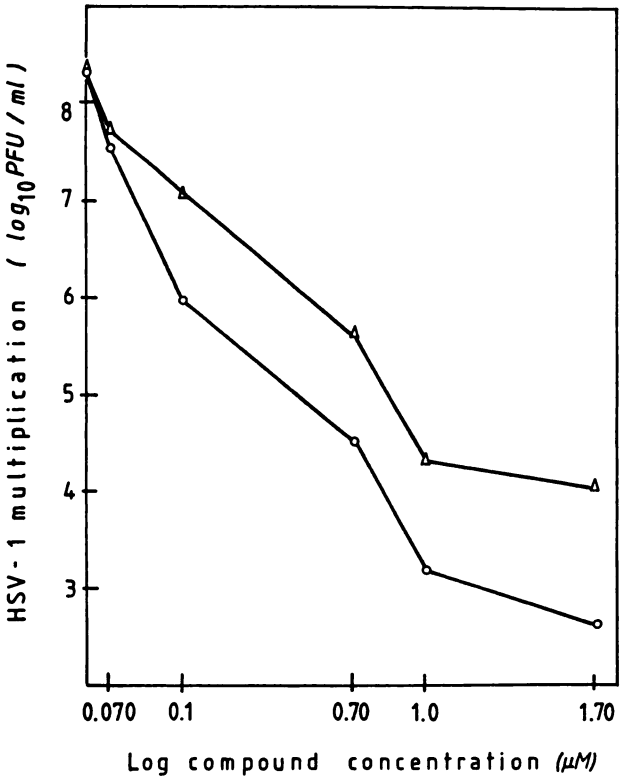


Fig. 2. Inhibitory effects of IVDU (○) and C-IVDU (△) on HSV-1 (KOS) multiplication in Vero cell cultures.

Effect of IVDU and C-IVDU on HSV-1 DNA synthesis in Vero cells. The effect of different concentrations of IVDU and C-IVDU on cellular and viral DNA synthesis was investigated in HSV-1 (KOS)-infected Vero cells in the presence of [^{32}P]orthophosphate. The data given in Fig. 3 represent the values of one representative experiment. However, although the efficiency of incorporation of ^{32}P into DNA may vary from one experiment to another (due to slight variations in the conditions of the cells, incubation, and extraction procedures), at least three to five other independent experiments were carried out in which comparable inhibitory effects of the test compounds were noted (Fig. 3). At a concentration of 0.5 μM, C-IVDU reduced viral DNA synthesis by approximately 65% (Fig. 3, *shadowed peaks* at high density in E and F). With IVDU, 50% inhibition of HSV-1 DNA synthesis was achieved at a concentration of 0.2 μM (Fig. 3, A and B). C-IVDU completely suppressed viral DNA synthesis at a concentration of 5 μM (Fig. 3 G). IVDU achieved an almost total inhibition of viral DNA synthesis at a concentration of 0.5 μM (Fig. 3C). At these concentrations (5 μM C-IVDU, 0.5 μM IVDU), neither compound interfered with cellular DNA synthesis. Only at doses as high as 20 μM (C-IVDU) or 2 μM (IVDU) was cellular DNA synthesis affected (Fig. 3, peaks at low density in D and H).

This inhibitory effect on cellular DNA synthesis was more pronounced for IVDU than C-IVDU.

Incorporation of IVDU and C-IVDU into viral and cellular DNA of HSV-1 (KOS)-infected Vero cells. Incorporation of [^{125}I] and C-[^{125}I]IVDU into viral and cellular DNA was investigated in HSV-1 (KOS)-infected Vero cells. The cell cultures were infected with HSV-1 (KOS) at a multiplicity of 0.1 PFU/cell and incubated with varying concentrations of the test compounds for 24 hr (Fig. 4). The higher the initial concentration of [^{125}I]IVDU, the more extensively [^{125}I]IVDU was incorporated into viral DNA rather than cellular DNA (Fig. 4, A, B, and C). C-[^{125}I]IVDU was more extensively incorporated into viral DNA at a concentration of 5 μM but less extensively at the concentration of 20 μM (Fig. 4, D, E, and F). At equivalent antiviral concentrations, i.e., 0.2 μM IVDU and 0.5 μM C-IVDU, and 2.5 μM IVDU and 6 μM C-IVDU (see Fig. 2), IVDU was incorporated into viral DNA to a much higher extent than C-IVDU [Fig. 4, A (0.2 μM IVDU) and C (2 μM IVDU), as compared with D (0.5 μM C-IVDU) and E (5 μM C-IVDU)].

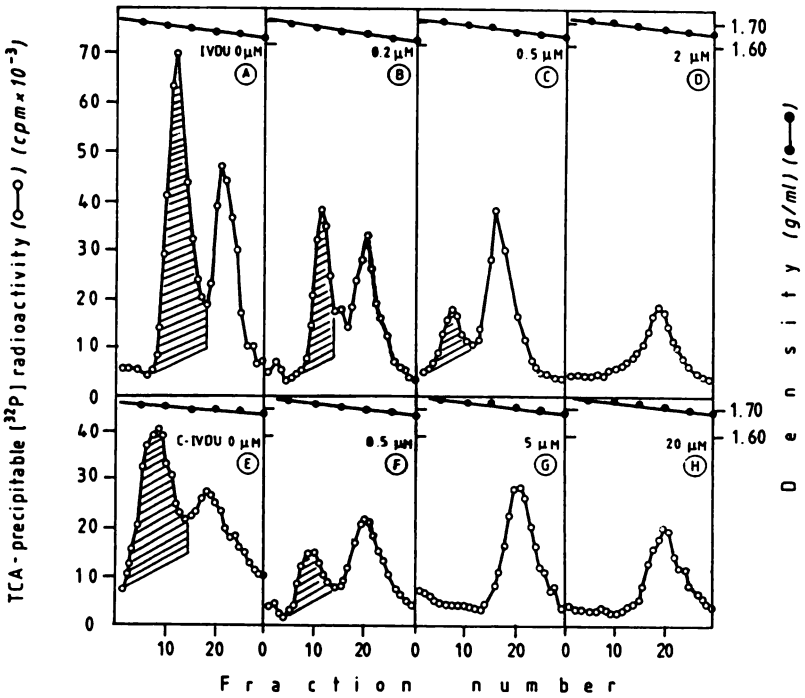


Fig. 3. CsCl equilibrium gradient analysis of DNA from HSV-1 (KOS)-infected Vero cells incubated for 24 hr in the presence of [^{32}P]orthophosphate at 25 μCi/cell culture. The concentration of IVDU was 0 μM (A), 0.2 μM (B), 0.5 μM (C), or 2.0 μM (D); the concentration of C-IVDU was 0 μM (E), 0.5 μM (F), 5 μM (G), or 20 μM (H). The results shown represent data from one experiment. Similar results were obtained in at least three to five independent experiments. *TCA*, trichloroacetic acid.

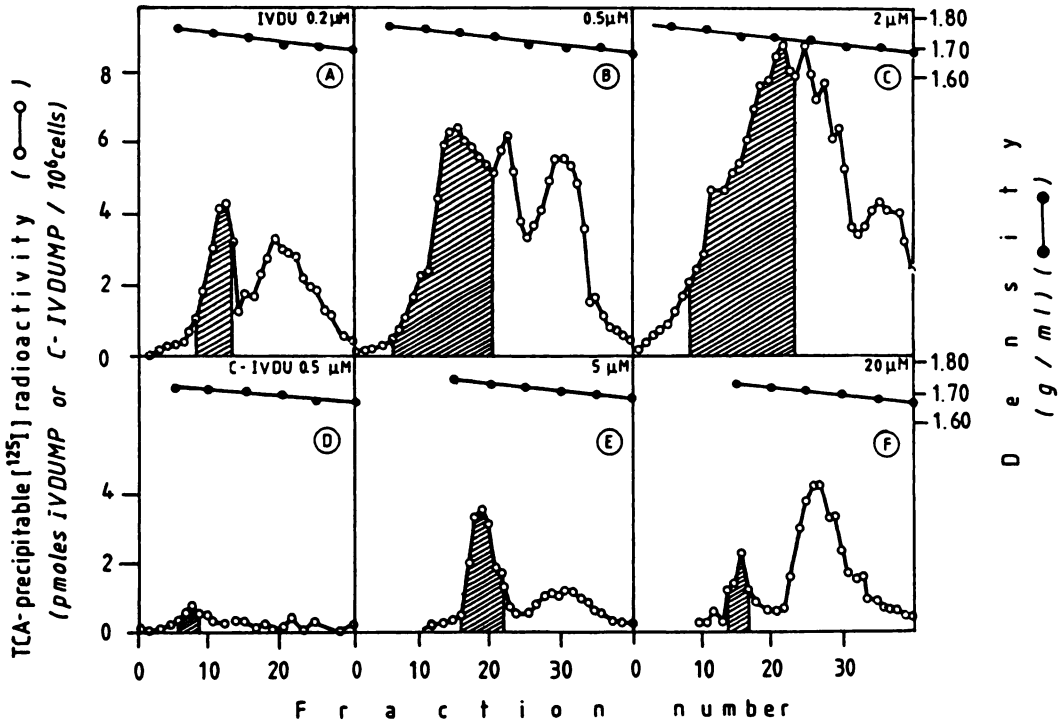


Fig. 4. CsCl equilibrium gradient analysis of DNA from HSV-1 (KOS)-infected Vero cells incubated for 24 hr in the presence of either [^{125}I]IVDU [at 0.2 μM (A), 0.5 μM (B), or 2.0 μM (C)] or C-[^{125}I]IVDU [at 0.5 μM (D), 5 μM (E), or 20 μM (F)]. The results shown represent data from one experiment. Similar results were obtained in at least three to five independent experiments. *TCA*, trichloroacetic acid.

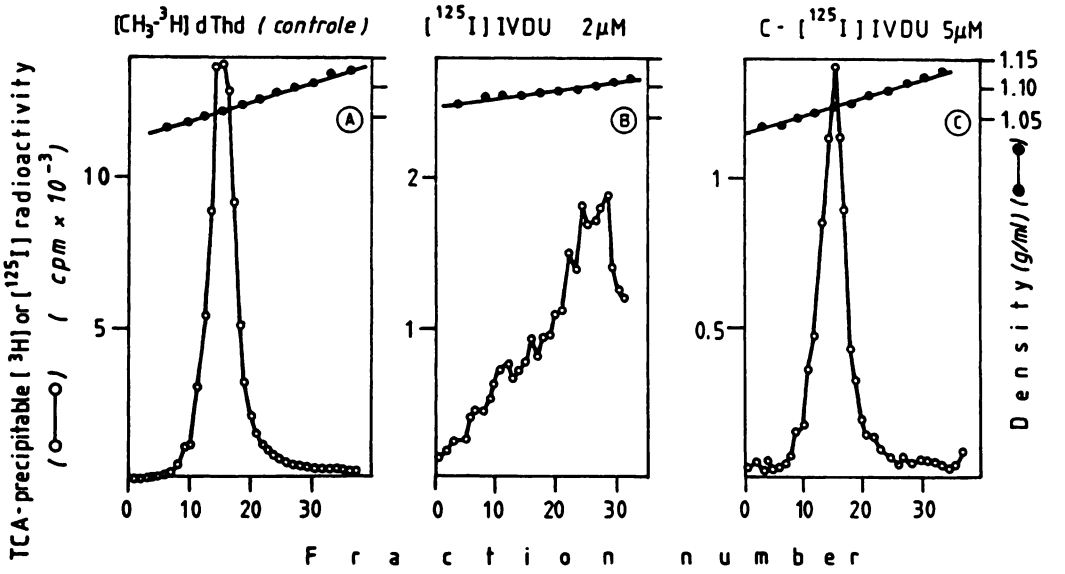


Fig. 5. Neutral sucrose linear gradient analysis of HSV-1 (KOS) DNA obtained upon CsCl equilibrium gradient analysis (Fig. 4, C and E). Viral DNA was labeled with [*methyl*-^{3}H]dThd (A), [^{125}I] IVDU (2 μM) (B), or C-[^{125}I]IVDU (5 μM) (C). The results shown represent data from one experiment. Similar results were obtained in at least three to five independent experiments.

Moreover, the incorporation of [^{125}I]IVDU into viral DNA resulted in a much broader viral DNA peak on the CsCl gradient diagram than the incorporation of C-[^{125}I]IVDU (Fig. 4, B and C, as compared with E and F).

When the viral DNA peaks (Fig. 4, C and E) were collected from the CsCl gradient (the *shadowed areas* under the viral DNA peaks represent the fractions that were collected for these experiments), desalted, and centrifuged in a neutral sucrose gradient, viral DNA recovered from the cells incubated with 2 μM [^{125}I]IVDU again showed a much broader peak than the viral DNA recovered from the cells incubated with 5 μM C-[^{125}I]IVDU (Fig. 5, B and C). The viral DNA labeled with C-[^{125}I]IVDU gave a band that was as narrow as the band of viral DNA labeled with [*methyl*-^{3}H]dThd (Fig. 5, A and C).

Discussion

IVDU and C-IVDU, in which the sugar moiety has been replaced by a cyclopentyl group, are potent and highly selective antiherpetic agents. When compared for their antiviral activity at the same concentration, IVDU invariably achieved a 10-fold greater reduction in virus yield than C-IVDU (Fig. 2); at a concentration of 0.5 μM C-IVDU afforded the same reduction in virus yield (about 99%) as did IVDU at a concentration of 0.2 μM. IVDU and C-IVDU completely suppressed virus replication at concentrations of 2 and 5 μM, respectively. The antiviral effects of IVDU and C-IVDU closely correlated with their inhibitory effects on viral DNA replication. IVDU at 0.2 μM and C-IVDU at 0.5 μM afforded a 50% reduction in viral DNA synthesis, whereas IVDU at 2 μM and C-IVDU at 5 μM completely suppressed viral DNA synthesis (Fig. 3). Cellular DNA synthesis was affected only at compound concentrations that were at least 10-fold (IVDU) or 40-fold (C-IVDU) higher than those required for inhibition of viral DNA synthesis. That higher concentrations of C-IVDU than of IVDU were required

to inhibit cellular DNA synthesis is in agreement with previous findings that C-IVDU is less cytostatic against different cell lines than IVDU (12, 13).

C-IVDU is effectively phosphorylated in HSV-1-infected cells and is eventually incorporated into both viral and cellular DNA of the infected cell (10). The phosphorylation was demonstrated by the detection of significant levels of C-[^{125}I]IVDU-monophosphate in the acid-soluble fraction of HSV-1-infected cells, whereas the incorporation of authentic C-IVDU into DNA was demonstrated by CsCl gradient analysis of viral and cellular DNA isolated from HSV-1-infected cells exposed to C-[^{125}I]IVDU (10). We have now established that [^{125}I]IVDU is incorporated much more efficiently in HSV-1 DNA than C-[^{125}I]IVDU, when compared at identical compound concentrations as well as equivalent antiviral concentrations (i.e., 0.2 μM IVDU versus 0.5 μM C-IVDU). It was estimated that, at antivirally comparable doses, IVDU is incorporated about 10-fold more efficiently than C-IVDU (Fig. 4, A and D). Interestingly, the higher the initial concentrations of IVDU and C-IVDU, the greater the extent to which IVDU and C-IVDU were incorporated into viral and cellular DNA, except for the highest concentration of C-IVDU (20 μM). The latter observation may be explained by the fact that at a concentration of 20 μM C-IVDU markedly inhibits viral DNA replication (Fig. 3, H) and, thus, limits its own incorporation at this concentration. Also, a substantial inhibition of viral ^{32}P-labeled DNA replication was noted at concentrations of IVDU (2 μM) and C-IVDU (5 and 20 μM) (Fig. 3, D, G, and H) that still allowed incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU compounds into viral DNA (Fig. 4, C, E, and F). At these concentrations of IVDU and C-IVDU, virus multiplication was reduced by at least 3 orders of magnitude (Fig. 2). At these antivirally effective doses of the compounds, no ^{32}P-labeled DNA could be detected. This is obvious, because at virus levels that are reduced by 3 orders of magnitude, one may expect radiolabeled virus DNA peak levels as low as 75 cpm, that is, 1000-fold less radioactivity than the untreated virus DNA control peaks (75,000 cpm) (Fig. 2). Apparently, such radiolabel is below background. The more extensive incorporation of [^{125}I]IVDU and C-[^{125}I]IVDU into viral than cellular DNA at the higher IVDU and C-IVDU concentrations may be related to a shift of cellular to viral DNA synthesis following infection of the cells with HSV-1.

Unlike [^{125}I]IVDU-labeled viral DNA (Fig. 5B), [*methyl*-^{3}H]dThd- and C-[^{125}I]IVDU-labeled viral DNA showed similar density profiles (d = 1.10, 1.06, and 1.07 g/ml, respectively). These data strongly suggest that, in contrast to C-IVDU-treated virus-infected cells, dThd is extensively substituted by IVDU in IVDU-treated virus-infected cells, resulting in a marked increase in density. This phenomenon has also been reported by several other laboratories (14, 15) and is in agreement with our findings shown in Fig. 4.

Analysis of [^{125}I]IVDU- and C-[^{125}I]IVDU-containing DNA by neutral sucrose gradient ultracentrifugation revealed a much greater heterogeneity for the DNA having incorporated [^{125}I]IVDU than for DNA having incorporated C-[^{125}I]IVDU (Fig. 5). These observations suggest that incorporation of IVDU, but not C-IVDU, results in extensive double-strand DNA breaks. This phenomenon has also been described for BVDU-containing varicella zoster virus DNA (14). With C-IVDU, however, no effect on double-strand DNA breaks was observed under our experimental conditions, even at C-IVDU concentrations that were antivirally equivalent to IVDU. Mancini *et al.* (15) demonstrated that the degree of HSV-1 inhibition by BVDU correlates well with the amount of BVDU substituted for dThd in HSV-1 DNA. These investigators also found that BVDU-substituted viral DNA is more labile than unsubstituted viral DNA, as demonstrated by a dose-dependent increase in single-strand breaks in alkaline medium. Although BVDU-containing DNA does not influence the DNA polymerase reaction (16), substitution of BVDU for dThd in a synthetic polydeoxynucleotide impairs the template affinity for RNA synthesis (17). Thus, the potent anti-HSV-1 activity of BVDU closely correlates not only with its incorporation into HSV-1 DNA but also with an altered stability and altered template properties of this DNA. Recently, Sagi *et al.* (18) reported that C-BVDU, when incorporated into synthetic DNA, alters the functioning of this DNA as template for both replication to DNA and transcription to RNA.

Taking together the data on IVDU, BVDU, C-IVDU, and C-BVDU, one may postulate that IVDU and BVDU behave differently from C-IVDU and C-BVDU in their rate of incorporation into viral DNA and effect on virus DNA integrity. IVDU and BVDU are extensively incorporated into viral DNA; this incorporation renders the DNA more labile and, in particular, susceptible to single- and double-strand DNA breakage. C-IVDU and C-BVDU are incorporated to a limited extent into viral DNA, and this incorporation apparently does not harm DNA integrity. All four compounds are inhibitory to viral DNA synthesis. The inhibitory effects of IVDU and BVDU on viral DNA synthesis may be the consequence of the single- and double-strand DNA breakage, resulting from the incorporation of the compounds into DNA. Although C-IVDU does not result in the occurrence of measurable single- and double-strand DNA breakage, it cannot be excluded that incorporation of C-IVDU into viral DNA may be much more detrimental to viral DNA replication than IVDU or BVDU incorporation. Alternatively, the inhibitory effects of C-IVDU and C-BVDU on DNA synthesis may result from an inhibition of the DNA polymerization reaction. Further studies with C-IVDU may clarify this issue.

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