In Vitro Susceptibility of Varicella-Zoster Virus to E-5-(2-Bromovinyl)-2'-Deoxyuridine and Related Compounds

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The in vitro susceptibility of eight strains of varicella-zoster virus (VZV) to E-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) was examined in human embryonic fibroblasts by the following techniques: inhibition of focus formation by either cell-free VZV (4-day assay) or cell-associated VZV (2-day assay), inhibition of viral antigen formation (2-day assay), and inhibition of viral cytopathogenicity (15-day assay). The 50% inhibitory dose (ID50) of BVDU ranged from 0.001 µg/ml (2-day assay) to 0.01 µg/ml (15-day assay). BVDU appeared highly selective in its anti-VZV activity since even at concentrations as high as 100 µg/ml, BVDU did not markedly affect the viability of the host cells. The ID50 of BVDU for VZV was comparable to that of IDVU (E-5-(2-iodovinyl)-2'-deoxyuridine). Both drugs inhibited the replication of VZV at a much lower concentration than did other antiviral compounds such as iododeoxyuridine, ethyldeoxyuridine, arabinosylcytosine, arabinosyladenine, phosphonoacetic acid, iododeoxyxycytidine, and acycloguanosine. BVDU and IDVU were virtually inactive against a thymidine kinase-deficient VZV mutant, suggesting that phosphorylation by the viral enzyme is responsible, at least in part, for the selective anti-VZV activity of the compounds.

Several nucleoside analogs exert a selective inhibitory effect on the replication of herpes simplex virus (HSV), be it type 1 (HSV-1) or type 2 (HSV-2). Typical examples are 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine, acyclovir [ACV]), 1-β-d-arabinofuranosylthymine (arabinosylthymeine [ara-T]), 5-ido-2'-deoxyctydine (IDC), S'-amino-5-ido-2',5'-dideoxyuridine (AIU), 1-(2-fluoro-2-deoxy-β-d-arabinofuranosyl)-5-iodocytosine (fluorooiodoaracynosine [FIAC]), 5,6-dihydro-5-aza-2'-deoxythymidine, and the halogenovinyl derivatives BVDU [E-5-(2-bromovinyl)-2'-deoxyuridine] and IDVU [E-5-(2-iodovinyl)-2'-deoxyuridine] (11). The selective antitherpesvirus activity of these compounds is, to an important extent, dependent on their phosphorylation by the HSV-induced thymidine kinase (TK). The latter differs from the host TK not only in physical properties but also in substrate specificity (4). The viral enzyme has a broader substrate affinity than the host enzyme, which means that some nucleoside analogs which are not recognized as substrate by the host TK may act as substrate for the viral TK; hence, their phosphorylation will be confined to the virus-infected cell.

Since varicella-zoster virus (VZV), like HSV, is capable of inducing a specific TK (5, 6, 12–14, 21) which resembles HSV-induced TK in substrate specificity, one may expect compounds like ACV, ara-T, IDC, AIU, and FIAC, which are all effective against HSV, to be similarly effective against VZV. Indeed, in vitro inhibition of VZV replication has been noted for ACV (2, 7), ara-T (20), IDC (12), AIU (15), and FIAC (18) at concentrations which were not toxic for the host cells. We have now extended these observations to the 5-halogenovinyl-substituted 2'-deoxyuridines BVDU and IDVU. These compounds were found to inhibit VZV replication in human diploid cells at a concentration of 0.001 to 0.01 µg/ml, which is comparable to the minimum effective dose reported for FIAC (18) but much lower than the concentrations at which the other compounds, i.e., ACV, AIU, and IDC, were found to inhibit VZV replication.

MATERIALS AND METHODS

Compounds. The origin of the antiviral compounds was as follows: IDU (5-ido-2'-deoxyuridine), Ludeco, Brussels, Belgium; 5-trifluoro-2'-deoxythymidine, Sigma Chemical Co., St. Louis, Mo.; 5-ethyl-2'-deoxyuridine and 5-propyl-2'-deoxyuridine, see reference 10; 5-propynoxyloxy-2'-deoxyuridine, see reference 25; ara-C (1-β-d-arabinofuranosylcytosine), Sigma Chemical Co.; ara-A (9-β-d-arabinofuranosyladenine), Parke Davis and Co., Ann Arbor, Mich. (courtesy of R. Wolf [Parke Davis Clinical Research Western Europe, München, Federal Republic of Germany]); ara-T, Terra-Marine Bioresearch, La Jolla,

**Cells.** Human embryo diploid fibroblast (HEF) cells were derived from a 3-month-old fetus at the Department of Gynecology of Fukushima Medical College Hospital. The HEF cells were grown in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (growth medium).

**Viruses.** Eight different VZV strains, seven wild-type clinical isolates, and one thymidine kinase-deficient (TK−) mutant were used for these studies. VZV isolates Batson and CaQu, both obtained from patients with herpes zoster, were provided by N. J. Schmidt (Viral and Rickettsial Disease Laboratory, Berkeley, Calif.). The VZV strains Ohtomo, Hirai, Kanno, and VEX 411 were isolated in our laboratories from patients with herpes zoster. VZV strain Oka, an attenuated virus vaccine strain, was provided by M. Takahashi (Osaka University, Osaka, Japan). The TK− variant Kanno-Kohmura was isolated from a persistently infected human breast cancer cell line after prolonged exposure to IDU (10 μg/ml). The viruses were used at the following passage level (in HEF, in our laboratory): Batson and CaQu, 5; Ohtomo, >30; Hirai and Kanno, 10; VEX 411 and Oka, 2; and Kanno-Kohmura, 3.

**Cell-free virus.** Cell-free virus was obtained by the method of Schmidt and Lenette (23). Briefly, HEF cell monolayers were infected at a multiplicity of infection of 0.16 and incubated for 36 h at 36°C. The cells were then dispersed with glass beads, suspended in growth medium containing 10% sorbitol, and sonicated for 20 s with a Tomy transonic disruptor (Tomy Seiko Co., Ltd.). The cell homogenates were centrifuged at 1,500 rpm for 15 min, and the supernatant was used as the cell-free virus stock.

**Cell-associated virus.** To obtain cell-associated virus, HEF cell monolayers were infected at a multiplicity of infection of 0.16 and incubated for 3 to 4 days at 36°C, until viral cytopathogenicity reached 90%. The cells were then washed with phosphate-buffered saline and treated with 0.01% trypsin in phosphate-buffered saline. The trypsinized cells were collected by centrifugation at 1,500 rpm for 15 min, suspended in growth medium containing 10% dimethyl sulfoxide, and frozen at −80°C until used as the cell-associated virus stock.

**Virus assays: focus formation by cell-free virus.** Confluent HEF monolayers were infected with 100 CCID50 of cell-free VZV (1 CCID50 being the cell culture infective dose for 50% of the cell cultures) in MEM with 3% FCS. The test compounds were added at varying concentrations simultaneously with the virus. After a 4-day (approximately 95- to 98-h) incubation period, the cell cultures were examined microscopically for the appearance of foci.

**Focus formation by cell-associated virus.** Confluent HEF monolayers were inoculated with 1,000 PFU of VZV-infected HEF cells in MEM with 3% FCS. The test compounds were added at varying concentrations simultaneously with the virus, and the foci were counted microscopically after a 2-day incubation period.

**Viral antigen formation.** Confluent HEF monolayers were infected with approximately 20 PFU of cell-free VZV, and the test compounds were added 90 min after virus inoculation. After a 2-day incubation period, the cells were fixed in acetone and stained by the direct immunofluorescence technique, using rabbit anti-VZV γ-globulin conjugated with fluorescein isothiocyanate (Flow Laboratories, McLean, Va.).

**Viral cytopathogenicity.** Confluent HEF monolayers were infected with 10 CCID50 of cell-free VZV for 2 h. Residual virus was removed, and the cell cultures were replenished with maintenance medium (MEM + 3% FCS) containing varying concentrations of the test compounds. The medium was renewed after 7 days, and virus-induced cell destruction was scored after 15 days, when it had reached about 50 to 75% of the entire cell monolayer.

**RESULTS**

In the first set of experiments we examined the inhibitory activity of BVDU, IVDU, IDU, and ara-C on VZV focus formation in HEF cells infected with cell-free virus. The 50% inhibitory dose (ID50) values of BVDU for six different clinical isolates of VZV ranged from 0.0012 to 0.008 μg/ml, with a mean ID50 of 0.0037 μg/ml (Table 1). The mean ID50 of IVDU for the six VZV strains was 2.6-fold lower than that of BVDU, whereas the mean ID50 value of ara-C was 13.5 times higher and that of IDU was even 160 times higher (Table 1). Thus, according to their inhibitory effects on VZV focus formation, the relative order of (decreasing) antiviral potency was IVDU > BVDU > ara-C > IDU. BVDU and IVDU were virtually inactive against a TK− mutant of VZV. IDU was about 30 times less inhibitory for the TK− variant than for the wild-type virus, and ara-C was only 4 times less inhibitory. Hence, of the four compounds tested, ara-C proved to be the most effective against TK− VZV (Table 1).

If VZV-infected cells (instead of cell-free virus) were used as the inoculum, the inhibitory effect of BVDU on VZV focus formation was even more dramatic. Figure 1 presents a characteristic dose-response curve for the inhibition of VZV focus formation by BVDU. A concentration of 0.001 μg of BVDU per ml sufficed to cause a 50% reduction in the number of foci; at a 0.01 μg/ml concentration BVDU effected a 90% reduction in focus formation, and, at a concentration of 0.1 μg/ml or higher, it suppressed focus formation completely (Fig. 1).

When viral antigen synthesis was determined as a parameter of virus growth, again BVDU was found to completely block VZV replication at a concentration of 0.1 μg/ml (Fig. 2). The BVDU concentration required to reduce viral antigen development by 50% was 0.003 μg/ml.
TABLE 1. Inhibitory effects of BVDU, IVDU, IDU, and ara-C on focus formation by cell-free VZV in HEF cell cultures

<table>
<thead>
<tr>
<th>VZV strain</th>
<th>ID50 (µg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BVDU</td>
</tr>
<tr>
<td>Batson</td>
<td>0.0013</td>
</tr>
<tr>
<td>CaQu</td>
<td>0.008</td>
</tr>
<tr>
<td>Hirai</td>
<td>0.002</td>
</tr>
<tr>
<td>Kanno</td>
<td>0.008</td>
</tr>
<tr>
<td>Oka</td>
<td>0.0016</td>
</tr>
<tr>
<td>Ohtomo</td>
<td>0.0012</td>
</tr>
<tr>
<td>Avg</td>
<td>0.0037</td>
</tr>
<tr>
<td>Kanno-Kohmura (TK⁻)</td>
<td>≥56</td>
</tr>
</tbody>
</table>

* Required to reduce focus formation by 50%. The number of foci was determined at 95 to 98 h postinfection by direct microscopic count. There were about 30 foci in the untreated virus-infected control cell cultures.

for ara-C it was 0.015 µg/ml, and for IDU it was 0.2 µg/ml. These ID50 values are comparable to those noted above for inhibition of VZV focus formation (Table 1), thus again establishing that the order of (decreasing) potency is BVDU > ara-C > IDU.

BVDU and IVDU were not only more potent than ara-C and IDU, they also surpassed various other antiviral compounds such as ara-A, ara-T, IDC, AIU, ACV, and phosphonoacetic acid in anti-VZV potency, as assessed by a 15-day viral cytopathogenicity assay (Table 2). In this assay several reference compounds were included which had already been examined for their VZV-inhibiting effects in previous studies. The ID50 values obtained for these compounds (Table 2) were markedly similar to those reported in the literature, namely, 1 to 2 µg/ml for ara-A (3), ≤0.5 µg/ml for ara-T (20), 36 µg/ml for AIU (15), 1 µg/ml for ACV (2, 7), and ≤20 µg/ml for phosphonoacetic acid (19, 26). In comparison with these compounds, BVDU and IVDU inhibited VZV replication at an ID50 of 0.01 µg/ml (Table 2). That BVDU is a more potent inhibitor of VZV replication in cell culture than other antiviral drugs such as ara-A and ACV has been confirmed by the observations of Y. Bryson, D. Hebblewaite, and E. De Clercq (12th International Congress of Chemotherapy, Florence, Italy, 19 to 24 July 1981, abstr. no. 131) and K. K. Biron, J. E. Noblin, J. A. Fyfe, and G. B. Elion (International Workshop on Herpesviruses, Bologna, Italy, 27 to 31 July 1981, abstr. no. 203).

BVDU was highly selective in its anti-VZV activity. Even when incubated with the cells at a concentration as high as 100 µg/ml for a period of 2 to 15 days, BVDU did not cause significant cytotoxicity for stationary (resting) human fibroblast cells, as assessed by either of the following parameters: (i) trypan blue dye exclusion, (ii) microscopic alteration of normal cell morphology, or (iii) cellular DNA synthesis ([methyl-3H]deoxythymidine incorporation), RNA synthesis ([5-3H]uridine incorporation), or protein synthesis ([4,5-3H]leucine incorporation) (data not shown). In addition (iv), Machida et al. (18a) have found that BVDU did not inhibit the proliferation of human fibroblasts (after 4 days of exponential cell growth) unless the drug concentration was raised to 150 µg/ml. Thus, based on these toxicity data (i to iv) and on the antiviral data presented in Tables 1 and 2 and Figs. 1 and 2, the selectivity index (ratio of the minimum cytotoxic dose to the minimum antiviral dose) of...
BVDU and VZV could be estimated at 10,000 to 100,000. This is even greater than the selectivity index of BVDU for HSV-1 (9, 11).

**FIG. 2.** Inhibitory effect of BVDU (○), ara-C (□), and IDU (△) on the formation of specific viral antigens in HEF cell cultures infected with cell-free VZV (strain Kanno). The appearance of the viral antigens was visualized with the direct immunofluorescence technique at 48 h postinfection.

**TABLE 2.** Inhibitory effects of different anti-herpes compounds on cytopathogenicity of cell-free VZV (strain VEX 411) in HEF cell cultures

<table>
<thead>
<tr>
<th>Compound</th>
<th>ID50 (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>IDU</td>
<td>0.4</td>
</tr>
<tr>
<td>TFI</td>
<td>1</td>
</tr>
<tr>
<td>EDU</td>
<td>7</td>
</tr>
<tr>
<td>PDU</td>
<td>1</td>
</tr>
<tr>
<td>PyODU</td>
<td>0.5</td>
</tr>
<tr>
<td>Ara-A</td>
<td>2.5</td>
</tr>
<tr>
<td>Ara-T</td>
<td>0.04</td>
</tr>
<tr>
<td>IDC</td>
<td>0.4</td>
</tr>
<tr>
<td>AIU</td>
<td>40</td>
</tr>
<tr>
<td>ACV</td>
<td>0.4</td>
</tr>
<tr>
<td>EHNAL</td>
<td>30</td>
</tr>
<tr>
<td>PAA</td>
<td>20</td>
</tr>
<tr>
<td>BVDU</td>
<td>0.01</td>
</tr>
<tr>
<td>IVDU</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a Abbreviations not previously identified: TFT, 5-trifluoro-2'-deoxythymidine; EDU, 5-ethyl-2'-deoxyuridine; PDU, 5-propyl-2'-deoxyuridine; PyODU, 5-propynoxy-2'-deoxyuridine; EHNA, erythro-9(2-hydroxy-3-nonyl)adenine; PAA, phosphonoacetic acid.

b Required to reduce viral cytopathogenicity by 50%. Viral cytopathogenicity was recorded at 15 days postinfection, when it reached 50 to 75% cell destruction in the untreated virus-infected control cell cultures.

**DISCUSSION**

VZV is the etiological agent of both a primary infection, varicella (chickenpox), and a recurrent infection, zoster (shingles). These infections are generally self-limited and uncomplicated. However, life-threatening complications may arise in immunocompromised patients when the VZV infection becomes disseminated. Clearly, there is a need for a selective antiviral agent that, when given systemically, would arrest the course of infection and prevent its complications.

Controlled studies have indicated that ara-A may be beneficial in the treatment of both localized and disseminated VZV infections (16, 27). ACV, BVDU, and FIAC appear at least as, if not more, promising than ara-A for the systemic treatment of VZV infections. In preliminary (uncontrolled) clinical trials, these compounds were found to block the progress of the infection promptly after treatment was started (8, 24; C. W. Young, B. Jones, R. Schneider, C. Tan, D. Armstrong, C. Lopez, K. A. Watanabe, J. J. Fox, and F. Philips, in Current Chemotherapy and Immunotherapy, in press).

The potentials of BVDU for the therapy of VZV infections appear particularly promising. (i) The minimum effective dose of BVDU required to inhibit VZV replication in cell culture is 0.001 to 0.01 µg/ml; this dose is comparable to the ID50 that has been reported for FIAC (18) but much lower than the ID50 values obtained for several other antiviral drugs such as IDU,
ara-C, ara-A, and ACV, when assayed in parallel with BVDU (Table 2). (ii) BVDU is readily absorbed when administered orally (i.e., at 7.5 to 15 mg/kg per day) to humans, achieving blood drug levels that are at least 100 times higher than the minimum effective dose required to inhibit VZV replication in cell culture (8; E. De Clercq, personal communication, 1981). (iii) When given orally to monkeys at a dosage (15 mg/kg per day) similar to that employed in humans, BVDU completely eliminates all manifestations of simian varicella virus infection (K. F. Soike, S. Gibson, and P. J. Gerone, Antiviral research, in press).

The selective anti-VZV activity of BVDU can at least partially be attributed to its phosphorylation by the virus-induced TK. Indeed, BVDU has a very high affinity for the VZV-induced TK ($K_I = 0.07 \mu M$, as compared to $>150 \mu M$ for the human cytosol TK) (5). Moreover, VZV mutant strains which have lost their ability to induce TK become resistant to the antiviral action of BVDU (Table 1).

In its anti-VZV activity, BVDU may follow the same sequence of events as postulated previously for its anti-HSV activity. Therefore, the compound should first be phosphorylated by the virus-induced TK (5), further processed by cellular kinases to the 5’-triphosphate, and then interfere at the DNA polymerization level. At this stage, it would inhibit the viral DNA polymerase to a greater extent than the cellular DNA polymerases $\alpha$, $\beta$, and $\gamma$ (1). The latter statement concerns HSV-1 DNA polymerase. Whether it also applies to VZV DNA polymerase remains to be determined.

Whether BVDU is capable of curing cells of VZV infection is another subject of further study. IDU and phosphonoacetic acid are apparently unable to eradicate VZV infection (22, 26), since infectious virus could be recovered from the cell cultures after removal of the compounds. With ACV it seems possible to rid the cultures of infectious virus if the drug is added at a sufficiently high concentration for a sufficiently long time (2).

ACKNOWLEDGMENTS

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