Analysis of the thymidine kinase of a herpes simplex virus type 1 isolate that exhibits resistance to (E)-5-(2-bromovinyl)-2'-deoxyuridine

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The mechanism responsible for the decreased sensitivity of a clinical herpes simplex virus type 1 (HSV-1) isolate, HSV-145, to (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) was examined. Measurements of 50% inhibitory doses of several drugs demonstrated that although HSV-145 was sensitive to phosphonoacetic acid, adenine arabinoside and acyclovir, its sensitivity to BVDU and 5-(2-chloroethyl)-2'-deoxyuridine was significantly less than that normally observed for HSV-1. Analysis of the thymidylate kinase (TMP-K) activity of HSV-145 thymidine kinase (TK) demonstrated a decreased level of TMP-K activity when compared to HSV-1 TK. The TMP-K activity of HSV-145 resembled that observed for HSV-2 and the TK-deficient strain HSV-1 TK-7. When the nucleotide sequence of the HSV-145 TK gene was compared to that of the HSV-1 strains CI(101) and SC16 a single nucleotide substitution (G changed to A at base position 502) was detected which would result in the substitution of threonine at amino acid position 168 for alanine. The substitution is the same as that for the laboratory-derived BVDU-resistant virus HSV-1 SC16B3. Collectively, these studies highlight the importance of amino acid conservation at position 168 of the HSV-1 TK in conferring efficient TMP-K activity and BVDU sensitivity.

The thymidine kinase (TK) of herpes simplex virus (HSV) is a multi-substrate enzyme (Jamieson et al., 1974) which possesses both TK and thymidylate kinase (TMP-K) activities (Chen & Prusoff, 1978). Activation of various anti-HSV nucleoside analogues by HSV TK (Fyfe et al., 1978; De Clercq et al., 1990; Fyfe, 1982), including (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU; De Clercq et al., 1979) is important in their mode of action. BVDU is a thymidine analogue that is monophosphorylated by HSV TK, diphosphorylated by HSV TMP-K and postulated to be triphosphorylated by cellular kinases (Cheng et al., 1981; Fyfe, 1982). Once activated to its triphosphate form, BVDU interacts with HSV DNA polymerase to terminate viral DNA synthesis, effectively inhibiting HSV replication (Allandeen et al., 1981; Ruth & Cheng, 1981).

Although BVDU is a potent inhibitor of HSV-1, it has been shown to inhibit the replication of HSV-2 to a much lesser degree (De Clercq et al., 1980). The difference between HSV-1 and HSV-2 in BVDU sensitivity is likely to be because of the decreased levels of TMP-K activity associated with the HSV-2 TK (Fyfe, 1982; Ayisi et al., 1985). This reduced ability to diphosphorylate BVDU is thought to account for the decreased sensitivity of HSV-2 to BVDU (Fyfe, 1982).

Limited sensitivity to BVDU because of reduced TMP-K activity has also been documented for certain laboratory strains of HSV-1 (Fyfe et al., 1983; Haarr & Flatmark, 1987; Coen et al., 1989). Following the selection of these HSV-1 strains in vitro, studies of their TK enzymes have aided in the identification of amino acid residues and protein regions involved in the TMP-K activity of HSV-1 (Darby et al., 1986; Haarr & Flatmark, 1987; Coen et al., 1989).

Recently, whilst characterizing a large number of clinical samples, we identified an HSV-1 variant, designated HSV-145, which exhibited decreased sensitivity to BVDU. Unlike the BVDU-resistant laboratory strains previously described, HSV-145 was characterized immediately after acquisition from a clinical infection and therefore did not result from in vitro selection (Docherty et al., 1991). To our knowledge, this was the first report of the isolation of a BVDU-resistant HSV-1 strain directly from a clinical source. The studies presented here were designed to determine the mechanism of resistance of HSV-145 to BVDU. Our results implicate a single amino acid substitution within the TK enzyme for the reduced BVDU sensitivity exhibited by HSV-145. Interestingly, this substitution is exactly the same as that for the laboratory-derived BVDU-resistant virus HSV-1 SC16B3 (Darby et al., 1986).

Because the antiviral activity of BVDU requires it to interact with viral TK and viral DNA polymerase, 50% inhibitory dose (ID₅₀) studies of several drugs were...
designed, to help determine which of the two enzymes was most likely to be involved in the resistance of HSV-145 to BVDU. The effects of adenine arabinoside (Ara-A; Sigma), phosphonoacetic acid (PAA; Sigma), acyclovir (ACV; a gift from Burroughs-Wellcome Research Laboratory, Research Triangle Park, N.C., U.S.A.), BVDU (a gift from E. De Clercq, Rega Institute for Medical Research, Leuven, Belgium) and 5-(2-chloroethyl)-2'-deoxyuridine (CEDU; a gift from B. Rosenwirth, Sandoz Forschungsinstutit, Vienna, Austria) on HSV-1, HSV-2 and HSV-145 replication were evaluated using the standard plaque assay. ID₅₀ values were then found to be comparable to that of HSV-1 (77 and 69 %) and HSV-2 (81 and 70 %).

To confirm and extend these results, enzyme dissociation (Kₘ) values for HSV-145, HSV-1 and HSV-2 TK were calculated. These values were determined by incubating 40 μg of the partially purified enzyme with the reaction mixtures described above, containing various concentrations of [³H]thymidine for 10 min. The Kₘ value (Lineweaver & Burk, 1934) for HSV-145 TK (0.78 μM) was not significantly different (P < 0.05) from those of HSV-1 (0.36 μM) or HSV-2 (0.63 μM), indicating that the affinity of HSV-145 TK for thymidine closely resembled that of HSV-1 and HSV-2 TK.

Although the above results indicated that the HSV-145 TK active site could bind and phosphorylate thymidine, it was possible that it could not interact with BVDU to produce BVDU-MP. Therefore, TK assays were performed as described above except that 0.3 mM-BVDU was also included in the reaction mixture as an inhibitor of TK activity. Thymidine phosphorylation in the presence of BVDU was assessed following 5, 15 and 30 min of incubation and compared to product formation in control reactions which did not contain BVDU. At 5 min, the presence of BVDU inhibited the phosphorylation of thymidine by HSV-145 by 53 %, at levels comparable to those for HSV-1 (55 %) and HSV-2 (65 %). The inhibition of thymidine phosphorylation by BVDU continued for 30 min with the percentage inhibition of HSV-1, HSV-145 and HSV-2 at 30 min being 54, 54 and 43 % respectively. These results suggested that, by inhibiting phosphorylation, BVDU was not only interacting with the TK of HSV-145, but doing so in a manner similar to HSV-1 and HSV-2.

Even though these studies suggested an interaction of HSV-145 TK with BVDU, more direct evidence was sought to determine whether HSV-145 could actually monophosphorylate BVDU. Therefore, TK assays, as described above, using BVDU as the substrate and [γ-³²P]ATP as the phosphate donor were undertaken. After 2 h incubation at 37 °C, samples from the reaction mix were spotted onto cellulose-PEI-TLC plates which were developed in 3 m-glacial acetic acid and 0.75 m-LiCl. Because the partially purified enzyme extracts used in this study contained not only the TK but other scintillation spectrometry and the percentage increase in thymidine phosphorylation by HSV-1, HSV-145 and HSV-2, above that observed for the negative control HSV TK⁻¹, was calculated.

Product formation by HSV-1, HSV-145 and HSV-2 at 5 min was observed to be 80, 80 and 86 % respectively, over the negative control. Additionally, HSV-145 was also found to phosphorylate thymidine over time with the increase in product formation at 15 and 30 min being 76 and 69 %. This level of substrate phosphorylation was found to be comparable to that of HSV-1 (55 %) and HSV-2 (65 %).
Table 1. Virus sensitivities to nucleoside analogues

<table>
<thead>
<tr>
<th>Drug</th>
<th>HSV-1 ID_{50} (µg/ml)</th>
<th>HSV-145 ID_{50} (µg/ml)</th>
<th>HSV-2 ID_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-A</td>
<td>17.33 ± 3.18†</td>
<td>8.07 ± 3.56</td>
<td>16.73 ± 4.85</td>
</tr>
<tr>
<td>PAA</td>
<td>26.33 ± 2.85</td>
<td>18.33 ± 2.73</td>
<td>17.30 ± 6.80</td>
</tr>
<tr>
<td>ACV</td>
<td>0.15 ± 0.06</td>
<td>0.03 ± 0.01</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>BVDU</td>
<td>0.03 ± 0.01</td>
<td>1.24 ± 0.84</td>
<td>0.62 ± 0.29</td>
</tr>
<tr>
<td>CEDU</td>
<td>0.45 ± 0.10</td>
<td>26.70 ± 22.30</td>
<td>24.67 ± 2.67</td>
</tr>
</tbody>
</table>

* ID_{50} values represent the average of three experiments, except the CEDU ID_{50} value for HSV-145 which represents the average of two experiments.
† ID_{50} mean ± s.E.M.

Fig. 1. Phosphorylation of BVDU by HSV-145. Reaction mixtures containing BVDU and [α-32P]ATP were incubated with enzyme extracts from mock-infected Vero cells or Vero cells infected with HSV-1, HSV-145 or HSV-2. Samples were removed and the reaction products were separated using TLC. The product profile of HSV-1, HSV-145 and HSV-2 are shown in lanes 1, 2 and 3 respectively. BVDU phosphorylation by enzyme extracts from mock-infected Vero cells was included as a negative control and is shown in lane 4. The migratory position of BVDU-MP was determined using a non-radioactive fluorescent standard, and is indicated.

The TK active site of HSV-145 was shown to be effective in phosphorylating not only its natural substrate thymidine but also BVDU. To be completely activated however, BVDU-MP must be phosphorylated to the diphosphate form by the viral TMP-K. To determine whether HSV-145 had an efficient TMP-K activity, the phosphorylation of TMP by HSV-145 TK was evaluated. Enzyme extracts were incubated with reaction mixtures containing 4 mM-[3H]TMP as the substrate. Samples were taken at 5, 10, 20 and 30 min, spotted on to Whatman DE81 discs and air-dried. The discs were washed as described by Chen & Prusoff (1974) except that they were washed for 30 min in 1 M-formic acid and 1 mM-ammonium formate and then washed for 5 min in 95% ethanol.

Following 5 min incubation of the reaction mixture, there was no significant difference in TMP-K activity between HSV-1, HSV-145, HSV-2 and HSV-1 TK^− (Fig. 2). However, at 10, 20 and 30 min, very little TMP-K activity was observed with HSV-145. The extent of phosphorylation by HSV-145 TMP-K from 10 to 30 min was comparable to the TMP phosphorylation by the TMP-K-deficient viruses HSV-2 and HSV-1 TK^−. These studies suggested that the TK enzyme of HSV-145 did not have effective TMP-K activity since it was not able to phosphorylate TMP efficiently.

In an effort to define any modifications within the TK gene that may be responsible for producing a TK enzyme with reduced TMP-K activity, the nucleotide sequence of the HSV-145 TK gene was determined. Plasmid pKSTK145 was constructed by isolating and subcloning the BamHI fragment of pBTK145, which contains the HSV-145 TK gene (Docherty et al., 1991), into the BamHI site of plasmid pBluescript KS (Stratagene). The
TK gene of HSV-145 in pKSTK145 was then sequenced using Sequenase 2.0 (USB) and oligonucleotide primers either purchased (National Biosciences) or synthesized using a Cyclone DNA synthesizer (Millipore).

Comparison of the nucleotide sequence of the HSV-145 TK gene to that of HSV-1 Cl(101) and HSV-1 SC16 (Wagner et al., 1981; Darby et al., 1986) revealed a single nucleotide substitution of an adenine for a guanine at base 502 (Fig. 3). This nucleotide substitution resulted in the alanine at amino acid position 168 (Ala) being changed to threonine (Thr; Fig. 3). This amino acid substitution was the only residue not comparable to the amino acids of either HSV-1 Cl(101) or HSV-1 SC16 TK, suggesting an involvement of Ala in conferring TMP-K activity.

The nucleotide and amino acid sequence of HSV-145 was also compared to that of a laboratory-derived BVDU-resistant virus, HSV-1 SC16B3 (Field & Neden, 1982; Darby et al., 1986). This comparison revealed that the substitution described above for HSV-145 was identical to the one that was reported for HSV-1 SC16B3 (Fig. 3). As previously observed (Darby et al., 1986), HSV-2 TK has the closely related amino acid serine at position 168, suggesting a resemblance at this position between HSV-145 TK and HSV-2 TK.

The results presented here on our clinical isolate, when combined with the characterization of the laboratory-generated BVDU-resistant strain HSV-1 SC16B3 (Darby et al., 1986), strongly suggest that amino acid position 168 of HSV-1 TK is essential for TMP-K activity and BVDU sensitivity. Because full activation of BVDU requires diphosphorylation by HSV TK, the observed reduced level of TMP-K activity associated with HSV-145 TK may result in decreased levels of BVDU-DP formation, subsequently reducing the concentration of BVDU-TP and the inhibition of viral DNA synthesis within the infected cell. These data provide information useful in identifying regions within the viral protein which are involved in drug interaction and also predict mutations that can emerge to reduce drug sensitivity in vivo. Such information can be helpful in the development of more efficient antiviral drugs.

References


Fyfe, J. A. (1982). Differential phosphorylation of (E)-5(2-bromovinyl)-2'-deoxyuridine monophosphate by thymidylate kin-


RUTH, J. L. & CHENG, Y.-C. (1981). Nucleoside analogues with clinical potential in antiviral chemotherapy: the effect of several thymidine and 2'-deoxycytidine analogue 5'-triphosphates on purified human (α, β) and herpes simplex virus (types 1, 2) DNA polymerases. *Molecular Pharmacology* **20**, 415-422.


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