Journal of General Virology (1994), **75**, 1743–1747. *Printed in Great Britain*

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 C1(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 (ID_{50}) 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 Forschungsinstitut, Vienna, Austria) on HSV-1, HSV-2 and HSV-145 replication were evaluated using the standard plaque assay. ID_{50} values were then calculated (Docherty *et al.*, 1971; Smith *et al.*, 1980).

HSV-145, like HSV-1 and HSV-2, was sensitive to the DNA polymerase drugs Ara-A and PAA (Table 1; Prusoff & Ward, 1976; Mao *et al.*, 1975), suggesting that the DNA polymerase of HSV-145 was functional and capable of interacting with these drugs. HSV-145 was also sensitive to ACV, a drug which is monophosphorylated by HSV TK before being fully activated by cellular kinases and interacting with HSV DNA polymerase (Elion *et al.*, 1977; Furman *et al.*, 1979). Sensitivity to ACV suggested that HSV-145 TK was able to monophosphorylate this drug.

In contrast, evaluation of viral inhibition by BVDU and CEDU revealed that HSV-145 and HSV-2 were relatively resistant to these drugs when compared to HSV-1. BVDU and CEDU ID_{50} values for HSV-145 were more closely related to those of HSV-2 than HSV-1 (Table 1). CEDU, like BVDU, requires diphosphorylation by the TMP-K activities of HSV TK, making it relatively ineffective against HSV-2, which has limited TMP-K activity (De Clercq *et al.*, 1990). Because HSV-145 was as sensitive to ACV as HSV-1, yet relatively resistant to BVDU and CEDU like HSV-2, it was possible that HSV-145 also contained reduced TMP-K activity.

Although the sensitivity of HSV-145 to ACV suggested the existence of a functional TK, studies were performed to examine whether the TK of HSV-145 retained the ability to phosphorylate its natural substrate, thymidine. This was determined by incubation of 50 μg of partially purified protein (Bernaerts *et al.*, 1989) from Vero cells infected with HSV-1, HSV-145, HSV-2 or HSV-1 TK^{-7} (a gift from J. Subak-Sharpe, MRC Virology Unit, Glasgow, U.K.; Javier *et al.*, 1987) with a reaction mixture consisting of 12 mM-ATP, 12 mM-$MgCl_2$, 20 mM-NaF, 0·5 mg/ml BSA and 0·6 μM-[^{3}H]thymidine (81·6 Ci/mmol; New England Nuclear/DuPont). During incubation at 37 °C, 25 μl samples were removed at 5, 15 and 30 min, spotted onto Whatman DE81 paper discs and air-dried. The discs were washed as described by Fyfe *et al.* (1978) except 2 mM-thymidine was substituted for 2 mM-guanosine. Product formation was assessed by 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^{-7}, 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 (77 and 69%) and HSV-2 (81 and 70%).

To confirm and extend these results, enzyme dissociation (K_m) 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 [^{3}H]thymidine for 10 min. The K_m 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-^{32}P]ATP as the phosphate donor were undertaken. After 2 h incubation at 37 °C, samples from the reaction mix were spotted onto cellulose-PEI-F 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

Table 1. *Virus sensitivities to nucleoside analogues*

Drug	ID_{50} (μg/ml)*		
	HSV-1	HSV-145	HSV-2
Ara-A	17·33 ± 3·18†	8·07 ± 3·56	16·73 ± 4·85
PAA	26·33 ± 2·85	18·33 ± 2·73	17·30 ± 6·80
ACV	0·15 ± 0·06	0·03 ± 0·01	0·20 ± 0·06
BVDU	0·03 ± 0·01	1·24 ± 0·84	0·62 ± 0·29
CEDU	0·45 ± 0·10	26·70 ± 22·30	24·67 ± 2·67

* 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.

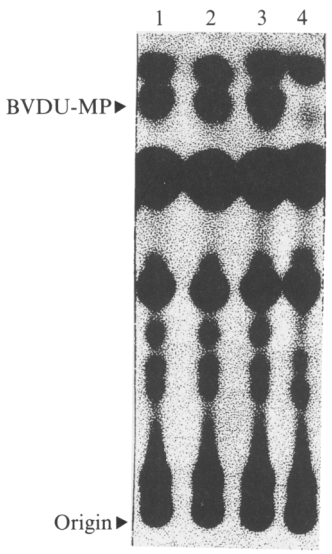

Fig. 1. Phosphorylation of BVDU by HSV-145. Reaction mixtures containing BVDU and [γ-^{32}P]ATP were incubated with enzyme extracts from mock-infected Vero cells or Vero cells infected with HSV-1, HSV-145 or HSV-2. Samples were removed and the reaction products were separated using TLC. The product profile 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.

enzymes capable of using ATP as a phosphate donor, multiple phosphorylated products were separated on the TLC plates (Fig. 1). A non-radiolabelled fluorescent standard was used for verification of the migratory position of BVDU-MP. Like those of HSV-1 and HSV-2, HSV-145 TK was able to produce BVDU-MP, demonstrating an effective interaction between BVDU and viral TK.

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

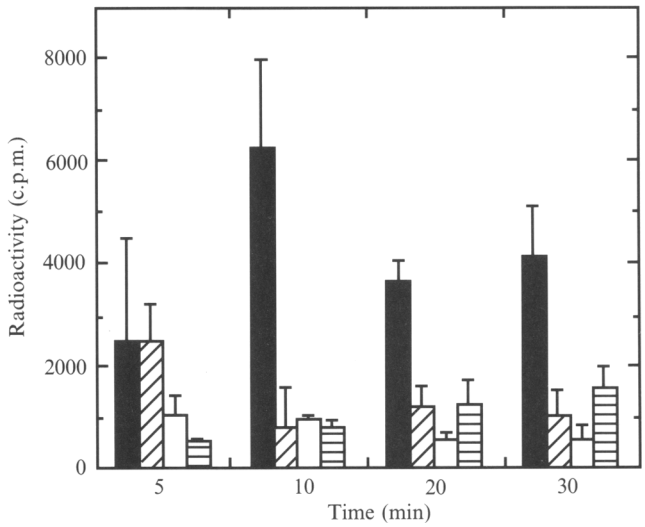

Fig. 2. Phosphorylation of TMP by HSV-145. Enzymatic assays were performed to evaluate the ability of HSV-145 to phosphorylate TMP. Samples were removed at 5, 10, 20 and 30 min from reaction mixtures containing enzyme extracts from HSV-1 (■), HSV-145 (▨), HSV-2 (□) and HSV-1 TK^{-7} (▤) infected Vero cells and [^{14}C]TMP. TMP phosphorylation was determined by the c.p.m. of each sample and the ability of each virus type to phosphorylate TMP was compared for each time point. Standard error bars are displayed.

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-[^{14}C]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^{-7} (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^{-7}. 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 *Bam*HI fragment of pBTK145, which contains the HSV-145 TK gene (Docherty *et al.*, 1991), into the *Bam*HI site of plasmid pBluescript KS (Stratagene). The

	gcc	ctc	acc	ctc	atc	ttc	gac	cgc	cat	ccc	atc	gcc	acc	ctc	ctg
145	A	L	T	L	I	F	D	R	H	P	I	A	T	L	L
C1(101)	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–
SC16	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–
SC16B3	–	–	–	–	–	–	–	–	–	–	–	–	**T**	–	–
HSV-2 333	–	–	–	–	–	–	–	–	–	–	–	–	**S**	–	–

Fig. 3. Nucleotide and amino acid sequence of a portion of the HSV-145 TK gene. These sequences were compared to those of the HSV-1 strains C1(101) (Wagner *et al.*, 1981) and SC16, the BVDU-resistant HSV-1 strain SC16B3 (Darby *et al.*, 1986) and the HSV-2 strain 333 (Swain & Galloway, 1983). The nucleotide region corresponding to amino acids 156 to 170 is shown at the top. The nucleotide substitution in the HSV-145 TK gene at base 502 and the corresponding amino acids at position 168 are marked in bold type.

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 C1(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^{168}) being changed to threonine (Thr^{168}; Fig. 3). This amino acid substitution was the only residue not comparable to the amino acids of either HSV-1 C1(101) or HSV-1 SC16 TK, suggesting an involvement of Ala^{168} 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.

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(Received 22 November 1993; Accepted 19 January 1994)