Effects of the Epipodophyllotoxin VP-16-213 on Herpes Simplex Virus Type 2 Replication

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SUMMARY

It has been recently shown that VP-16-213, a semi-synthetic derivative of podophyllotoxin, inhibits the function of mammalian DNA topoisomerase II. In the present study, we examined the effects of VP-16-213 on the replication of herpes simplex virus type 2 (HSV-2). The compound did not inhibit the synthesis of early viral polypeptides at concentrations at which viral DNA synthesis was strongly suppressed, but induced double-strand breaks in newly synthesized HSV DNA. Electron microscopic examination of treated cells revealed the presence of a number of capsids with empty or partial cores. The level of topoisomerase II activity remained unaltered after infection, and all attempts to isolate VP-16-213-resistant mutants of HSV-2 have failed in spite of extensive efforts. It is suggested therefore that the mode of action of VP-16-213 may be inhibition of viral DNA synthesis by impairing the function of host cell topoisomerase II.

It is known that initiation of herpes simplex virus (HSV) DNA replication requires prior synthesis of immediate early (α) and early (β) polypeptides. Some of the early peptides, such as viral DNA polymerase and the 130000 mol. wt. (130K) major DNA-binding protein, have been demonstrated to be involved in viral DNA replication by using temperature-sensitive and drug-resistant mutants (Purifoy & Benyesh-Melnick, 1975; Hay & Subak-Sharpe, 1976; Conley et al., 1981; Littler et al., 1983; Leinbach et al., 1984). The alkaline nuclease induced by HSV also may play a role in viral DNA replication (Francke & Garrett, 1982; Banks et al., 1983). It seems reasonable to assume that more than three proteins, whether of viral or cellular origin, are involved in the replication of HSV DNA, since it is known that nearly ten proteins are required for replication of the Escherichia coli chromosome in vitro (Kaguni & Kornberg, 1984). One approach to identifying such unknown proteins and to determining their functions is to use compounds that inhibit DNA synthesis. Recently, a semi-synthetic derivative of podophyllotoxin, VP-16-213, which can induce DNA breaks in cultured mammalian cells, has been shown to interfere with the function of mammalian DNA topoisomerase II (Wozniak & Ross, 1983; Chen et al., 1984; Long et al., 1984; Ross et al., 1984). It has also been shown that VP-16-213 does not intercalate or bind to DNA (Ross et al., 1979). It has therefore been suggested that DNA topoisomerase II is the target of VP-16-213 in vivo (Chen et al., 1984; Ross et al., 1984). In this study, we examined the effects of VP-16-213 on the replication of HSV type 2 (HSV-2) in human embryonic fibroblasts.

Human embryonic fibroblasts (HEF) were prepared as described previously (Nishiyama et al., 1983), grown in Eagle's MEM supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin. HSV-2 strain 186 was obtained from Dr Fred Rapp, Pennsylvania State University College of Medicine, Hershey, Penn., U.S.A. The virus stock was prepared in HEF by infecting at low multiplicities (0.01 to 0.1 p.f.u./cell) as described...
Fig. 1. Effect of VP-16-213 on the replication of HSV-2. The sensitivity of viruses to VP-16-213 was measured by plaque reduction assays (a) and yield reduction assays (b) on monolayers of HEF as described in the text. The results are expressed as a percentage of the values obtained in control cultures that received no drug.

Fig. 2. Effect of VP-16-213 on protein synthesis in infected (a) and mock-infected cells (b). Confluent monolayers of HEF were infected with HSV-2 at 20 p.f.u./cell, treated with the various concentrations (μg/ml) of VP-16-213 indicated between 1 and 3 h post-infection and then labelled with [35S]methionine (10 μCi/ml) for 1 h in the presence of the drug. The radioactive polypeptides were analysed by PAGE, followed by autoradiography.

previously (Nishiyama & Rapp, 1981). VP-16-213 was obtained from Bristol-Myers Co. and Nippon Kayaku Co. [Me-3H]Thymidine (25 Ci/mmol), [Me-3H]thymidine 5′-triphosphate (29 Ci/mmol) and [35S]methionine (600 Ci/mmol) were purchased from Amersham. Plasmid ColE1 DNA was from Wako, Osaka, Japan. The assays of DNA topoisomerase and DNA polymerase activity were done as described previously (Liu, 1983; Nelson et al., 1984; Nishiyama et al., 1984).

To assess the antiviral effect of VP-16-213, both plaque reduction and yield reduction assays were performed in confluent cultures of HEF. Plaque formation with HSV-2 was inhibited by 50% at 5 μg/ml of VP-16-213 and completely suppressed at 15 μg/ml (Fig. 1a). In yield reduction assays, cells were infected with HSV-2 at a multiplicity of approximately 10 p.f.u./cell and treated with the drug after 1 h adsorption. As shown in Fig. 1(b), virus production in cultures treated with 20 μg/ml VP-16-213 was inhibited by more than 99%. The cytotoxicity of the
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inhibitor for confluent HEF was monitored for 48 h by microscopy. No significant morphological changes were observed in cultures treated with 20 μg/ml VP-16-213. However, treatment with 100 μg/ml resulted in cell rounding within 24 h.

Next, experiments were performed to determine the effect of VP-16-213 on HSV-2-induced protein synthesis. Cells infected with HSV-2 were treated with VP-16-213 for 2 h after 1 h adsorption and labelled with [35S]methionine between 3 and 4 h post-infection in the presence of the drug. As shown in Fig. 2, addition of 20 μg/ml VP-16-213 had little effect on the synthesis of early viral polypeptides. In cultures treated with 100 μg/ml VP-16-213, however, protein synthesis was inhibited by more than 95%.

To examine the effect of VP-16-213 on viral and cellular DNA synthesis, CsCl density gradient centrifugation was performed as described previously (Nishiyama & Rapp, 1981). Confluent monolayers of HEF were infected with HSV-2 at a multiplicity of approx. 20 p.f.u./cell, and labelled with [3H]thymidine (10 μCi/ml) between 2-5 and 3-5 h post-infection in the presence or absence of VP-16-213. When the inhibitor was added to cultures 30 min before labelling at 20 μg/ml, the incorporation of [3H]thymidine into viral DNA was almost completely inhibited. Cellular DNA synthesis was also inhibited by the drug, but appeared less sensitive than viral DNA synthesis in this system. Therefore, the sensitivity of cellular DNA synthesis to VP-16-213 was measured independently in actively growing HEF, and compared with that of viral DNA synthesis. As shown in Fig. 3, the inhibition curve of

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Fig. 3. Effect of the dose of VP-16-213 on inhibition of viral and cellular DNA synthesis. The sensitivity of viral DNA synthesis to VP-16-213 (●) between 2.5 and 3.5 h post-infection was determined by CsCl density gradient equilibrium centrifugation analysis. The sensitivity of cellular DNA synthesis (○) was determined in actively growing HEF by measuring [3H]thymidine incorporation into acid-insoluble fractions. The drug was added to cultures 30 min before labelling and cells were labelled with [3H]thymidine (10 μCi/ml) for 1 h in the presence of various concentrations of VP-16-213.

Fig. 4. Neutral sucrose sedimentation profiles of radioactive DNA from HSV-2-infected cells treated with VP-16-213. Confluent monolayers of HEF were infected with HSV-2 (20 p.f.u./cell), labelled with [3H]thymidine (10 μCi/ml) between 3 and 3.5 h post-infection, and chased for 1 h in the presence of 20 μg/ml VP-16-213 (●) or 10 mM-hydroxurea (○), or in the absence of drugs (▲). The cells were harvested, and digested by the addition of 200 μg/ml proteinase K. A sample was layered onto neutral sucrose gradients (5 to 20%), and centrifuged in an SW27 rotor at 12000 r.p.m. for 16 h. Fractions were collected by puncturing the bottom of the tube. The arrow indicates the position of virion DNA (55S) in a parallel gradient.
cellular DNA synthesis was quite similar to that of viral DNA synthesis, indicating that viral and cellular DNA syntheses are equally sensitive to this drug.

It has been shown that VP-16-213 can induce single-strand and double-strand DNA breakages in vivo (Long et al., 1984). In simian virus 40 (SV40)-infected cells, treatment with epipodophyllotoxins results in conversion of supercoiled SV40 DNA into linear and nicked forms (Chen et al., 1984). In order to test whether VP-16-213 induced double-strand DNA breaks in HSV DNA, the sedimentation behaviour of newly synthesized viral DNA was examined in neutral sucrose gradients. Infected cells were pulse-labelled with [3H]thymidine for 30 min between 3 and 3.5 h post-infection since more than 85% of the DNA synthesis during this period was viral (data not shown). The cells were then chased for 1 h in the presence of 20 µg/ml VP-16-213 or 10 mM-hydroxyurea, or in the absence of these drugs. To avoid DNA breakage, the cells were carefully treated with SDS, digested with proteinase K, and a sample was layered directly onto neutral sucrose gradients. The size distribution profiles are illustrated in Fig. 4. In the gradient of DNA from control cultures, a single peak of radioactivity was observed in the position that corresponded to that of mature viral DNA (55S) in a parallel gradient. The distribution profile was not affected by inhibition of DNA synthesis with hydroxyurea. In contrast, the labelled DNA from VP-16-213-treated cells sedimented in the gradient as a broad peak with a shoulder in the lower molecular weight region.

We also measured the level of DNA topoisomerases I and II, and viral DNA polymerase activities at various times after infection. The activity of viral DNA polymerase became detectable by 2 h post-infection and increased until 8 h. However, the level of DNA-relaxing activity of both topoisomerases I and II remained constant for at least 10 h after infection (data not shown). Although we examined the level of topoisomerase II under several assay conditions, no detectable change was observed.

The data described above indicated that VP-16-213 inhibited the replication of HSV-2 at the stage of viral DNA synthesis, and it was inferred that the inhibition may be due to interference in a function of DNA topoisomerase II. If the target of VP-16-213 is encoded by the viral genome, it should be possible to isolate drug-resistant mutants. To test this possibility, we tried to isolate such mutants but in spite of extensive efforts all attempts have failed.

To determine whether VP-16-213 has any inhibitory effect on HSV-2 morphogenesis, electron microscopical studies were also performed. Cultures were treated with 20 µg/ml VP-16-213 from 1 h post-infection and fixed with glutaraldehyde 9 h post-infection. A number of viral capsids were observed even in the nuclei of infected cells treated with VP-16-213, but most were incomplete with empty or partial cores (data not shown).

Many investigators have studied the effects of podophyllotoxin and its derivatives on the growth of mammalian cells (Keller-Juslen et al., 1971; Grieder et al., 1974). Earlier studies have shown that two derivatives, VP-16-213 and VM-23, have a different mechanism of action from that of the parent compounds. Neither derivative, unlike podophyllotoxin, affects microtubule assembly but both induce DNA strand breakage (Loike & Horwitz, 1976a, b). Recently, Chen et al. (1984) have reported that VP-16-213 and VM-23, neither of which intercalate or bind to DNA, interfere with the breakage–reunion reaction of mammalian DNA topoisomerase II by stabilizing a cleavable complex, and suggested that the DNA breakage activity in vivo of these epipodophyllotoxins is mediated by topoisomerase II. Using SV40-infected cells they have also shown that the epipodophyllotoxins induce both single-strand and double-strand breaks in viral DNA. HSV DNA is known to have single-strand nicks and gaps naturally (Wilkie, 1973), but neutral sucrose gradient sedimentation analysis showed that VP-16-213 induced double-strand DNA breaks only in newly synthesized HSV DNA in vivo. Our results and previous observations taken together suggest that DNA topoisomerase II is involved in the replication of HSV DNA and that VP-16-213 inhibits viral DNA synthesis by interfering with the function of topoisomerase II.

To our knowledge there are three reports on HSV-related topoisomerases. Biswal et al. (1983) have reported that a DNA-relaxing enzyme which has a strict requirement for Mg2+ and ATP co-purified with HSV-1-induced DNA polymerase. Leary & Francke (1984) have described an enzyme activity induced in HSV-1-infected cells which is capable of catenating closed circular
DNA in the presence of Mg\(^{2+}\), but this enzyme did not require ATP. Muller et al. (1985) have recently shown that a type I topoisomerase which did not require ATP or Mg\(^{2+}\) for activity was associated with purified virions of HSV-1. At present, however, there is no clear evidence that these DNA topoisomerases are virus-coded. Although we cannot present direct evidence, our observations suggest that the topoisomerase II involved in the replication of HSV DNA may be of host cell origin. The reasons are as follows: (i) the sensitivity of viral DNA synthesis to VP-16-213 was very similar to that of cellular DNA synthesis, (ii) there was no change in the level of topoisomerase II activity following infection with HSV and (iii) extensive efforts to isolate HSV mutants resistant to VP-16-213 failed, whereas a number of viral DNA polymerase mutants resistant to inhibitors were isolated in parallel experiments. In order to resolve this issue directly, it will be necessary to examine the antiviral effect of VP-16-213 in VP-16-213-resistant cells that have an altered cellular topoisomerase II.

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REFERENCES


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