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Identification and characterization of a benzothiophene inhibitor of herpes simplex virus type 1 replication which acts at the immediate early stage of infection

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Abstract

Analysis of a large compound library in a high throughput virus infection assay screen identified the benzothiophene PD146626 as a potent and specific inhibitor of herpes simplex virus type 1 (HSV-1) replication. PD146626 possessed an EC_{50} and EC_{90} against HSV-1 of 0.1 and 1 μM, respectively, and mediated no detectable cytotoxicity in cells at concentrations up to 1 μM. Western blot analyses and time of addition experiments demonstrated that in the presence of PD146626 HSV-1 underwent a specific block in viral gene expression at the immediate early stage. However, several observations indicated that a cellular function rather than a viral immediate early transactivator protein represented the molecular target for PD146626, including the lack of resistance of VP16 and ICP0 mutant viruses to the compound, the inability to select resistant strains of HSV-1 following exhaustive serial passaging of virus in the presence of the compound, and the sensitivity of human cytomegalovirus, which lacks VP16 and ICP0 homologs, to the compound. Moreover, kinetic studies suggested an unusual pattern of responsiveness of the host cell to PD146626, in that the compound could induce an extended antiviral state in cells after only a brief exposure. Together these results suggest that PD146626 targets a novel cellular function that is critical for the expression of HSV-1 immediate early genes but not host cell genes.

Keywords: Antiviral; Benzothiophene; Herpes; HSV-1; Immediate early gene; PD146626

1. Introduction

The life cycle of herpes simplex virus type 1 (HSV-1) is exceedingly complex and affords numerous opportunities for the development of a novel antiviral therapy. Successful infection by HSV-1 requires the virus particle to attach to and penetrate into cells; uncoat its DNA and deposit it into the nucleus; transcribe its genes sequentially to produce immediate early, early, and late mRNAs; synthesize regulatory proteins, DNA replication enzymes, and structural viral proteins from these mRNAs; replicate its DNA genome; assemble new capsids and virions; and release

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these new particles from the infected cell (Roizman, 1996). Each of these steps represents an opportunity for the creation of new intervention strategies. To date, the only aspect of the HSV-1 life cycle for which antiviral therapies have been successfully developed is the process of DNA replication, which has been targeted by a small group of nucleoside analogues that include acyclovir, valacyclovir, penciclovir, and famciclovir. While these drugs have been clinically successful in treating acute infections, they fail to modulate reactivation of latent virus and can be rendered ineffective by resistance mutations (Coen, 1994; Field and Biron, 1994). Thus, the continued discovery and development of new and improved treatments remains essential for the future management of herpes infections in humans.

Immediate early gene expression represents one stage of the HSV-1 life cycle that could be targeted by a novel antiviral therapy to deliver a significant reduction in virus replication in both acute and latent infections. Immediate early gene expression plays not only an indispensable regulatory role in the expression of all classes of viral genes during lytic infection, but also appears to be the key initiating event in the process by which reactivation of the latent HSV-1 genome takes place. Low levels of immediate early transcripts can be identified in latently infected neurons, suggesting that activation of the promoters encoding these mRNAs above a threshold level triggers reactivation from latency (Preston, 2000). Moreover, ICP0 mutant viruses that are defective in immediate early transactivation have been shown to reactivate very poorly from latent infections (Clements and Stow, 1989; Leib et al., 1989). Thus, small molecules which act as inhibitors of HSV-1 immediate early gene expression have the potential to impact clinical disease to a far greater extent than currently marketed nucleosides. Recently, several compounds were described which appeared to inhibit HSV-1 immediate early gene expression, although the exact mechanism of action of their activity was not determined (Albin et al., 1997). In this study, the benzothiophene HSV-1 inhibitor PD146626 was characterized and found to inhibit viral immediate early gene expression through a cellular rather than viral mechanism of action. Although the pharmacokinetic properties of this compound and others within its class were sufficiently poor to preclude their further development as clinical candidates (data not shown), the host cell function which they target appears to be a novel component required for HSV-1 immediate early gene expression. As such, this cellular target has the potential to be exploited in the identification of additional classes of HSV-1 immediate early inhibitor molecules.

2. Materials and methods

2.1. Viruses, cells, and reagents

HSV-1 employed in this study included HSV-1(US3::Tn5-*lac*Z), which contains an HSV-1 glycoprotein C promoter-*lac*Z gene fusion inserted into the US3 gene of the viral genome (Hendricks et al., 1991; Fink et al., 1992); V422, which expresses a transactivation-defective version of VP16 (kindly provided by J. Smiley, University of Alberta) (Smiley and Duncan, 1997); dl1403, an ICP0 null mutant (kindly provided by R. Everett, MRC Virology Unit) (Stow and Stow, 1986); d120, an ICP4 null mutant (kindly provided by N. DeLuca, University of Pittsburgh) (DeLuca et al., 1985); and wild type HSV-1 (strain 17). Each of these viruses were propagated in African green monkey (Vero) cells that were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, with the exception of d120, which was propagated in ICP4-expressing Vero-derived E5 cells (DeLuca et al., 1985). The HCMV strain used in this study was the *lac*Z-expressing recombinant RC256 (kindly provided by E. Mocarski, Stanford University) (Spaete and Mocarski, 1987). This virus was propagated in human foreskin fibroblast (HFF) cells that were purchased commercially (Clontech) as low passage stocks and cultured in modified Eagle's medium supplemented with 5% fetal bovine serum. Acyclovir was purchased commercially as acycloguanosine (Sigma) and the benzothiophene PD146626 was synthesized as described previously (Connor et al., 1997).

2.2. Antiviral activity assays

2.2.1. HSV-1-lacZ replication inhibition assay

Vero cells were seeded into 96 well plates at a density of 2×10^4 cells per well and incubated overnight. On the following day, the compound to be tested was resuspended in DMSO to create a 10 mM stock solution. In a separate 96 well plate, 5 μl of this stock was added to 245 μl media to create an initial 1:50 dilution, and a series of additional 1:5 dilutions were prepared by adding 40 μl of compound to 160 μl media. The media was then removed from the 96 well plate containing the overnight culture of Vero cells. Each well was given 100 μl fresh media, 25 μl compound dilution (representing a minimum DMSO dilution of 1:400), and 75 μl HSV-1(US3::Tn5-*lac*Z) suspension (equivalent to 2×10^3 PFU per well). After overnight incubation, the cells were lysed and assayed for β-galactosidase activity by the addition of 100 μl of 2× Z buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgSO_4$, 5 mM EDTA, and 0.2% Triton X-100) supplemented with CPRG (0.8 mg/ml; Roche) per well. Following color development, the absorbance of the plates was determined at 575 nm. Percent inhibition was determined by comparison to a set of control reactions which employed dilutions of DMSO lacking compound. All assays were performed in triplicate and EC_{50}s were determined from logarithmic plots of compound concentration versus mean percent inhibition.

2.2.2. HSV-1 yield reduction assay

Vero cells were seeded into six well plates at a density of 5×10^5 cells per well and incubated overnight. On the following day, the compound to be tested was resuspended in DMSO to create a 10 mM stock solution, which was then used to create a series of 1:5 dilutions in DMSO. The media was then removed from the six well plate containing the overnight culture of Vero cells. Each well was given 3 ml fresh media containing 5×10^3 PFU HSV-1 and 7.5 μl compound dilution (representing a DMSO dilution of 1:400). After overnight incubation, the cells were lysed by freeze-thawing and centrifuged briefly at low speed to remove cellular debris. The titers of the virus yield in each cell free supernatant were then determined by plating dilutions on fresh six well dishes of cells and then enumerating plaques which formed after 3 days under media supplemented with methylcellulose. Percent inhibition was determined by comparison to a set of control infections which employed DMSO lacking compound. All assays were performed in duplicate and EC_{50}s were determined from logarithmic plots of compound concentration versus mean percent inhibition. Within duplicate experiments, the range in data was never greater than 20% of the mean, and in many cases was substantially less than this percentage.

2.2.3. HCMV-lacZ replication inhibition assay

This assay was identical to the HSV-1-*lac*Z replication inhibition assay described above, except that HFF cells and the HCMV-*lac*Z recombinant virus RC256 were used, and the infection was allowed to proceed for 7 days before the cells were lysed for β-galactosidase activity determinations.

2.3. Cytotoxicity assays

2.3.1. XTT assay

Vero or HFF cells were seeded into 96 well plates at a density of 2×10^3 cells per well and incubated overnight. On the following day, the compound to be tested was resuspended in DMSO to create a 10 mM stock solution and diluted sequentially in media as described in the HSV-1-*lac*Z replication inhibition assay above. The media was then removed from the 96 well plate containing the overnight culture of cells. Each well was given 175 μl fresh media and 25 μl compound dilution (representing a minimum DMSO dilution of 1:400). After incubation for 5 days, the cells were assayed for their ability to generate XTT formazan by the addition of 50 μl XTT reagent (XTT-PMS stock (200 mg/ml XTT (Sigma) and 2.45 mg/ml phenazine methosulfate (Sigma) in DMSO) diluted 1:600 in media) per well. Following color development for 4 h, the absorbance of the plates was determined at 450 nm. Percent toxicity was determined by comparison to a set of control reactions which employed

dilutions of DMSO lacking compound. All assays were performed in triplicate and TC_{50}s were determined from logarithmic plots of compound concentration versus mean percent toxicity.

2.3.2. Five day proliferation assay

Vero or HFF cells were seeded into six well plates at a density of 5×10^4 cells per well, which was sufficiently low to allow them to proliferate in the absence of contact inhibition for six days. On the following day, the compound to be tested was resuspended in DMSO to create a 10 mM stock solution, which was then used to create a series of 1:5 dilutions in DMSO. The media was then removed from the 6 well plate containing the overnight culture of cells. Each well was given 3 ml fresh media and 7.5 μl compound dilution (representing a DMSO dilution of 1:400). The plates were incubated for 5 days and then either stained with crystal violet or subjected to the XTT assay described above. All assays were performed in duplicate and the maximum nontoxic concentration (MNTC) was determined to be the highest concentration at which no alteration in cellular proliferation could be detected by either inspection of cell density following crystal violet staining or XTT formazan production.

2.4. Western and Southern blot procedures

Western blots were performed as described previously (Spatz et al., 1996) and employed monoclonal antibodies that were specific for the HSV-1 proteins ICP4, glycoprotein B, glycoprotein D (all purchased from ABI), and ICP0 (antibody 11060 (Everett et al., 1993), a generous gift from R. Everett, MRC Virology Unit). Southern blotting was performed using a digoxigenin labelling and detection kit (Boerhinger-Mannheim) according to the recommended protocols of the manufacturer, and employed a labeled 4.2 kb HindIII/SalI fragment of pIGA15 (Gelman and Silverstein, 1985) containing the HSV-1 ICP0 gene as a probe. DNA was isolated from nuclei purified from infected cells using a genomic DNA isolation kit (Qiagen) according to the recommended protocols of the manufacturer.

3. Results

3.1. Identification and initial characterization of a benzothiophene inhibitor of HSV-1 replication

The antiherpesviral activity of benzothiophenes was discovered through random screening of a large compound library using an HSV-1-*lac*Z replication inhibition assay. This simple high throughput colorimetric assay employed a recombinant HSV-1, HSV-1(US3::Tn5-*lac*Z), which contained a *lac*Z gene driven by the late promoter for the viral glycoprotein C gene (Fink et al., 1992 Hendricks et al., 1991). Since the *lac*Z gene in this virus is expressed with late kinetics, normal β-galactosidase expression was dependent upon the successful completion of a number of steps in the viral life cycle, including virion entry, subsequent expression of viral immediate early, early, and late gene products, and unimpaired DNA replication. Inhibition of any of these stages of HSV-1 replication resulted in significantly diminished *lac*Z expression. By employing a 24 h infection in Vero cells at a multiplicity of 0.1 in a 96 well plate format, the HSV-1-*lac*Z replication inhibition assay was able to generate the readily detectable and highly reproducible levels of β-galactosidase activity that were required for its adaptation into a high throughput screen for inhibitors of HSV-1 replication.

The benzothiophene PD146626 (Fig. 1) was a potent inhibitor discovered in the HSV-1-*lac*Z replication inhibition assay. This compound became the prototype for the benzothiophene series and is the principal focus of the mechanism of action experiments presented in this study. Additional chemical syntheses aimed at exploring structure–activity relationships within the benzothiophene series resulted in the generation of several hundred derivatives of PD146626 (data not shown); their characterization will be de-

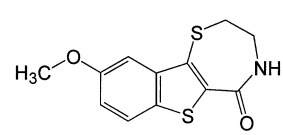

Fig. 1. Chemical structure of PD146626.

Table 1
Antiviral and cytotoxic properties of PD146626

Assay	PD146626	Acyclovir
HSV-1 assays		
EC_{50} (μM) in HSV-1-*lac*Z replication inhibition assay[a]	0.1	2.0
EC_{50} (μM) in HSV-1 yield reduction assay	0.2	0.1
TC_{50} (μM) for Vero cells in XTT assay[b]	10	>25
MNTC (μM) for Vero cells in 5 day proliferation assay[c]	1	>25
HCMV assays		
EC_{50} (μM) in HCMV-*lac*Z replication inhibition assay[a]	0.1	nd[d]
TC_{50} (μM) for HFF cells in XTT assay[b]	>25	nd
MNTC (μM) for HFF cells in 5 day proliferation assay[c]	1	nd

[a] Concentration resulting in 50% inhibition of β-galactosidase synthesis during infection by either the HSV-1-*lac*Z recombinant HSV-1(US3::Tn5-*lac*Z) or the HCMV-*lac*Z recombinant RC256.

[b] Concentration resulting in 50% inhibition of XTT formazan production in either Vero or HFF cells.

[c] Maximum nontoxic concentration (MNTC) allowing unaltered proliferation of Vero or HFF cells in the absence of contact inhibition over a 5 day period.

[d] nd, not done due to known inactivity of acyclovir against HCMV.

scribed elsewhere. PD146626 was found to have an EC_{50} of 0.1 μM against HSV-1(US3::Tn5-*lac*Z) in the HSV-1-*lac*Z replication inhibition assay and an EC_{50} of 0.2 μM against wild type HSV-1 in a standard virus yield reduction assay (Table 1). The close correlation of the EC_{50} values between the two assays for not only PD146626 but also other benzothiophene inhibitors (data not shown) underscored the reliability of the HSV-1-*lac*Z replication inhibition assay as a rapid alternative to the yield reduction assay in the assessment of antiviral activity in this compound series. The potency of PD146626 was comparable to that of acyclovir, which gave an EC_{50} of 0.1 μM in yield reduction assays (Table 1). Interestingly, the EC_{50} for acyclovir in the HSV-1-*lac*Z replication inhibition assay was an order of magnitude higher at 2.0 μM (Table 1), indicating that β-galactosidase expression was less sensitive to this compound than infectious virion production during HSV-1(US3::Tn5-*lac*Z) infection.

Cytotoxicity of PD146626 for Vero cells was initially assessed in an XTT assay, where this compound was found to possess a TC_{50} of 10 μM and therefore a 100-fold difference between antiviral activity and toxicity (Table 1). Since other unrelated compounds have been observed to arrest cell proliferation without affecting production of XTT formazan in this assay (data not shown), the issue of cytotoxic potential was addressed more rigorously by analyzing the effects of PD146626 on Vero cell proliferation in the absence of contact inhibition over a 5 day period. In this assay, Vero cells were found to exhibit normal proliferation in the presence of PD146626 concentrations up to 1 μM (Table 1), even when fresh compound was added to the cells for each of the 5 days of the incubation time (data not shown). This dose, termed the maximum nontoxic concentration (MNTC), was therefore the upper limit of PD146626 used in all subsequent experiments. The fact that 1 μM also represented the EC_{90} of the compound in both the HSV-1-*lac*Z replication inhibition and yield reduction assays confirmed that PD146626 could specifically inhibit HSV-1 replication by up to 90% through a mechanism which had no detectable consequences to the host cell.

Since other benzothiophenes have been previously implicated as inhibitors of inflammation (Boschelli et al., 1995) as well as the replication of other viruses such as HIV (Butera et al., 1995), PD146626 was examined in a variety of additional assays to ascertain whether it possessed similar inhibitory properties. PD146626 was found to lack the anti-inflammatory properties observed in other benzothiophenes, as the concentrations that were required to mediate downregulation of the expression of adhesion molecules such as VCAM and E-selectin were found to be indistinguishable from the concentrations at which cytotoxicity was observed (data not shown). Moreover, this compound lacked any detectable antiviral activity against both HIV and influenza virus (data not shown). However, PD146626 was found to inhibit the replication of the betaherpesvirus human cytomegalovirus (HCMV) in a manner that was

nearly identical to that of HSV-1, possessing antiviral activity and cytotoxicity properties that were quite comparable between viruses (Table 1). Consistent with all of these findings, other benzothiophenes which had been previously shown to possess anti-inflammatory and anti-HIV properties were found to exhibit very weak if any antiviral activity against either HSV-1 or HCMV (data not shown). Thus, despite obvious structural similarities with these previously characterized benzothiophenes, the distinct antiherpesviral activities of PD146626 indicate that this compound most likely represents a novel pharmacophore.

3.2. PD146626 inhibits HSV-1 replication by interfering with viral immediate early gene expression

Although the HSV-1-*lac*Z replication inhibition assay was able to readily identify PD146626 as an HSV-1 inhibitor when utilized in a high throughput screening format, it nevertheless was unable to provide any information regarding the mechanism of action of this compound. A series of experiments was therefore carried out to determine the stage in the viral life cycle in which PD146626 acted. Prior to conducting these studies, a standardized set of infection conditions were established that were employed in all mechanism of action studies. To eliminate any concerns about the potential contribution of compound cytotoxicity to inhibition of virus replication, PD146626 was never used in mechanism of action experiments at concentrations which exceeded its MNTC (1 μM, Table 1). Additional experiments were carried out to explore a possible multiplicity dependence of PD146626 antiviral activity. This was investigated by comparing the effect of increasing the multiplicity of infection in yield reduction assays on the antiviral activity of EC_{90} concentrations of PD146626 (1 μM) and acyclovir (2.5 μM). As expected, for both of these compounds, 24 h virus yields were reduced by 90% or greater when the multiplicity of infection used was 0.01 (Fig. 2). However, slight reductions in antiviral activity were observed for PD146626 when the multiplicity of infection was increased to 0.1, and the antiviral activity of this compound was completely abrogated when the multiplicity of infection was increased to 1 (Fig. 2). In contrast, the antiviral activity of acyclovir remained unchanged for all three multiplicities of infection (Fig. 2). These results indicated that the potency of PD146626 was indeed multiplicity-sensitive, which suggested that the ability of this compound to interfere with viral replication could be overcome by the elevated levels of one or more viral proteins synthesized during a high multiplicity infection. It should be noted that this property was not a concern for the development of this compound series as an antiviral therapy, since high multiplicity infections are an artificial situation which can be readily created in cell culture but not in vivo. However, it did warrant that all of the infections employed in the mechanism of action studies described below necessarily did not exceed a multiplicity of 0.01.

In order to initially assess the effect of PD146626 on HSV-1 infection, Western blot analysis of lysates of viral infected cells was performed using antibodies specific for HSV-1 polypeptides

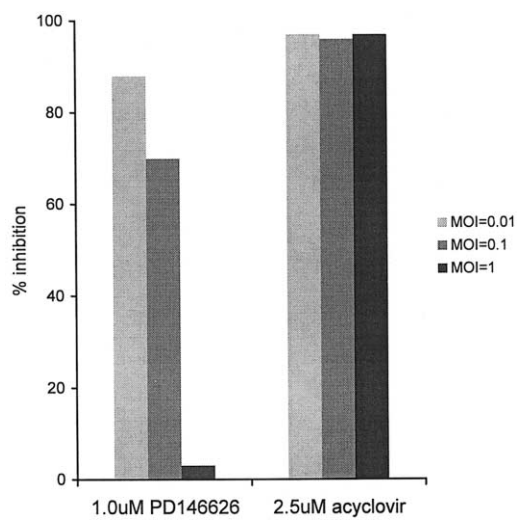

Fig. 2. Effect of multiplicity of infection on antiviral activity of PD146626. HSV-1 yield reduction assays were performed using the indicated concentrations of either PD146626 or acyclovir at the indicated multiplicities of infection. Percent inhibition represented the reduction in virus yield observed in infections containing compound compared to infections containing DMSO as a solvent control. The bars in the histograms correspond to the means of duplicate experiments.

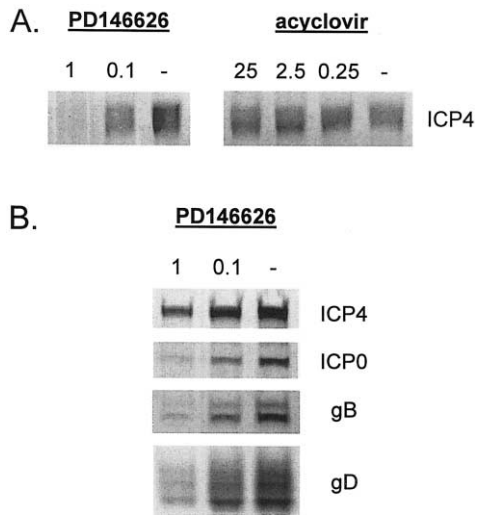

Fig. 3. Effect of PD146626 on HSV-1 gene expression. Cells were infected at a multiplicity of 0.01 in the presence of the indicated concentrations of either PD146626 or acyclovir and lysed at either 7 h (A) or 18 h (B) postinfection. Control infections carried out in the presence of DMSO lacking compound (-) were also lysed. Western blot analyses were then performed on the lysates using monoclonal antibodies specific for the HSV-1 polypeptides ICP4, ICP0, gB, and gD.

of all kinetic classes. These experiments revealed that PD146626 promoted a significant inhibition of immediate early protein synthesis during infection. For example, the immediate early protein ICP4 could be readily detected in cells which had been infected with HSV-1 at a multiplicity of infection of 0.01 and lysed after just 7 h (Fig. 3A). However, ICP4 levels were reduced in a dose–responsive fashion in identical infections which had been carried out in the presence of EC_{50} and EC_{90} concentrations (0.1 and 1 μM, respectively) of PD146626 (Fig. 3A), such that the decreases in ICP4 observed in these lysates were directly proportional to the reductions in virus yields that were observed at 24 h postinfection. In contrast, acyclovir had no effect on ICP4 expression at all concentrations tested (Fig. 3A), which was consistent with its ability to act only late in the HSV-1 replication cycle as a DNA replication inhibitor long after the immediate early stage. Since the expression of later classes of viral proteins is known to be strictly dependent upon the prior synthesis of immediate early proteins such as ICP4 (DeLuca et al., 1985), the significant reductions in the levels of these regulatory proteins that are mediated by PD146626 early in infection would be expected to result in a global inhibition of viral gene expression by late times. Analyses carried out at 18 h postinfection confirmed this prediction, revealing a comparable dose-responsive inhibition of the expression of all classes of viral proteins by PD1246626, including reductions in the levels of the immediate early proteins ICP4 and ICP0, the early protein glycoprotein B, and the early–late protein glycoprotein D (Fig. 3B). Thus, PD146626 is able to prevent infectious particle formation through the widespread disruption of viral gene expression resulting from the absence of adequate levels of immediate early proteins at the onset of infection.

Although the most obvious explanation for the failure of HSV-1 to synthesize immediate early proteins in the presence of PD146626 is that this compound acts as a direct inhibitor of viral immediate early gene expression, other mechanisms of action are possible. For example, PD146626 could inhibit the attachment or penetration of virions to host cells, or the uncoating of virions to release viral DNA into the nucleus, either of which would also be expected to result in a lack of immediate early gene expression. However, identical levels of inhibition were observed in yield reduction experiments where PD146626 and virus were added simultaneously to cells versus comparable experiments where the cells were infected with virus first, washed to remove any unbound virions, and then treated with PD146626 (data not shown). This observation, together with the results of time of addition experiments presented below, indicated that the observed reduction in immediate early gene expression was not an indirect result of inhibition of virion attachment or entry into cells.

The potential effect of PD146626 on the state of virion DNA in the early stages of infection was also investigated. Cells which had been infected with HSV-1 at a multiplicity of 0.01 in the presence or absence of an EC_{90} concentration (1 μM) of PD146626 were lysed at 4 h post infection. At this point in the infection, significant levels of immediate early gene expression should have been initiated in these infected cells based on the results

from Western blot analysis (Fig. 3), but the input DNA derived from virions should not have begun replication in either set of cells. Nuclei were extensively purified from these lysates and DNA was isolated for analysis on Southern blots using a probe specific for the ICP0 gene of HSV-1 that should hybridize to 5.9 kb BamHI and 2.6 kb BamHI/SalI fragments of the viral genome (Fig. 4A). Since the input viral DNA had not yet undergone amplification due to DNA synthesis this early in the infection, the signals of the two ICP0-specific bands on the blot were understandably weak; nevertheless it was clear that there were no detectable differences in the levels of nuclear virion DNA present in PD146626-treated and untreated infected cells (Fig. 4B). These results demonstrated that in the presence of PD146626, virions are able to enter cells, uncoat, and deposit their DNA into the nucleus with normal efficiency. Thus, the failure of HSV-1 to adequately express immediate early proteins in these cells is most likely the result of transcriptional inhibition of the promoters associated with the genes encoding these polypeptides.

While the results described above demonstrated that PD146626 was able to mediate inhibition of viral immediate early gene expression, it was unclear whether this was the result of specific inhibition of transcription from viral immediate early promoters or generalized inhibition of transcription from all viral promoters. To address this question and to further explore the kinetics of immediate early stage inhibition by PD146626, time of addition experiments were carried out. In these analyses, yield reduction assays were carried out in which EC_{90} concentrations of inhibitor compounds were added to identically infected cells at progressively later times in infection, and virus yields for each experiment were then determined at 24 h postinfection. As expected, 1 μM PD146626 inhibited virus yields by 90% when present from 0 to 24 h (Fig. 5A). However, when the addition of PD146626 was delayed for increasing lengths of time after the initiation infection, its antiviral activity was proportionally reduced and ultimately completely abrogated (Fig. 5A). Remarkably, PD146626 had no effect on virus replication when added past 7 h postinfection, even though this meant it was still present during the final 17 h of infection. The latter results were entirely consistent with PD146626 being a specific inhibitor of immediate early transcription, since the viral requirement for the presence of immediate early proteins during infection is limited to the earliest stage of infection and

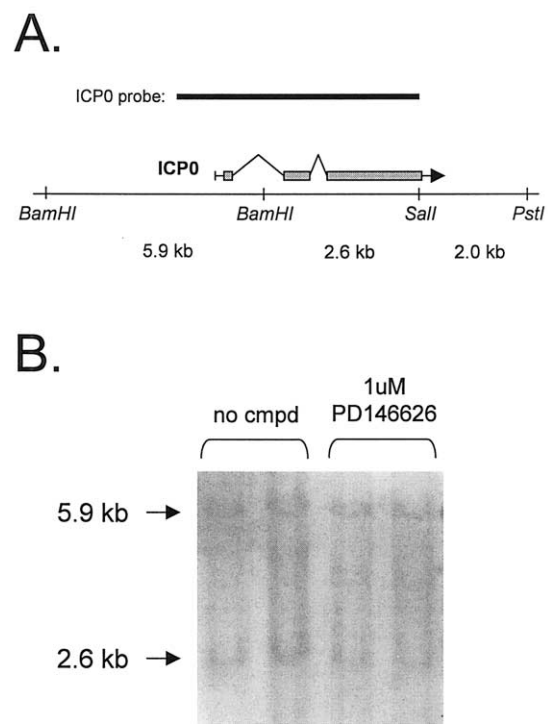

Fig. 4. Effect of PD146626 on entry of HSV-1 DNA into the nucleus. (A) Restriction map of region of HSV-1 genome hybridizing to Southern blot probe. Positions of sites recognized by BamHI, SalI, and PstI and sizes of the fragments generated by these enzymes are shown below the map, and the location of the ICP0 gene and the sequences comprising the Southern blot probe are shown above the map. (B) Southern blot analysis of input genome DNA in infected cell nuclei. Cells were infected at a multiplicity of 0.01 in the presence of 1 μM PD146626 or DMSO lacking compound and lysed at 4 h postinfection. DNA isolated from purified nuclei was then digested with BamHI, SalI, and PstI, electrophoresed on an agarose gel, and subjected to Southern blot analysis using the probe shown in (A). For each compound treatment, duplicate infections were prepared and their nuclear DNA analyzed in separate gel lanes. The expected mobilities of the 5.9 kb BamHI and 2.6 kb BamHI/SalI fragments of ICP0 that are expected to hybridize to the probe are indicated by arrows at left.

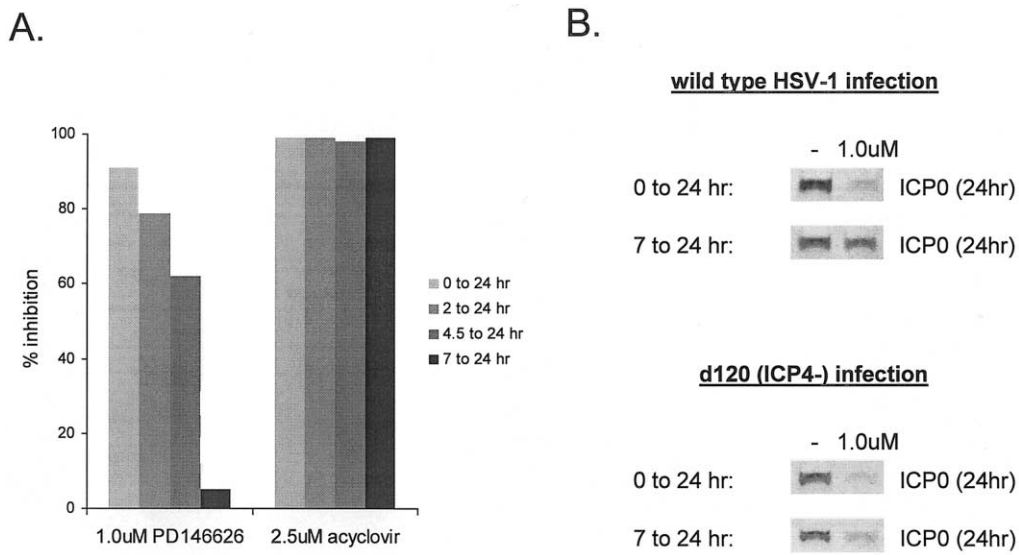

Fig. 5. Effect of varying the time of addition on the antiviral activity of PD146626. (A) Virus yield in time of addition experiments. HSV-1 yield reduction assays were performed at a multiplicity of infection of 0.01 using the indicated concentrations of either PD146626 or acyclovir; however, the compounds were only present in the infections during the time intervals shown. Percent inhibition represented the reduction in virus yield observed in infections containing compound compared to infections containing DMSO as a solvent control. The bars in the histograms correspond to the means of duplicate experiments. (B) Virus gene expression in time of addition experiments. Cells were infected as in (A) and lysed at 24 h postinfection. Western blot analyses were then performed on the lysates using a monoclonal antibody specific for the HSV-1 polypeptide ICP0. Results are shown for cells infected with either wild type HSV-1 or the ICP4 null mutant d120 in the presence of PD146626 (1.0 μM) or DMSO lacking compound (-) that was added to cells at 0 or 7 h postinfection.

would therefore be expected to be dispensable once an infection has been established (i.e. after 7 h postinfection). Conversely, these results were inconsistent with PD146626 being a generalized transcription inhibitor, since this should have negatively impacted on early and late gene expression during the 17 h in which the inhibitor was present during this experiment. The latter results were in fact observed when the time of addition experiments employed acyclovir, a known inhibitor of viral DNA replication and therefore late gene expression. In this case, delaying the addition of acyclovir to as late as 7 h postinfection had no effect on the antiviral activity of the compound (Fig. 5A).

3.3. PD146626 inhibits HSV-1 immediate early gene expression through a cellular rather than viral target

The results described above demonstrated that PD146626 mediated a specific downregulation of HSV-1 immediate early gene expression, but the mechanism by which this inhibition was effected remained unclear. Studies with mutant HSV-1 have revealed that there are only two viral proteins known to be required for efficient immediate early gene expression, VP16 and ICP0 (Smiley and Duncan, 1997; Stow and Stow, 1986). Viruses encoding transactivation-defective versions of either of these proteins possess phenotypes similar to each other and to wild type HSV-1 replicating in PD146626-treated cells, exhibiting significant inhibition of immediate early gene expression and reductions in viral yields of up to two orders of magnitude in low multiplicity infections. With respect to the latter property, it is interesting to note that while PD146626 was incapable of inhibiting virus yield by more than an order of magnitude due to the equivalence of its EC_{90} and MNTC, more potent derivatives of PD146626 were identified that were able to match the two order of magnitude reduction seen with

these mutant viruses; moreover, these compounds were not able to exceed this level of inhibition, even at compound concentrations in significant excess (data not shown). Thus, VP16 and ICP0 represented two logical candidates for a viral-encoded molecular target of PD146626.

Conveniently, the transactivation functions of either of these proteins could be individually assayed in the absence of other viral polypeptides using transient expression assays employing co-transfection of the VP16 or ICP0 gene with a reporter gene under the control of an HSV-1 immediate early promoter. However, when the inhibitory effects of PD146626 were analyzed in these experiments, reductions in VP16- or ICP0-mediated transactivation were observed only at toxic concentrations of the compound (data not shown). While these results would seem to suggest that PD146626 fails to exert any specific effects on the function of either of these two proteins, it is important to note that the level of VP16 or ICP0 protein expressed in these transfection assays is likely to be orders of magnitude greater than that generated in a viral infection carried out at low multiplicity (Spatz et al., 1997). As demonstrated earlier, the antiviral activity of PD146626 disappears when the multiplicity of infection is raised above 0.1 (Fig. 2), a phenomenon which most likely results from overwhelming the compound-induced block in immediate early gene expression by the elevated levels of VP16 and/or ICP0 within the infected cell. Thus, the presence of significantly increased protein expression made it impossible to unequivocally dismiss VP16 or ICP0 as potential targets for PD146626 using assays carried out in transfected cells.

However, the availability of recombinant HSV-1 expressing transactivation-defective versions of either VP16 or ICP0 permitted the development of an alternate strategy for assessing the role of these two proteins in the mechanism of action of PD146626. The HSV-1 derivatives used in these experiments were V422, which expresses a VP16 protein lacking the carboxy-terminal acidic transcriptional activation domain but retaining the amino-terminal structural domains required for virion assembly (Smiley and Duncan, 1997), and dl1403, which expresses a severely truncated null mutant derivative of ICP0 (Stow and Stow, 1986). In low multiplicity infections in Vero cells, both of these viruses typically generated yields of progeny virus that were approximately two orders of magnitude lower than that of wild type HSV-1. While this represented a significant reduction in virus yield, enough progeny virus was still produced by these viruses to enable their utilization in yield reduction assays at the low multiplicity of infection required for the detection of antiviral activity in PD146626. Thus, V422 and dl1403 could be used to directly determine the roles of the VP16 and ICP0 proteins in the mechanism of action of PD146626. If either protein was specifically targeted by this compound, then their cognate mutant virus would be expected to be fully resistant to the antiviral activity of PD146626, in much the same way that thymidine kinase mutants of HSV-1 have lost all sensitivity to acyclovir (Coen, 1994; Field and Biron, 1994). When yield reduction assays were performed to compare the effects of EC_{90} concentrations of PD146626 (1 mM) and acyclovir (2.5 mM) on the replication of wild type HSV-1, V422, and dl1403, it was determined that all three viruses were completely sensitive to both compounds (Fig. 6). These results demonstrated that PD146626 was still able to inhibit HSV-1 replication in the absence of either VP16- or ICP0-mediated transactivation, so that neither of these proteins were directly targeted by this compound. Thus, PD146626 most likely acts to antagonize a host cell pathway of HSV-1 immediate early expression, since these experiments served to eliminate the two known viral pathways of immediate early promoter activation. The hypothesis that PD146626 acted through a cellular mechanism of action was further supported by the failure to select for resistant mutants which could overcome the compound-induced block in immediate early gene expression following exhaustive serial passaging of virus in the presence of EC_{90} concentrations of inhibitor (data not shown).

3.4. Characterization of the antiviral state induced in the host cell by PD146626

The results of the experiments described above indicated that the mechanism of action of PD146626 involved the induction of one or more alterations in the host cell which resulted in the suppression of HSV-1 immediate early gene expression. Additional experiments were therefore carried out to further characterize the kinetics with which these cellular events took place. One kinetic study, the time of addition experiments described earlier, had already clearly demonstrated that PD146626 failed to inhibit virus replication when added after immediate early times. The simplest interpretation of these results would be that PD146626 still inhibited immediate early gene expression when added at 7 h postinfection but that this had no effect on virus replication by 24 h postinfection, as threshold levels of immediate early proteins had already been synthesized by the time the inhibitor was added. In order to directly test this hypothesis, the levels of immediate early proteins within time of addition experiments were determined by Western blot analysis. When an EC_{90} concentration of PD146626 (1 μM) was added at the time of infection and was present for the full 24 h of the experiment, accumulation of immediate early proteins such as ICP0 was significantly inhibited at 24 h postinfection (Fig. 5B). This was entirely consistent with western blot analyses carried out at earlier times in infection (Fig. 3). Remarkably, when PD146626 was added at 7 h postinfection and was present for the remaining 17 h of the experiment, accumulation of ICP0 was essentially unchanged at 24 h postinfection (Fig. 5B). These findings demonstrated that a host cell which had been infected for 7 h was surprisingly no longer capable of responding to PD146626 to prevent the expression of viral immediate early genes.

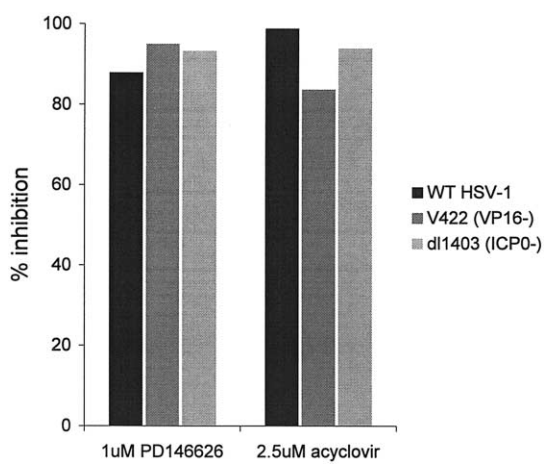

Fig. 6. Sensitivity of mutant HSV-1 expressing transactivation-defective versions of VP16 and ICP0 to PD146626. HSV-1 yield reduction assays were performed at a multiplicity of infection of 0.01 using the indicated concentrations of either PD146626 or acyclovir and the indicated viruses. Percent inhibition represented the reduction in virus yield observed in infections containing compound compared to infections containing DMSO as a solvent control. The bars in the histograms correspond to the means of duplicate experiments.

To explore this phenomenon further, the time of addition experiment was repeated using d120, a recombinant HSV-1 containing deletions of both copies of its ICP4 genes (DeLuca et al., 1985). Since it is an ICP4 null mutant, d120 is completely defective in the expression of all early and late viral genes and should express only immediate early proteins during the 24 h infection used in this experiment. As with wild type HSV-1, addition of PD146626 for the full 24 h of infection resulted in significant inhibition of ICP0 expression in d120-infected cells (Fig. 5B). However, unlike wild type HSV-1, this inhibition remained when PD146626 was added at 7 h postinfection and was present for the remainder of the infection (Fig. 5B). Thus, there was a fundamental difference in the ability of host cells to respond to PD146626 in the presence of infection by either of these two viruses. The simplest explanation of all of these results is that there likely exists a defined window at the onset of infection for PD146626 to induce an antiviral state within the host cell. However, by 7 h postinfection certain viral early proteins are synthesized (or certain cellular proteins are lost) during infection by wild type HSV-1 but not d120 which reverse the potential to create this state if the compound had not already been added by this time.

In order to investigate the length of PD146626 exposure required for induction of this antiviral state, time of removal experiments were carried

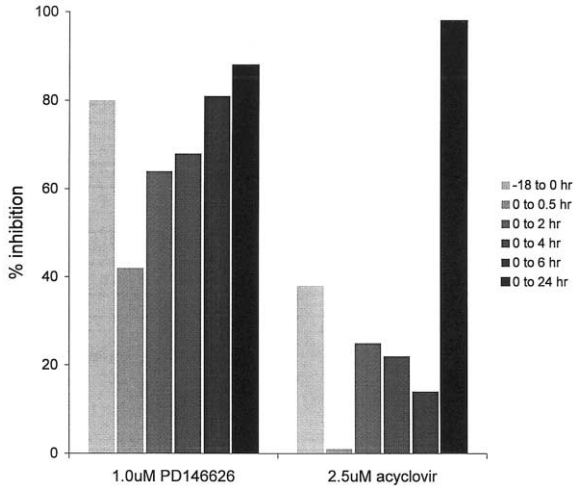

Fig. 7. Effect of varying the time of removal on the antiviral activity of PD146626. HSV-1 yield reduction assays were performed at a multiplicity of infection of 0.01 using the indicated concentrations of either PD146626 or acyclovir; however, the compounds were only present in the infections during the time intervals shown. Percent inhibition represented the reduction in virus yield observed in infections containing compound compared to infections containing DMSO as a solvent control. The bars in the histograms correspond to the means of duplicate experiments.

out. In these analyses, yield reduction assays were performed in which identical sets of cells were infected and washed to remove unbound virus, then exposed to EC_{90} concentrations of inhibitor compounds which were subsequently removed with further washing after progressively increasing lengths of time of infection. Virus yields for each experiment were then determined at 24 h postinfection. As expected, PD146626 inhibited virus yields by 90% when present for the full 24 h of the experiment (Fig. 7). However, exposures to PD146626 for much shorter lengths of time also yielded significant levels of antiviral activity: the presence of compound during only the first 6 h of infection was nearly as effective as the full 24 h, while just 120 and 30 min exposures of PD146626 at the onset of infection resulted in 64% and 42% declines in virus yield at 24 h, respectively (Fig. 7). Perhaps most remarkable of all was the finding that an 18 h preexposure of cells to an EC_{90} dose of PD146626 resulted in nearly normal levels of inhibition of virus replication (Fig. 7), so that the compound did not even need to be present during the infection provided that it had already induced an antiviral state within the cells. In contrast, acyclovir required a full 24 h exposure of compound during infection to exhibit its maximum antiviral activity, as lesser exposures were found to reduce virus yield by less than 30% in this same series of experiments (Fig. 7). Thus, while the opportunity for PD146626 to induce an antiviral state may be limited to the onset of infection, it nevertheless required only a brief exposure of compound to achieve this effect, and once the inhibitory activity had been induced it appeared to be long-lasting.

4. Discussion

The benzothiophene PD146626 was found in this study to act as a potent and specific inhibitor of HSV-1 immediate early gene expression. The principal lines of evidence to support this conclusion were: (a) PD146626 mediated a significant reduction in immediate early protein synthesis, resulting in a corresponding decrease in early and late gene expression and infectious virus production by late times in the virus life cycle (Fig. 3 and Table 1); (b) PD146626 exhibited no effect on virus replication when added past the immediate early stage (i.e. at 7 h postinfection or later), consistent with a specific inhibition of immediate early but not early or late gene expression (Fig. 5); and (c) the antiviral activity of PD146626 was overcome by increasing the multiplicity of infection, which acts to increase the available levels of VP16 and ICP0 transactivators that are necessary for efficient immediate early gene expression at the onset of infection (Fig. 2). However, despite this last piece of evidence, a viral protein involved in immediate early transactivation did not appear to represent the molecular target for PD146626. The experimental results which support this conclusion were: (a) mutant viruses encoding transactivation-defective versions of potential PD146626 targets such as VP16 or ICP0 failed to exhibit any detectable resistance to the compound (Fig. 6); (b) human cytomegalovirus, a herpesvirus which lacks sequence homologs of both the VP16 and

ICP0 proteins, possessed a sensitivity to PD146626 that was indistinguishable from that of HSV-1 (Table 1); and (c) no resistant mutants which could overcome the compound-induced block in immediate early gene expression were selected for following exhaustive serial passaging of virus in the presence of EC_{90} concentrations of inhibitor (data not shown).

The absence of a demonstrable viral target protein indicated that the mechanism of action of PD146626 is likely exerted at the level of the host cell. To this end, it is important to note that at least three lines of experimental evidence ruled out indirect inhibition of virus replication through nonspecific host cell cytotoxicity as a possible mechanism of action. First, PD146626 was subjected to the most stringent cytotoxicity tests available, including a 5 day cellular proliferation assay, and was found to inhibit virus replication by up to 90% at concentrations that failed to promote any detectable adverse events within cells (Table 1). Second, expression profiling experiments using DNA chip technology revealed that less than a dozen out of 6800 HeLa cell genes exhibited alterations in expression 3-fold or greater after overnight treatment with 1 μM PD146626 (data not shown), corroborating the excellent cytotoxicity profile of this compound. Finally, during the course of structure–activity relationship analysis of the benzothiophene series, a number of derivatives of PD146626 were identified which exhibited significant reductions in EC_{50}s but not TC_{50}s. Perhaps the most remarkable of these was a compound which possessed an EC_{50} of only 0.0002 μM, a full three orders of magnitude lower than that of PD146626, while retaining the parental compound's MNTC of 1 μM in a 5 day proliferation assay (data not shown). This dramatic improvement in activity without any increase in detectable toxicity demonstrated that a single chemical modification introduced into the PD146626 structure was capable of significantly enhancing the selectivity of its antiviral activity, and presumably its affinity for its molecular target, with little if any negative effect on the host cell.

These findings indicate that PD146626 promotes a highly specific alteration to the host cell which results in the selective inhibition of viral gene expression with very little impact on host cell gene expression or proliferation. There are a number of possible mechanisms which could generate such an effect. For example, PD146626 may act to only partially inhibit the cellular target in question; this would then be of little consequence to the host cell, but could be catastrophic to HSV-1 if full activity of the target was required for immediate early gene expression. Alternatively, the target may be one of several proteins that share redundant functions, so that the host cell could compensate for its inhibition through the action of the other proteins; however, the expression of HSV-1 immediate early genes may have evolved in such a way that it cannot substitute the inactivated target with a replacement protein. Nevertheless, these are most likely to be overly simplified explanations of the mechanism of action of PD146626 in light of several observations that were made during the course of this study. For example, kinetic experiments revealed that a window exists within the first few hours of infection during which PD146626 must be present in order to induce an antiviral effect. After this time it seems probable that certain viral early proteins are synthesized or cellular proteins are lost which abrogate the potential to create this state if the compound had not already been added by this time (Fig. 5B). While these properties would seem to limit the utility of PD146626 as an antiviral agent, it should be noted that other experiments demonstrated that cells required only transient exposures to PD146626 to achieve a state that was inhibitory to HSV-1 infection for extended periods of time (Fig. 7). Thus, the putative cellular protein targeted by PD146626 possesses several unique properties, including accessibility only at the onset of infection and the ability to maintain an antiviral state long after the compound has been removed from the environment.

Although the actual target and mechanism of action of PD146626 was not identified in this study, a number of additional experiments were carried out which eliminated some obvious possibilities (data not shown). First, although ICP0 was ruled out as a direct target for PD146626

based on results obtained with the dl1403 mutant (Fig. 6), this transactivator interacts with several host cell proteins which are themselves likely to be involved in the process of creating a host cell state conducive to HSV-1 immediate early gene expression (Everett, 1999). However, PD146626 was shown to have no effect on the stability or organization of ND10, whose ICP0-induced and proteasome-dependent disruption is an important event early in the viral life cycle. Consistent with these results, PD146626 did not act as a proteasome inhibitor, did not affect the state of SUMO modification of the PML protein in ND10, and did not alter the concentration or localization of the HAUSP (herpesvirus-associated ubiquitin-specific protease) protein in cells. Second, although the antiviral state brought on by PD146626 was reminiscent of that induced by interferons, comparative studies demonstrated that this compound was found to be far more potent than these cytokines, lacked the elevated cytotoxicity and antiproliferative properties of interferons, and failed to induce PKR/p68 dsRNA-activated protein kinase in cells following exposure. Third, PD146626 did not appear to modify the host cell transcription machinery itself, insofar as it failed to alter the phosphorylation state of RNA polymerase II, which is known to change during HSV-1 infection. In addition, the mechanism of action of PD146626 did not appear to involve transcriptional silencing of input viral genomes by histone deacetylase, inasmuch as treatment of infected cells with the histone deacetylase inhibitor trichostatin A did not reverse the effects of PD146626. Finally, the possibility that PD146626 mediates its antiviral effect by inducing an apoptotic state in infected but not uninfected cells was explored, but no evidence to support this hypothesis was obtained. Together, the results of all of these experiments indicate that PD146626 possesses a mechanism of action that cannot be determined in the context of the current knowledge of HSV-1 immediate early gene expression, and is likely to target a novel cellular function required for this process. Although the PD146626 series is no longer being pursued for antiviral development due to significant pharmacokinetic problems associated with this class (data not shown), continued studies with this compound should shed further light on the complex mechanisms by which HSV-1 initiates its transcriptional program in newly infected cells.

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References

Albin, R., Chase, R., Risano, C., Lieberman, M., Ferrari, E., Skelton, A., Buontempo, P., Cox, S., DeMartino, J., WrightMinogue, J., JirauLucca, G., Kelly, J., Afonso, A., Kwong, A.D., Rozhon, E.J., O'Connell, J.F., 1997. SCH 43478 and analogs: in vitro activity and in vivo efficacy of novel agents for herpesvirus type 2. Antiviral Res. 35, 139–146.

Boschelli, D.H., Kramer, J.B., Khatana, S.S., Sorenson, R.J., Connor, D.T., Ferin, M.A., Wright, C.D., Lesch, M.E., Imre, K., Okonkwo, G.C., et al., 1995. Inhibition of E-selectin-, ICAM-1-, and VCAM-1-mediated cell adhesion by benzo[b]thiophene-, benzofuran-, indole-, and naphthalene-2-carboxamides: identification of PD 144795 as an antiinflammatory agent. J. Med. Chem. 38, 4597–4614.

Butera, S.T., Roberts, B.D., Critchfield, J.W., Fang, G., McQuade, T., Gracheck, S.J., Folks, T.M., 1995. Compounds that target novel cellular components involved in HIV-1 transcription. Mol. Med. 1, 758–767.

Clements, G.B., Stow, N.D., 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. J. Gen. Virol. 70, 2501–2506.

Coen, D.M., 1994. Acyclovir-resistant, pathogenic herpesviruses. Trends Microbiol. 2, 481–485.

Connor D.T., Gracheck S.J., Weber P.C., 1997. Method for inhibiting and controlling viral growth. US Patent 5,703,069

DeLuca, N.A., McCarthy, A.M., Schaffer, P.A., 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate early protein ICP4. J. Virol. 56, 558–570.

Everett, R.D., 1999. A surprising role for the proteasome in the regulation of herpesvirus infection. Trends Biochem. Sci. 24, 293–295.

Everett, R.D., Cross, A., Orr, A., 1993. A truncated form of herpes simplex virus type-1 immediate-early protein Vmw110 is expressed in a cell type dependent manner. Virology 197, 751–756.

Field, A.K., Biron, K.K., 1994. The end of innocence' revisited: resistance of herpesviruses to antiviral drugs. Clin. Microbiol. Rev. 7, 1–13.

Fink, D.J., Sternberg, L.R., Weber, P.C., Mata, M., Goins, W.F., Glorioso, J.C., 1992. In vivo expression of beta-galactosidase in hippocampal neurons by HSV-mediated gene transfer. Hum. Gene Ther. 3, 11–19.

Gelman, I.H., Silverstein, S., 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA 82, 5265–5269.

Hendricks, R.L., Weber, P.C., Taylor, J.L., Koumbis, A., Tumpey, T.M., Glorioso, J.C., 1991. Endogenously produced interferon alpha protects mice from herpes simplex virus type 1 corneal disease. J. Gen. Virol. 72, 1601–1610.

Leib, D.A., Coen, D.M., Bogard, C.L., Hicks, K.A., Yager, D.R., Knipe, D.M., Tyler, K.L., Schaffer, P.A., 1989. Immediate early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. 63, 759–768.

Preston, C.M., 2000. Repression of viral transcription during herpes simplex virus latency. J. Gen. Virol. 81, 1–19.

Roizman, B., 1996. The function of herpes simplex virus genes: a primer for genetic engineering of novel vectors. Proc. Natl. Acad. Sci. USA 93, 11307–11312.

Smiley, J.R., Duncan, J., 1997. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. J. Virol. 71, 6191–6193.

Spaete, R.R., Mocarski, E.S., 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA 84, 7213–7217.

Spatz, S.J., Nordby, E.C., Weber, P.C., 1996. Mutational analysis of ICP0R, a transrepressor protein created by alternative splicing of the ICP0 gene of herpes simplex virus type 1. J. Virol. 70, 7360–7370.

Spatz, S.J., Nordby, E.C., Weber, P.C., 1997. Construction and characterization of a recombinant herpes simplex virus type 1 which overexpresses the transrepressor protein ICP0R. Virology 228, 218–228.

Stow, N.D., Stow, E.C., 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw 110. J. Gen. Virol. 67, 2571–2585.