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The antiviral xanthate compound D609 inhibits herpes simplex virus type 1 replication and protein phosphorylation

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Abstract

The mechanism of antiviral action of tricyclodecan-9-yl-xanthogenate (D609) was investigated in vitro. D609 inhibited herpes simplex virus type 1 (HSV-1) replication without apparent cytotoxicity. It reduced phosphorylation of virus-infected cell polypeptides and inhibited the HSV-1 encoded protein kinase (US3 PK) and, to a lesser extent, cellular protein kinase C in vitro. Virus production was reduced by D609 at concentrations greater than 3.8 μM, with complete inhibition at 75.2 μM at an MOI of 1 PFU/cell or less. Addition of D609 could be delayed until 7 h post-infection and still inhibit virus replication. Phosphorylation of infected cell viral polypeptides of 34 (similar molecular weight to the substrate of the viral US3 protein kinase) and 69 kDa was inhibited at 18.4 μM. Treatment of infected or uninfected cells with 37.6 μM D609 reduced protein phosphorylation to background levels. A concentration of 1.9 μM D609 in vitro inhibited the viral US3-encoded PK, which had been purified from infected cell lysates by affinity chromatography and identified by specific antibody. Purified cellular protein kinase C was inhibited at 75.2 μM D609 whereas other cellular kinases including casein kinase 1 and cAMP dependent kinase were not inhibited at concentrations as high as 188 μM D609. Collectively these data indicate that the mechanism of antiviral action of D609 is by inhibition of protein kinases and protein phosphorylation affecting a late step in HSV replication. © 1997 Elsevier Science B.V.

Keywords: HSV-1; Protein kinase; US3 PK; Antiviral drug; D609; Protein phosphorylation

1. Introduction

The antiviral xanthate compound D609 (tricyclodecan-9-yl-xanthogenate) inhibits a wide vari-

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ety of enveloped and non-enveloped DNA and RNA viruses without inhibiting the growth of the cells (Sauer et al., 1984). In addition to its antiviral activity, D609 also inhibits the growth of certain tumors in animal models without toxicity (Amtmann et al., 1985; Amtmann and Sauer, 1987; Fürstenberger et al., 1989). Such a broad spectrum of activity suggests that D609 inhibits a fundamental metabolic process required for virus replication and tumor growth. The work of Müller-Decker (1989) showed that phospholipid turnover was inhibited by D609 suggesting that phospholipase C activity may be inhibited by D609.

Protein phosphorylation was suggested as a target for D609 by the demonstration that phosphorylation, but not synthesis, of the regulatory protein NS of vesicular stomatitis virus (VSV) was inhibited following treatment of VSV-infected cells with D609 (Müller-Decker et al., 1987). D609 treatment also inhibits the phosphorylation of the P protein of respiratory syncytial virus (Villanueva et al., 1991).

HSV proteins are phosphorylated by viral and cellular protein kinases and protein phosphorylation appears to be important for virus replication (Wilcox et al., 1980). HSV-1 encodes two protein kinase activities, US3 PK and UL13 PK (Purves et al., 1987b, 1991; Smith and Smith, 1989). The US3 PK is not essential for viral growth in tissue culture but is important for growth in neurons (Fink et al., 1992; Glorioso et al., 1994) and primary cell lines (unpublished data).

The replication of herpes simplex virus type 1 (HSV-1) is inhibited by D609, but by an unknown mechanism (Sauer et al., 1984). Thus, the primary aim of this study was to determine whether D609 inhibits viral and cellular protein kinases (PKs) thereby identifying the mechanism of antiviral action. Proteins are synthesized and phosphorylated at several times during the replication of HSV-1 (Wilcox et al., 1980), thus an additional aim was to determine when the drug-sensitive step occurs during the replication cycle of HSV-1. A model for D609 activity is presented.

2. Materials and methods

2.0.1. Cells and viruses

Vero cells were obtained from the American Type Culture Collection and were maintained in Modified autoclavable minimum essential medium with Earle's salts (EMEM) from GIBCO supplemented with 10% newborn calf serum, 20 U/ml penicillin and 20 μg/ml streptomycin and 29.2 μg/ml L-glutamine. HSV type 1 strain F (which contains and expresses the US3 gene) and strain R7041 (a US3 deletion mutant which does not express the US3 gene) (Longnecker and Roizman, 1987) was obtained from Dr B. Roizman, University of Chicago; stocks were prepared in Vero cells. Virus was quantitated by plaque assay on Vero cells using an overlay of EMEM containing 0.5% methyl cellulose.

2.0.2. Chemicals

Tricyclodecan-9-yl-xanthogenate (D609), molecular weight of 266.46, was synthesized by Merz (Frankfurt, Germany) and is currently available from Calbiochem. Stock solutions of D609 were prepared fresh in distilled water, sterilized by filtration using 0.22 μm pore size filters and diluted 1:1000 in medium which had been adjusted to pH 6.8. Assay of the antiviral activity of D609 was performed at pH 6.8, as per Sauer et al. (1984).

2.1. Protein kinases

2.1.1. Cellular protein kinases

Protein kinase C and casein kinase I were obtained from Upstate Biotechnology. The catalytic subunit of cAMP-dependent PK was obtained from Sigma.

2.1.2. Extraction of total PKs from infected and uninfected cells

PKs were extracted from cells according to the procedure of Blue and Stobbs (1981). Mock-infected or virus-infected cells were harvested in phosphate-buffered saline (PBS) and pelleted by low-speed centrifugation at $1000 \times g$ for 10 min. The cells were resuspended in extraction buffer containing: 10 mM Tris–HCl pH 8.0, 1% Nonidet

P-40, 0.5 mM dithiothreitol, 0.2 M NaCl, 0.2 mM ethylene glycol-bis (β-aminoethylether)-*N*,*N*-tetraacetic acid (EGTA) and 10% glycerol. The cells were sonically disrupted and cellular debris was removed by centrifugation at 27 000 × *g*. The supernatants were dialyzed overnight against extraction buffer without EGTA and NaCl.

2.1.3. Purification of US3-PK

The PK encoded by the US3 gene of HSV-1 (US3-PK) was extracted and isolated from infected cells according to the procedure of Purves et al. (1987a). Briefly, the post-ribosomal supernatant obtained from cells infected with HSV-1 strain F for 18 h was applied to a DEAE-cellulose column and eluted with a linear gradient of 0–0.4 M KCl in buffer A (20 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 40 μg/ml PMSF and 10% glycerol). The fractions were collected and assayed for PK activity utilizing protamine sulfate as exogenous phosphate acceptor. The US3-PK which eluted at 0.2 M KCl was pooled and dialyzed versus buffer A. The US3-PK was applied to a threonine-Sepharose column preequilibrated in buffer A and eluted with a linear gradient of 0.4–1.0 M KCl in buffer A. A 10 μl aliquot of each fraction was spotted onto nitrocellulose and assayed for immunoreactivity to an antiserum generated against the C-terminal eight amino acids of the predicted US3 protein sequence as per Frame et al. (1987). The PK activity eluting at approximately 0.7 M KCl and reacting with anti-US3-PK peptide serum was pooled and dialyzed versus 20 mM Tris–HCl pH 7.6 and 10% glycerol prior to in vitro testing in the presence of D609.

2.1.4. Assay of protein kinases

The standard assay mixture contained: 10 mM Tris–HCl pH 8.0, 0.5 mM DTT, 10 mM $MgCl_2$, 0.1% NP-40, 25 μg lysine-rich histones or other substrate (as indicated), and 3 mM [γ-^{32}P]ATP (2000–5000 cpm/pmol) in a final reaction volume of 100 μl. The protein kinase sample was added (25 μl) and the mixture was incubated at 37°C for 15 min.

PKC activity was measured using a PKC enzyme assay system (Amersham) which monitors the incorporation of ^{32}P into a peptide which is a specific substrate for PKC. Each reaction contained 25 ng purified protein per 25 μl reaction volume. The reaction was incubated at 25° for 15 min.

Casein kinase 1 activity was measured by incubating 25 ng of purified enzyme in 25 μl reaction mixture containing 25 mM MES, pH 6.5, 50 mM NaCl, 15 mM $MgCl_2$, 2 mg/ml autoclaved, partially dephosphorylated casein, 2 mM EGTA, and 100 μM [γ-^{32}P]ATP at 37°C for 20 min.

The catalytic domain of cAMP-dependent protein kinase was measured by incubating 25 ng of purified enzyme in 70 μl reaction mixture containing 50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 3 mM [γ-^{32}P]ATP (2000–5000 cpm/pmol), and 0.3 mg type IIA histone.

Following incubation, samples were applied to phosphocellulose filters and washed batchwise three times in 75 mM phosphoric acid, twice in absolute ethanol, air-dried and their radioactivity measured by liquid scintillation spectrometry.

2.1.5. Preparation of ^{32}P-labeled cell lysates

Vero cells grown in 6-well culture dishes were infected with 10 PFU of virus per cell in EMEM. After 5 h, the medium was replaced with EMEM adjusted to pH 6.8 and the indicated amount of D609 was added. After 10 h infection, cells were labeled with 100–200 μCi of $^{32}P_i$ (carrier-free; ICN Biomedicals). Cells were harvested at 24 h post-infection by rinsing once with PBS and lysed by sonication in PBS containing 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone, 0.1 mM tosylsulfonyl lysyl chloromethyl ketone, 1.0% (vol/vol) Nonidet P40 and 1.0% (wt/vol) sodium deoxycholate.

2.1.6. Polyacrylamide gel electrophoresis

Samples were prepared for SDS-PAGE by placing approximately 50 μg protein in sample buffer containing 1.0 M urea, 2% SDS, 3% 2-β-mercaptoethanol, 0.1 M Tris–HCl pH 6.8, 20% glycerol, and bromophenol blue and boiling for 5 min. The polypeptides were separated by electrophoresis in 7–15% linear gradient SDS-polyacrylamide gels utilizing 5% stacking gels (Laemmli, 1970). Electrophoresis was at 10–20 mA and was monitored

by the migration of bromophenol blue. Proteins were visualized by staining with Coomassie brilliant blue. Radiolabeled proteins were visualized in dried gels by exposure to Kodak XAR-5 film utilizing intensifying screens. Densitometric analysis of autoradiographs was performed using an LKB densitometer. The autoradiographic density was determined by weighing the traces of the peaks corresponding to the bands. Molecular weight estimations were based on the relative migrations of marker proteins (Bio-Rad) ranging in size from 14 to 200 kDa.

2.1.7. Protein determinations

Protein concentrations of infected cell lysates were determined using the Bio-Rad microassay (Bradford, 1976).

2.1.8. Viral DNA quantification

Confluent monolayers of Vero cells were infected with HSV-1 strain F at an MOI of 10 PFU/cell. The cells were left untreated or treated with 10 μg/ml D609 at 0 h post-infection or 6 h post-infection. Beginning at 7 h post-infection the viral DNA was isolated from the infected cells. Cells rinsed with PBS were extracted with 100 μl of extraction buffer containing 100 mM NaCl, 10 mM Tris–HCl, pH 8, and 0.5% SDS. The DNA was phenol extracted according to standard procedures (Maniatis et al., 1982) and spotted onto 0.45 μm nitrocellulose membrane using the DOT-Blot Apparatus (BioRad, Hercules, CA). The pBTK145 plasmid which contains DNA specific for the BamHI Q fragment of the HSV genome was isolated from transformed *E. coli* (a gift from B. Jennings, NEOUCOM, Rootstown, OH) (Docherty et al., 1991) using the Qiagen Plasmid isolation kit (Qiagen, Chatsworth, CA). The viral insert was isolated and purified using the Qiaex Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The probe was prepared by labeling the Bam HI Q fragment with α-^{32}P(dCTP) (ICN Biochemicals, Irvine, CA) using a commercially available Nick Translation Kit (United States Biochemical Corporation, Cleveland, OH). Hybridization was performed overnight at 42°C using the Stratagene Quik-Hyb Kit (La Jolla, CA). Radioactivity was detected by autoradiography following 2–8 h exposure periods. The autoradiogram was analyzed using an Acuscan scanner and Photo-Look Program; the image was generated with the Photoshop and LDraw-7 programs.

3. Results

3.0.1. D609 inhibits HSV-1 production

Vero cells infected at different multiplicities of infection (MOI) were treated with several concentrations of D609 at levels well below toxicity (Rosenthal et al., 1987; Sauer et al., 1984). The amount of virus produced after 24 h was determined by plaque assay on Vero cells.

The extent of anti-herpetic activity of D609 was dependent upon the MOI of virus (Table 1). At an MOI of 1 PFU/cell or less, concentrations of D609 as low as 3.8 μM reduced virus production by 50% and 75.2 μM D609 completely inhibited virus production. Total inhibition represented a 10^5 reduction in virus titer. At an MOI of 10 PFU/cell, virus production was reduced by 50% at 18.4 μM D609. Maximum inhibition at an MOI of 10 PFU/cell, representing a 10^5 reduction in virus production, was observed at 150.1 μM of D609. We observed no significant reduction in the number or the viability of Vero cells incubated for 24 h in the presence of 75.2 μM D609 in either the confluent or semi-confluent state.

The time of addition of D609 was varied to determine when the drug-sensitive step occurs during virus replication. HSV-1 replication is temporally regulated in three phases referred to as the

Table 1
D609 inhibits HSV-1 replication

MOI	IC_{50} (μM)
0.01	1.9
0.1	3.8
1.0	3.8
10.0	18.4

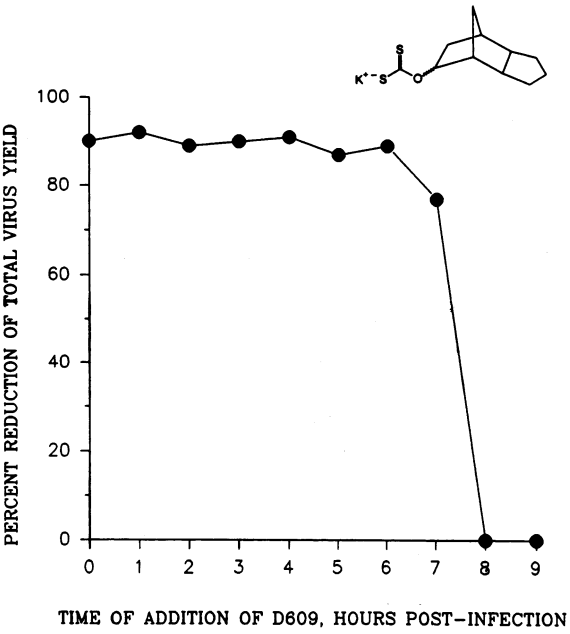

Fig. 1. D609 inhibits virus production when added during the initial 7 h post-infection. D609 was added at a final concentration of 37.6 μM at hourly intervals following infection of Vero cells with HSV-1 strain F at an MOI of 10 PFU/cell. The amount of virus produced after 24 h infection was determined by plaque assay on Vero cells. The percent reduction was calculated relative to the amount of virus produced in the absence of D609. The average of three experiments are shown.

immediate early, early and late phases. Newly synthesized DNA is detectable beginning at 3 h post-infection, during the early phase of gene expression, and extracellular virus appears after 12 h post-infection. Replicate monolayers of Vero cells were infected with HSV-1 strain F at an MOI of 10 PFU/cell and treated with 37.6 μM D609, final concentration, at hourly intervals following infection. An MOI of 10 PFU/cell was used to ensure complete infection of the monolayer. The infection was allowed to proceed for a total of 24 h at which time the cells and overlying medium were harvested and the amount of virus produced was determined by plaque assay (Fig. 1). D609 inhibited virus production when added at any time during, but not after, the first seven hours of infection. These results suggest that the target of D609 antiviral activity occurs in the late phase of viral replication.

3.0.2. DNA synthesis is not affected by D609 treatment

In order to determine whether D609 treatment affected viral DNA synthesis, we isolated viral DNA from infected cells that were untreated or treated at various times during infection with D609. The viral DNA was spotted onto nitrocellulose and reacted with a radiolabeled probe specific for the BamH1 Q fragment of the HSV-1 genome. These results shown in Fig. 2 demonstrate that infected cells treated with D609 at 6 h post-infection synthesize viral DNA at levels comparable to untreated, infected cells even though previous experiments demonstrated that virus production is reduced by 80–90% when D609 is added at 6 h post-infection. Viral DNA synthesis is slightly less in cells treated with D609 at the time of infection compared to untreated, infected cells but not enough to explain the 80–90% reduction in virus production. Collectively, these data suggest that D609 antiviral activity occurs late in viral replication, following DNA replication.

3.0.3. D609 inhibits protein phosphorylation

Phosphorylation of viral proteins was suggested as a target for D609 (Müller-Decker et al., 1987). Although protein phosphorylation occurs throughout HSV replication (Wilcox et al., 1980),

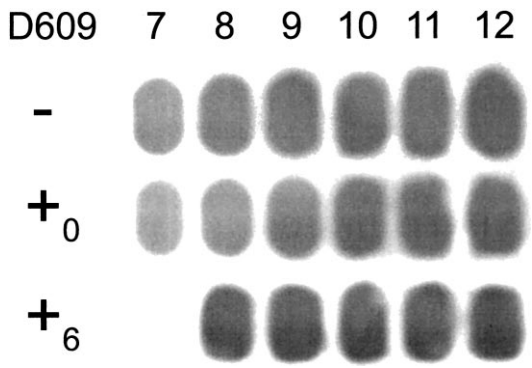

Fig. 2. Comparison of HSV DNA production in D609-treated and untreated cells. Replicate monolayers of Vero cells were infected with HSV-1 strain F at an MOI of 10 PFU/cell in the absence (−) or presence (+) of 37.6 μM D609. D609 was added at 0 h post-infection ($+_0$) or 6 h post-infection ($+_6$). At hourly intervals beginning at 7 h post-infection, the viral DNA was extracted from the cells and detected by Southern hybridization as described in the Methods.

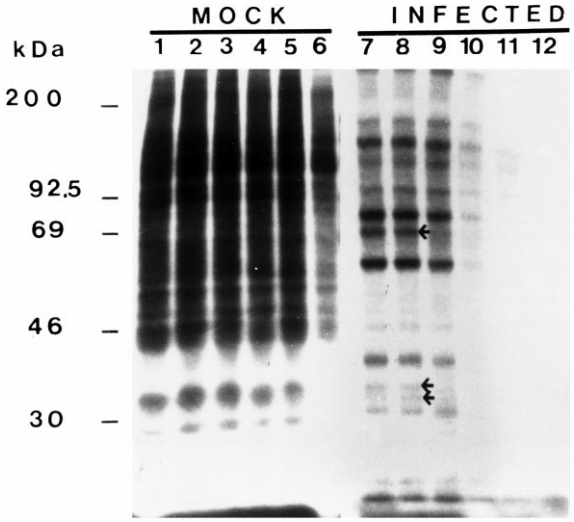

Fig. 3. D609 inhibits phosphorylation of polypeptides in virus-infected cells. Vero cells were infected with HSV-1 strain F at an MOI of 10 PFU/cell. D609 was added at 5 h post-infection at the following concentrations (Lanes 1 and 7, 0; Lanes 2 and 8, 3.8; Lanes 3 and 9, 18.4; Lanes 4 and 10, 37.6; Lanes 5 and 11, 75.2; Lanes 6 and 12, 188 μM D609). $^{32}PO_4$ was added at 10 h post-infection. Cells were harvested at 24 h post-infection. Phosphorylated proteins in cell lysates (50 μg) were analyzed by SDS-PAGE on a 7.5–15% gel and visualized by autoradiography. The migrations of molecular weight markers are indicated. Arrows identify polypeptides of 32, 34 and 69 kDa which were selectively reduced in their phosphorylation by D609 treatment.

D609 inhibits virus replication when added late in infection; thus the effect of D609 on protein phosphorylation was investigated late in HSV replication. Vero cells were mock-infected or infected with HSV-1 strain F at an MOI of 10 PFU/cell. D609 was added at 5 h post infection at several concentrations from 0 to 188 μM and $^{32}PO_4$ was added 5 h later. Under these conditions, virus replication is initiated resulting in the shutdown of host cell protein synthesis (Fenwick and Walker, 1978). The cells were harvested at 24 h post-infection and the phosphorylated polypeptides were examined by SDS-PAGE and autoradiography (Fig. 3).

Several polypeptides were phosphorylated in untreated infected cells (lane 7). These phosphorylated polypeptides were not present in uninfected control cells (lanes 1 through 6). The phosphorylation of polypeptides of 32, 34, and 69 kDa was noticeably inhibited at 18.4 μM D609 (lane 9). Protein phosphorylation was reduced to background levels in infected cells treated with D609 concentrations equal to or greater than 37.6 μM (lanes 10, 11, and 12).

The effect of D609 on the phosphorylation patterns of viral proteins was analyzed further by comparing the phosphoprotein species in cell lysates prepared from cells infected with HSV-1 strain F (US3$^+$ PK) and strain R7041, a mutant which lacks the US3 PK gene (US3$^-$ PK) (Fig. 4). The phosphorylation of polypeptides at 69 and 34 kDa was inhibited by D609 in cells infected with HSV-1 strain F. In contrast, the phosphorylation of higher molecular weight polypeptides was not affected by D609 treatment of cells infected with either virus. The 34 kDa phosphopeptide has an electrophoretic mobility similar to the substrate for US3, as described by Purves et al. (1991). The 34 kDa is also phosphorylated by cellular kinases at sites other than for US3 (Purves et al., 1991). The ratio of autoradio-

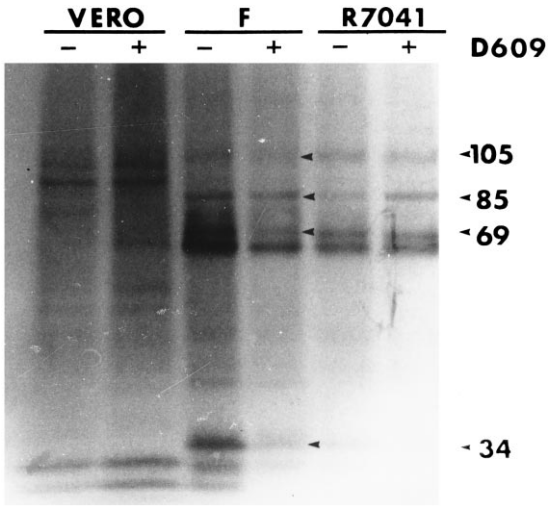

Fig. 4. Analysis of phosphoprotein species in D609-treated Vero cells infected with US3$^+$ PK (strain F) or US3$^-$ PK (strain R7041) of HSV-1. Replicate monolayers of Vero cells were mock-infected (Vero) or infected at an MOI of 10 PFU/cell with strain F or R7041. Cells were left untreated (–) or treated with 37.6 μM D609 at 5 h post-infection (+) and $^{32}PO_4$ was added at 10 h post-infection. Cell lysates were prepared at 24 h post-infection and phosphoprotein analysis performed according to the procedures given in the Methods.

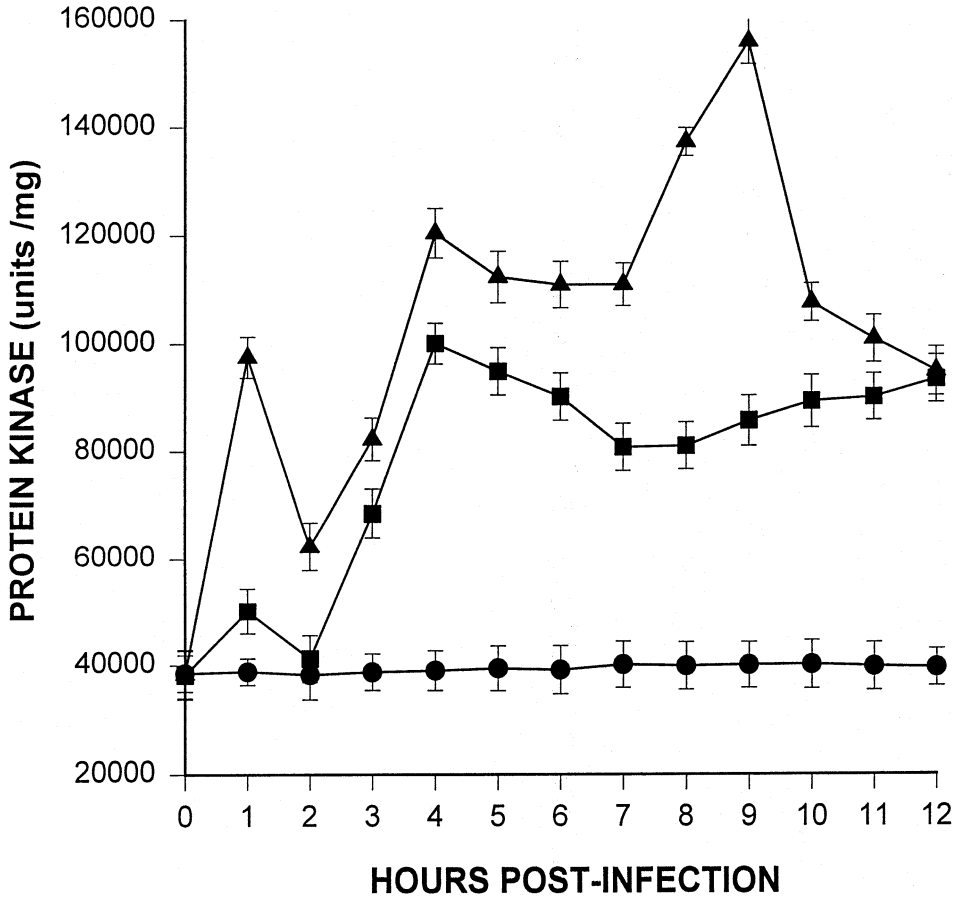

Fig. 5. D609 inhibits infected cell protein kinase activity. Replicate monolayers of Vero cells were mock-infected (●) or infected with HSV-1 strain F at an MOI of 10 PFU/cell in the absence (▲) or presence (■) of 28.6 μM D609. Cells were harvested and assayed for protein kinase activity as described in Methods. One unit of protein kinase specific activity is defined as the amount of enzyme which catalyzes the incorporation of 1 pmol of ^{32}P into trichloroacetic acid-insoluble product in 30 min at 37°C.

graphic density for the bands representing the 34 kDa peptide was 1.0:0.6:0.3:0.8 for the untreated US3$^+$ PK, treated US3$^+$ PK, untreated US3$^-$ PK and treated US3$^-$ PK lysates, respectively. The reduction in intensity upon D609 treatment may result from inhibition of phosphorylation of one or both of the sites of the 34 kDa protein due to one or more protein kinases (viral or cellular).

3.0.4. Effect of D609 treatment on protein kinase activity in infected cells

The level of protein kinase activity in extracts prepared from untreated infected, D609-treated infected, and untreated mock-infected cells were compared. Protein kinase activity was measured using lysine-rich histones as substrate, a substrate previously shown to be utilized by the viral US3 protein kinase (Katan et al., 1985). The level of protein kinase specific activity of mock-infected cells changed little during the 12 h of the experiment (Fig. 5). Untreated infected cells showed increases in protein kinase specific activity at 1, 4, and 9 h post-infection. The triphasic pattern of induction of protein kinase specific activity following HSV-1 infection was altered by treatment with 28.6 μM of D609. Less protein kinase activity was induced in D609-treated infected cells at 1

and 4 h post-infection than in untreated, infected cells. These results show that D609 reduces the enzyme activities of selected virus-encoded or virus-induced PKs during infection.

3.0.5. D609 inhibits the activity of selected protein kinases

The inhibitory activity of D609 toward several cellular PKs and the viral US3-PK was tested at concentrations ranging from 0.1 to 150 μM (Table 2). Concentrations of D609 of 1.9 μM reduced the purified US3 PK enzyme activity by 98% and concentrations of 3.8 and 7.6 μM reduced enzyme activity by greater than 98%. Concentrations greater than 18.4 μM reduced the US3 PK enzyme activity to background levels. PKC was inhibited by 30 and 80% in the presence of 75.2 and 150 μM D609, respectively. Cellular PKs, including casein kinase I and the catalytic domain of the cAMP-dependent PK, were not inhibited by D609 at these concentrations.

4. Discussion

The ability of D609 to inhibit the replication of RNA and DNA, enveloped and naked capsid viruses as well as certain tumors suggests a common target which is selectively important for their growth and replication. The inhibition of HSV-1 replication and protein phosphorylation with similar concentration dependence and the inhibitory activity of D609 on purified viral and cellular protein kinases indicates that protein phosphorylation is the target for D609 anti-HSV activity and that the target activity is important for HSV replication. Protein kinase activities are important for replication of many viruses including VSV and respiratory syncytial virus (Leader, 1993).

Table 2
Effect of D609 on PK activity

D609, μM	Enzyme[a]			
	US3 PK	PKC	CK1[b]	PKA[c]
0	1.48×10^6	1.57×10^6	1.17×10^6	1.61×10^6
0.1	1.78×10^6	ND[d]	ND	ND
1.9	2.89×10^3	ND	ND	ND
3.8	1.97×10^3	1.48×10^6	1.12×10^6	1.60×10^6
7.6	835	ND	ND	1.38×10^6
18.4	0	1.67×10^6	1.11×10^6	1.48×10^6
37.6	0	1.65×10^6	1.26×10^6	1.55×10^6
75.2	0	1.10×10^6	1.25×10^6	1.64×10^6
150.0	0	3.10×10^5	1.24×10^6	1.36×10^6

[a] The average of three experiments each done in duplicate are reported as cpm's above background.
[b] Casein kinase 1.
[c] The regulatory subunit of cAMP-dependent protein kinase.
[d] Not done.

Demonstration that D609 inhibits the immunoaffinity purified HSV-1 US3 encoded protein kinase defines the drug as a protein kinase inhibitor and also identifies this viral enzyme as a target. D609 must also have selective activity towards cellular protein kinases, based on its antitumor activity, its broad spectrum of antiviral activity and its ability to inhibit virus production and protein phosphorylation of the US3$^-$ PK mutant, R7041. The limited toxicity towards African green monkey kidney cells (Sauer et al., 1984; Rosenthal et al., 1987) and in animal models (Fürstenberger et al., 1989) indicates a selective activity of the drug towards a subset of protein kinases and potentially towards a limited number of substrates for phosphorylation. PKC is an example of a cellular enzyme which can be inhibited by D609 whereas casein kinase I and the catalytic domain of the cAMP-dependent PK are examples of enzymes which are not inhibited by D609. The selectivity of D609 is also suggested by its ability to inhibit phosphorylation of one site of the EGF receptor of human epidermoid A431 carcinoma cells without inhibiting the phosphorylation at a second site (Müller-Decker et al., 1988). The actual cellular protein kinase(s) which are inhibited by D609 are not known.

In order to inhibit virus replication, the target(s) for D609 inhibition must be important for HSV-1 replication but not for viability of the host cell. The US3 PK or its substrate is a likely candidate for the target of D609 inhibition. The natural substrate for the US3 PK is the 34 kDa protein and this was the primary target for D609 inhibition of protein phosphorylation (Fig. 4). Phosphorylation of specific sites on the 34 kDa protein is essential for virus production (Purves et

al., 1991). This protein was phosphorylated in Vero cells infected with virus encoding the US3 PK (F strain) and virus lacking the US3 PK (R7041 strain). This indicates that the 34 kDa protein is also phosphorylated by cellular kinases present in Vero cells (see Fig. 4) but not in all cells (Purves et al., 1991). D609 reduced the phosphorylation of the 34 kDa protein in cells infected by either of these viruses but the reduction in phosphorylation levels to background for the R7041 virus is further indication that D609 inhibits the viral and a subset of cellular protein kinases which have a very defined substrate specificity.

The time at which D609 manifests its activity and its effects on viral replicative events provide insight to its mechanism of action. D609 is inhibitory when added prior to the eighth hour post-infection, a period which includes most of the steps in viral replication. Examining the intracellular events more closely, D609 treatment prevented the induction of specific PK activities early and late in infection. Normally, protein kinase activity is induced in three phases during HSV-1 infection (Blue and Stobbs, 1981). Selective inhibition of the early and late phases of PK activity could result from D609 inhibiting cellular PKs in addition to the US3 PK. The cellular PKs may be expressed at different times in virus replication or the virus may activate or induce the synthesis of other PKs. Although inhibition of protein phosphorylation can have pleiotropic effects on the infected cell and affect more than one step in virus replication, the lack of inhibition of viral DNA synthesis indicates that the relevant D609 sensitive step occurs late in HSV replication.

The following model is suggested by these results: D609 inhibits the US3 PK and a related cellular protein kinase which are capable of activating the 34 kDa protein by phosphorylation of a specific sequence. The lack of appropriate phosphorylation blocks a late step in virus replication thereby inhibiting virus production. This is consistent with the time course of inhibition and the lack of inhibition of DNA synthesis.

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