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Antiviral properties of isoborneol, a potent inhibitor of herpes simplex virus type 1

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Abstract

Isoborneol, a monoterpene and a component of several plant essential oils, showed dual viricidal activity against herpes simplex virus 1 (HSV-1). First, it inactivated HSV-1 by almost 4 $\log_{10}$ values within 30 min of exposure, and second, isoborneol at a concentration of 0.06% completely inhibited viral replication, without affecting viral adsorption. Isoborneol did not exhibit significant cytotoxicity at concentrations ranging between 0.016% and 0.08% when tested against human and monkey cell lines. Isoborneol specifically inhibited glycosylation of viral polypeptides based on the following data: (1) the mature fully glycosylated forms of two viral glycoproteins gB and gD were not detected when the virus was replicated in the presence of isoborneol, (2) no major changes were observed in the glycosylation pattern of cellular polypeptides between untreated and isoborneol treated Vero cells, (3) isoborneol did not affect the glycosylation of gB produced from a copy of the gB gene resident in the cellular genome, and (4) other monoterpenes such as 1,8-cineole and borneol, a stereoisomer of isoborneol, did not inhibit HSV-1 glycosylation.

Keywords: Anti-herpes simplex virus agents; Antiviral agents; Borneol; Essential oil; Glycoproteins of HSV-1; Glycosylation; Glycosylation inhibitors; Herpes simplex virus 1; HSV-1; Isoborneol; Monoterpenes; *Salvia fruticosa*; Specific inhibitors of viral glycosylation; Transformed Vero cells

1. Introduction

Herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2) are common human pathogens that cause localised skin infections of the mucosal epithelia of the genitals, the oral cavity, the pharynx, the oesophagus and the eye, depending upon the type involved. However, HSV infections may also cause severe problems to infected individuals due to the following virus properties. (1) The virus establishes latent infections that can be periodically reactivated. (2) Under certain circumstances the virus can produce serious infections of the central nervous system including acute necrotising encephalitis and meningitis; the viruses may also produce fatal infections in patients with immune deficiencies (Whitley, 1990). (3) The immediate-

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early genes of HSV-1 can stimulate the activation of genes belonging to different viruses such as human immunodeficiency virus (Ostrove et al., 1987), varicella-zoster virus (Felser et al., 1988) or human papillomavirus type 18 (Gius and Laimins, 1989).

Consequently there is a need for effective antiviral therapy. Prospective antiviral drugs should exhibit inhibition specific to viral functions and be non-toxic to eukaryotic cells. The antiviral drugs used today are targeted against two viral enzymes, thymidine kinase and DNA polymerase, whose action can be differentiated from their cellular counterparts. A problem common to current antivirals is the emergence of resistant viral mutants which can easily be isolated in vivo and in vitro (Sasadeusz et al., 1997). Thus, the search for new antiviral agents emerges as an imperative need.

Recently interest in the biological activities of plant extracts has been rekindled and has been the subject of intense scientific investigation. Thus several plant extracts have shown antiviral activities against some RNA and DNA viruses (DeTommasi et al., 1990; Rusak et al., 1997; Serkedjieva and Hay, 1998), while recently we have described the strong neutralizing activity of *Salvia fruticosa* essential oil against HSV-1 (Sivropoulou et al., 1997). Among the components of *Salvia fruticosa* essential oil, isoborneol exhibits relatively low levels of cytotoxicity and was thus investigated in this study for its antiviral properties against HSV-1.

2. Materials and methods

2.1. Solutions and buffers

Disruption buffer consisted of 0.05 M Tris (pH 7.0), 8.5% (w/v) sucrose, 5% 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate (SDS) and was supplemented with 0.01 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; Sigma Chemical Co., St. Louis, MO) and 0.01 mM L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK; Sigma). Phosphate buffered saline (PBS) consisted of 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl and 2.5 mM KCl. Buffer A consisted of PBS supplemented with 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate and 10^{-5} M TPCK and TLCK.

2.2. Cell culture

The human cell lines Hep-2, HeLa and 143tk$^-$ as well as the monkey Vero cell line were grown in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 0.1% antibiotics (penicillin and streptomycin). Transformed cell lines V5 and V6 were derived from Vero cells and were grown in culture medium supplemented with 10 mM histidinol (histidinol; Sigma).

2.3. Cytotoxicity assay

Hep-2, HeLa, 143tk$^-$ and Vero cells were first synchronized by incubation for 48 h in DMEM supplemented with 0.5% FCS, and were then seeded into 48-well plates at a density of 4×10^4 cells/well in DMEM supplemented with 10% FCS. The appropriate concentration of isoborneol (Aldrich, Chemical Co., Milwaukee, WI) or ethanol, which served as control, was then added. The isoborneol stock was initially diluted 1:10 (v/v) in ethanol and further dilutions were made in DMEM. After the appropriate time of incubation in the presence of isoborneol (24, 48, or 72 h) 50 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; M2128, Sigma) was added. MTT was prepared as a 5 mg/ml stock solution in PBS and was filter sterilized to remove insoluble matter. The plates were incubated at 37°C for an additional 2.5 h, the growth medium was removed and the dark blue formazan crystals, formed only by metabolically active cells, were dissolved in 200 μl of acid–isopropanol (0.04 N HCl in isopropanol). To ensure that all crystals were dissolved the plates were incubated for an additional hour at 37°C with shaking at 120 rpm. Finally the optical density of each well was measured using an automatic plate reader (Bio-Tech, EL311SX) at a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance measured at 630 nm was automatically subtracted from the absorbance at 570 nm, so as to eliminate

the effects of non-specific absorption. Blanking was carried out directly on the plates with the first column wells, which contained all reagents except cells. The data represent the average values obtained from triplicate wells for each treatment, and the results were confirmed by three independent experiments.

2.4. Viruses and viral plaque assay

The properties of wild type herpes simplex virus 1 (HSV-1) strain F and of the R3678 recombinant, carrying a 500 bp deletion within the thymidine kinase (tk^-) gene have been previously described (Ejercito et al., 1968; Arsenakis and Roizman, 1990). Virus stocks were prepared in Vero cells and the infected cell lysates were sonicated and clarified of gross debris by low speed centrifugation before use. For titration, the viral samples were serially diluted in DMEM and aliquots of each dilution were adsorbed for 1 h at 37°C on Vero cell monolayers. For inhibition studies, the virus and the appropriate amount of isoborneol or ethanol which served as control, were adsorbed for 1 h on Vero cell monolayers. At the end of the adsorption period the virus inoculum was removed and the cells were replenished with fresh DMEM containing 1% (v/v) FCS and 0.1% (v/v) human immunoglobulins. After a 72-h incubation period, the monolayers were stained with crystal violet (0.5% w/v in 20% v/v ethanol) and the viral plaques were counted.

2.5. Virus inactivation

Tubes containing 2×10^9 PFU (plaque forming units) of HSV-1 strain F were incubated for 30 min at 37°C in DMEM supplemented with varying concentrations of isoborneol. Since the initial dilution of isoborneol was done in ethanol, an additional tube containing the virus and the appropriate amount of ethanol, was used as control. At the beginning and at the end of the incubation period 100 μl viral samples were removed from each tube. Tenfold serial dilutions of these samples from 10^{-4} to 10^{-8} were assayed for remaining infectivity on Vero cells by plaque assay. The effective concentration of isoborneol at these dilutions (10^{-4} to 10^{-8}%) was several orders of magnitude below the cytotoxic levels of > 0.08%.

2.6. HSV-1 growth curve

Vero cells seeded in 50-mm culture dishes were infected with HSV-1(F) at a multiplicity of infection (MOI) of 5 PFU per cell. After the 1 h adsorption period, cell monolayers were rinsed with PBS, were overlaid with DMEM containing 1% (v/v) FCS and either 0.03% or 0.06% (v/v) of isoborneol, and incubated at 37°C. Since the initial dilution of isoborneol was done in ethanol, an additional plate containing the appropriate amount of ethanol was included as control. At specific times post-infection 100 μl viral samples were removed from each treatment and were assayed for remaining infectivity on Vero cells by plaque assay.

2.7. Labeling infected cell polypeptides/Western blot

HSV-1(F) infected and mock infected cells were pulse labeled for 2 h with [^{35}S]methionine at 4, 6 or 8 h post-infection, in medium containing one-tenth the normal concentration of methionine and 25 μCi of [^{35}S]methionine per ml of medium (specific activity 1260 Ci/mmol; New England Nuclear Corp., Boston, MA). The infected cell polypeptides were either used for immunoprecipitation studies or were separated on 8.5% SDS–polyacrylamide gels (Laemmli, 1970) and transferred to a nitrocellulose sheet as previously described (Towbin et al., 1979). The labeled separated polypeptides were either directly visualized by autoradiography on Fuji X-ray film, or used for Western immunoblotting as previously described (Braun et al., 1983).

2.8. Immunoprecipitation

Infected cells were disrupted in buffer A and were then sonicated and clarified from particulate matter by centrifugation at $15{,}000 \times g$ for 1 h at 4°C. The supernatant fluids were mixed with 5 μl of monoclonal antibody HD-1 specific for the glycoprotein D (Pereira et al., 1980) or mono-

clonal antibody H 233 specific for the glycoprotein B (Kousoulas et al., 1984) of HSV-1, and allowed to react overnight at 4°C. The immune complexes were collected on protein A–Sepharose beads (Sigma), washed extensively with buffer A, and released from the beads by boiling for 5 min in disruption buffer. The released immune precipitates were separated on 8.5% SDS–polyacrylamide gels (Laemmli, 1970) and electrically transferred to a nitrocellulose sheet as previously described (Towbin et al., 1979). For autoradiography the nitrocellulose sheet containing the immobilized polypeptides was exposed for the appropriate time to Fuji X-ray film at −80°C.

2.9. Detection of glycoproteins on isoborneol treated Vero cells

Equal numbers of synchronized Vero cells seeded in 50-mm dishes were overlaid with growth medium supplemented with varying concentrations of isoborneol (0.016%, 0.025%, 0.03%, 0.05%, or 0.06% v/v) or with the appropriate amount of ethanol, which served as control, and incubated for 24 h at 37°C. The cell lysates were electrophoretically separated on an 8.5% SDS–polyacrylamide gel and electrically transferred to a nitrocellulose sheet. The detection of glycoproteins on the nitrocellulose sheet was done with the DIG Glycan Detection Kit (Boehringer Mannheim, Germany) after minor modifications. Briefly, the nitrocellulose sheet was incubated in 10 mM sodium metaperiodate dissolved in 0.1 M sodium acetate buffer (pH 5.5) for 30 min at 4°C in the dark. The membrane was then washed three times with PBS and incubated in 1 μM Dig-hydrazide in 0.1 M sodium acetate buffer for 1 h followed by three washes with PBS. The membrane was then washed three times (20 min each wash) with blocking buffer consisting of 3% bovine serum albumin, 1% Ficoll and 1% polyvinyl pyrolidone, in PBS. After the blocking step, the membrane was incubated for 1 h at room temperature with anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (750 U/ml) at 1:5000 dilution in blocking buffer. After washing the membrane three times with PBS containing 0.1% Tween 20, the glycoproteins were detected by incubating the filter in freshly prepared substrate solution consisting of 100 μl of NBT/X (4-nitroblue tetrazolium chloride prepared in phosphate solution) in 10 ml buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

2.10. Construction of plasmid pMA 8800

Plasmid pMA 8000 contains the *Bam*HI G fragment of HSV-1 strain S (a spontaneous syncytial mutant derived from the prototype HSV-1(F)) cloned into the *Bam*HI site of plasmid pUC18. The 3449 bp *Bam*HI/*Xho*I fragment of pMA 8000 containing the regulatory and coding regions of gB (Pellet et al., 1985) was cloned into the *Bam*HI/*Xho*I sites of pREP8 (Invitrogen Co, San Diego, CA) and the resulting plasmid was designated as pMA 8800.

2.11. Construction of gB expressing Vero cell lines

Vero cells were transfected with plasmid pMA 8800 and selected for resistance to histidinol (10 mM) as previously described (Arsenakis and Roizman, 1990). Cells resistant to histidinol were pooled, expanded and tested for constitutive expression of gB by Western blot with monoclonal antibody H336 specific for HSV-1 gB. Two of these cell lines designated as V5 and V6 were selected for further study.

3. Results

3.1. Effect of isoborneol on cell proliferation

Salvia fruticosa essential oil and its component isoborneol were previously shown to have strong virucidal activity against herpes simplex virus 1 (Sivropoulou et al., 1997). In these studies we report on the antiviral properties of isoborneol. The first issue to be addressed was to examine the possible cytotoxicity of this substance. For that purpose, four cell lines were used, three of human origin (Hep-2, HeLa and 143tk$^-$), since HSV-1 is a human pathogen, and one of monkey origin (Vero) included for comparison as it is commonly

used in HSV studies. Synchronized Vero, Hep-2, HeLa or 143tk$^-$ cells were exposed for 24, 48 or 72 h to various concentrations of isoborneol and the metabolically active cells were quantitated by the MTT assay. As shown in Fig. 1, isoborneol at concentrations up to 0.08% had trivial effects on Hep-2 and Vero cell proliferation, and exactly the same results were obtained with the other two cell

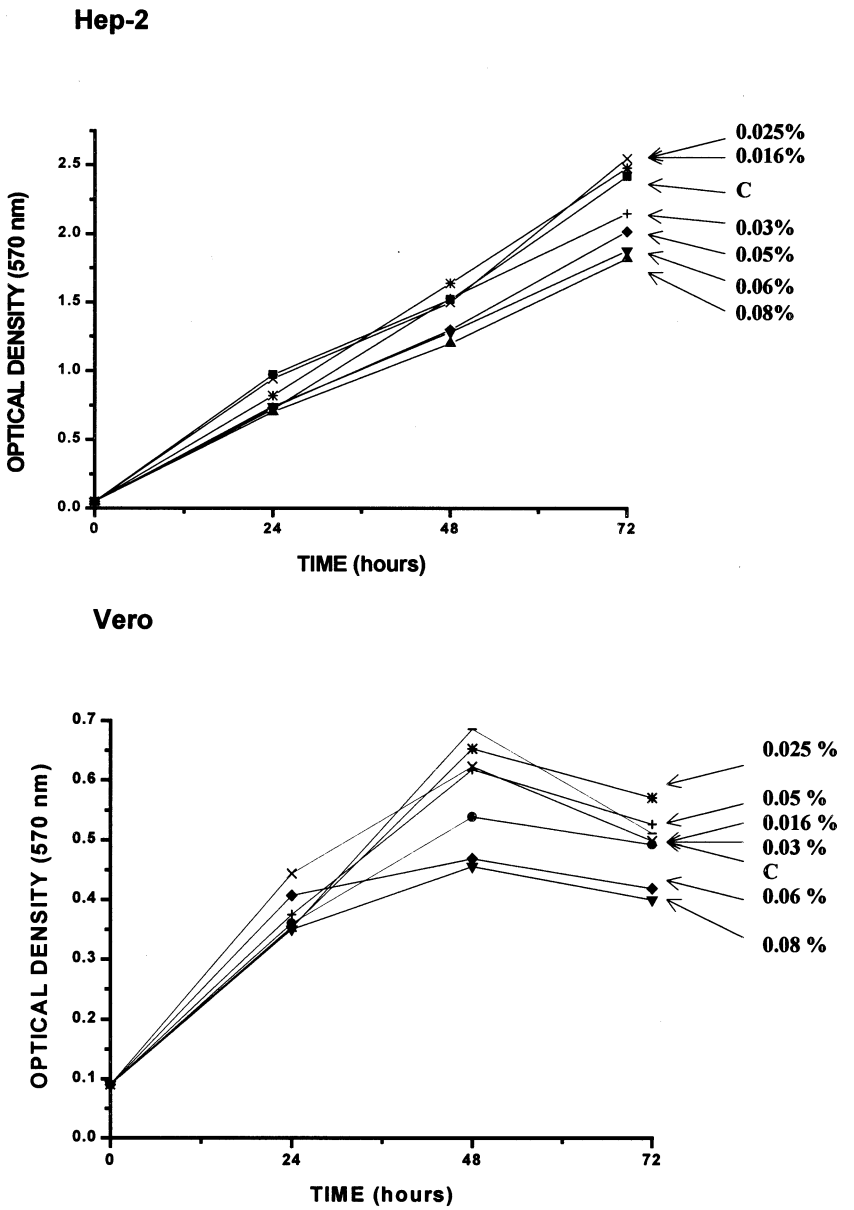

Fig. 1. Time- and concentration-dependent effect of isoborneol on the rate of Hep-2 and Vero cell growth. Equal numbers of synchronized Hep-2 or Vero cells were grown in the presence of varying concentrations of isoborneol (0.016%, 0.025%, 0.03%, 0.05%, 0.06% and 0.08%) and at the times indicated cell growth was evaluated by the MTT assay (see Section 2). For controls (C), cells were exposed to the appropriate concentration of ethanol alone. The data represent the average values obtained from triplicate wells for each treatment, and the results were confirmed by three independent experiments.

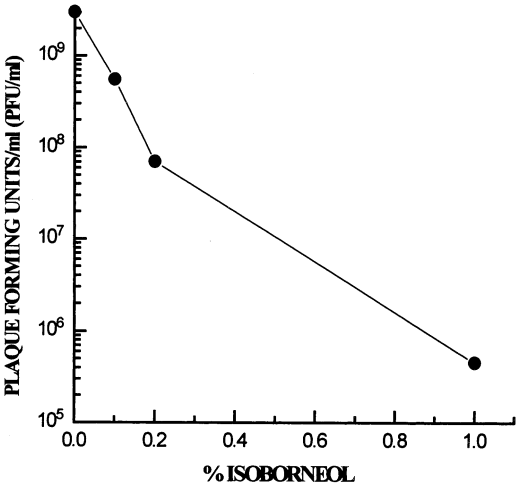

Fig. 2. Concentration-dependent effect of isoborneol on HSV-1 inactivation. A 1 ml aliquot of virus inoculum containing 2×10^9 PFU of HSV-1 strain F in DMEM was exposed for 30 min at 37°C to various concentrations of isoborneol or to the appropriate concentration of ethanol alone which served as control. At the beginning and at the end of the exposure period, 100 μl samples were drawn from each treatment and after suitable dilution (10^{-4} to 10^{-8}) were titrated on Vero cells for remaining infectivity. The data represent the average values obtained from triplicate wells for each treatment, and the results were confirmed by three independent experiments.

lines HeLa and 143tk$^-$ (data not shown). Thus, in all subsequent experiments Vero cells were used since virus propagation and titration is more efficient in this cell line.

3.2. Antiviral action of isoborneol

In previous studies isoborneol was shown to posses some viricidal activity against HSV-1. In order to extend those initial observations, HSV-1 strain F inoculum was exposed for 30 min to varying concentrations of isoborneol and the residual, in each case, infectivity was counted after suitable dilution (10^{-4} to 10^{-8}; see Fig. 2) that reduced the effective isoborneol concentration several orders of magnitude below the observed cytotoxic levels. Isoborneol at a concentration of 0.1% inactivated 86% of infectious virus within 30 min, while at higher concentrations (1%) this effect is accelerated to almost 4 $\log_{10}$ values.

3.3. Effect of isoborneol on viral replication

The next issue to be addressed was the effect of isoborneol on virus replication in infected cells. For this purpose, Vero cells were infected with 5 PFU/cell and the virus was allowed to replicate in the absence or in the presence of different concentrations (0.03% or 0.06%) of isoborneol. In all cases the virus growth kinetics were monitored by titration of virus samples removed from the infected cultures at various times post-infection on Vero cells. As shown in Fig. 3, isoborneol at a concentration of 0.06% completely suppressed viral growth by 24 h post-infection, while at the lower concentration of 0.03% caused an initial 96% reduction on viral growth rates by 8 h post-infection, but thereafter growth rates increased and by 24 h post-infection attained values similar to the control. Since viral replication was inhibited by isoborneol we examined whether this could be attributed to effects on early events of

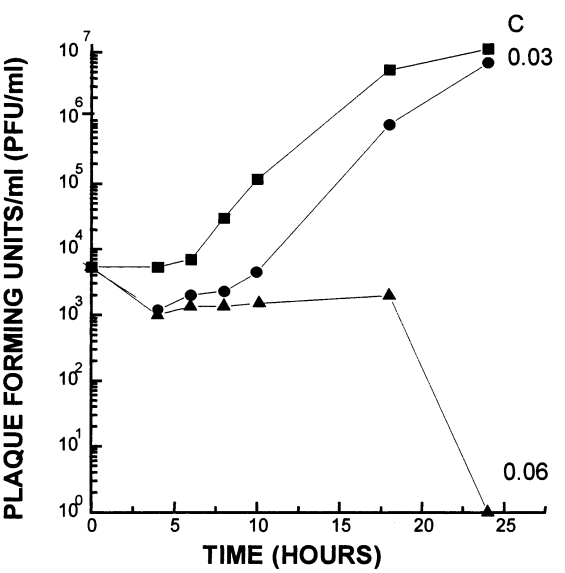

Fig. 3. Growth curve of HSV-1 in the presence or in the absence of isoborneol. Vero cells were infected with 5 PFU/cell of HSV-1 strain F. After 1 h adsorption period the virus was removed, the plates were washed and overlaid with medium supplemented with either 0.03% or 0.06% isoborneol or the appropriate amount of ethanol alone, which served as control. At the indicated times post-infection 100 μl samples were removed from each treatment and titrated on Vero cells.

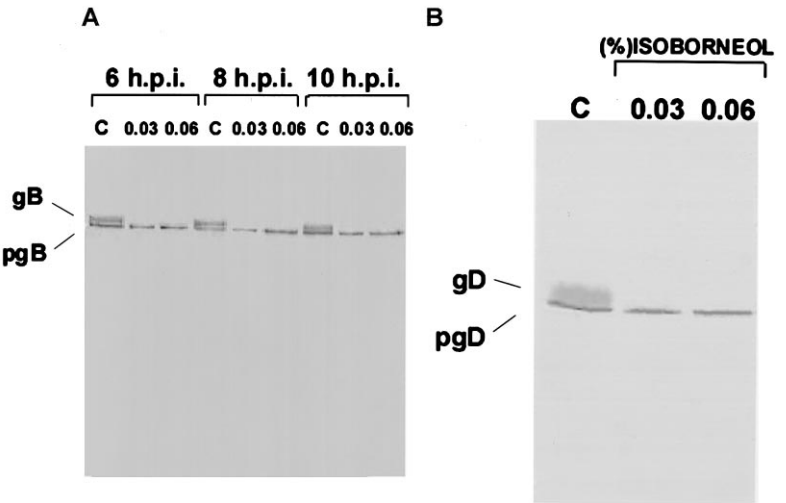

Fig. 4. Detection of gB by Western Blot (A) and of gD by immunoprecipitation (B) in lysates of HSV-1 infected Vero cells grown in the absence or in the presence of isoborneol. Vero cells were infected with 5 PFU/cell of HSV-1 strain F and were maintained in the presence of either 0.03% or 0.06% isoborneol or for control purposes in the appropriate amount of ethanol alone (C, control). Infected cell polypeptides were labeled with [^{35}S]methionine for 2 h at 4, 6 or 8 h post-infection, and then harvested. (A) Cell lysates were elecrophoretically separated on 8.5% SDS–polyacrylamide gels followed by electrical transfer to a nitrocellulose sheet. The nitrocellulose sheet was immunoblotted with monoclonal antibodies H1397 specific for the HSV-1 gB. (B) Lysates of infected cells labeled with [^{35}S]methionine from 6 to 8 h post-infection were immune precipitated with monoclonal antibody HD-1, specific for HSV-1 gD. The immune precipitates were elecrophoretically separated on 8.5% SDS–polyacrylamide gels, electrically transferred to a nitrocellulose sheet and exposed to Fuji X-ray film for autoradiography. gB, mature fully glycosylated form of gB; pgB, precursor form of gB; gD, mature fully glycosylated form of gD; pgD, precursor form of gD.

HSV infection. Thus, Vero cells were exposed to varying concentrations of isoborneol 0, 3, 6 or 12 h prior to HSV-1 infection (taken as time 0 h) and the amount of adsorbed virus was evaluated by counting the viral plaques at 72 h post-infection. Significant effect on virus plaquing efficiency was not observed for any of the treatments. Therefore, isoborneol does not appear to affect the adsorption of the virus to the cell membrane.

3.4. Effect of isoborneol on viral protein synthesis

Because isoborneol suppressed virus replication, we examined its effect on viral protein synthesis. Thus, Vero cells were infected with HSV-1 (5 PFU/cell) and after the adsorption period isoborneol was added at concentrations of 0.03% or 0.06%. Viral polypeptides were radiolabeled at 4, 6 or 8 h post-infection with [^{35}S]methionine, separated on SDS–polyacrylamide gels and transferred to a nitrocellulose sheet that was initially processed for autoradiography and was then used for the detection of the glycoprotein B (gB) by Western immunoblotting (Fig. 4A). Isoborneol affected moderately the quantity of some viral polypeptides and this was more pronounced at the higher concentration (data not shown). More interestingly, as shown in Fig. 4A, isoborneol completely inhibits the appearance of the mature fully glycosylated form of gB at both concentrations. In order to extend this observation to other viral glycoproteins, we examined its effect on glycoprotein D (gD). As shown in Fig. 4B, isoborneol had a similar effect on gD glycosylation as it inhibited the appearance of the mature fully glycosylated form of the glycoprotein. Next, we examined whether the effects of isoborneol on the glycosylation of viral polypeptides were permanent or reversible. Thus, Vero cells were infected with HSV-1 at a multiplicity of 5 PFU/cell and the virus was allowed to adsorb for 1 h. At the end of the adsorption period the virus inoculum

was removed, isoborneol was added at a concentration of 0.06% and the cultures were incubated for 10 h. At that time, the cell monolayers were washed extensively with PBS to remove the isoborneol and were replenished with fresh medium. After an additional incubation of 2 h the cells were harvested and the glycosylation pattern of gB was studied by Western immunoblotting. As shown in Fig. 5, the inhibition of gB glycosylation by isoborneol is reversible since the mature fully glycosylated form of gB appeared after the withdrawal of isoborneol.

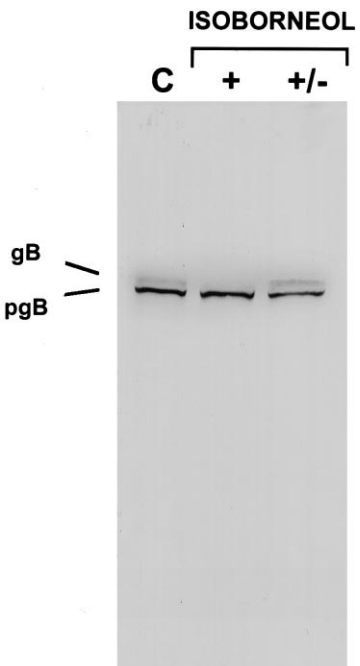

Fig. 5. The glycosylation pattern of gB after the withdrawal of isoborneol. Vero cells were infected with 5 PFU/cell with HSV-1(F) in the presence of 0.06% of isoborneol or in the appropriate amount of ethanol for control (C). Isoborneol either remained in infected cells for 12 h incubation period (+) or was removed at 10 h post-infection, and then the infected cells were further incubated for additional 2 h in medium lacking isoborneol (+/−). In all cases the cell lysates were harvested at 12 h post-infection, elecrophoretically separated on 8.5% SDS–polyacrylamide gels and electrically transferred to a nitrocellulose sheet. The nitrocellulose sheet was immunoblotted with monoclonal antibody H1397 specific for HSV-1 gB. gB, mature fully glycosylated form of gB; pgB, precursor form of gB.

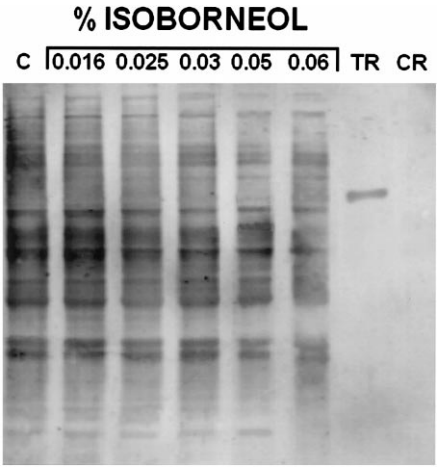

Fig. 6. Detection of glycoproteins in untreated and isoborneol treated Vero cells. Equal numbers of synchronized Vero cells were overlaid with growth medium supplemented with various concentrations of isoborneol (0.016%, 0.025%, 0.03%, 0.05% or 0.06%) or with the appropriate amount of ethanol, which served as control, and incubated for 24 h. The cell lysates along with aliquots of purified transferrin and creatinase were elecrophoretically separated on 8.5% SDS–polyacrylamide gels and electrically transferred to a nitrocellulose sheet. The glycosylated polypeptides on the nitrocellulose were oxidized, labeled with digoxigenin, and were finally visualized with alkaline phosphatase conjugated anti-digoxigenin antibodies. T, transferrin; CR, creatinase; C, control.

3.5. Glycosylation of cell proteins in the presence of isoborneol

Since isoborneol inhibited the glycosylation of viral proteins, we examined its effect on the glycosylation of cellular proteins. For that purpose synchronized Vero cells were exposed for 24 h to different concentrations (0.016%, 0.025%, 0.03%, 0.05% and 0.06%) of isoborneol. The cell lysates as well as transferrin (a glycoprotein) and creatinase (a non-glycosylated protein), which served as positive and negative controls respectively, were separated on SDS–polyacrylamide gels, and then electrically transferred to a nitrocellulose sheet. The glycosylated polypeptides were oxidized, labeled with digoxigenin, and finally visualized with anti-digoxigenin antibodies conjugated to alkaline phosphatase. As shown in Fig. 6, no major changes in the glycosylation pattern of cellular polypeptides were detected between untreated and

isoborneol treated Vero cells. In addition, the pattern of cellular polypeptides labeled with [^{35}S]methionine remained unaffected by the presence of isoborneol (data not shown).

3.6. Effect of isoborneol on the glycosylation of HSV-1 gB constitutively expressed in transformed Vero cell lines

The results cited above showed that isoborneol inhibited specifically the glycosylation of viral polypeptides in infected cells while that of the cellular polypeptides remained largely unaffected. Since viral glycoproteins expressed in transformed cells in the absence of other viral gene products are glycosylated (Arsenakis et al., 1985; Johnson and Smiley, 1985; Sivropoulou and Arsenakis, 1993), we investigated the effect of isoborneol on the glycosylation of gB expressed in the cellular genome in the absence of other viral gene products. Vero cells were transfected with pMA 8800 plasmid DNA carrying the gB gene of HSV-1 and the histidinol resistance gene. The transfected cells were selected in medium containing histidinol, and surviving colonies in each dish were pooled, expanded and tested for constitutive expression of gB by immunoprecipitation using gB specific monoclonal antibodies. Two of these cell lines, designated as V5 and V6, expressed gB and were used to investigate the effect of isoborneol on gB glycosylation. Thus, synchronized V5 cells were incubated for 2 h, in the presence or in the absence of 0.06% isoborneol, and were then radiolabeled with [^{35}S]methionine for 1 h in the continued presence or absence of isoborneol. The cell lysates were immunoprecipitated with gB specific monoclonal antibody. The results, presented in Fig. 7, showed that V5 cells express the mature fully glycosylated form of gB and that this glycosylation is not affected by the presence of isoborneol. This experiment was repeated with V6 cells and yielded the same results (data not shown). Therefore, taken together these results show that the inhibitory effect of isoborneol on the glycosylation of viral glycoproteins is only manifested in the context of virus infection. These data suggest the involvement of some viral product(s) in the glycosylation of HSV-1 polypeptides. In an effort to map this putative function on the viral genome we attempted to isolate virus resistant to isoborneol. After numerous attempts using repeated cycles of exposure and selection to 0.03% or 0.05% isoborneol, we isolated a virus designated FD. The FD isolate is considerably less sensitive (approximately 100-fold) than the parental HSV-1 strain F to the inactivation and inhibition of viral replication effects of isoborneol, but curiously was not fully resistant to inhibition of viral glycosylation (data not shown).

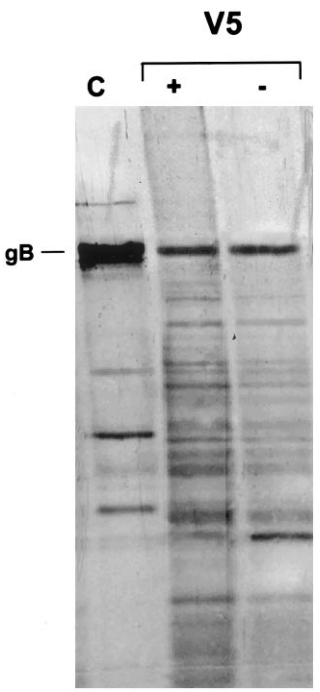

Fig. 7. Autoradiographic image of gB immune precipitated from V5 cells and from HSV-1 infected Vero cells. Transformed V5 cells were incubated in the presence of 0.06% isoborneol (+) or in the appropriate amount of ethanol alone as control (−). The V5 cell polypeptides were labeled 2 h after exposure to isoborneol and the infected cell polypeptides 10 h post-infection, with [^{35}S]methionine for 1 h. The cell extracts were immune precipitated with monoclonal antibody H233 specific for gB, and the immune precipitates were analyzed in 8.5% SDS–polyacrylamide gels, electrically transferred to a nitrocellulose sheet, and finally exposed to Fuji X-ray film.

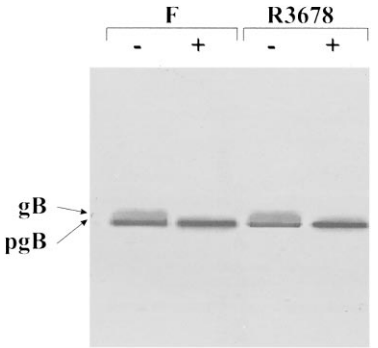

Fig. 8. Detection of gB by Western Blot in Vero cells infected with HSV-1 strain F and virus recombinant R3678 deficient in thymidine kinase (tk −). Vero cells were infected at a multiplicity of 5 PFU/cell with either HSV-1 strain F or recombinant R3678 either in the presence (+) of 0.06% isoborneol or in the appropriate amount of ethanol for control (−). The infected cell lysates were harvested at 10 h post infection, elecrophoretically separated on 8.5% SDS–polyacrylamide gels and electrically transferred to a nitrocellulose sheet. The nitrocellulose sheet was immunoblotted with monoclonal antibodies H1397 specific for the HSV-1 gB. gB, mature fully glycosylated form of gB; pgB, precursor form of gB

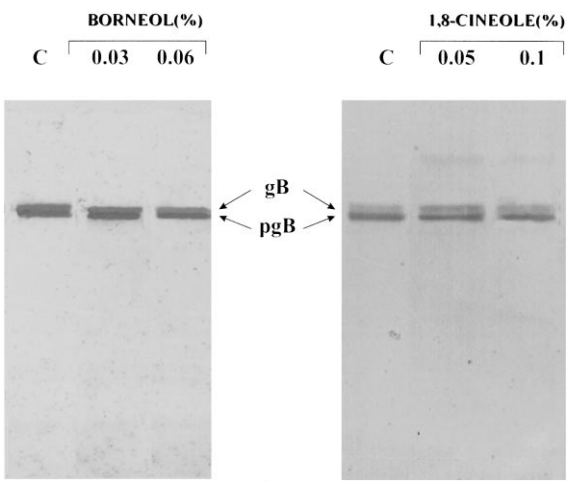

Fig. 9. Effect of borneol and 1,8 cineole on gB glycosylation. Vero cells were infected with HSV-1 strain F at a multiplicity of 5 PFU/cell in the presence of borneol (0.03% and 0.06%) or 1,8-cineole (0.1% and 0.05%) or in the appropriate amount of ethanol for controls (C). The cell lysates were harvested at 10 h post-infection, elecrophoretically separated on 8.5% SDS–polyacrylamide gels and electrically transferred to a nitrocellulose sheet. The nitrocellulose sheet was immunoblotted with monoclonal antibody H1397 specific for HSV-1 gB. gB, mature fully glycosylated form of gB; pgB, precursor form of gB.

3.7. Effect of isoborneol on a thymidine kinase negative HSV-1 mutant

Since our repeated efforts to isolate virus resistant to isoborneol-induced inhibition of viral glycosylation were not successful, the possibility was considered that this effect may be attributed to viral functions for which cellular counterparts exist. Relating to this, BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, a nucleoside analogue, has been shown to exert specific inhibition on viral glycosylation after undergoing specific phosphorylation by the viral thymidine kinase (Olofsson et al., 1988). Viral thymidine kinase can also phosphorylate alcohol groups (EC 2.7.1.114) and thus isoborneol, a monoterpenoid alcohol, could potentially be phoshorylated by the viral thymidine kinase. In that event the putative phosphorylated isoborneol could exert specific inhibition on viral glycosylation, in a way similar to that of BVdU. In order to investigate this possibility we examined the effect of isoborneol on gB glycosylation in Vero cells infected with the HSV-1 recombinant R3678 carrying a deletion in the thymidine kinase gene (Arsenakis and Roizman, 1990) resulting in abolishment of enzyme activity. The results presented in Fig. 8, show that isoborneol inhibits the glycosylation of gB in the thymidine kinase deficient mutant to the same extent as the wild type virus. Consequently, the specific action of isoborneol on viral glycosylation is independent of the presence of a functional thymidine kinase gene.

3.8. Effect of other monoterpenes on viral glycosylation

The results cited above showed specific inhibition of HSV-1 glycosylation by isoborneol. Since isoborneol is a monoterpenoid alcohol, we examined whether other monoterpenes exhibit similar activity. For that purpose we selected two monoterpenes, 1,8-cineole and borneol, a stereoisomer of isoborneol differing only in the orientation of the OH group, that exhibit comparable neutralizing activity against HSV-1 (3 and 4 $\log_{10}$ values at 1% concentration, respectively). Inhibition of viral glycosylation was tested in HSV-1 infected Vero cells in the presence of non-

cytotoxic concentrations of borneol (0.03% and 0.06%) or 1,8-cineole (0.1% and 0.05%; Sivropoulou et al., 1997). The results presented in Fig. 9 show that both compounds do not affect gB glycosylation, since the mature fully glycosylated form of gB could be detected in infected cells grown in the presence of either compound.

4. Discussion

4.1. Isoborneol as an antiviral agent

Isoborneol showed dual viricidal action against HSV-1. First, it inactivated the virus by almost 4 $\log_{10}$ values within 30 min of exposure without affecting viral adsorption. The inactivation activity of isoborneol, a monoterpenoid alcohol, is significant only at high concentrations of the compound owing perhaps to interactions between its alcoholic moiety and the lipids present on the virus envelope resulting in loss of infectivity. The following data support this notion. (1) The interactions of alcohols with proteins and cell membranes are well documented in the literature. For example binding of ethanol to phospholipid bilayers promotes lipid headgroup solvation, which at high concentrations causes formation of mixed lipid–alcohol micelles (Vierl et al., 1994). (2) HSV-1 acquires its envelope from cellular membranes where the viral glycoproteins are embedded (Roizman and Sears, 1990). In addition, cell membrane disruption and massive protein release was observed in Vero cells treated with high concentrations (1%) of isoborneol (data not shown). (3) Other monoterpenoid alcohols and particularly borneol, a stereoisomer of isoborneol, showed equivalent levels of HSV-1 inactivation. Second, isoborneol at a concentration of 0.06% completely inhibits virus replication, and moreover specifically inhibits viral glycosylation. Since the concentration of isoborneol needed for complete inhibition of viral multiplication is not toxic to three human cell lines tested, and despite repeated efforts, virus completely resistant to isoborneol could not be isolated, isoborneol appears a good candidate for antiviral therapy.

4.2. Inhibition of viral glycosylation by isoborneol

This study shows that isoborneol acts as a specific inhibitor of HSV-1 glycosylation based on the following results. (1) Isoborneol inhibits the glycosylation of HSV-1 proteins but it does not appear to grossly affect the glycosylation of cellular proteins. (2) When a viral glycoprotein gene is resident in the cellular genome the glycosylation of its product is not affected by isoborneol. (3) Inhibition of viral glycosylation was not observed with borneol, a stereoisomer of isoborneol, or with other monoterpenes. The specific inhibition of viral glycosylation by isoborneol may be attributed to the following:

(1) HSV-1 may encode a protein that specifically participates in glycosylation of viral proteins and isoborneol may directly or indirectly interfere with its function, resulting in inhibition of viral glycosylation. This hypothesis is supported by the fact that isoborneol-induced inhibition of gB glycosylation was not observed when the gB gene was resident in the cellular genome. According to this hypothesis it should be possible to isolate virus resistant to isoborneol and glycosylation of its proteins should be unaffected by isoborneol. Furthermore, there are reports in the literature supporting the possible participation of viral product(s) in glycosylation of HSV-1 proteins. Thus, Tognon et al., (1984) reported the specific inhibition of HSV-1 glycosylation by benzhydrazone [BH; 1*H*-benz[*f*]indene-1,3(2*H*)-dionebis-(amidinohydrazone)]. Furthermore a mutant virus resistant to BH, designated HSV-1(13)S11 was isolated, and BH resistance was mapped to the *Bam*HI 'L' and *Bam*HI 'SP' fragments of HSV-1(13)S11 DNA, spanning coordinates 0.707–0.745 and 0.81–0.85 respectively (Tognon et al., 1988). In this study, after repeated rounds of selection and plaque purification in increasing concentrations of isoborneol, we isolated a virus designated FD that was less sensitive than the parental strain F, to isoborneol-mediated virus inactivation and inhibition of replication. However, the FD isolate was as sensitive as the parental F strain to inhibition of viral glycosylation by isoborneol (data not shown). Alternatively isoborneol may become activated, with

respect to glycosylation inhibition, through modification by a virus-specified function. An analogous example is provided by another specific inhibitor of viral glycosylation, the nucleoside analogue, BVdU, which attains its specific activity only after undergoing specific phosphorylation by the viral thymidine kinase (Olofsson et al., 1988). The results reported here show that isoborneol's specific inhibition on viral glycosylation is independent of the viral thymidine kinase gene.

(2) The observed inhibition of viral glycosylation by isoborneol may also involve physiological and structural changes that ensue in the cell during the course of HSV-1 infection. The cellular membranes of infected cells are more fragile and permeable than those of uninfected cells, and it is likely that isoborneol penetrates them more easily, affecting enzymes responsible for protein glycosylation located in the endocellular membranic system (Golgi, endoplasmic reticulum). Furthermore, significant differences regarding K_m values and specificity of the acceptor molecules were reported for sialyl and galactosyl transferases between uninfected and HSV-1 infected cells (Olofsson et al., 1980). Alcohols have been reported to reduce the activity of membrane bound enzymes (Kitagawa et al., 1995) and activities involved in the hepatic mitochondrial and microsomal electron-transport systems (Sanchez-Amate et al., 1995). Similarly, monoterpenes such as α-limonene and perillyl alcohol were shown to inhibit enzymes responsible for post-translational modification of cellular proteins (Gould, 1997; Ren et al., 1997). Alternatively, ethanol has been reported to induce the GRP78 protein, a molecular chaperon involved in glycoprotein trafficking (Hsieh et al., 1996). In addition GRP78 and calnexin, also a membrane bound chaperone, have been reported to associate and retard the traffic of partially glycosylated forms of HSV-1 glycoproteins (Navarro et al., 1991; Yamashita et al., 1996). Finally, in HSV-1 infected cells host macromolecular synthesis is blocked by a virus-specified function (Roizman and Sears, 1990), this effect may be exacerbated in isoborneol treated virus infected cells. At present we can not differentiate between these possibilities

4.3. Effect of isoborneol on cell proliferation

Isoborneol at concentrations ranging from 0.016% to 0.08% and over a 72 h incubation period did not affect significantly the proliferation of four different cell lines. In relation to that, the isolation of UDP-glucuronosyltransferase 2 (UGT2) from rat liver was recently reported (Green et al., 1995). Genes encoding UGT2 have been isolated from many species and their products have been shown to catalyze the glucuronidation of monoterpenoid alcohols such as isoborneol, borneol, menthol and nopol, as well as phenolic compounds such as estrogen, androgens and *p*-nitrophenol (Boutin et al., 1987; Green et al., 1995). Northern blot analysis has demonstrated the presence of UGT2 mRNA in tissues other than liver (Green et al., 1995).

The results presented here should stimulate studies relating to isoborneol's action as a specific inhibitor of HSV-1 glycosylation and its therapeutic efficacy in animal model systems.

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