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Antiviral Research 43 (1999) 103–112

Antiviral Research

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Antiviral effects of 28-deacetylsendanin on herpes simplex virus-1 replication

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Received 20 April 1998; accepted 20 April 1999

Abstract

The compound purified from the fruit of *Melia azedarach* exerted an antiviral effect on herpes simplex virus-1 (HSV-1) in Vero cells. It was identified as 28-deacetylsendanin (28-DAS). The 50% inhibitory concentration (IC_{50}) of 28-DAS was 1.46 μg/ml without cytotoxicity at 400 μg/ml on Vero cells. Electron microscopy showed that low electron-dense cores of newly synthesized nucleocapsids remained in swollen nuclei and no extracellular virus particles were observed at 15 h p.i. Consistent with this result, it was confirmed by a plaque assay that few infectious progeny viruses were released from the 28-DAS-treated virus-infected cells at 24 h p.i. Intracellular viruses in 28-DAS-treated virus-infected cells were 23% of untreated and infected cells. The synthesis of thymidine kinase (TK) was reduced by 28-DAS at early stage. In conclusion, 28-DAS inhibited the replication of HSV-1, reduced the synthesis of HSV-1 TK, and led to the formation of defective nucleocapsids.

Keywords: Herpes simplex virus-1; Fruit of *Melia azedarach*; 28-Deacetylsendanin

1. Introduction

Since the discovery of methisazone, much effort has been devoted to the development of safe and effective antiviral agents, and as a result, a number of drugs have been newly discovered and developed. Most are synthetic compounds such as acyclovir (ACV) and azidothymidine. However, it has been reported that compounds from certain plants may be suitable antiviral agents; these include camptothecin (from *Camptotheca acuminata*) and pokeweed antiviral protein (from *Phytolacca americana*) (Horwitz and Brayton, 1972; Irvin, 1983; Wachsman et al., 1987; Locher et al., 1996). Moreover, the use of medicinal plants or extracts of herbs seems to offer advantages for the development of safe and efficacious antiviral agents.

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0166-3542/99/$ - see front matter
PII: S0166-3542(99)00037-6

Melia azedarach, the Chinaberry or Persian lilac tree, has long been recognized as an insecticidal and medicinal plant, and in particular, its fruit has been used traditionally for the treatment of a variety of diseases, especially dermatitis and rubella. In addition, a number of potent pharmaceutical limonoids and triterpenoids have been isolated from its fruits and bark (Lee et al., 1987; Champagne et al., 1992). It has also been reported that partially purified extracts from leaves of *M. azedarach* exert a broad range of antiviral effects on DNA and RNA viruses (Wachsman et al., 1987).

In this study, the most active compound from methanolic extract of the fruit of *M. azedarach* was identified on the basis of spectral data after purification, and investigated for its antiviral effect on herpes simplex virus-1 (HSV-1).

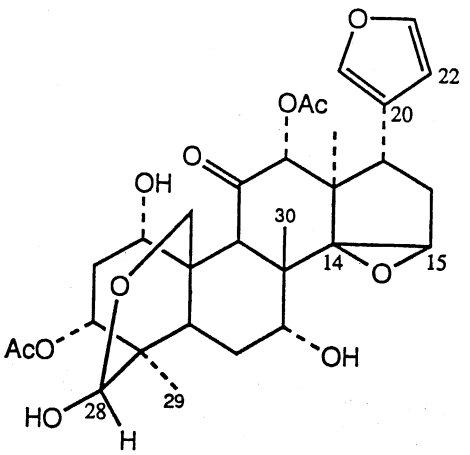

Fig. 1. Chemical structure of 28-deacetylsendanin purified from the fruit of *Melia azedarach*.

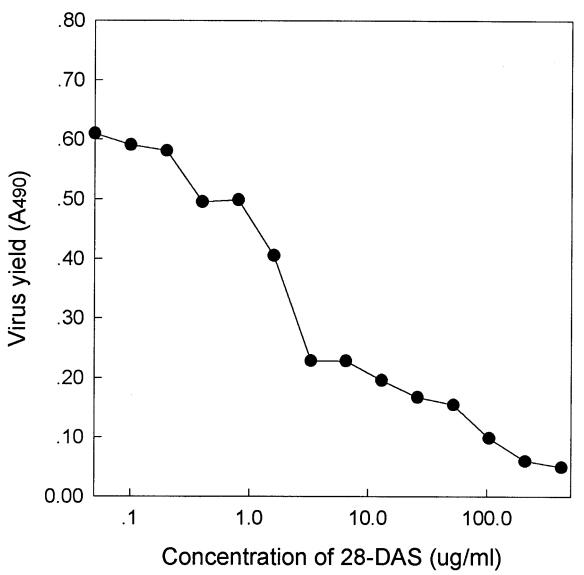

Fig. 2. Antiviral effect of 28-DAS in HSV-1-infected Vero cells. HSV-1 (1000 PFU/well) was adsorbed onto confluent monolayers of Vero cells for 1 h at 37°C. Infected cells were incubated with maintenance media in the presence of various concentrations of 28-DAS. After 1 day, virus yield was determined by in situ ELISA. and expressed as the optical density at 490 nm and the concentration of 28-DAS on the *X* axis ($\log_{10}$). Each point represents the mean of two samples.

2. Materials and methods

2.1. Cells and viruses

Vero cells were grown at 37°C in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere. For cell maintenance, serum concentration was reduced to 1%. To obtain virus stocks, confluent monolayers of Vero cells were adsorbed with HSV-1 (McIntyre) for 1 h and incubated in maintenance medium for 18 h at 37°C. The supernatant was harvested, clarified, and stored at −70°C (Burleson et al., 1992). Virus titration was carried out by plaque assay using DMEM containing 0.8% gum tragacanth, 4% FBS, and antibiotics as the overlay medium (Burke and Mulcahy, 1980).

2.2. Compounds

2.2.1. Extraction and isolation of 28-deacetylsendanin

The fruit of *M. azedarach* was cut into slices and extracted three times with MeOH at 50°C and concentrated under reduced pressure. The concentrated extract was partitioned between hexane and H_2O. The $CHCl_3$ fraction showing an antiviral activity was subjected to silica-gel column chromatography using only benzene,

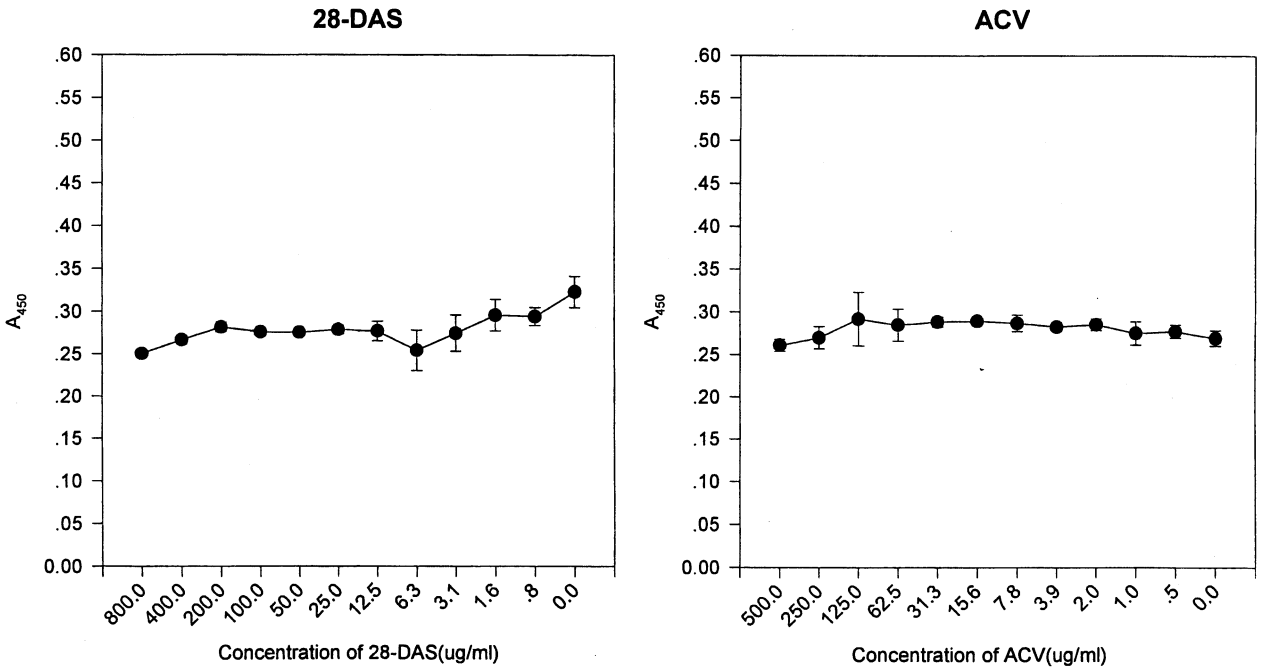

Fig. 3. Cytotoxicity assay for 28-DAS. Subconfluent Vero cell monolayers grown for 1 day in a 96-well culture plate were incubated with maintenance media in the absence or presence of two-fold diluted 28-DAS (0–800 μg/ml) and ACV (0–500 μg/ml) for 1 day. XTT assay was then performed. Most of the absorbances measured were within 0.25–0.3.

$CHCl_3$, or MeOH, or a gradient composed of them as an eluting system to give four fractions. These fractions were then partitioned into 17 subfractions with benzene, benzene–$CHCl_3$ (20:1 → 1:1), $CHCl_3$, $CHCl_3$:EtOAc (20:1 → 1:1), EtOAc, and EtOAc:MeOH (5:1 → 1:1). Among them, the most active fraction was further chromatographed on a silica gel column and eluted with $CHCl_3$, EtOAc, or a gradient to give four fractions. The fraction showing the highest antiviral activity was further repeatedly subjected to silica-gel column chromatography using $CHCl_3$ and $CHCl_3$:EtOAc (40:1 → 1:1) gradient solution.

2.2.2. Determination of structure of the compound

The structure of the compound was deduced from ^{1}H-nuclear magnetic resonance (NMR) (Varian Gemini-200), ^{13}NMR (Varian Gemini-200), infrared (IR) (Unicam Mathon-1000), ultraviolet (UV) (Perkin Elmer Lambda 15) scanning, electron ionization-mass spectrometry (EI-MS) (VG Biotech, Quattro-4000). MP, 260–262°C (decomposed); UV λ_{max} (MeOH) (nm), 195, 264; IR υ_{max} (KBr) (cm^{-1}), 3590, 3460, 3150, 1750, 1735, 1720, 1510, 1270, 885; ^{1}H-NMR (CD_3OD, 200 MHz) δ, 0.84, 1.13, 1.38 (each 3H, s, $-CH_3$), 1.96, 2.08 (each 3H, s, OAc), 1.74 (1H, br d, 15.2Hz, H-6), 1.87 (1H, d, $J = 15.4$Hz, H-2), 2.02 (2H, H-6 and H-16), 2.14 (1H, dd, $J = 13.4$, 6.2Hz, H-16), 2.75 (2H, H-2 and H-5), 2.88 (1H, dd, $J = 11.4$, 6.2Hz, H-17), 3.59 (1H, br s, H-7), 3.84 (1H, s, H-15), 4.24 (1H, d, $J = 12.5$Hz, H-19), 4.33 (1H, d, $J = 12.5$Hz, H-19), 4.70 (1H, s, H-9), 4.85 (1H, s, H-28), 5.20 (1H, d, $J = 3.6$Hz, H-3), 5.34 (1H, s, H-12), 6.17 (1H, s, H-22), 7.20 (1H, s, H-21), 7.41 (1H, s, H-23); ^{13}C-NMR (CD_3OD, 50MHz) δ: 210.67 (C-11), 174.55 ($COCH_3$), 173.88 ($COCH_3$), 145.19 (C-21), 143.48 (C-23), 125.63 (C-20), 114.45 (C-22), 98.67 (C-28), 81.21 (C-12), 76.32 (C-3), 75.12 (C-14), 72.60 (C-1), 72.24 (C-7), 66.89 (C-19), 61.45 (C-15), 51.73 (C-9), 48.32 (C-13), 45.34 (C-10), 44.34 (C-8), 42.63 (C-4), 41.26 (C-17), 38.61 (C-2), 36.23 (C-16), 31.05 (C-5), 27.59 (C-6), 24.61 ($COCH_3$), 22.78

($COCH_3$), 22.40 ($-CH_3$), 21.38 ($-CH_3$), 17.30 ($-CH_3$); LC-MS (m/z): 597 $(M+Na)^+$, 574 (M^+).

Due to its low water solubility, 28-deacetylsendanin (28-DAS), purified as stated, was dissolved in DMSO (100 μg/μl) and diluted to more than 100-fold in cell culture media when used.

2.3. Evaluation of antiviral activity

Antiviral activities of each active fraction and purified 28-DAS were assayed by in situ enzyme-linked immunosorbent assay (ELISA). Confluent monolayers of Vero cells grown in a 96-well microtiter plate were infected with HSV-1 (1000 PFU/well). Virus adsorption was allowed for 1 h at 37°C, and the inoculum was then removed and incubated in maintenance media with or without antiviral agents at 37°C. Next day, the medium was removed from the microtiter wells, and 100 μl phosphate-buffered saline (PBS) containing 2% skim milk was added for 1 h at 37°C. This was replaced by 50 μl HSV-1 specific monoclonal antibody cocktail (MHSVI-06, 66, and 116) (Cha et al., 1988) for 1 h at 37°C. The wells were then washed three times with PBS. To each well, 50 μl peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Sigma), diluted to 1:1000 in PBS containing 2% skim milk, was then added. The plate was incubated for 1 h at 37°C and washed three times with PBS, and 50 μl substrate, 40% *o*-phenylenediamine (OPD) (Sigma) in phosphate–citrate buffer (pH 6.0) with 0.05% hydro-

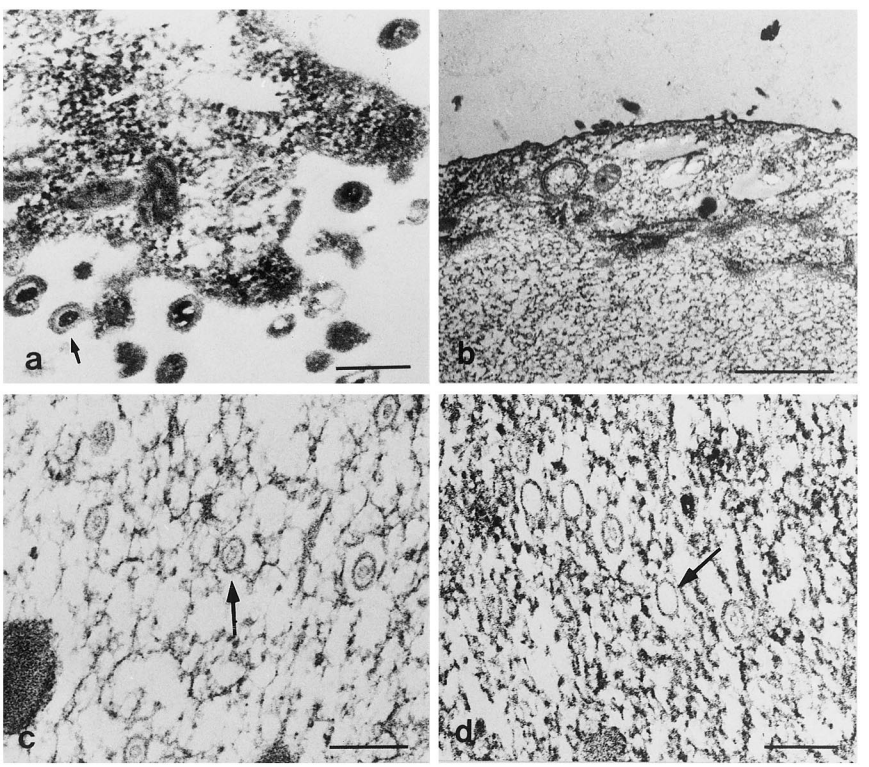

Fig. 4. HSV-1-infected Vero cells 15 h p.i., untreated (a), treated with 28-DAS (b, c), or ACV (d). HSV-1 (MOI of 1) was adsorbed onto monolayers of Vero cells for 1 h at 37°C. 28-DAS (400 μg/ml) or ACV (250 μg/ml) was added at the time of virus adsorption. At 15 h p.i., the ultrastructures of untreated and treated virus-infected cells were observed under transmission electron microscopy. (a) Extracellular viral particles exhibit dense inner cores and coats (arrow). (b) No progeny virus particles are released from the intact cell membrane. (c): Numerous unenveloped capsids have low electron-dense cores (arrow), indicating insufficient DNA content. (d) Most capsids are empty (arrow). Bars indicate 200 nm in (a, c, d) and 1 μm in (b).

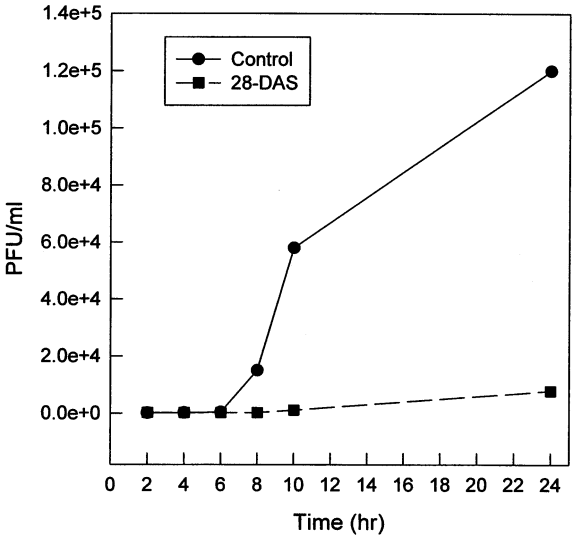

Fig. 5. Growth curve of HSV-1 in the presence(●) and absence (■) of 28-DAS (400 μg/ml). HSV-1 (MOI of 1) was adsorbed onto confluent monolayers of Vero cells. 28-DAS was added at the time of virus adsorption. Culture media were harvested at the indicated times and titrated by plaque assay for extracellular viruses. Each point represents the mean of two samples.

gen peroxide, was added to each well. After 15 min at room temperature, 12.5% H_2SO_4 was added to stop the enzyme reaction. Using an MR 700 Microplate Reader (Dynatech Laboratories; Kaufman et al., 1995), optical density was read at a wavelength of 490 nm.

In order to titrate extracellular and intracellular viruses after the treatment of antiviral agents, plaque assay was performed throughout the replication cycle of HSV-1 and at 15 h p.i., respectively. Confluent monolayers in 60-mm Petri dishes were infected with HSV-1 (MOI of 1) and virus adsorption was allowed for 1 h in the presence and absence of 28-DAS. Culture media were removed for titration of extracellular viruses at various times. At 15 h p.i., infected cells were harvested with cell scrapers for titration of intracellular viruses. Harvested cells were resuspended in serum-free DMEM and repeatedly frozen and thawed three times, and the media containing intracellular viruses were obtained after centrifugation at 10 000 × *g* for 3 min. The virus solutions were used for plaque assay (Burke and Mulcahy, 1980).

ACV (Zovirax) was purchased from Wellcome Foundation and used as a reference anti-HSV-1 drug. It was dissolved in sterile distilled and deionized water (μg/μl), and used after dilution in cell culture media.

2.4. Cytotoxicity test

28-DAS was evaluated for its cytotoxicity by the XTT assay. Subconfluent Vero cell monolayers grown for 1 day in a 96-well microtiter plate were incubated with maintenance media in the absence or presence of two-fold diluted 28-DAS (0–800 μg/ml) for 1 day. XTT reagents (Boehringer Mannheim) were then added to the monolayers of Vero cells. After an incubation for 4 h at 37°C, absorbance (450 nm) was measured to evaluate the activity of the mitochondrial dehydrogenase as a parameter for the viable cell count. Each experiment was carried out in triplicate and repeated three times.

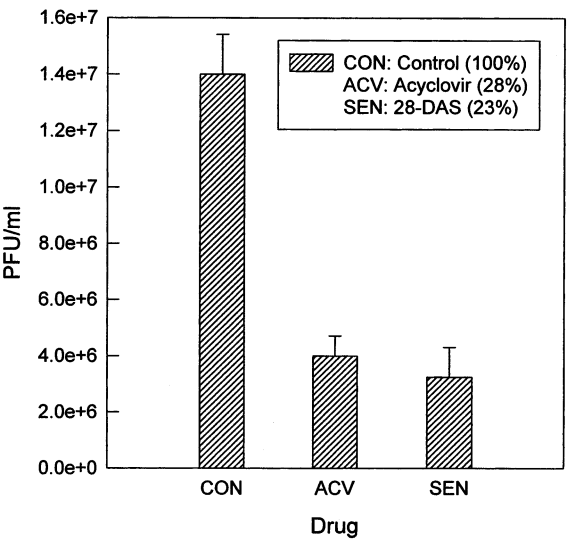

Fig. 6. Titration of intracellular HSV-1 in the presence and absence of 28-DAS (400 μg/ml) or ACV (250 μg/ml). HSV-1 (MOI of 1) was adsorbed onto confluent monolayers of Vero cells for 1 h at 37°C. Drugs were added at the time of virus adsorption. At 15 h p.i., infected cells were washed twice with PBS and harvested. The harvested cells in 1 ml of DMEM were prepared by repeated freezing and thawing, and centrifuged to remove cell debris. Supernatants were titrated by plaque assay. Each point represents the mean of two samples.

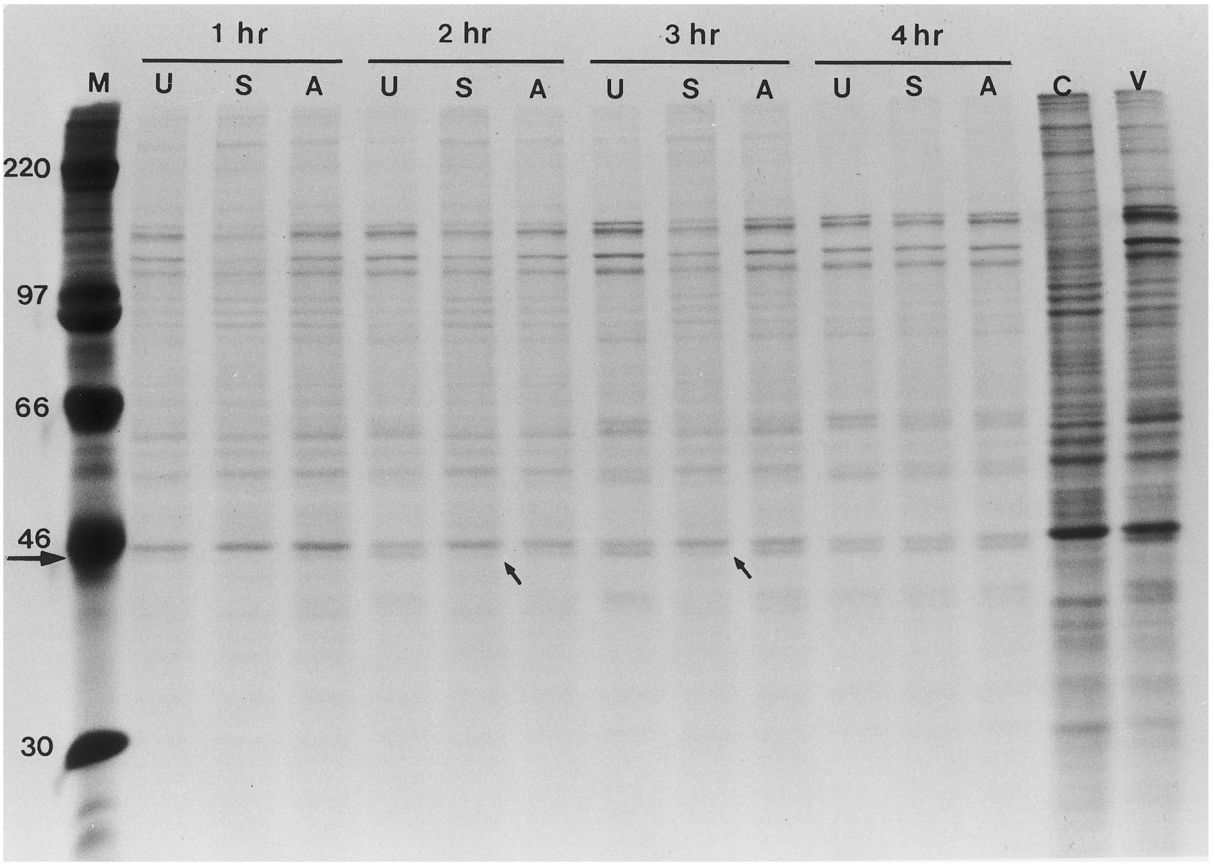

Fig. 7. Effect of 28-DAS and ACV on the synthesis of HSV-1-induced and host cellular proteins. HSV-1 (MOI of 1) was adsorbed onto Vero cell monolayers for 1 h at 37°C. 28-DAS (400 μg/ml) (S) or ACV (250 μg/ml) (A) was added at the time of virus adsorption, or the cells were left untreated (U). Labelling with [^{35}S]methionine was carried out for 1 h at 1, 2, 3, and 4 h p.i. Untreated uninfected cells (C) and untreated virus-infected cells (V) were labelled with [^{35}S]methionine for 5 h. Lane M indicates molecular weights of proteins (220, 97, 66, 46, 30, and 22 kDa). Arrows indicate the band which had almost disappeared (44 kDa).

2.5. Electron microscopy

Confluent monolayers of Vero cells were infected with HSV-1 (MOI of 1). Then 28-DAS (400 μg/ml) or ACV (250 μg/ml) was added simultaneously with virus inoculum. After 1 h, the cells were incubated for 15 h at 37°C. At that time, infected cells ($\sim 1 \times 10^8$) were harvested with cell scrapers and settled in the culture media. The pellets were fixed in glutaraldehyde and osmium tetraoxide. Sections, treated in a routine manner, were stained with uranyl acetate and lead citrate, and images on a transmission electron microscope (TEM) (Jeol JEM-1200EX II) were photographed.

2.6. [^{35}S]methionine pulse labelling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Effect on virus-induced protein synthesis was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after [^{35}S]methionine pulse labelling. Confluent monolayers of Vero cells grown in a 24-well tissue culture plate were infected with HSV-1 (MOI of 1) and simultaneously treated with 400 μg/ml 28-DAS. After 1 h at 37°C, media were removed and cells were washed with PBS. Cells were then incubated in maintenance media at 37°C. At the indicated times, the media were replaced by 0.2 ml of methionine-free MEM supplemented with 1%

FBS and 5 μCi [^{35}S]methionine (Amersham) per well. Each labelling was continued for 1 h at 37°C, and cells were washed twice with 1 ml PBS and dissolved in 0.1 ml of sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Samples were heated to 100°C for 5 min and analysed by electrophoresis through a 10% polyacrylamide gel (Otero and Carrasco, 1987). For fluorography, the gel was sequentially treated with DMSO for 1 h and with 2,5-diphenyloxazole dissolved in DMSO (200 g/l) for 3 h. The gel was washed with water for 1 h, dried, and then exposed to AGFA film (CP BU) at −70°C (Hames and Rickwood, 1981).

2.7. Radioimmunoprecipitation

Confluent monolayers of Vero cells in 60-mm Petri dishes were infected at a MOI of 1. 28-DAS

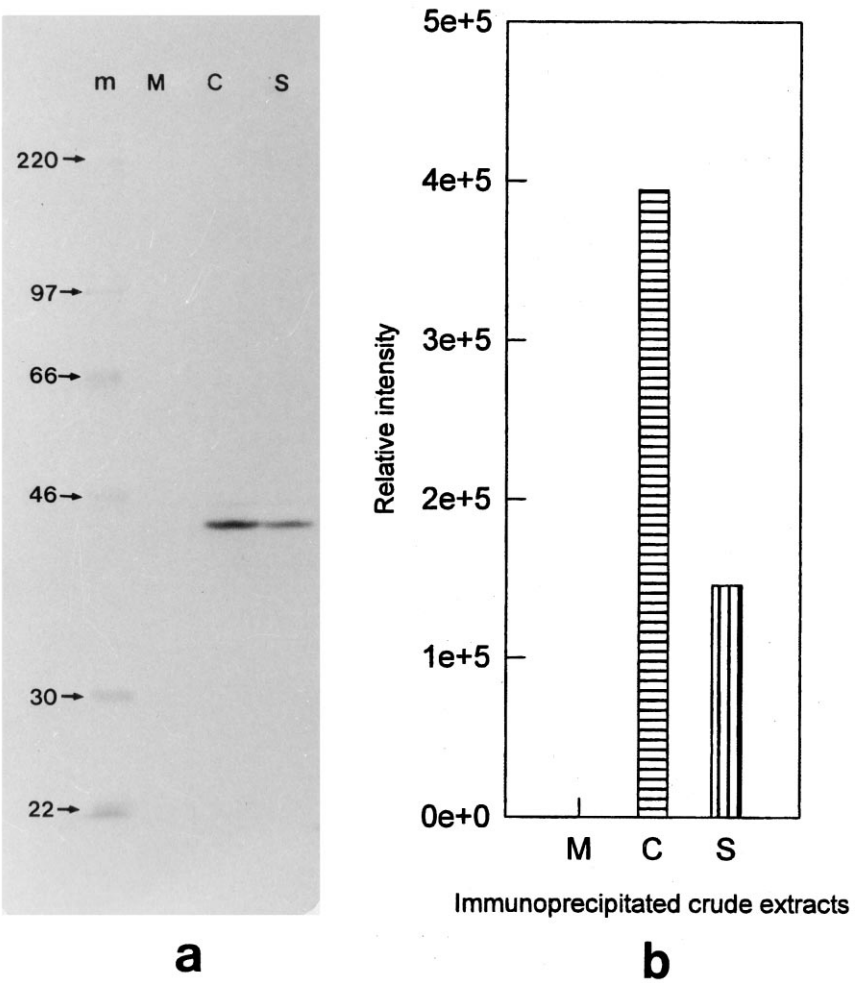

Fig. 8. Polypeptide immunoprecipitated by HSV-1 TK antiserum. HSV-1 (MOI of 1) was adsorbed onto Vero cell monolayers for 1 h at 37°C. 28-DAS (400 μg/ml) was added at the time of virus absorption. Labelling with [^{35}S]methionine was carried out for 1 h at 2 h p.i. Proteins in lysates from [^{35}S]methionine-labelled mock-infected (M) and infected Vero cells treated (S) or not treated (C) with 400 μg/ml 28-DAS were immunoprecipitated with HSV-1-specific TK antiserum and separated on 10% SDS-PAGE. (m) indicates molecular weights of proteins (220, 97, 66, 46, 30, and 22 kDa) (a). Relative intensity (b) was measured by BIO-PROFIL (Viber Lourmat).

(400 μg/ml) was added at the time of virus adsorption. At 2 h p.i., the growth media were removed and washed once with PBS. The cells were incubated in MEM lacking methionine, and supplemented with 1% FBS and 100 μCi/ml [^{35}S]methionine for 1 h. The radiolabelling media were replaced with ice-cold PBS and the cells were dislodged with rubber scrapers. Cells were collected by centrifugation at 12 000 × *g*, 4°C for 30 s, and resuspended in 1 ml lysis buffer (RIPA buffer supplemented with 1 mM *N*α-ρ-tosyl-L-lysin chloromethyl ketone; 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 1% SDC, 0.1% SDS, 0.5% aprotinin, 0.1% sodium azide, and 1 mM *N*α-ρ-tosyl-L-lysin chloromethyl ketone). After incubation on ice for 20 min, clear lysates were obtained by centrifugation as already stated. Nonspecific precipitates were removed from the lysate by incubation with normal rabbit serum and swollen Protein A Sepharose at 4°C for 16–18 h. HSV-1 thymidine kinase (TK) antiserum (generously provided by Dr Peter Collins, GlaxoWellcome Research and Development) and swollen Protein A Sepharose were added to the supernatants and incubated at 4°C for 16–18 h. After centrifugation as already stated, the pellets were sequentially washed with RIPA, high salt buffer (2 M NaCl, 10 mM Tris–HCl, pH 7.5, 1% IGEPAL CA-630, 0.5% SDC, 0.1% sodium azide), low salt buffer (3 mM KCl, 1.5 mM KH_2PO_4, 14 mM NaCl, 4 mM NaH_2PO_4, 0.5% IGEPAL CA-630, and 0.1% SDS), and, finally, 10 mM Tris–HCl (pH 7.0). The pellets were resuspended in electrophoresis sample buffer and heated to 100°C for 5 min (Irmiere et al., 1989). The supernatants were analyzed by electrophoresis through a 10% polyacrylamide gel. The gel was processed for fluorography and exposed at −70°C as already described. The relative intensity of the immunoprecipitated band was measured by BIO-PROFIL (Viber Lourmat).

3. Results

3.1. Structure of the compound

Among several extracts of the fruit of *M. azedarach*, the methanolic extract had the highest antiviral activity on HSV-1 in Vero cells. The active compound was isolated from the extract by repeated silica-gel chromatography and examined for its structure.

The structure of the compound was determined to be identical with 28-DAS (12-acetoxyamoorastatin) by comparing their physical and spectral data with published data (Fig. 1). 28-DAS obtained as colorless crystals, m.p. 260–262°C, exhibited the following spectral data: UV λ_{max} (CH_3OH), 264 nm; IR υ_{max}(KBr) (cm^{-1}), 3590, 3460 (hydroxyl group), 1750, 1735 (six-memberedring ketone), 885 (furan); ^{1}H-NMR indicated the presence of three aromatic protons (δ 6.17, 7.20, and 7.41), two methyl protons of acetate groups (δ 1.96 and 2.08), and methyl protons (δ 0.84, 1.13, and 1.38); ^{13}C-NMR showed 30 carbon signals, among which three carbon signals of β-substituted furan ring and ketone, four quaternary carbons, three methylene carbons, and three methyl carbons were observed.; The molecular weight was deduced as 574 from LS-MS at a retention time of 8.88 min. 28-DAS existed as isomers.

3.2. Antiviral activity

Antiviral activity of 28-DAS was assessed by in situ ELISA on monolayers of Vero cells. 28-DAS exerted an antiviral effect on HSV-1, with a 50% inhibitory concentration (IC_{50}) of 1.46 μg/ml. The antiviral activity reached the maximal inhibition (plateau) at a concentration of 200–400 μg/ml, respectively (Fig. 2). According to this result, experiments to investigate the antiviral mechanism were performed at the maximal inhibition concentration of 400 μg/ml. As a reference drug, ACV was used also at the maximal inhibition concentration of 250 μg/ml.

3.3. Cytotoxicity

Cytotoxicity of 28-DAS on Vero cells was examined by XTT assay. Cytotoxic effect was not observed up to the concentration of 400 μg/ml; however, mitochondrial activity of Vero cells decreased slightly at the concentration of 800 μg/ml (Fig. 3).

3.4. Electron microscopy

To investigate which step of the HSV-1 infection cycle was blocked by 28-DAS, an ultrastructural study was performed in drug-treated virus-infected cells at 15 h p.i. The cytoplasmic membrane of 28-DAS-treated virus-infected cells was maintained without releasing progeny viruses. Characteristically numerous nucleocapsids with low electron-dense cores remained within the swollen nuclei, whereas the cell membrane of untreated virus-infected cells had ruptured, producing extracellular virus particles with high electron-dense cores. There were empty capsids and low electron-dense cores in the swollen nucleus of ACV-treated virus-infected cells (Fig. 4). The findings that extremely few extracellular viruses were released following 28-DAS treatment, and that the low electron-dense particles produced following 28-DAS treatment were not infectious, could be easily corroborated by plaque assays. Much fewer infectious intracellular viruses were formed in ACV- or 28-DAS-treated virus-infected cells as compared with the untreated virus-infected cells (Figs. 5 and 6).

3.5. Effect on viral protein synthesis

To investigate whether 28-DAS inhibits viral protein synthesis, cell extracts from 28-DAS-treated and untreated virus-infected cells, which had been pulsed for 1 h with [^{35}S]methionine at various times p.i., were analyzed by SDS-PAGE. A slight overall reduction of protein synthesis was observed in 28-DAS-treated virus-infected cells up to 4 h p.i. Especially, 28-DAS inhibited the synthesis of 44 kDa protein from 2 to 3 h p.i. In contrast, HSV-1 protein synthesis was not affected by ACV (Fig. 7). At later stages, there was no change in late viral protein synthesis (data not shown).

Because the 44 kDa polypeptide of HSV-1 has been known as TK, we carried out immunoprecipitation using HSV-1 TK antiserum to identify the protein band. In 28-DAS-treated virus-infected cells, TK synthesis was reduced by about 50% (Fig. 8).

4. Discussion

Chemotherapy against herpes infections has received considerable attention and has been facilitated by the presence of several viral enzymes amenable as targets for antiviral drugs; the enzymes most commonly involved are viral TK and DNA polymerase (Zuckerman, 1985).

28-DAS purified from the fruit of *M. azedarach* had a hydrophobic structure and inhibited the replication of HSV-1 in a dose-dependent manner with an IC_{50} of 1.46 μg/ml without cytotoxicity. At 15 h p.i., the cores of newly assembled nucleocapsids in 28-DAS-treated virus-infected cells were low electron-dense compared with those of untreated virus-infected cells. On the basis that low electron-density may imply insufficiently packaged genome (Gibson and Roizman, 1972), the result might suggest that the formation of mature virus particles was blocked by 28-DAS. Empty capsids accumulating in the nucleus of virus-infected cells are not transported into the cytoplasm (Roizman and Furlong, 1974; Vlazny et al., 1982). Numerous empty and low electron-dense nucleocapsids were observed in ACV-treated virus-infected cells. This result is consistent with the known antiviral mechanism of ACV which inhibits viral DNA synthesis.

Marked inhibition of 44 kDa viral protein was observed. It was proved to be an early protein, thymidine kinase. The inhibition of the 44 kDa protein synthesis may be related to the formation of low electron-dense cores in 28-DAS-treated virus-infected cells. Although the mechanism underlying the selective transport of capsids containing full-size viral DNA is unknown, our results might suggest that the formation of defective nucleocapsids by 28-DAS may be related to incomplete DNA synthesis and lack of envelopment, leading to marked reduction of extra- and intracellular viruses. Transient suppression of the 44kDa protein early after infection, which is a quite critical period in viral genome replication, will affect the whole viral replication cycle. However, it may be necessary to examine whether the effect of 28-DAS on TK synthesis is primary or secondary, to verify the involvement of 28-DAS in DNA synthesis of HSV-1-infected cells, and to determine more clearly the specific steps at which

the expression of TK in the transcription–translation cascade is inhibited.

Acknowledgements

This study was supported by the Ministry of Health and Welfare, grant number HMP-96-M-2-1063 and, in part, G-7 project. We gratefully acknowledge the award by the Korea Science and Engineering Foundation and Korea Research Foundation of a postdoctoral fellowship to Dr Min Kim. We thank Dr D.H. Hwang for electron microscopy. In addition, we are grateful to Dr Kenneth L. Powell, who introduced Dr Peter Collins, and to Dr Peter Collins, himself, who generously provided HSV-1 TK antiserum.

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