

Original Research Article

Development of a Broad-Spectrum Antiviral Agent with Activity Against Herpesvirus Replication and Gene Expression

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Abstract

Purpose: To evaluate the broad-spectrum antiviral activity of peptide H9 (H9) *in vitro* in order to gain insight into its underlying molecular mechanisms.

Method: Antiviral activity against Herpes simplex virus type 1 (HSV-1) was determined using thiazolyl blue (MTT) assay. Polymerase Chain Reaction (PCR) was employed to assay H9 antiviral activity against human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV). The inhibitory effect of H9 on the replication of these viral genes including early genes was assayed by real time-Polymerase chain reaction (RT-PCR) and Western blot.

Results: H9 possessed significant inhibitory effect on the four different herpesviruses with 50 % inhibitory concentration (IC_{50}) of 1.21 ng/mL (HSV-1). AD169 infection was strongly inhibited with an EC_{50} value of 0.46 ng/ml. The anti-herpesviral activity of H9 was dose-dependent. The peptide acted primarily during the early stage of infection by detection of the early genes.

Conclusion: The results demonstrate that H9 can inhibit the infection of HSV-1, EBV and HCMV. Furthermore, H9 has a broad-spectrum anti-herpesviral effect *in vitro* based on targeted killing of infected cells expressing genes.

Keywords: Antagonist, Trapping receptor/ligand, Broad-spectrum, Anti-herpesvirus, H9 peptide, Gene expression

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INTRODUCTION

In the last 20 years, herpesviruses have increased, leading to increase in the number of immunocompromised individuals. Members of the herpesviridate families encode Viral G-protein-coupled receptors (vGPCRs) that mimic chemokines and chemokine receptors to evade the human immune system. vGPCRs make significant contributions to viral lifecycle and associate pathologies. As is already known, US28 encoded by human cytomegalovirus is the best investigation viral chemokine receptors.

US28 has been reported to form multiple signaling pathways via G proteins, and various kinases are activated to regulate transcription factors such as nuclear factor κ B (NF κ B) and activating protein 1 (AP1) [1-2].

In the foregoing cases, cellular signal pathways from vGPCR provide invaluable perspectives on virus-associated pathologies and may be hopeful drug targets that treat or prevent virus-associated pathologies. Latent infection of herpesvirus increases difficulties in treatment. Unfortunately, therapy approaches against herpesvirus with

conventional anti-herpesvirus drugs have been mostly ineffective [3]. Currently, clinically anti-herpesvirus drugs, in the form of nucleoside analogs, demonstrate significant effect in the therapy of herpesvirus. Several drugs, such as ganciclovir and cidofovir, are known to exhibit anti-herpesvirus activity during viral replication with normal molecular mechanisms *in vitro* by influencing gene expression [4,5]. However, drug resistance, development of drug-related toxicity and side effects limit their clinical use in patients [3,6].

A critical feature of herpesvirus-mediated pathogenesis is the replication of the virus in infected tissue and the overt disease caused by viral replication [7]. For a productive infection, herpesvirus expresses three ordered classes of viral genes in a tightly regulated cascade, designated as immediate-early (IE), early (E) and late (L), which are synthesized during infection [8]. On account of the fact that genes are essential for viral replication, it is imperative to research into herpesvirus pathogenesis, and as well as investigate the mechanisms of their regulation.

We had reported previously [9,10] that the mechanism of H9 action on chemokine receptor US28 of human cytomegalovirus indicates antiviral activity against HCMV. The objective of the present study was to determine the ability of H9 to inhibit herpesviruses, EBV and HSV-1 genes required for viral DNA replication expression with a view to gaining insight into underlying molecular mechanisms.

EXPERIMENTAL

Cells and viruses

Vero cells and Helf cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal bovine serum (FBS). B95-8 cells and Raji cells were cultivated in RPMI 1640 with 10 %v/v fetal calf serum (FCS). The infected Vero cells were overlaid with medium containing 1 % of methylcellulose, and incubated for 3 days before it was fixed with formalin.

HCMV AD169 virus (5-10 μ l) was added to Helf cells at the concentration of 1×10^6 /ml in logarithmic phase in logarithmic phase. Cytopathicity of cells was observed daily with inverted microscope (Olympus SZX16, Japan), and pp65 was monitored every 3 days. EBV production was induced chemically by the addition of 40nM TPA (phorbol 12-myristate 13-acetate, Sigma) for 7 days and 3mM butyric acid for 3 days in medium containing 2 % FCS.

Cytotoxic assay

IC₅₀ was determined using MTT (Sigma–Aldrich) assay. Briefly, Vero cells was incubated in 96-well plates (100 μ l per well) . After a period of incubation, MTT solution (0.15 mL, 5 mg/mL in 0.01 M PBS) was added to each well. Subsequently, the cells were incubated for 4 h at 37 °C by removing MTT solution, then 100 μ l/well DMSO was added to each well, and the samples measured at 590 nm in a microplate reader.

Evaluation of anti-EBV activity of H9

Raji cells were incubated with or without H9 (from 0.01 to 100 ng/ml or 1000 ng/ml) for 48 h. Cell viability was determined using MTT assay as previously prepared. A standard curve was constructed based on H9 concentration and % cell survival.

Real-time RT-PCT quantification of EBV mRNA

The effect of H9 treatment on EBV on EBV of H9 treatment was determined by RT-PCR (Applied Biosystems). Simply, total RNA was prepared using TRizol reagent (Invitrogen). Reverse transcription was performed as follows: 11.5 μ l total RNA and 2 μ l Oligo(dT)₁₅ were added to the tube. The mixture was heated to 70 °C for 5 min and quickly quickly to an ice bath for 5 min. Thereafter, 4 μ l 5 \times RT Reaction Buffer, 0.5 μ l 400U/ μ l RNase Inhibitor and 1 μ l M-MLV were added. The tubes were incubated at 42 °C for 60 min and 95 °C for 10 min. The primer pairs for BamH1-W were BamH1-WF (5'-GCCAGAGGTAAGTGGACTTT-3') and BamH1-WR (5'-TGGAGAGGTCAGGTTACTTA-3'); and for GAPDH were GAPDH-F (5'-AATCCCA TCACCATCTTCCA-3') and GAPDH-R (5'-CCTGCTTACCACCTTCTTG-3') [11]. The reaction mixture (20 μ l) contained 1 μ l of cDNA template, 2 μ l Taq Buffer and 1 μ l of each primer. Briefly, it was pre-incubated at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, annealing at 59 °C for 30 s, and polymerization at 72 °C for 30 s, and a final extension of 7 min at 72 °C followed the last amplification cycle.

Western blot detection of viral protein

Monoclonal antibodies to the protein products of the EBV early genes have been described [12,13]. Anti-EBV antibodies to MAb-BALF2 (anti-EA; Chemicon, Temecula, CA) and PAb-BZLF1 were used for analysis by standard Western blot technique [14]. Briefly, cell lysates were harvested in RIPA (radio immunoprecipitation assay) lysis buffer, and

SDS-loading buffer added. The mixture was denatured at 95 °C for 10 min. The lysates were separated (15 µl) on 12.5 % polyacrylamide gels followed by Western blot transfer and immunostaining with EBV specific antibodies [15].

Evaluation of anti-HCMV activity of H9

Helf cells were cultivated on 24-well plates and used for infection with AD-169 HCMV and counted for the number of syncytial cytopathic effects (CPEs). CPE inhibitory rate and EC₅₀ (50 % effective concentration) were calculated according to Reed-Muench method [16]. H9 were added either after virus adsorption (post) or pre-incubation of the cells for 1 h.

The UL83 sequence encoding pp65 was isolated by PCR with primers specific for pp65 5'-GTCAGCGTTCGTGTTTCCCA-3' and 5'-GGGACACAACACCGTAAAGC-3'. For qPCR, a FAM-TAMRA-labeled TaqMan probe was used. qPCR was performed on a ABI 7300 sequence detection system using SYBR Green Kit (Invitrogen) under the following conditions: 2 min at 50 °C, 3 min at 95 °C, followed by 10 cycles of 45 s at 95 °C and 55 s at 60 °C. Inhibitory rate was calculated as $\{(A-B)/(A) \times 100\}$, where A and B denote the copy number of HCMV control group and H9 control group, respectively.

Assessment of anti-HSV-1 activity of H9

Vero cells were incubated in 96-well plates (100 µl per well) . After a period of incubation, cell viability was determined using MTT assay as previously described. A standard curve was constructed based on H9 concentration and % cell survival. IC₅₀ (50 % effective concentration) was determined by Reed-Muench method [16].

RT-PCR of HSV-1 genes

The effect of H9 on IE(UL54), E(UL30) and L(US6) genes from HSV of H9 treatment was determined by RT-PCR method. Simply, total RNA was prepared using TRizol reagent (Invitrogen). Reverse transcription was performed as described above. Primers and probes were added as outlined in Table 1. The reaction mixture (20 µl) contained 1 µl of cDNA template, 2µl Taq Buffer and 1 µl of each primer. Briefly, it was pre-incubation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, annealing at 59 °C for 30 s, and polymerization at 72 °C for 30 s, followed by a final extension at 72 °C for 7 min.

Table 1: Primers and probes used in the multiplex real-time PCRs specific to the polymerase gene of HSV-1

Gene	Primers Sequence(5'-3')	Amplicon size (bp)
UL54	5'- CCAGGACCCTATCATCGGAACG- 3'	303
	5'- AGTATTTCAATGAGACCCGCCAT- 3'	
UL30	5'- GAAGCGCAGCAAGATAAAGG-3'	309
	5'- ATGGTQCGGGTGATGTTAAT-3'	
US6	5'- GGTTTACTACGCCGTGTTGG-3'	342
	5'- ATCTTACGAGCCGCAGGTA-3'	
GAPDH	5'- AATCCCATCACCATCTTCCA-3'	580
	5'- CCTGCTTCACCACCTTCTTG-3'	

Statistical analysis

The results were presented as mean ± standard deviation. The data were analyzed by SPSS software, version 16. Significant differences ($p < 0.01$) between groups were determined using unpaired Student's t-test.

RESULTS

Cytotoxic and optimum drug concentrations

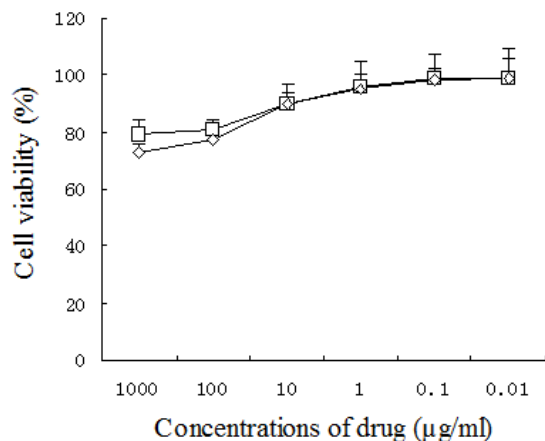
H9 were examined for cytotoxicity to Vero cells and for detection of drug concentration screening using Raji cells by MTT assay. Fifty percent toxic concentration (TC₅₀) of H9 was > 1000 ng/ml. To investigate the optimum drug concentrations for RT-PCR studies, Raji cells were incubated with H9 from 0.01 ng/ml to 1000 ng/ml. The data are shown in Fig 1.

Inhibitory effect of H9 on EBV RNA expression

Fig 2A reveal that the levels of EBV RNA decreased with elevation of H9 concentration, unlike the control group ($p < 0.05$).

Inhibitory effect of H9 on viral proteins

Fig 2B shows that the levels of early viral proteins decreased with increase in H9 dose, unlike the control group ($p < 0.05$). It also revealed that anti-EBV protein activity of H9 was dose-dependent.



Figures 1: Cytotoxicity (◇) and drug concentration (□) of H9 based on Vero and Raji cells.

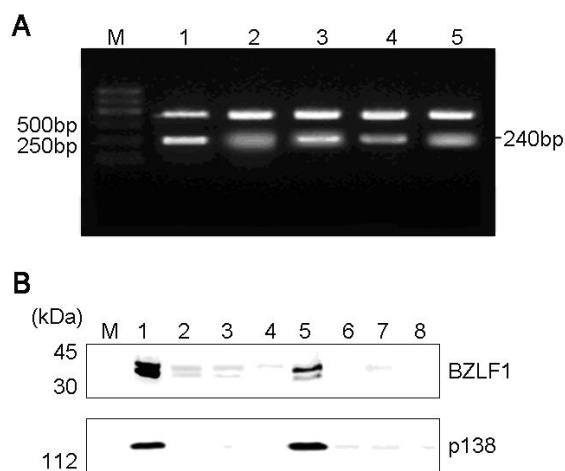


Figure 2: Effect of H9 on (A) EBV DNA replication (M = Marker; 1 = EB virus; 2 = ACV; 3 = 1 ng/mL H9; 4 = 5 ng/mL H9; 5 = 10 ng/mL H9), and (B) the levels of EBV viral immediate early protein and early protein (M = Marker; 1 = EB virus, 48 h; 2 = ACV, 48 h; 3 = 5 ng/mL H9, 48 h; 4 = 10 ng/mL H9, 48 h; 5 = EB virus, 72 h; 6 = ACV, 72 h; 7.5 ng/mL H9, 72 h; 8.10 ng/mL H9, 72 h)

Anti-HCMV activity

H9 inhibited the production of pp65 in HCMV-infected Helf cell culture supernatant in a dose-dependent manner. EC_{50} of H9 (0.46 ng/ml) was greater than that of the standard ganciclovir (GCV) group (0.68 ng/ml). As shown in Fig 3A, with increasing dose of H9, the numbers of helf cells treated with H9 gradually increased. Furthermore cytotoxicity was low.

With regard to the inhibitory effect of HCMV, Fig 3B shows that HCMV infection rate had no significant effect ($p < 0.05$) at 24 and 48 h, compared with control group. However, after 72 h, H9 inhibited HCMV spreading in helf cells in a

dose-dependent fashion, and reach a peak value of 60 % at 10 ng/mL.

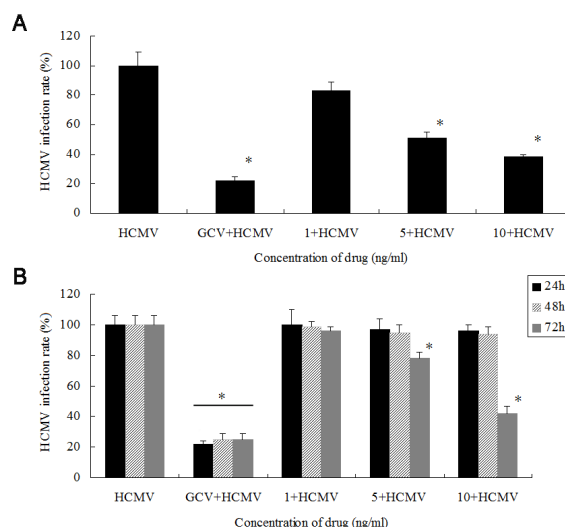


Figure 3: Effect of H9 on HCMV replication (A) and diffusion (B); * $p < 0.05$ compared with control

Anti-HSV-1 activity

As shown in Fig 4A, It is noteworthy that HSV-1 virus was effectively inhibited by H9 at 1.25 ng/ml.

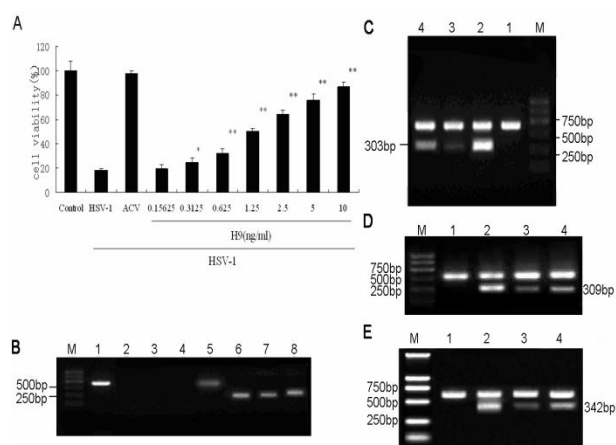
Inhibitory effect on HSV-1 genes replication

To determine if there was decrease in gene expression, we examined changes in steady-state gene mRNA level by RT-PCR. As shown in Fig 4, B-E, ACV and H9 (10ng/mL) significantly ($p < 0.05$, $p < 0.01$) decreased and delayed UL54, UL30 and US6 mRNA expression. Mock-treated control cells exhibited normal levels of UL54, UL30 and US6 mRNA expression. Primers specific for GAPDH were used to confirm that equal amounts of cDNA were used in each PCR.

DISCUSSION

Several parts of the chemokine system have been hijacked and wrecked by herpesviruses, including ligands and the receptors. vGPCRs are important for herpesviruses during viral life cycle to display different healing mechanisms. In a previous study, it was observed that there were significant differences in absorbance between H9-treatment and negative BSA-treatment groups, which is strongly suggests that H9 interacts with the US28 receptor. Moreover, after interaction with US28 receptor, the level of Ca^{2+} concentration induced by hMIP-1 β could be efficiently prevented by H9, which further demonstrates that H9 is an effective antagonist of US28 [9,10]. These results suggested that

anti-HCMV mechanism of H9 seems to involve binding initially with HCMV membrane.



Figures 4: Effect of H9 on HSV-1; (A) inhibitory effect of H9 on MTT; (B) expression of target genes in normal cells and cells infected by HSV-1 (M = marker; lanes 1 - 4 = normal Vero cells (1 = GAPDH; 2 = UL54; 3 = UL30; 4 = US6; lane 5 - 8 = HSV group; 5 = GAPDH, 6UL54; 7 = UL30; 8 = US6); (C) effect of H9 on specific expression of HSV-1 UL54 mRNA (M = Marker; 1 = Vero cells; 2 = HSV-1; 3 = ACV; 4 = H9); (D) effect of H9 on specific expression of HSV-1 UL30 mRNA (M = Marker; 1 = Vero cells; 2 + HSV-1; 3 = ACV; 4 = H9); (E) effect of H9 on specific expression of HSV-1 US6 mRNA (M = marker; 1 = Vero cells; 2 = HSV-1; 3 = ACV; 4 = H9); * $p < 0.05$, ** $p < 0.01$ compared with control

Like ORF74 and BILF1, US28 displays constitutive signaling pathway through Gai and Gq as well as NFAT by CREB, which is important for the expression of viral genome, and specifically by stimulating immediate-early gene promoter [1, 17,18]. We hypothesize that H9 changed cellular signaling to influence the level of gene expression specifically, including early gene, by interfering with vGPCRs, resulting in inhibition of viral replication.

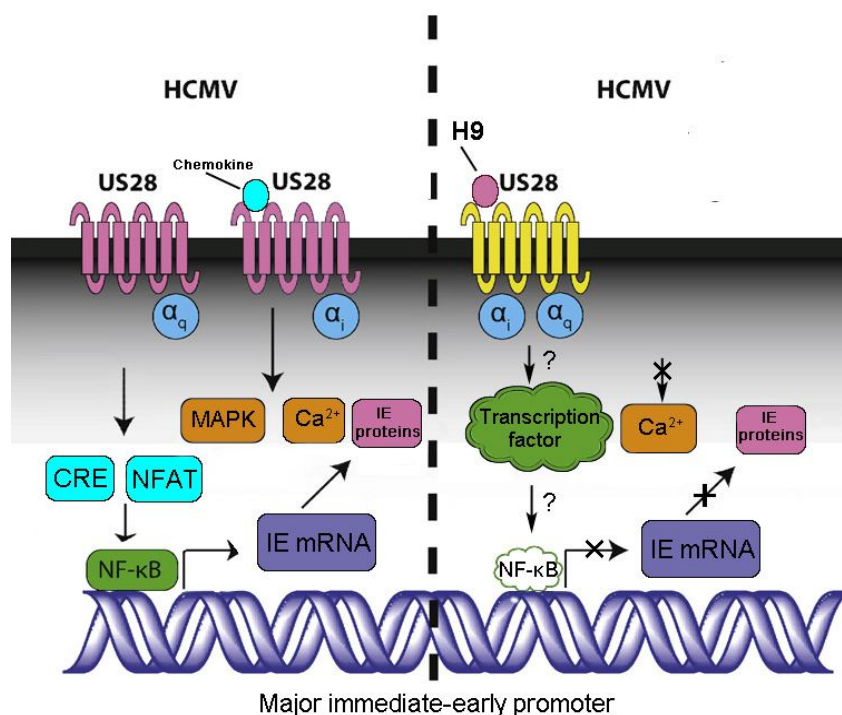
Small molecular peptides are widely expressed and gradually become the research focus. US28 receptor is highly homologous to many receptors encoded by viruses such as ORF74 and U12, which can interact with a wide variety of human chemokines, such as CC and CX3C chemokine. The initial purpose of the present study on the screening was to develop a small peptide acting as a broad spectrum chemokine antagonist, which combines with various chemokine receptors, and compare it with some small peptides that interact with human receptors. We had studied on acting as a broad spectrum chemokine antagonist, H9, which combines with various chemokine receptors, and displays inhibitory activity against various herpesviruses.

The experimental results showed that H9 possessed significant inhibitory effects on the three different herpesviruses. Inhibitory concentration (IC_{50}) values was 1.21 ng/mL (HSV-1). AD169 infection was strongly inhibited with an EC_{50} value of 0.46 ng/ml.

During virus infection and pathogenicity, viral replication plays a key role, and viral replication suppression is a critical feature to exploit novel drug. To gain insight into the underlying molecular mechanisms, we analyzed the ability of H9 to inhibit EBV viral DNA replication and gene expression by EBV protein-specific Western blots. This was confirmed by results obtained by detection of viral genomes by RT-PCR, and they indicated suppression of viral genome replication with an intermediate concentration of H9 (10 ng/mL). BILF1 encoded by EBV mainly take part in immune escape and inhibits the phosphorylation of RNA-dependent protein kinase (PKR) to help EBV by preventing acellular antiviral response [19]. vGPCRs displays constitutive signaling that is important for the regulation of viral genome, specifically by stimulating immediate-early gene promoter *in vitro* from infection cell. A major immediate-early promoter (MIEP) regulated IE gene expression and required productive viral replication as well as immediately early (IE) gene transcription [20]. Like anti-EBV activity, H9 could down-regulate the transcriptional expression of UL54, UL30 and US6, resulting in the inhibition HSV-1 infection. Based on these findings, it is proposed that H9 changes vGPCRs cellular pathway, like NF- κ B activity, and thus causes efficient replicative block at the level of viral gene expression, including specifically early gene, by interfering with vGPCRs (Fig 5). All of these results indicate that H9 may be a broad-spectrum therapeutic antagonist for herpesviruses.

CONCLUSION

H9 inhibited the infection of herpes simplex virus type 1 (HSV-1), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV), and demonstrated a broad-spectrum anti-herpesvirus effect *in vitro*. At the same time, the level of immediate-early gene expression was decreased, resulting in the inhibition of viral replication. These results demonstrate that H9 is an efficient broad-spectrum antiviral agent with activity against herpesvirus replication and genes expression including early genes.



Figures 5: Schematic illustration of the effect of US28 cellular signaling without or with H9 treatment, *in vitro*. From left to right: US28 displays constitutive signaling manner through G α_i and G α_q as well as NFAT by CREB which is important for the expression of the viral genome, specifically by stimulating the immediate-early gene promoter. H9 peptide may change cellular signaling to influence the level of immediate-early gene expression by interfering with vGPCRs, resulting in the inhibition of viral replication (Illustration is adapted from Slinger *et al* [21]).

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