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ORIGINAL ARTICLE

A trapping ligand antagonist peptide H22-LP inhibition of human cytomegalovirus infection

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Background: Human cytomegalovirus (HCMV) can cause acute or chronic diseases, especially in immunocompromised patients. Currently, most drugs licensed for the treatment of the herpes virus are nucleoside analogs that have been developed over the past 25 years. Drug resistance, development of drug related toxicity, and side effects limit their clinical use in patients. In a previous study, we found a trapping ligand H22-LP (the conservative sequence is NAHCALL) from a random phage library according to the broad-spectrum trapping receptor H22, which derived from the residue 14-35 near the N-terminal region of receptor US28 on HCMV. Here, the aim was to evaluate the anti-HCMV activity of H22-LP.
Methods: Antivirus activity of H22-LP on HCMV replication was visualized by fluorescence microscopy. We determined the effects of H22-LP on the expressions of HCMV late protein using q-PCR and Western blot. Comprehensive analysis of the characteristics of H22LP-mediated inhibition of HCMV were quantitatively analyzed by flow cytometry.
Results: H22-LP showed a 65.4% inhibition of viral infection at a concentration of 10 ng, and 50% inhibition at concentrations of 5 ng. The levels of mRNA and proteins were also found to have decreased by H22-LP in a concentration-dependent manner. The mode of antiviral action is based on a block of viral entry cells during HCMV cell adsorption/entry.
Conclusion: These results demonstrated that H22-LP could inhibit HCMV by direct interaction with the viral particle.

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Introduction

Human cytomegalovirus (HCMV) is a ubiquitous, worldwide virus with a high clinical relevance. Infection with the virus can cause mild to severe diseases, especially in immunocompromised patients.[1] Herpes simplex virus fuses directly with the plasma membrane. During its life cycle, HCMV is first adsorbed in the cell membrane. After penetration of the adsorbed virus, the incoming nucleocapsids are transported to the nuclear pore where the DNA is released into the nucleus. Herpes viruses are dependent on the host cytoskeleton for efficient entry, replication, and egress. Herpes viruses have developed a variety of strategies [e.g., members of the herpes viruses encode Viral G-protein-coupled receptors (vGPCRs)] to evade the immune system, which refers to the communication between numerous virus genes and cells. vGPCRs that show homology to human chemokine receptors make significant contributions to the viral lifecycle and associated pathologies.[2] During infection, viral entry cells play a key role in viral spread. US28 encoded by HCMV is the best characterized 7TM/GPCR and is suggested to enhance cell to cell fusion. US28 apparently shares properties with the cellular chemokine receptors CCR5 and CXCR4, which behave as CD4-associated coreceptors for human immunodeficiency virus (HIV)-1 or HIV-2.[3,4] US28 can form multiple signaling pathways to activate the immediate early HCMV promoter, which generally leads to the regulation of transcription factors such as nuclear factor κB (NFκB).[5,6]

DNA polymerase has been extensively studied as a target for antiviral drugs. Currently, several drugs such as ganciclovir (GCV) and cidofovir (CDV) have been tested for their ability to inhibit herpes viruses with normal molecular mechanisms *in vitro*.[7,8] However, such antiviral therapy of HCMV-associated diseases is generally complicated by a number of clinical problems, such as drug resistance, development of drug related toxicity, and side effects.[1,9] A recent review focused on the targets, used or under investigation, for the treatment of HCMV infections.[1] Many herpes viruses probably recognize multiple entry receptors, any one of which may be sufficient for viral entry. Several drugs are known to exhibit anti-herpes virus activity during viral replication and show significant resistance to herpes viruses activity with novel molecular mechanisms by targeting attachment and entry (e.g., by heparin), virion fusion (e.g., by Roscovitine).[8] These examples support the hypothesis that inhibitors that target and disrupt the lipid interfaces mediating viral entry can be developed as broad-spectrum antivirals.[9]

In previous studies, antiviral strategies have been targeted at inhibiting or killing the herpes virus based on the expression of vGPCR. A peptide (sequences NAHCALL, known as H22-LP), based on the broad-spectrum chemokine binding activity of US28, was designed to act as an analogous site of vGPCR of the herpes virus, which was selected from the N-terminal active site and the transmembrane domain of US28 (H22), referred to as the trapping receptor.[10,11] In this report, we present the discovery of the H22-LP antiviral that shows activity against HCMV.

Methods

Cells and viruses

Human embryonic lung fibroblast (HELF) cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS). The Towne strain of HCMV containing the green fluorescent protein (GFP) expression cassette was propagated in HELF cells. The 50% tissue culture infectious dose ($TCID_{50}$) measurements were done in triplicates using eight serial four-fold dilutions of each HCMV. For each dilution, 100 mL of diluted HCMV was added to every well of a 96-well culture plate and then trypsinized HELF cells were added. Plates were incubated at 37°C, 5% CO_2 for 48 hours. An endpoint virus titer of $TCID_{50}$ was calculated by the Spearman-Karber method.[12] $TCID_{50}$ was $10^{-4.67}$/100 μL for HCMV.

Cytotoxic assay

The cytotoxicity of H22-LP was determined by the MTT method. Briefly, HELF cells were incubated in 96-well plates. After a period of incubation, MTT solution was added to each well. Subsequently, cells were incubated for 4 hours at 37°C then DMSO was added to each well. Samples were measured at 590 nm in a microplate reader.

Antiviral effect assay of H22-LP against HCMV

To test the antiviral effect of H22-LP against HCMV, a strain of virus containing green fluorescent protein (GFP) was used.[13] Briefly, HFLF cells (3.5×10^5 cells/well) were seeded in 24-well plates 24 hours prior to infection. HELF cells were washed with 1× DPBS and then treated with mixture of 200 $TCID_{50}$ of Towne-GFP strain of HCMV (20 μL/well) and H22-LP (100 μL/well) in 24-well plates, which was immediately added to the mixture without incubation. The total volume was 200 μL/well. After infection, the virus was removed and Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 mg/mL), and GlutaMAX (2 mM) were added to each well and cells were incubated at 37°C for 48 hours. GFP-positive cells were visualized 48 hours postinfection by fluorescence microscopy and then quantified using flow cytometry.

Quantitative real-time PCR

H22-LP-treated and GCV-treated groups were prepared as follows. HFLF cells (1×10^6 cells/well) were seeded in 24-well plates 24 hours prior to infection. HELF cells were washed with 1× DPBS and then treated with mixture of 200 $TCID_{50}$ of Towne-GFP strain of HCMV (20 μL/well) and H22-LP (100 μL/well), which was immediately added to the mixture without incubation. The cells were treated with the indicated concentrations ranging from 5 ng to 25 ng of H22-LP for 7 days, 9 days, and 11 days (4 days following the end of exposure to the drug) after infection and were harvested for reverse transcription-polymerase chain reaction (RT-PCR) analysis. In a further trial, the cells were

treated with concentrations ranging from 50 μm to 150 μm of GCV in the same conditions after infection.[14–16]

To analyze the viral RNA synthesis, a quantitative real-time RT-PCR (qRTPCR) was conducted to determine the expression level of the HCMV *UL83* gene. Total RNA was prepared using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed as follows: 11.5 μL total RNA and 2 μL Oligo(dT)$_{15}$ were added to the tube. The mixture was heated to 70°C for 5 minutes and immediately transferred to an ice bath for 5 minutes. Thereafter, 4 μL 5× RT reaction buffer, 0.5 μL 400 U/μL RNase inhibitor, and 1 μL Moloney murine leukemia virus reverse transcriptase (M-MLV) were added. The tubes were incubated at 42°C for 60 minutes and 95°C for 10 minutes. Primers and probes were designed for the *UL83* gene of HCMV using Primer Express (Applied Biosystems, Foster City, CA, USA); forward primer (5-GTC AGCGTTCGTGTTTCCCA-3), reverse primer (GGGACACAACA CCGTAAAGC), and probe (CCGGCCCTCGGTTCTCTGCTG). Primers were manufactured by Qiagen (Qiagen, Valencia, CA, USA), and FAM/TAMRA-labeled probes by Biosearch Technologies (Novato, CA, USA). RT-PCR was performed on the ABI 7300 Sequence Detection System using the SYBR Green Kit (Invitrogen) under the following conditions: 2 minutes at 50°C, 3 minutes at 95°C, followed by 10 cycles of 45 seconds at 95°C and 55 seconds at 60°C. After completion of the PCR amplification, the relative fold change was calculated based on the $2^{-\Delta\Delta Ct}$ method[17] (where Ct is cycle threshold).

Western blot

HFLF cells (1 × 10^5 cells/well) were seeded in a 24-well plate 24 hours prior to infection. HELF cells were washed with 1× DPBS and then treated with a mixture of 200 TCID$_{50}$ of Towne-GFP strain of HCMV (20 μL/well) and H22-LP (100 μL/well), which was immediately added to the mixture without incubation. The cells were treated with the indicated concentrations ranging from 5 ng to 25 ng of H22-LP for 7 days after infection and were harvested for Western blotting analysis with the antibody against the late protein (p65). In a further trial, the cells were treated with concentrations ranging from 50 μm to 150 μm of GCV in the same conditions following infection.

Cell samples were lysed in a radio-immunoprecipitation assay buffer, and proteins were measured by the Bradford assay. Cultures of the fibroblasts were harvested for Western blot analysis using a Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were mixed with 2× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer [125 mM Tris (pH 7.0), 20% glycerol, 10% -mercaptoethanol, 6% SDS, and 0.2% bromophenol blue], boiled for 5 minutes, and were then separated by PAGE on 12% acrylamide gels. After blotting on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) and blocking for 2 hours with 5% nonfat dry milk in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween20, the membranes were probed with one of the following primary antibodies: anti-p65 antibody (1:1000 dilution, Santa Cruz, Santa Cruz Biotechnology, CA, USA), anti-β-actin antibody (Millipore, Bedford, MA, USA). Secondary antibodies used included goat anti-mouse or -rabbit horseradish peroxidase-conjugated (SouthernBiotech, Birmingham, AL, USA). Antibody-tagged protein bands on the probed membranes were detected using diaminobenzidine peroxidase substrate kit (Vector, Burlingame, CA, USA).[18]

Action mode of the peptides to HCMV

To determine the possible inactivated mechanism of H22-LP, a series of experiments containing four different treatment groups were designed for comparison with the antiviral effect as follows. (1) H22-LP pretreated group. HELF cells (3.5 × 10^5 cells/well) were pretreated with H22-LP (10 ng/mL; 100 μL/well) at 37°C for 120 minutes in 24-well plates and then 200 TCID$_{50}$ of HCMV (20 μL/well) were incubated. The total volume was 200 μL/well. (2) Infection first group. HELF cells (3.5 × 10^5 cells/well) were infected with HCMV (20 μL/well) at 37°C for 120 minutes in 24-well plates and then the infected cells were treated with H22-LP (100 μL/well). The total volume was 200 μL/well. (3) H22-LP+HCMV mixture group. HELF cells (3.5 × 10^5 cells/well) were treated with a mixture of HCMV (20 μL/well) and H22-LP (100 μL/well) in 24-well plates, which was immediately added to the mixture without incubation. The total volume was 200 μL/well. (4) H22-LP+HCMV preincubation group. HCMV (20 μL/well) and H22-LP (100 μL/well) preincubated at 37°C for 120 minutes, then the mixture was added to the HELF cells (3.5 × 10^5 cells/well) in 24-well plates.[19] Cells were measured by GFP assay after 48 hours incubation, as described above, and the inhibitory rate was quantified.

Next, to determine the interaction time of the infection, three other treatment groups were designed for comparison with the antiviral effect as follows. (1) Post group. HELF cells (3.5 × 10^5 cells/well) were incubated with HCMV (20 μL/well) for 120 minutes, then H22-LP (100 μL/well) was added immediately after the adsorption phase of the virus. (2) Pre-post, HELF cells (3.5 × 10^5 cells/well) were treated with a mixture of HCMV (20 μL/well) and H22-LP (100 μL/well) in 24-well plates, but which was immediately added to the mixture without incubation. (3) Pre group, HELF cells (3.5 × 10^5 cells/well) were pretreated with the mixture of H22-LP (100 μL/well) and HCMV (20 μL/well) at 37°C for 120 minutes, during which H22-LP was exclusively preincubated on cells and removed after virus adsorption.[20] Cells were measured by GFP assay following 48 hours of incubation, as described above, and the inhibitory rate was quantified.

Statistical analysis

Data were presented as mean ± SD (standard deviation). Multiple comparisons were statistically analyzed using SAS software (statistica analysis system, SAS Institute INC., NC, USA) version 8.0. Significant differences ($p < 0.05$) between groups were determined using unpaired Student *t* test.

Results

H22-LP acted sensitively and rapidly against HCMV

To confirm whether anti-HCMV activity of H22-LP was dependent on dose, several doses of H22-LP anti-HCMV

activity were measured according to the number of GFP positive cells. The cytotoxic assay has been described by Liu et al.[11] As shown in Fig. 1A, the antiviral-activity of H22-LP against HCMV was dose-dependent and an inhibition of 50% was achieved at a concentration of 5 ng/mL. Up to 85.15% inhibition was achieved when the concentration rose to 50 ng/mL. To determine the effective time needed for H22-LP to act on HCMV, 10 ng/mL H22-LP was incubated with 200 TCID50 of HCMV for 10 minutes, 30 minutes, 60 minutes, 120 minutes and 240 minutes, respectively. As shown in Fig. 1B, with increasing time, higher inhibition was observed. However, there was no significant difference between the 120 minute treatment group and the 240 minute treatment group. Yet, inhibition was as high as 47.5% for the 10 minute treatment group. Therefore, the time taken for H22-LP to act on HCMV is extremely short with activity eventually peaking at 120 minutes. These results show that the anti-HCMV activity of H22-LP is rapid.

Inhibition of HCMV replication and cellular gene expression

After treatment with H22-LP, HELF cells were harvested for RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B). The levels of HCMV DNA were measured for the control group, GCV group, and H22-LP group, respectively. After treatment, the levels of HCMV DNA decreased with elevation of H22-LP compared to the control group in a concentration dependent manner ($p < 0.05$). As shown in Fig. 2A, the inhibition effect of H22 LP-treated cultures reached a peak value of 88.3% at 25 ng on Day 9 and kept to approximately 54% at Day 11 (4 days after the end of exposure to the drug). Interestingly, the inhibition rates of H22-LP on HCMV DNA at Day 11were lower than those observed on Day 9. However, they were higher than those on Day 7. At the same time, the inhibition values of GCV for HCMV RNA declined and were lower than the values on Day 7. Next, to determine if the decrease in protein expression was regulated at the protein level, we examined the changes in steady-state late protein (p65) levels by Western blot analysis. As shown in Fig. 2B, the levels of protein were reduced with elevation in H22-LP and GCV concentrations. The levels of protein expression in H22-LP treatment groups were higher than the levels of protein expression in GCV treatment groups at Day 7. The antiviral effect analyzed by Western blot was consistent with the antiviral effect analyzed by RT-PCR at Day 7. These results indicate that the anti-HCMV protein activity of H22-LP is concentration-dependent and may have a persistent effect on suppressing HCMV.

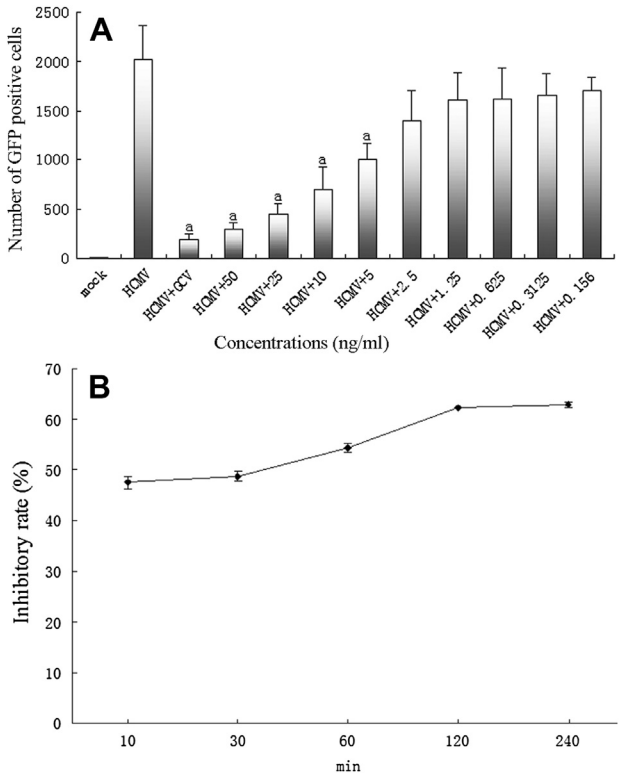

Figure 1. Dose- and time-dependent effects of H22-LP against human cytomegalovirus (HCMV). (A) Dose-dependent effect of H22-LP. *$p < 0.05$ versus the corresponding HCMV group and (B) time-dependent effect of H22-LP.

Characterization of H22LP-mediated inhibition of HCMV replication

HELF cells were varied in seven different groups as described in the Methods section. As shown in Fig. 3A, H22-LP (10 ng/mL) incubation of HCMV for 120 minutes prior to inoculation of HELF cells (H22-LP+HCMV preincubation group) showed a prominent inhibitory effect. A mixture of HCMV and H22-LP (10 ng/mL), which were not preincubated (H22-LP+HCMV), also showed significant inhibition. Because of the same treatment conditions, a similar antiviral effect of H22-LP (10 ng/mL) was demonstrated (Fig. 1A). However, the pretreated group and infection first group did not show significant antiviral effect (Fig. 3A). Importantly, the pre-group, in which H22-LP (10 ng/mL) was exclusively preincubated on cells and removed after the virus adsorption, and the pre-post group, in which H22-LP (10 ng/mL) was pretreated with HCMV prior to inoculation, were both essential to obtain the antiviral effect. However, no significant difference was observed in the post group when H22-LP (10 ng/mL) was added to the mixture of HCMV and cells following virus adsorption. By contrast, when GCV was exclusively preincubated on cells and removed after virus pre-adsorption, no inhibition was observed (Fig. 3B). The results of these treatments using H22-LP (10 ng/mL) indicates that HCMV cell adsorption/entry is inhibited by H22-LP.

Discussion

Currently, nucleoside analogs play an important role in the therapy of HCMV. However, some disadvantages of these agents include side effects, drug resistance, and expense, which limit their clinical use in HCMV-infected patients. However, an important factor to be taken into account when considering anti-virus therapies is that H22-LP, as a small molecule peptide, binds to virus encoded chemokine receptors but does not initiate cytoplasmic signal

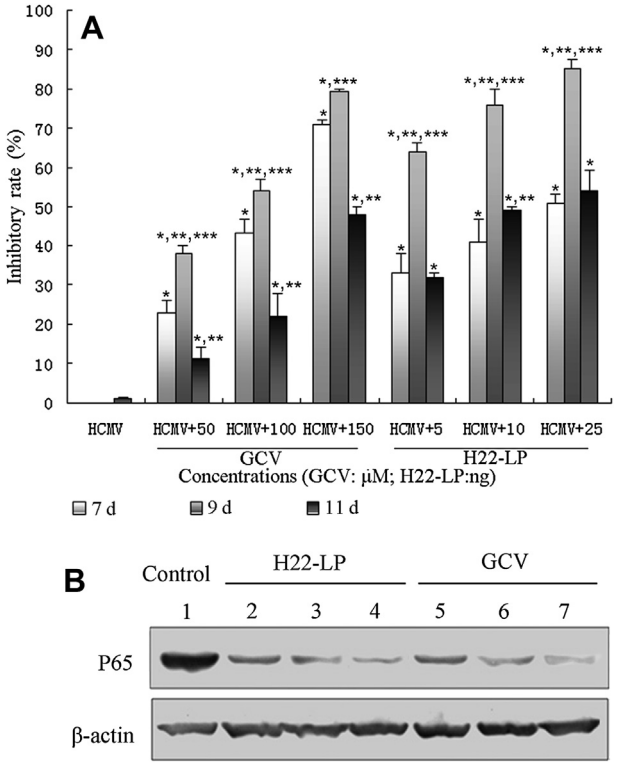

Figure 2. The inhibitor effect on the levels of mRNA and proteins in GCV groups and H22-LP groups at different concentrations. (A) The inhibitor effect on the levels of mRNA. (B) The inhibitor effect on the levels of proteins. β-Actin was used as the internal reference. Lane 1: negative control; lane 2: H22-LP (5 ng); lane 3: H22-LP (10 ng); lane 4: H22-LP (25 ng); lane 5: GCV (50 μM); lane 6: GCV (100 μM); and lane7: GCV (150 μM). *$p < 0.05$ versus the corresponding negative control. **$p < 0.05$ versus the corresponding 7 days outcome of the same dose. ***$p < 0.05$ versus the corresponding 11 days outcome of the same dose.

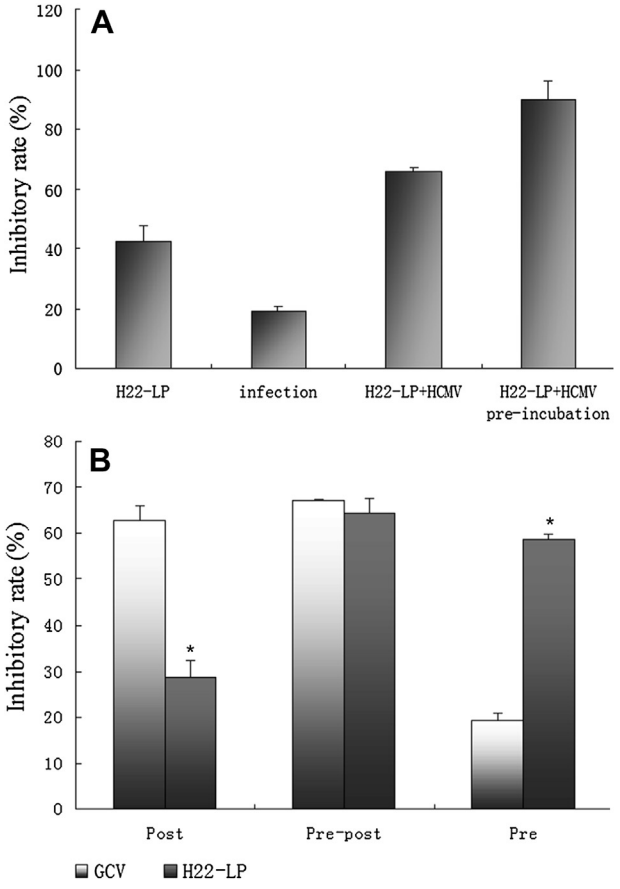

Figure 3. The mechanism of H22-LP against HCMV. (A) (i) H22-LP pretreated group: HELF cells were pretreated with 10 ng/mL H22-LP at 37°C for 120 minutes prior to infection; (ii) infection first group: HELF cells were infected with HCMV at 37°C for 120 minutes and then treated with 10 ng/mL H22-LP; (iii) H22-LP+HCMV mixture group: HELF cells were treated with mixture, without incubation, of HCMV and 10 ng/mL H22-LP; (iv) H22-LP+HCMV preincubation group: HCMV and 10 ng/mL peptides preincubated at 37°C for 120 minutes, then the mixture was added to the HELF cells. H22-LP directly targeted HCMV. (B) H22-LP inhibits HCMV cell adsorption/entry. (i) Post group: HELF cells were incubated with HCMV for 120 minutes, then 10 ng/mL H22-LP was immediately added after the adsorption phase of the virus; (ii) pre-post group: HELF cells were pretreated with 10 ng/mL H22-LP at 37°C prior to infection; (iii) Pre group: HELF cells were pretreated with the mixture of H22-LP and HCMV at 37°C for 120 minutes, when H22-LP was exclusively preincubated on cells and removed after virus adsorption. *$p < 0.05$ versus the GCV groups.

transduction cascades. The N-terminal region of a chemokine, which initiates a series of signal cascades by binding to receptors, can therefore be considered as a candidate site of antagonists. Besides CCR5 and CXCR4, several chemokine receptors and related orphan GCRs have been found to mediate CD4-dependent HCMV entry, with various efficacies.[21,22] US28 can also enhance cell—cell fusion by a mechanism apparently distinct from HIV coreceptor activity. Thus, a small molecule peptide H22-LP was designed, which derived from the US28 N-terminal region of HCMV.[10,11]

Many parts of the chemokine system have been hijacked and wrecked by herpes viruses, including the ligands and the receptors. A great number of chemokine receptor antagonists have been applied to animal experiments and clinical studies in recent years.[23] In a previous study, H22-LP was found to bind to the US28 receptor on NIH/3T3 cells, with an emphasis on H22-LP being an effective antagonist for US28. This agrees with previous findings that CC chemokines can increase the intracellular concentration of Ca^{2+}.[10,11]

In this study, we examined the activities of H22-LP against HCMV *in vitro* and the possible antiviral mechanism using the Towne strain of HCMV, which proved to be useful and reliable for the determination of the HCMV lytic replication. The experimental results showed H22-LP provided significant inhibitory effects to HCMV. The activity of H22-LP against HCMV was dose dependent with an inhibition rate of 50% achieved at a concentration of 5 ng/mL. Compared with the 10 minute treatment group and 60 minute treatment group, no significant difference was observed between the 120 minute treatment group and 240 minute treatment group. The results show that the anti-

HCMV activity of H22-LP is rapid and the possible antiviral mechanism is similar to that of mucroporin, which is a host defense peptide of bacteria. Interaction time between adsorption and membrane fusion continues to be a current focus of research.[24]

Herpes viruses engage multiple receptors during viral entry. Inhibition of the target and disruption of the lipid interfaces mediating virus—cell fusion could be developed as broad spectrum antivirals. As reported earlier, lysophosphatidylcholine can stabilize positive spontaneous curvature of membranes and prevent entry of several viruses[25]. We assumed that H22-LP may implement the inhibition of HCMV using a similar mechanism. Research using four different treatment groups employing H22-LP to HCMV, in which viral infectivity was inhibited only when H22-LP was able to interact directly with HCMV, provided us with a clue indicating H22-LP may exert its antiviral activity by damaging the HCMV virus particle itself. We, therefore, set up three different treatment groups using H22-LP to test this assumption. When H22-LP and GCV were added to the infected cells after the adsorption phase (post group), the antiviral effect of H22-LP was lower than the antiviral effect of GCV. As a nucleoside analog that is activated by phosphorylation, GCV can inhibit the integration of dGTP by viral DNA polymerase, resulting in the termination of the elongation of viral DNA.[7] Thus, although H22-LP and GCV were removed following virus adsorption (pre group), the inhibition values of H22-LP remained around 41.3%, which was higher than the inhibition values of GCV. The results show that the time needed for H22-LP to act on HCMV is short and HCMV cell adsorption/entry is inhibited by H22-LP. In the experiments examining the effective action time of H22-LP on HCMV, it was observed that 10 minutes of interaction allowed H22-LP to show almost maximum inhibition of HCMV (Fig. 2B). We may deduce the active principles as follows: once H22-LP encounters HCMV, H22-LP binds to US28 of HCMV directly and immediately. The attached H22-LP aggregates and inserts into viral entry receptors so that US28 is unable to bind with other receptors, which then inhibits the triggering events for viral entry. In summary, we have described a small molecule inhibitor that prevents enveloped virus entry. However, further research will be required to clarify our findings with regard to whether H22-LP can inhibit virus entry between virus binding and virus—cell fusion.

These results demonstrate that H22-LP exhibited resistance to HCMV *in vitro* and could inhibit HCMV by direct interaction with the viral particle.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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