A Cationic TAT Peptide Inhibits Herpes Simplex Virus Type 1 Infection of Human Corneal Epithelial Cells

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Abstract

Purpose: To determine if a peptide, TAT-Cd⁰, inhibits Herpes simplex virus type 1 infection of human corneal epithelial cells.

Methods: TAT-Cd⁰ and a control peptide, E_{50,51}TAT-Cd⁰, were added at various times throughout infection with the *lacZ*-expressing hrR3 virus, and viral replication was measured by β -galactosidase activity. Toxicity was assessed using a dye reduction assay.

Results: The CC₅₀ value for TAT-Cd⁰ was $\sim 100 \mu\text{M}$. In assays with peptide present at all times, TAT-Cd⁰ was 150-fold more active than E_{50,51}TAT-Cd⁰ (EC₅₀ 0.2 vs. 30.0 μM). The EC₅₀ values of TAT-Cd⁰ for entry inhibition, cell protection, virus inactivation, and inhibition of attachment were 0.1, 0.4, 9.5, and 3.0 μM , respectively. TAT-Cd⁰ was less effective when added 1 h postinfection (EC₅₀ = 30.0 μM).

Conclusions: TAT-Cd⁰ is an effective inhibitor of Herpes simplex virus type 1 infection in human corneal epithelial cells and affects multiple steps before, or very early, in infection. The peptide has potential as an antiviral and further studies are warranted.

Introduction

HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) is a significant human pathogen, infecting 70%–90% of the population.¹ An estimated 400,000 people in the United States suffer from HSV ocular infection, with 50,000 new and recurrent cases each year. HSV keratitis is the leading cause of blindness due to infection in developed countries.^{2,3} On the basis of the Herpetic Eye Disease trial, the current recommended therapy is topical corticosteroid with a prophylactic antiviral.⁴ In addition, prophylaxis with oral acyclovir reduces the rate of recurrence.⁵ Although these therapies are effective, they are not always successful and there are problems associated with the use of steroids.⁴ As a result, there is a need for additional therapeutic approaches.

HSV rapidly establishes a latent infection, and this presents significant problems in dealing with the virus. Reactivation can lead to recrudescence, increasing the damage to the cornea with each event. Currently available antivirals can reduce the establishment of latency but only when added within the first few hours of the primary infection, which is difficult to achieve in practice.^{6,7} Therapeutic strategies that prevent the primary infection or eliminate latent virus would be useful.

We have previously identified several peptides that are broad-spectrum inhibitors of virus infection. These peptides act to either inhibit attachment of virus to cells or inhibit entry at low micromolar concentrations.^{8–13} In addition, we have shown that these peptides have little, if any, toxicity for a number of cell types, including a human trabecular meshwork cell line and primary keratocyte cultures at antiviral concentrations.¹⁴ Moreover, they were not toxic when applied topically to mouse corneas at concentrations up to 1,000 times the EC₅₀ values.¹⁴ These studies raise the possibility that these antiviral peptides may be therapeutically useful for ocular HSV infections. One of our cationic peptides, TAT-Cd⁰, is particularly attractive as a candidate as it contains only 11 residues and is highly soluble and stable in aqueous solutions.¹¹

In another study testing the *in vivo* efficacy of a modified cationic θ -defensin, RC-2, we found that the peptide had modest activity.¹⁵ One potential explanation for the modest efficacy is that θ -defensin and other cationic antivirals may have had reduced antiviral activity in corneal epithelial cells. Therefore, we believed that it was necessary to show that the TAT-C peptide was an effective antiviral in corneal epithelial cells before conducting studies in animal models of ocular HSV infection. The goals of this study were to determine if

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the TAT-Cd⁰ peptide was toxic for a corneal epithelial cell line and to confirm that the peptide acts to inhibit the entry of HSV-1 into these cells.

Methods

Cells and viruses

An immortalized human corneal epithelial (HCE) cell line¹⁶ was grown in 50% Dulbecco's modified Eagle's medium: 50% F12 medium supplemented with 5% fetal bovine serum, 5% calf serum, penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (2.5 µg/mL). Assays were done with HEPES buffered medium to which no fungicide or antibiotic was added (S+, medium with serum; S-, medium without serum). S+ and S- Dulbecco's modified Eagle's medium without carbonate buffer was used if the plate was incubated at room temperature, at 4°C, or at 37°C in the absence of CO₂ (Attachment, entry blocking and virus inactivation assays). High titer stocks of the HSV-1 KOS mutant hrR3, which expresses *Escherichia coli* β-galactosidase from the early ICP6 promoter,¹⁷ were prepared in Vero cells¹⁸ and further purified on sucrose gradients as described previously.¹⁹

Peptide synthesis and analysis

The TAT-Cd⁰ and the E_{50,51}TAT-Cd⁰ (2 glutamic acid residues substituted for lysine at positions 50 and 51) peptides were synthesized in the Peptide Synthesis Facility at the University of Wisconsin-Madison Biotechnology Center (>95% purity) as described previously.^{8,9,11} Peptide concentrations were determined by measuring absorbance at 215 and 225 nm and are reported as the monomeric peptide concentration.²⁰

MTS assay

Peptide cytotoxicity was determined using a commercially available assay (Celltiter 96[®] AQueous One Solution cell proliferation assay reagent, #3582; Promega) as described previously.¹⁴ Briefly, HCE cells (4.0 × 10⁴ cells/well) were seeded in a 96-well plate and incubated for 24 h at 37°C. After incubation, the medium was aspirated from each well and replaced with 40 µL of S+ medium and the plate incubated at 37°C. Fifty microliters of S+ medium with the desired concentration of peptide was added to each well. Control wells received the medium only. The plate was incubated at 37°C for 6 h. After incubation, the cells were examined visually for cytotoxicity and refed with 100 µL S+ medium and 20 µL of the 96 AQueous One Solution cell proliferation assay reagent per well. The plate was then incubated for 1.5 h at 37°C, and at the end of incubation 25 µL of lysis buffer (2.5% IGEPAL CA-630; Sigma, #I3021) was added to each well. Absorbance at 490 nm was determined using a 96-well plate reader (Biotek Instruments, Inc.).

Antiviral assays

HCE cells (2 × 10⁴ cells/well), seeded in 96-well plates and incubated for 2 days at 37°C, were infected with hrR3 at a multiplicity of infection (MOI) of 0.5. All rinses with S+ medium, 1 × phosphate-buffered saline (PBS), and citrate buffer treatment used 100 µL/well. At 6 h postinfection, β-galactosidase activity was measured by ELISA as we de-

scribed previously.¹² After lysing the cells with 0.5% NP-40 (Sigma Chemical Co.), 2 mM MgCl₂ in PBS (50 µL/well) 10 mM Chlorophenol Red-β-D-galactopyranoside, 50 µL/well (Roche Applied Science) was added to each well and the absorbance was measured at 570 nm in a 96-well plate reader (Biotek Instruments). All assays were done in duplicate or triplicate. Note that the peptides tested were oxidized and therefore dimers. We have previously shown that dimerization improves the antiviral activity.¹¹

Comprehensive antiviral assay. Various concentrations of the TAT-Cd⁰ or the E_{50,51}TAT-Cd⁰ peptides were mixed with virus (1 × 10⁶ PFU/mL) in 70 µL of S+ medium and incubated for 1 h at 37°C. HCE cells in 96-well plates were treated with various concentrations of peptide (40 µL/well) and incubated for 1 h at 37°C. After incubation, the medium was aspirated from the plate, the virus/peptide mixture was added (40 µL/well), and the plate was incubated at 37°C. At 6 h postinfection, β-galactosidase activity was measured as described above.

Virus inactivation assay. Twenty-five microliters of virus (1 × 10⁶ PFU/mL) was mixed with various concentrations of peptide in 25 µL of S- medium and incubated for 1 h at 37°C. The virus/peptide mixture (40 µL) was then added to 1 mL of ice-cold S+ medium and spun at 13,200 RPM in an Eppendorf 5415 microcentrifuge for 90 min at 4°C. The supernatant was aspirated and the pellet was resuspended in 200 µL of ice-cold S+ medium. Virus was resuspended by sonication using 10 pulses from a microtip at 30% duty cycle (Branson SLPe cell disruptor; Branson Corporation). Fifty microliters of the sonicated solution was diluted in 150 µL of S+ medium and 40 µL of the dilution was applied to a 96-well plate seeded with HCE cells. The plate was incubated at 37°C for 1 h, the cells were washed with 1 × PBS, and extracellular virus was inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The cells were rinsed with 1 × PBS, fed with S+ medium, and incubated at 37°C. The β-galactosidase activity was measured 6 h later.

Cell protection assay. HCE cells were treated with various concentrations of peptide (40 µL/well) and incubated at 37°C for 1 h. The cells were then washed with S+ medium, infected with 40 µL/well of hrR3 (MOI 0.5), and incubated at 37°C. After 1 h, the cells were rinsed with S+ and washed with 1 × PBS, and any remaining extracellular virus was inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The infected cells were rinsed again with 1 × PBS, fed with S+ medium, and placed in a 37°C incubator, and the β-galactosidase activity was measured 6 h later.

Entry inhibition assay. HCE cells were fed with S+ medium and precooled at 4°C for 30 min. The medium was removed, and hrR3 (40 µL/well, MOI 0.5) was allowed to attach to cells at 4°C. After 1 h, cells were rinsed with S+ medium. Various concentrations of peptide were then added (40 µL/well) and the cultures were incubated at 4°C for 15 min before shifting them to 37°C. After 1 h, the cells were washed with 1 × PBS and extracellular virus was inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The infected cells were rinsed with 1 × PBS, fed with S+ medium, incubated at 37°C, and β-galactosidase activity was measured 6 h later.

Attachment assay. HCE cells were fed with S+ medium and precooled at 4°C for 30 min. The hrR3 virus (5×10^7 PFU/mL) was mixed with various concentrations of peptide in S+ and 40 μ L of the mixture was added to each well. The plate was then incubated at 4°C for 1 h. After rinsing with 100 μ L S–, the cells were fixed with 4% formalin in PBS (100 μ L/well) and left at room temperature for 1 h. The cells were washed 3 $\times$ with S– medium (100 μ L/well) and blocked for 1 h with 3% bovine serum albumin (BSA) in S– medium (100 μ L/well). Absorbed virus was detected with a 1:100 dilution of polyclonal rabbit anti-HSV-1 IgG (#B0014; Dako, 90 μ L per well) in 0.05% BSA S– medium. After 1 h incubation at room temperature, the cells were rinsed 3 $\times$ with 5 min washes of 0.05% BSA in S– medium (100 μ L/well). An alkaline phosphatase-conjugated goat-anti rabbit IgG (#A3687; Sigma-Aldrich) was diluted 1:2,000 in 0.05% BSA S– medium and 90 μ L was added per well. After 1 h incubation at room temperature, the cells were rinsed 3 $\times$ with 5 min washes of 0.05% BSA in S– medium (100 μ L/well). The medium was aspirated and an alkaline-phosphatase substrate (BCIP tablet dissolved in 10 mL ddH₂O, #B5655; Sigma) was added (50 μ L/well). Absorbance (405 nm) was read in a 96-well plate reader (Biotek Instruments, Inc.) for 20 min at 1 min intervals. The amount of binding was determined from the slope of the initial rate, and the signal from mock-infected cultures was subtracted from the data.

Post-entry assay

HCE wells were infected with hrR3 (40 μ L/well, MOI 0.5) and incubated at 37°C. After 1 h, cells were rinsed with S+ medium and washed with 1 $\times$ PBS, and extracellular virus was inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The infected cells were rinsed again with 1 $\times$ PBS, fed with S+ medium, and incubated at 37°C for 1 h. S+ medium was aspirated and various concentrations of peptide in S+ medium were then added (50 μ L/well) and the plate was incubated at 37°C. The β -galactosidase activity was measured 6 h later.

Results

Toxicity

To determine if TAT-Cd⁰ was toxic, HCE cells were incubated with peptide for 6 h and toxicity was measured using a dye reduction assay. As shown in Fig. 1, the CC₅₀ value was $\sim 100 \mu$ M, indicating little, if any, toxicity. This result was in the same range as other cell lines tested previously with TAT peptides.¹⁴

Comprehensive antiviral assay

Antiviral activity was first tested in a comprehensive assay where peptides were incubated with virus before addition of the cells and were present for the entire assay. The sequences and EC₅₀ values of the TAT-Cd⁰ and E_{50,51}TAT-Cd⁰ control peptides are shown in Table 1 and the data are shown in Fig. 2. The EC₅₀ value for the control peptide was $\sim 30 \mu$ M compared to 0.20 μ M for the TAT-Cd⁰ peptide. Thus, the antiviral activity was sequence specific and the peptide was effective in preventing the infection of HCE cells by HSV-1. On the basis of these results, the control peptide was not included in subsequent assays.

FIG. 1. MTS assay. The TAT-Cd⁰ peptide is not cytotoxic to HCE cells. Monolayers of HCE cells in a 96-well plate were treated with various concentrations of the peptides for 6 h at 37°C. After incubation, 20 μ L of Celltiter 96 AQ^{aqueous} One Solution cell proliferation assay reagent (Promega) was added into each well, and 90 min later, the absorbance at 490 nm was read in a 96-well plate reader. Each point and error bar represents the mean $\pm$ standard error of triplicate samples. HCE, human corneal epithelial.

TAT-Cd has multiple antiviral activities in HCE cells

Previous studies with TAT peptides showed that, depending on the peptide, they could be virucidal, inhibit entry, inhibit infection when cells were pretreated with the peptide, and interfere with attachment to cells. To determine if TAT-Cd⁰ was virucidal, virus was incubated with various concentrations of peptide for 1 h at 37°C and then added to cells. As shown in Fig. 3A and Table 2, TAT-Cd⁰ was virucidal with an EC₅₀ value of 9.5 μ M. Pretreatment of HCE cells with peptide followed by exposure to virus revealed that TAT-Cd⁰ could induce a state of resistance to infection (Fig. 3B and Table 2) with an EC₅₀ value of 0.4 μ M. To determine if TAT-Cd⁰ was a specific entry inhibitor, virions were attached to cells at 4°C, peptide was added, and the cells were shifted to 37°C. Viral titers were then assayed. As shown in Fig. 3C and Table 2, TAT-Cd⁰ blocked entry with an EC₅₀ value of 0.1 μ M. The TAT-Cd⁰ peptide also inhibited attachment of virus to cells (Fig. 3D and Table 2). At lower concentrations, there appeared to be a slight enhancement of attachment, but inhibition was evident at concentrations above 1.0 μ M. The EC₅₀ value for inhibition of attachment was $\sim 3.0 \mu$ M. When considered together, the results indicate that TAT-Cd⁰ has multiple activities that inhibit infection of HCE cells by HSV-1.

TAT-Cd does not act at a post-entry step

To confirm that the peptide acted at an early step in infection, cells were infected with virus and peptide was added at the end of the adsorption period (1 h after addition of virus). Figure 4 shows that the peptide was not effective when added after virus had entered cells. The EC₅₀ value in the post-entry assay was $\sim 30 \mu$ M, which was over 150-fold higher than the EC₅₀ for the comprehensive assay (Table 1), suggesting that TAT-Cd⁰ was predominantly acting before entry of virus into the cells.

TABLE 1. PEPTIDE SEQUENCES AND EC₅₀ VALUES OF THE TAT PEPTIDES IN THE COMPREHENSIVE ASSAY

Peptide	Sequence	EC ₅₀ (μM)
TAT-Cd ⁰	NH ₂ -GRKKRRQRRRC-amide	0.20
E _{50,51} TAT-Cd ⁰	NH ₂ -GREERRQRRRC-amide	~30.0

Numbers are means of the EC₅₀ values determined from the comprehensive antiviral assay.

Discussion

Cell-penetrating peptides and peptides derived from them are effective antivirals with broad-spectrum activity. These characteristics raise the possibility that such peptides might be useful therapeutic agents. The TAT-C peptides have not been tested for antiviral efficacy in animal models, and this is a necessary step if these peptides are to be useful in treating or preventing infections. Previous work with these peptides used Vero cells, fibroblasts, and MDCK cells to test antiviral activity, which is not necessarily representative of ocular cells that might be targeted *in vivo*.^{8-11,13} Before testing TAT-Cd⁰ peptide *in vivo*, it was necessary to show that the peptide was active in protecting corneal epithelial cells. The data presented here demonstrate that TAT-Cd⁰ inhibits infection of HCE cells and that the peptide acts in multiple ways early in infection, including inhibition of entry, protection of pre-treated cells, interference with attachment, and killing virions in solution (virucidal activity). The peptide affects multiple activities, and this might be an advantage compared to other antivirals that have a single target.

Previous studies using a number of other cell types¹⁴ indicated that CC₅₀ values for the TAT-C peptide were >400 μM. The CC₅₀ value of ~100 μM with HCE cells suggests that these cells may be more sensitive than other cell types for reasons that are not known. Nevertheless, the TAT-C peptide showed no evidence of toxicity when applied

topically to mouse corneas at concentrations >1,000 times the EC₅₀ value.¹⁴ Further, the activity of TAT-C peptides is not chiral¹¹ and that the presence of a charged (TAT-Cd⁻) or neutral (TAT-Cd⁰) carboxy-terminus has little, if any, effect on the antiviral activity of the peptide.¹¹ Thus, it is unlikely that differences in toxicity are due to the use of slightly different peptides.

In a previous study where TAT-Cd⁰ was tested using Vero cells, the EC₅₀ values for entry inhibition, cellular resistance, and virucidal activity were 0.9, 0.4, and 34 μM, respectively.¹¹ These values compare favorably with the EC₅₀ values of 0.1, 0.4, and 9.5 μM obtained with HCE cells for these activities. The observation that TAT peptides appear to have multiple activities raises questions as to which activity is most important and whether common or different mechanisms are involved. In both Vero and HCE cells, the EC₅₀ values for entry inhibition and cellular protection are very similar; thus, both of these activities may be equally important. The EC₅₀ values for virucidal activity are substantially higher, suggesting that this activity may be less critical, but the EC₅₀ values suggest that all of these activities would occur at therapeutically relevant concentrations.

In terms of mechanism of action, preliminary data suggest that TAT-Cd⁰ binds to sialic acid residues on the virus (unpublished data) and cellular uptake (cell penetration) involves binding to heparan sulfate on the cell surface.^{21,22} It is not surprising that the cationic TAT-Cd⁰ peptide would bind to negatively charged carbohydrates, but it is not clear how this might be relevant to the antiviral activities or whether this represents a common mechanism. Clearly, binding of TAT-Cd⁰ to one or more of the HSV-1 fusion complex proteins could affect its assembly or function, but additional studies are needed to address this. Such an activity could be responsible for both the entry inhibition and virucidal activity. Cellular proteins could also involve TAT-Cd⁰ binding to sialic acids on surface proteins such as gD, gB, and gH co-receptors,²³ but, again, further studies are needed. The binding of TAT-Cd⁰ to heparan sulfate is probably not involved, because we previously showed that TAT-C peptides do not interfere with HSV-1 binding.¹¹

The virucidal activity of TAT-Cd⁰ raises the possibility that the peptide could be used to prepare an inactivated virus vaccine. However, we previously showed that inactivation was reversible to a level of 1% when TAT-Cd⁰-treated virions were incubated overnight in the peptide-free medium. This might allow for some level of replication, which might not be acceptable. Whether TAT-Cd⁰ peptides are immunogenic has not been rigorously addressed, but TAT-coupled moieties have been used for delivery *in vivo* and there are no reports of TAT-specific immune responses to our knowledge.²⁴

Numerous antiviral peptides have been described in the literature, but few, if any, have been tested for efficacy in animal models. In a previous study, we tested a modified cationic θ-defensin (RC-2) in our murine model of HSV-1

FIG. 2. Comprehensive antiviral assay. The antiviral activity of TAT-Cd⁰ (●) and E_{50,51}TAT-Cd⁰ (○) in the comprehensive assay. Cells and virus were incubated with various concentrations of peptides for 1 h at 37°C. After incubation, the medium was replaced with virus/peptide mixtures and the cultures were incubated further. Six hours later, β-galactosidase activity was determined as a measure of infectivity. Each point and error bar represents the mean ± standard error of triplicate samples.

TABLE 2. EC_{50} VALUES (μM) OF TAT-Cd⁰ PEPTIDES IN VARIOUS ANTIVIRAL ASSAYS

Peptide	Entry block	Postentry	Cell protection	Virus inactivation	Attachment
TAT-Cd ⁰	0.1	~30.0	0.4	9.5	3.0

Numbers are means of the EC_{50} values determined for each of the assays.

FIG. 3. Antiviral assays. Antiviral activities of the TAT-Cd⁰ peptides. **(A)** Inactivation of virions in solution. Virus was incubated with various concentrations of peptide at 37°C for 1 h. The mixture was then spun for 90 min; the pellet was resuspended and sonicated and then applied to HCE cells in a 96-well plate. After 1 h of incubation at 37°C, the cells were washed with PBS, treated with citrate buffer (pH 3.0), washed with PBS, and refed with the medium. After 6 h at 37°C, β -galactosidase activity was measured. **(B)** Cell protection assay. HCE cells were treated with various concentrations of peptide and then infected with hrR3 (MOI 0.5) and incubated at 37°C for 1 h. The cells were then rinsed with the medium, washed with PBS, treated with citrate buffer (pH 3.0), washed with PBS, and refed with the medium. After 6 h at 37°C, β -galactosidase activity was measured. **(C)** Entry inhibition assay. HCE cells were precooled at 4°C for 30 min and hrR3 (MOI 0.5) was allowed to attach to cells at 4°C. After 1 h, cells were rinsed with S+ medium and various concentrations of peptide were then added. The cultures were incubated at 4°C for 15 min before shifted to 37°C. After 1 h, the cells were washed with PBS, treated with citrate buffer (pH 3.0), washed with PBS, and refed with the medium. After 6 h at 37°C, β -galactosidase activity was measured. **(D)** Viral attachment assay. HCE cells were cooled at 4°C for 1 h. The cultures were fixed with 4% formalin and blocked with 3% bovine serum albumin in the medium for 1 h at room temperature. Primary and secondary antibodies were polyclonal rabbit anti-Herpes simplex virus type 1 antibody (Dako) and goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma), respectively. Absorbance at 405 nm was read and recorded as percent of viral attachment. Each data point and error bar represents the mean $\pm$ standard error of duplicate or triplicate samples for all panels. MOI, multiplicity of infection; PBS, phosphate-buffered saline.

FIG. 4. TAT-Cd⁰ is not active post-infection. HCE wells were infected with hrR3 (MOI 0.5) and incubated at 37°C. After 1 h, cells were rinsed with the medium, washed with PBS, treated with citrate buffer (pH 3.0), washed with PBS, and refed with the medium for 1 h. Various concentrations of peptide were added and after 6 h at 37°C, β-galactosidase activity was measured. Each data point and error bar represents the mean ± standard error of duplicate or triplicate samples for all panels.

keratitis.¹⁵ The peptide showed modest efficacy when pre-incubated with peptide, but was not effective in reducing clinical scores when treatment was started 4 h postinfection, even though reductions in titer were seen in this treatment paradigm.¹⁵ The activity, or lack thereof, could be affected by the formulation of the test article or pharmacokinetic properties of the peptide in the cornea. We used 2% methylcellulose in PBS, which likely increased the residence time on the corneal surface, but may not have enhanced penetration into the cornea. Because the TAT-Cd⁰ peptide is also cationic, proceeding with *in vivo* efficacy testing might yield the same result seen with RC-2. Before animal work, studies, such as this one, as well as formulation and pharmacokinetic studies may be necessary to design the optimum test of efficacy.

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Author Disclosure Statement

No competing financial interests exist for Dr. Brandt or Ms. Larsen

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