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Characterization of a Proteolytically Stable D-Peptide That Suppresses Herpes Simplex Virus 1 Infection: Implications for the Development of Entry-Based Antiviral Therapy

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Uncontrolled herpes simplex virus 1 (HSV-1) infection can advance to serious conditions, including corneal blindness or fatal encephalitis. Here, we describe a highly potent anti-HSV-1 peptide (DG2) that inhibits HSV-1 entry into host cells and blocks all aspects of infection. Importantly, DG2 is highly resistant to proteases and shows minimal toxicity, paving the way for prophylactic or therapeutic application of the peptide *in vivo*.

Herpes simplex virus 1, a double-stranded DNA virus, infects the majority of the world's populations (1). HSV-1 infections can remain dormant (viral latency); however, reactivation from latency can cause various conditions, ranging from cold sores to critical herpes keratitis that may advance to corneal blindness (2, 3). HSV-1 is the leading infectious cause of corneal blindness worldwide (4). Additionally, HSV-1 infections of the central nervous system can lead to fatal encephalitis (5, 6). Therefore, treatment of HSV infections is a cardinal health care concern.

The first line of therapy for HSV-1 infection comprises primarily acyclovir and its pharmaceutical analogues (7); however, these suffer two major drawbacks: ineffectiveness in preventing emergence of drug-resistant strains and toxicity that also includes serious side effects (1, 8). Therefore, we decided to exploit recent knowledge of virus infection mechanisms and essential interactions with the host to develop more-effective anti-HSV-1 therapeutics. From this study, we report a novel highly effective D-peptide that targets HSV-1 entry and suppresses the infection while showing hydrolytic stability with respect to proteases. Thus, we describe a valuable tool for future clinical application and a significant step toward the development of an *in vivo* therapy for treatment of herpes infections. In addition, it will be a good reagent to study HSV-1 entry.

Results and methods. Virus infection is facilitated via binding of envelope glycoprotein gD to host cell receptors, such as 3-O-sulfated heparan sulfate (3-OS HS) (9, 10). Thus, we reasoned that targeting cell surface 3-O HS might provide a tool to prematurely block the infection by interference with a virus-host interaction essential for viral entry. Our preliminary work provided a proof of principle that targeting 3-OS HS can block entry and infection (11, 12). However, a serious obstacle to the *in vivo* application of such a treatment is the fact that peptides are easily hydrolyzed through the proteolytic and peptidolytic action of body proteases, making peptide therapy usually infeasible (13). Therefore, we reasoned that a D-peptide might be resistant to the action of proteases. Thus, using the procedure outlined in Fig. 1A, we designed the arginine-rich D-peptide as shown (Fig. 1B).

To investigate the proteolytic stability of DG2, and its effect on the HSV-1 infection-blocking activity, we incubated DG2, a scrambled control peptide, or an L peptide with a model protease, trypsin, followed by assessing the entry activity of the trypsin-digested peptides with human corneal epithelial (HCE) cells (9). After treating cells with the peptides for 1 h, β-galactosidase-expressing HSV-1 (KOS) gL86 was used to infect cells. Using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate, β-galactosidase activity was quantified as an indication of viral entry. We observed that the L peptide exerted entry-blocking activity which was abolished upon peptide digestion (Fig. 1C); however, interestingly, the DG2 peptide retained its full activity even after pretreatment with trypsin (Fig. 1D). To further characterize the protease stability of the peptide, we incubated DG2 and L peptide with trypsin for various digestion times (30 and 60 min) and determined the viral entry-inhibiting activity relative to the results seen with untreated cells (no peptide). We also found that DG2 peptide, in contrast to the L peptide, showed remarkable stability under all digestion durations (Fig. 1E). Furthermore, DG2 also could resist all concentrations of the protease (Fig. 1F). Taking the data together, we concluded that DG2 is a proteolytically stable peptide that may be suitable for future trials of antiherpetic therapy *in vivo*.

After establishing the proteolytic stability of DG2, we then sought to test the efficacy of DG2 in comparison to that of the L peptide. Cells were infected with HSV-1 gL86 after various concentrations of each peptide were incubated with cells for 1 h, and a viral-entry assay was performed as described above. We found that DG2 showed higher efficacy than the L peptide (Fig. 2A and B). The estimated in-culture 50% inhibitory concentration (IC_{50}) was also much lower than that of the L peptide (Fig. 2C).

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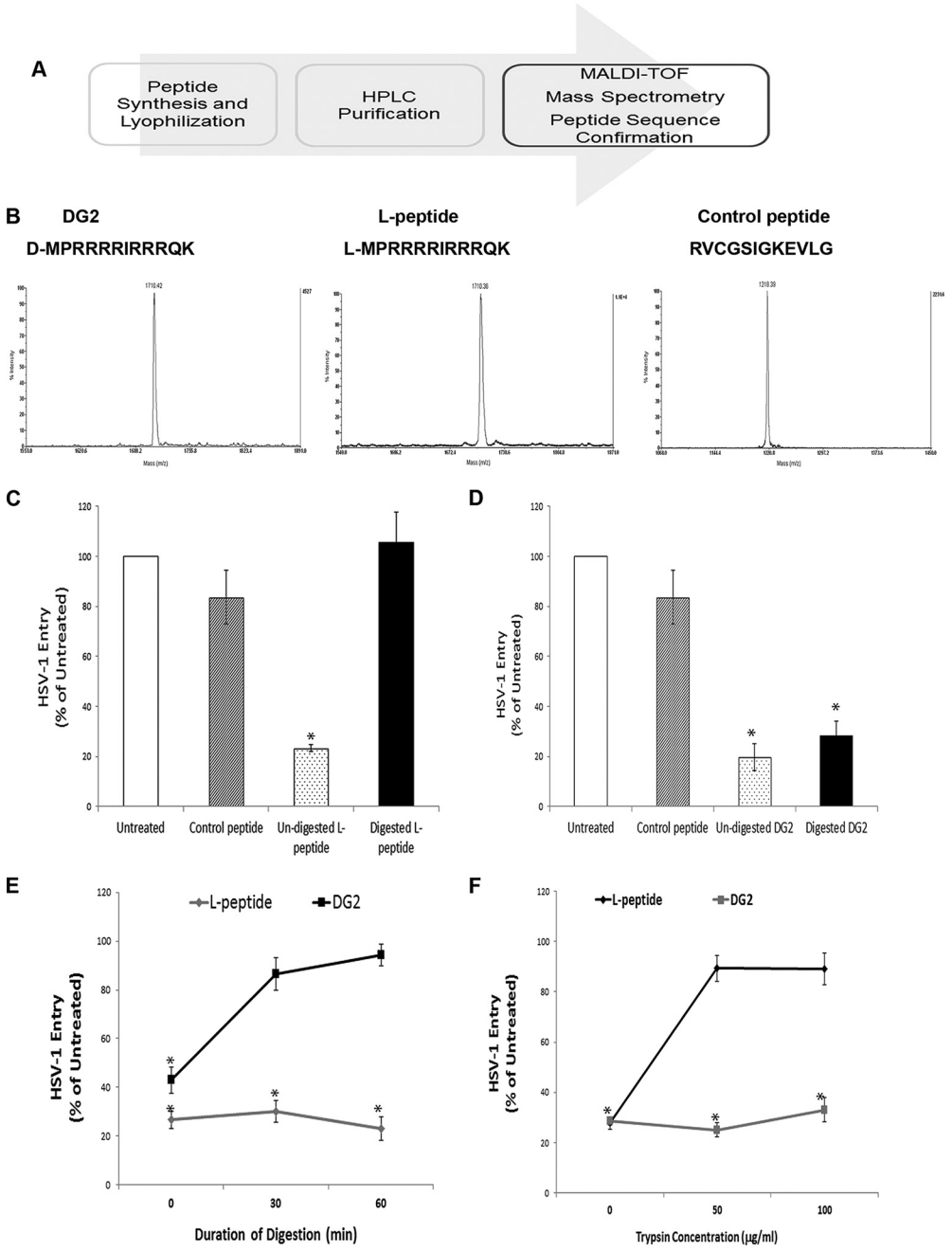
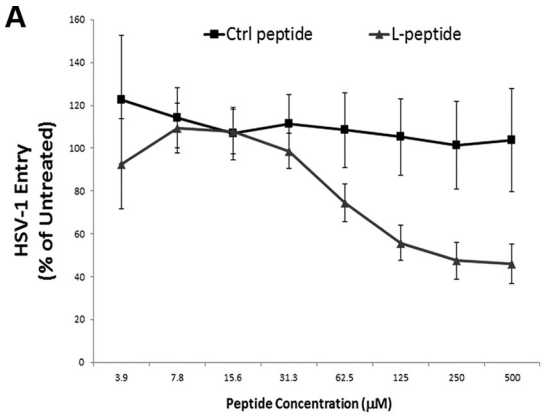
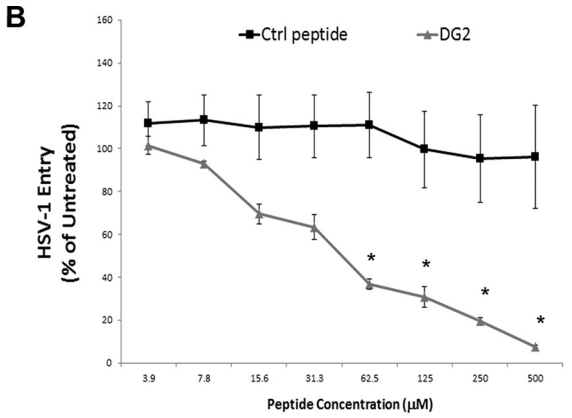


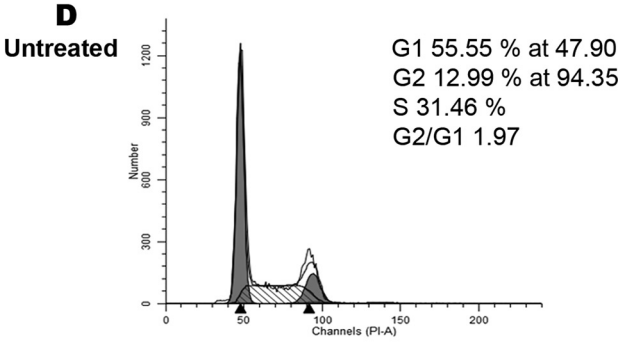
FIG 1 Synthesis and validation of a protease-stable anti-HSV-1 peptide (DG2). (A) Peptide synthesis scheme. Peptide synthesis was performed by a solid-phase synthesis method using 9-fluorenylmethoxy carbonyl (Fmoc)-amino acid-Wang resin. Peptide is synthesized in cycles, starting with the removal of the Fmoc group with 20% piperidine–*N*,*N*-dimethylformamide (DMF; Sigma) followed by washing the resin with DMF. The Fmoc-protected first amino acid (L or D form; purchased from AnaSpec) was added in the presence of 0.2 M HBTU [2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; AnaSpec]–0.4 M 4-methylmorpholine–DMF. After excess reagents in DMF were washed away, this process was continued and the C-terminal to N-terminal synthesis took place. Removal of the side groups of amino acids remained protected during synthesis, and the cleavage of peptide from the resin was effected by the treatment of the resin with trifluoroacetic acid (TFA):thioanisole:water:phenol:ethanedithiol (EDT). Peptide was precipitated from the TFA solution by the use of ethyl ether and then dissolved in 50% acetonitrile–water and lyophilized. The crude peptide was purified via a standard high-performance liquid chromatography (HPLC) procedure. The pure peptide fraction was identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). (B) Sequence (top) and MALDI-TOF MS spectra (bottom) of DG2, an L peptide, or a control peptide (scrambled sequence). Spectra confirm the peptide sequence. (C and D) Proteolytic stability of DG2. L peptide (C), DG2 (D), or scrambled control peptide was digested with trypsin (Gibco) for 30 min, and then the peptides were added to HCE cells and the cells were infected with HSV-1 gL86 at a multiplicity of infection (MOI) of 5 for 6 h. ONPG (Thermo Scientific) was added to measure β-galactosidase activity (optical density at 405 nm [OD_{405}]) using a plate reader (Tecan GENios Pro). Readings were normalized to an untreated control. (E) DG2 or control peptide was incubated and digested with trypsin for the indicated durations of time. The cells were then incubated with the digested peptides and infected, and viral entry was determined as described above. (F) DG2 or control peptide was digested with various concentrations of trypsin. The cells were then treated with digested peptides and infected, and viral entry was measured as described above. Data are representative of the results of three independent experiments. Statistical significance was evaluated using Student's *t* test. $P < 0.05$ was considered significant.

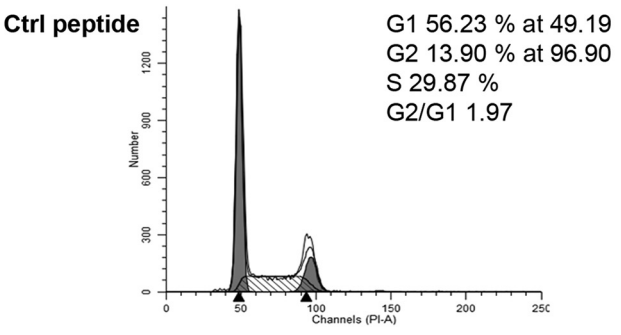


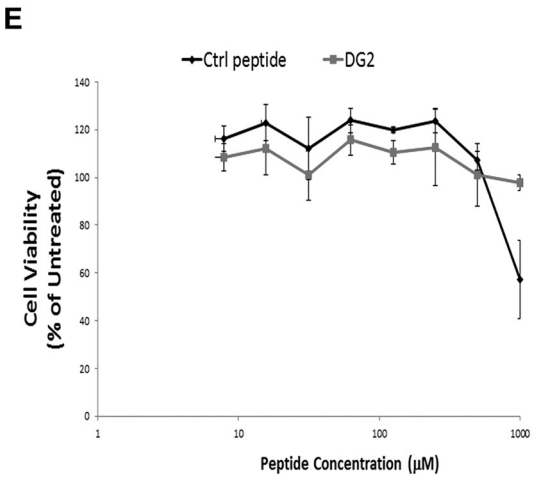


C

Peptide	IC_{50}
L-peptide	~ 200 μM
DG2	~ 45 μM







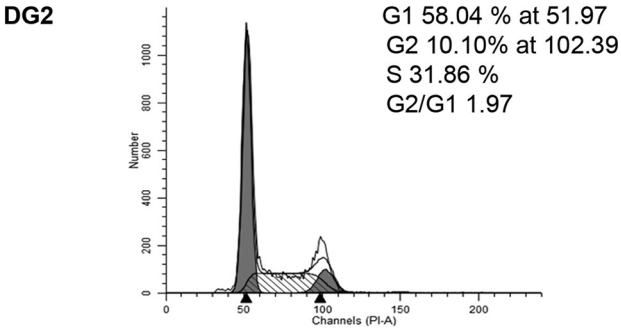


FIG 2 Assessment of the anti-HSV-1 activity and toxicity of DG2. (A and B) Dose-response curves of an L peptide (A) or DG2 (B) compared to a control (Ctrl [scrambled]) peptide. HCE cells were treated with the indicated concentrations of DG2, L peptide, and control peptide. The cells were then infected with HSV-1 gL86 at an MOI of 5. ONPG was added at 6 hpi. β-galactosidase activity (OD_{405}) was measured using a plate reader. Readings were normalized to an untreated control. (C) Estimated IC_{50} values, as calculated from the dose-response viral entry curves. (D) Cell cycle analysis with indicated treatments was performed after gating via height and area parameters. Six hours after treatment, cells were fixed with cold 100% ethanol, treated with RNase (Thermo Scientific) (10 μg/ml), and stained with PI (Fluka) (50 μg/ml)–PBS for 30 min. Samples were analyzed using an LSR Fortessa (BD) flow cytometer. ModFit LT3.2 software was used for data analysis. (E) Cells were left untreated or treated with DG2 for 24 h. Cell viability was then determined via a standard MTT (Sigma) assay. OD_{562} values were measured using a plate reader (Tecan). Data are representative of the results of three independent experiments. Statistical significance was evaluated using Student's *t* test. $P < 0.05$ was considered significant.

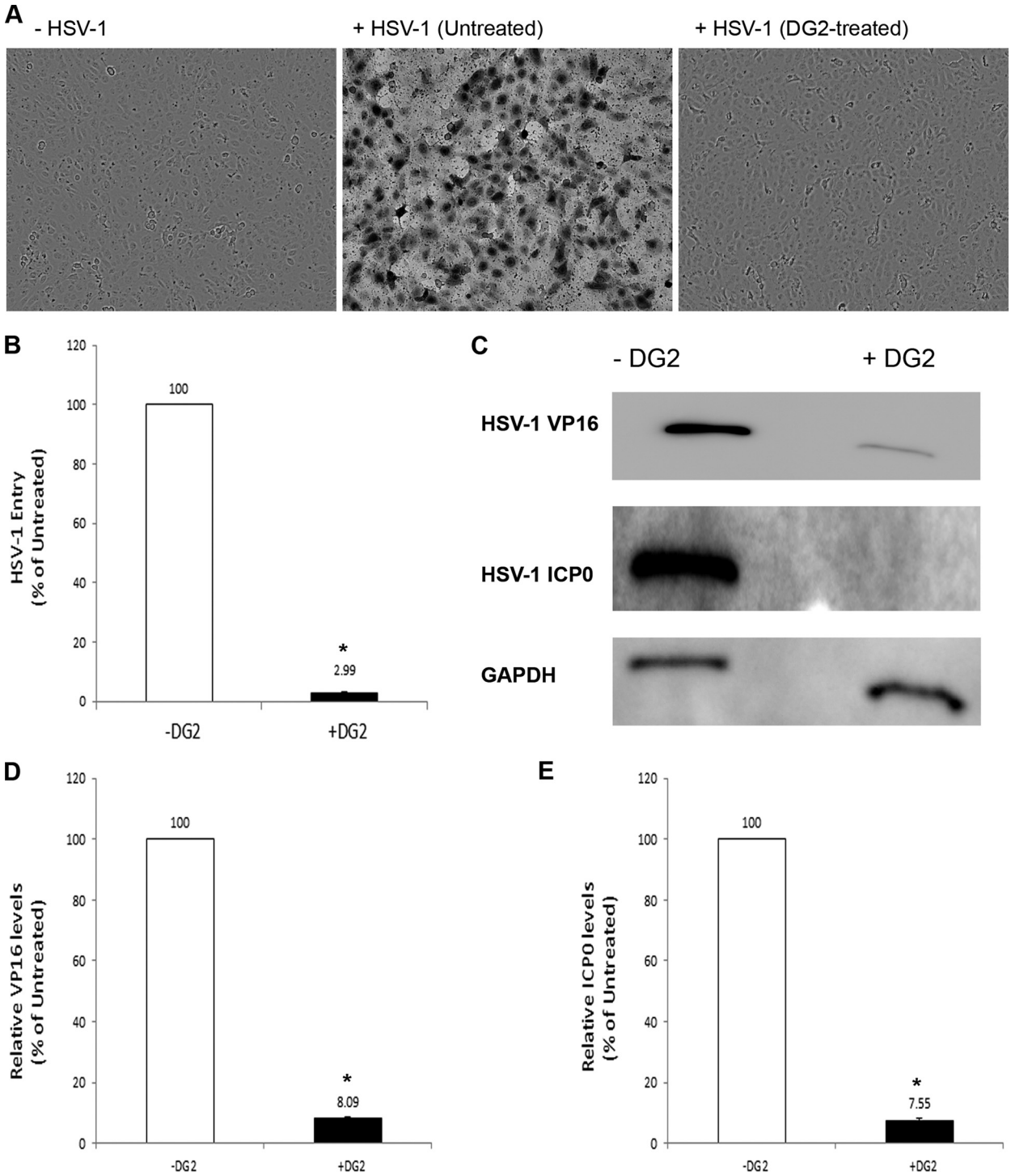


FIG 3 In-depth characterization of the entry-blocking activity of DG2. (A and B) X-Gal images of and data from untreated (-DG2) or DG2-treated (+DG2) infected or uninfected cells. HCE cells were pretreated for 1 h with DG2. Cells were infected with HSV-1 gL86 at an MOI of 5 for 6 h. Cells were then fixed, permeabilized, and treated with X-Gal (Invitrogen)-ferricyanide buffer for 2 h. The cells were imaged with a Zeiss Axiovert 200 microscope. Image analysis was performed using Metamorph software (Zeiss). (C) Immunoblotting of untreated or DG2-treated infected cells. Untreated or DG2-treated cells were infected with HSV-1 at an MOI of 10. Cell lysates were collected in radioimmunoprecipitation assay (RIPA) buffer with proteinase and phosphatase. Lysates were electrophoresed on a 4% to 12% Bis-Tris gel (NuPage) and transferred to a polyvinylidene difluoride (PVDF) membrane (Novex). After nonspecific blocking in 5% nonfat milk, the membrane was incubated with primary (VP16 mouse monoclonal [Santa Cruz]; GAPDH [glyceraldehyde-3-phosphate dehydrogenase] rabbit polyclonal [Santa Cruz]; ICP0 mouse monoclonal [Abcam]) and secondary horseradish peroxidase (HRP)-conjugated (Jackson) antibodies. Development then followed (ECL kit; Pierce). The density of the bands was quantified using ImageQuant TL image analysis software. (D and E) Quantification of the immunoblots shown in panel C. Data are representative of the results of two independent experiments. Statistical significance was evaluated using Student's *t* test. $P < 0.05$ was considered significant.

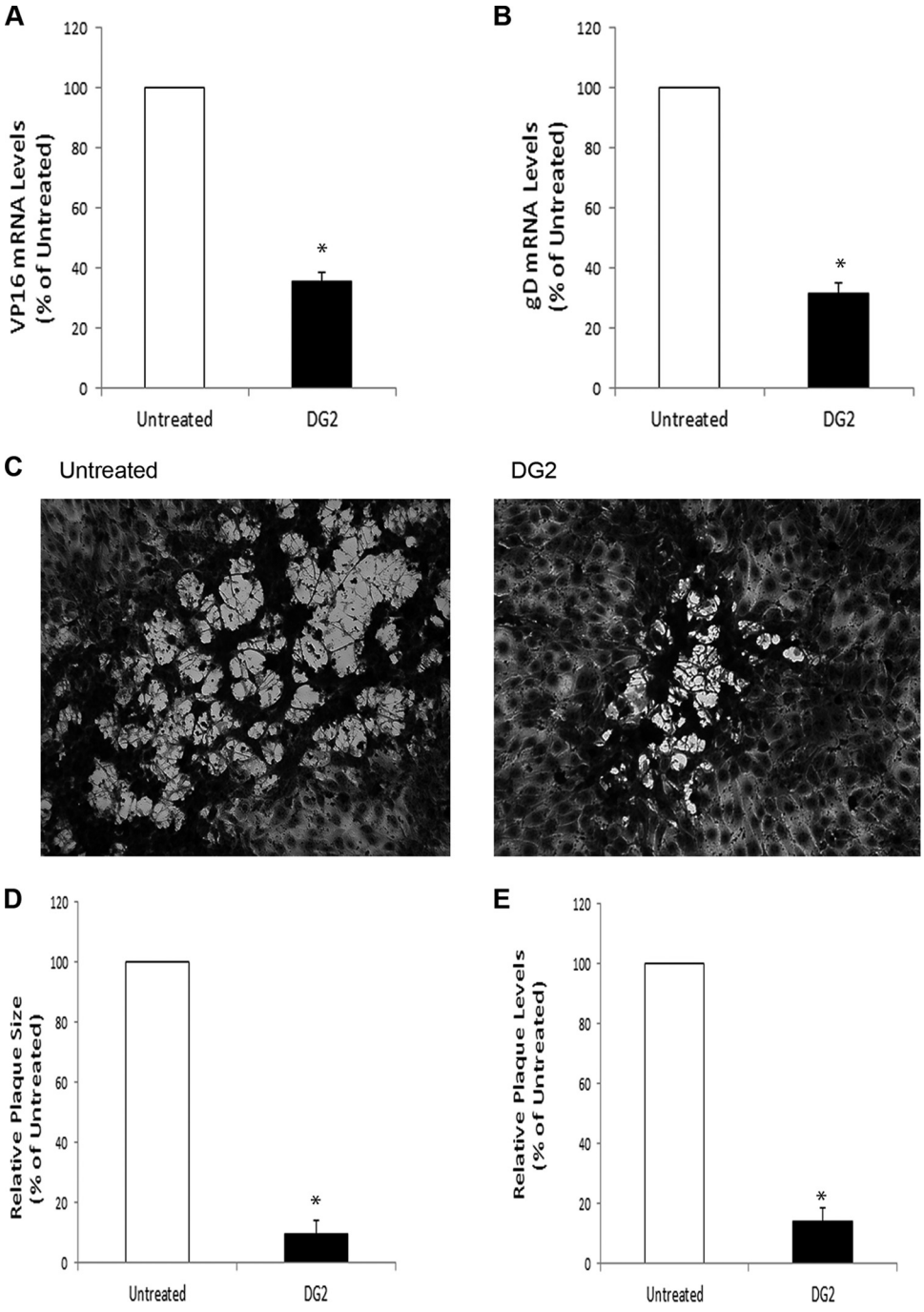


FIG 4 DG2 effectively suppresses all aspects of infection in infected cells. (A and B) Relative mRNA levels of viral VP16 (A) or gD (B) from infected untreated or DG2-treated cells. The cells were infected with HSV-1 at an MOI of 10 for 2 h. RNA was extracted using TRIzol (Life Technologies) at 6 hpi, and cDNA was synthesized using a High-Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was performed using Fast SYBR green (Applied Biosystems), and primers specific to gD and VP16 were used. (C to E) Cells were left untreated or treated with DG2 for 1 h, after which they were infected with HSV-1 for 2 h. Medium containing 1% methylcellulose (Sigma) was added to the cells for 72 h. The cells were then fixed and stained with crystal violet. Plaques were counted and imaged via the use of an AxioVert microscope (Zeiss). Data are representative of the results of three independent experiments. Statistical significance was evaluated using Student's *t* test. $P < 0.05$ was considered significant.

Having observed a high level of efficacy of DG2, we then wanted to rule out any possible cytotoxicity of the peptide. We investigated the effect of DG2 treatment on cell cycle progression. Cells were left untreated, treated with control peptide, or treated with DG2 for 6 h, after which they were harvested, fixed, treated with RNase, stained with propidium iodide (PI), and subjected to fluorescence-activated cell sorter (FACS) analysis. FACS analysis demonstrated that DG2 treatment had no significant effect on the cell cycle progression of the division dynamics of the cells (Fig. 2D). We also determined the toxicological profile of DG2 using a

standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (14). Cells were incubated with increasing doses of DG2 or control peptide, and after 24 h, MTT was added and quantified using a plate reader. We found that DG2 did not produce significant toxicity at any of the concentrations used (Fig. 2E). Collectively, these data rule out any possible cytotoxicity or undesirable effects of DG2 treatment.

After observing the entry-blocking activity of DG2 based on the results of the ONPG assay, we sought to further establish this activity using multiple assays. First, we performed an X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) assay. Cells were left untreated or treated with DG2 for 1 h and then infected with HSV-1 gL86. Untreated, uninfected cells were used as a negative control. We found that DG2 treatment significantly blocked viral entry (Fig. 3A and B). Then, to confirm the effect of DG2 treatment on viral entry and infection, we determined the levels of the VP16 viral tegument protein that is immediately released into the cells upon viral entry (15, 16) or the levels of the ICP0 protein that marks successful initiation of infection (17–19). Infection with HSV-1 (KOS) was done with untreated cells or with cells treated with DG2 for 1 h. VP16 and ICP0 were detected by Western blot analysis from cell lysates obtained at 6 h postinfection (hpi). DG2-treated cells showed a significant reduction of VP16 and ICP0 levels (Fig. 3C, D, and E).

To further characterize the antiviral activity of DG2, we then investigated the effect of DG2 on all aspects of the infection process. Upon entry, the viral genome undergoes transcription of various genes. Thus, we monitored the mRNA levels of different HSV-1 genes upon viral entry. Untreated cells or cells treated with DG2 for 1 h were infected with HSV-1. At 6 hpi, RNA was extracted and quantified by quantitative reverse transcription-PCR (qRT-PCR). DG2 suppressed transcription of the different viral genes, such as VP16 and gD (Fig. 4A and B). Moreover, we also tested the ability of DG2 to suppress viral cell-cell spread. Cells were left untreated or treated with DG2 for 1 h and were infected with HSV-1. Methyl cellulose-containing medium was added to prevent secondary plaque formation. We observed a strong reduction of cell-cell spread by DG2 (Fig. 4C), manifested through diminished plaque size (Fig. 4C and D) and number (Fig. 4E). Taking the data together, it is evident that DG2 blocks all aspects of HSV-1 infection.

In this study, we discovered for the first time a protease-resistant anti-HSV-1 D-peptide and characterized its activity using various methods. This treatment, we propose, is a fundamental step toward development of an anti-HSV-1 therapy for *in vivo* applications. Peptide therapeutics are currently on the rise due to several advantages such as high potency and selectivity and ease of synthesis and modification (20–22) and, most importantly, a lack of the significant side effects and toxicity associated with conventional antiviral drugs (23–25). However, the major limitations of peptide therapeutics are their rapid clearance and susceptibility to proteases (13, 22, 26–28). DG2, however, overcomes a major obstacle to *in vivo* peptide therapy: stability with respect to proteases. Thus, we present DG2 as a promising tool for future *in vivo* applications, including use as a novel reagent to study viral entry.

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