Dendrimers functionalized with membrane-interacting peptides for viral inhibition

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Abstract: This contribution reports the synthesis of a poly(amide)-based dendrimer functionalized at the termini with a membrane-interacting peptide derived from the herpes simplex virus (HSV) type 1 glycoprotein H, namely gH625-644. This peptide has been shown to interact with model membranes and to inhibit viral infectivity. The peptidodendrimer inhibits both HSV-1 and HSV-2 at a very early stage of the entry process, most likely through an interaction with the viral envelope glycoproteins; thus, preventing the virus from coming into close contact with cellular membranes, a prerequisite of viral internalization. The 50% inhibitory concentration was 100 and 300 nM against HSV-1 and HSV-2 respectively, with no evidence of cell toxicity at these concentrations. These results show that the functionalization of a dendrimer with the peptide sequence derived from an HSV glycoprotein shows promising inhibitory activity towards viruses of the Herpesviridae family.

Keywords: peptidodendrimer, antiviral activity, membranotropic peptides

Introduction

Herpes simplex viruses (HSV) are responsible for a wide variety of clinical manifestations and represent a significant worldwide disease and economic burden. There are two serotypes of HSV; HSV-1 and HSV-2, which can infect either oral or genital sites respectively. For some populations, between 60% and 95% are infected with HSV-1 and between 6% and 50% with HSV-2.¹ Even if HSV infections are often subclinical, their incidence and severity have increased over the past decades due to the increasing number of immunocompromised patients. In particular, the impact of genital herpes as a public health threat is amplified because of its epidemiological synergy with the human immunodeficiency virus (HIV). Synthetic nucleoside analogs targeting viral DNA polymerase (eg, acyclovir) are routinely used as standard treatment of symptomatic HSV infections;² however, their clinical use in immunocompromised patients receiving long-term treatments may lead to treatment failures due to the emergence of antiviral-resistant strains.³ Thus, it is imperative to develop new anti-HSV agents with antiviral activity based on alternative mechanisms of action. Inhibition of HSV attachment and/or entry represents a particularly attractive antiviral strategy since it may prevent the establishment of infection. Target compounds with this mode of action could provide a starting point for the development of topical microbicides that block transmission at the mucosal surface, thereby providing a method of prophylactic intervention.⁴

Dendrimers have been extensively studied as vehicles for the delivery of therapeutics or as carriers for in vivo imaging.⁵⁻¹⁰ Dendrimers are highly branched macromolecules
with well defined three-dimensional architectures. The appeal of dendrimers lies in their unique perfectly branched architectures which affords them different properties than corresponding linear polymers of the same composition and molecular weights. As dendrimers increase in generation, they exponentially increase the number of termini, while only linearly increasing in radius; thus, the termini become more densely packed giving the entire structure a globular shape, where the termini radiate outwards from a central core. The globular structure of a dendrimer has a significant effect on its rheological properties with important biological implications; for example, a linear analog of a dendrimer will generally see much reduced blood circulation times.12

“Click reactions,” such as the copper catalyzed 1,3-dipolar alkyne/azide cycloaddition (CuAAC),13 have been used extensively for polymer functionalizations due to their high yielding character.14,15 Using CuAAC, dendrimers have been functionalized with amines, peptides, and polysaccharides and investigated as antiviral agents.16–19 While the mechanisms of action of the antiviral activity is not fully understood, it is thought that the interactions used by viruses to infect cells can be inhibited due to the dendrimer physically blocking or interfering with the fusion mechanism between the virus and the cell.20 Notable is the poly(lysine)-based dendrimer SPL7013,21 which is a sulfonate terminated dendrimer gel that has been shown to act as a topical virucide against HIV-1,22–24 and is now undergoing Phase II clinical trials as VivaGel® (Starpharma, Melbourne, Australia). Peptide-functionalized dendrimers have also been investigated as antiviral agents. Both Luganini et al and Donalisio et al have synthesized dendritic structures based on a poly(lysine) core containing four short (∼10 mer) peptide sequences.20,25,26 These dendrons were shown to have antiviral activity. Using a linear analog greatly diminishes antiviral activity, suggesting the dendritic structure is necessary for the antiviral effect. The peptides used by Hunter are lysine-rich, giving the outer surface of the dendron a positive charge to interact with biological membranes. However, poly(cationic) structures generally show cytotoxic effects, which limits their usefulness.27

Herein, the functionalization of a dendrimer terminated with a membranotropic peptide sequence, the gH(625–644) domain derived from viral fusion proteins, has been shown to exhibit antiviral properties is reported.28 The current authors have previously identified several regions of the herpes simplex virus type I glycoprotein H that have membrane-interacting capabilities.29–33 Of these, the gH(625–644) domain proved to be a good candidate for the purposes of this study: it is mainly hydrophobic in nature and displays an amphiphilic character when in an α-helical form. Furthermore, it folds easily into a helix in a membrane-mimetic environment.29–31 Finally, the authors of this present paper previously reported the ability of gH625 to inhibit HSV entry.31 The gH625 peptide was therefore used to functionalize dendrimers and their properties as antiviral agents were investigated.

**Experimental section**

**Materials**

Fmoc-protected amino acids, coupling reagents, and Rink-amide p-methylbenzhydrylamline (MBHA) resin were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Fmoc-L-propargylglycine (Fmoc-PrA-OH) was purchased from NeoSystem (Tysons Corner, VA, USA). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), or TCI International (Portland, Oregon, USA). Dialysis membranes (SpectraPor 6) were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA) and used after rinsing the membrane in water for 30 minutes. Size-exclusion chromatography was performed using a 1 cm × 18 cm Sephadex G-50 column. The octadecaaizidedendrimer (1) was synthesized as described in the literature.34

**Solid-phase peptide synthesis**

Peptides were synthesized on a Syro I Multisynthetic GmbH (Witten, Germany) automatic synthesizer. Syntheses were performed using standard Fmoc solid-phase techniques on a 20 µmol scale as previously reported.30 Peptide purification was performed on LC8 Shimadzu (Kyoto, Japan) high-performance liquid chromatography with a Phenomenex (Torrance, CA, USA) C18 column (300A, 250 mm × 21.20 mm, 5 µ) and a Waters (Milford, MA, USA) ultraviolet (UV) Lambda-Max model 481 detector. Peptide purity was analyzed by a Thermo Electron (San Jose, CA, USA) Finnigan Surveyor MSQ single quadrupole ESI LC-MS with a Phenomenex C18 column and the same eluent system (NH2-GLASTLTRWAHYNLIRAFX-CONH2, X = PrA, Rt = 9.41 minutes, molecular weight = 2392.7 [M + 2H]+/2 = 1197 amu).

**Peptide functionalization of 1**

To obtain compound 2, to a solution of 1 (50 µg, 0.0146 µmol) in 208 µL of water/methanol (1:1) was added 660 µL of a 1:1 methanol water solution of the peptide gH625-PrA (30.33 eq), 10 µL of a water solution of CuSO4·5H2O (1.46 mM, 1 eq), and 50 µL of a water solution of sodium ascorbate.
(1.17 mM, 4 eq). The mixture was stirred for 12 hours. The reaction mixture was then concentrated and peptide functionalized dendrimer 2 was purified by size-exclusion chromatography. The amount of peptide functionalization on 2 was confirmed by UV analysis ($\varepsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 280 \text{ nm}$).

Cells and viruses
African green monkey kidney cells (Vero) (ATCC CCL-81) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. HSV-1 (strain SC16) and HSV-2 (strain 333), both carrying a $\beta$-galactosidase driven by the CMV IE-1 promoter to express $\beta$-galactosidase, were propagated on Vero cells monolayers.

Virus entry assays
For all experiments below, compounds 1, 2, and peptide gH625 were dissolved in DMEM without serum and used at concentrations of 0, 5.5, 55, 280, and 550 nM. The concentrations used for the peptide correspond to the quantity present on each dendrimer molecule, ie, 18 times the concentrations used for the peptide correspond to the quantity present on each dendrimer molecule, ie, 18 times the concentrations of the dendrimer. All experiments were conducted in triplicate. The percentage of infectivity inhibition was calculated by setting the number of plaques obtained in positive controls where no antiviral compounds were added to the cell monolayers to 0% inhibition. To assess the effect of the dendrimer and the peptidodendrimer on viral attachment, two experimental procedures were carried out. Briefly, prechilled Vero cell monolayers (12-well plates) were infected with precooled HSV-1 or HSV-2 at an MOI of 0.01 pfu/cell for 2 hours at 4°C to allow viral attachment. Cells were then washed three times with cold MEM to remove unattached virus and treated with various concentrations of the above compounds or heparin for 3 days at 37°C. The virus inocula were mixed with the antiviral compound to be tested in each experiment as stated above. Non-penetrated viruses were inactivated by citrate buffer at pH 3.0 after the 45 minute incubation with cells at 37°C. The cells were then incubated for 24 hours at 37°C in DMEM supplemented with carboxymethyl cellulose (CMC). Monolayers were fixed, stained with X-gal, and plaque numbers were scored.

d. Attachment
To assess the effect of the dendrimer and the peptidodendrimer on viral attachment, two experimental procedures were carried out. Briefly, prechilled Vero cell monolayers (12-well plates) were infected with precooled HSV-1 or HSV-2 at an MOI of 0.01 pfu/cell for 2 hours at 4°C to allow viral attachment. Cells were then washed three times with cold MEM to remove unattached virus and treated with various concentrations of the above compounds or heparin.

b. Posttreatment assay
Vero cell monolayers (12-well plates) were incubated with HSV-1 or HSV-2 for 45 minutes at 37°C. The above compounds were then added to the inoculum followed by an additional incubation period of 30 minutes at 37°C. For all treatments, non-penetrated viruses were inactivated by citrate buffer at pH 3.0 after the 45 minute incubation with cells at 37°C. The cells were then incubated for 24 hours at 37°C in DMEM supplemented with carboxymethyl cellulose (CMC). Monolayers were fixed, stained with X-gal, and plaque numbers were scored.

c. Cytopathic effect inhibition assay
Confluent Vero cell monolayers in a 96-well plate were infected with HSV-1 and HSV-2 at an MOI of 0.01 pfu/cell at 37°C for 1 hour. Following removal of virus inocula, the infected cells were washed with citrate buffer at pH 3.0 followed by a PBS wash and covered with medium containing the above compounds for 3 days at 37°C. The cytopathic effect was monitored daily, and an arbitrary score was assigned at the end of the incubation period in consideration of the protection from the cytopathic effect observed.

e. Cell pretreatment
Prechilled Vero cell monolayers were treated as above with the above compounds or heparin for 30 minutes at 4°C and infected with precooled HSV-1 or HSV-2 at an MOI of 0.1 pfu/cell for 2 hours at 4°C. Cells were then washed three times with cold MEM to remove unattached...
virus and compounds, overlaid with CMC, and incubated for 2 days at 37°C. Plates were then fixed, stained with X-gal, and the number of plaques was scored.

f. Virucidal assay
The aforementioned compounds were added to aliquots of HSV-1 or HSV-2 (10^4 pfu) and incubated at either 4°C or 37°C for 2 hours. After incubation, the samples were diluted with medium to reduce the concentration of the antiviral compound to one that was not active in an antiviral assay. The MOI of HSV-1 and HSV-2 after dilution was of 0.01 pfu/cell. The viruses were then titrated on Vero cell monolayers. Plates were then fixed, stained with X-gal, and the number of plaques was scored.

Cytotoxicity
Vero cells were exposed to increasing concentrations of compounds, and the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay that is based on the reduction of the yellowish MTT to the insoluble and dark blue formazan by viable and metabolically active cells. Vero cells were subcultured in 96-well plates at a seeding density of 2 × 10^4 cells/well and treated with compounds 1 or 2 at 0.28, 0.55, 1.1, 2.8, or 5.5 µM for 3 and 24 hours. The medium was then gently aspirated, MTT solution (5 mg/mL) was added to each well, and cells were incubated for a further 3 hours at 37°C. The medium with MTT solution was removed, and the formazan crystals were dissolved with dimethyl sulfoxide. The absorption values were measured at 570 nm using a Bio-Rad Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The viability of Vero cells in each well was presented as a percentage of control cells.

Flow cytometry analysis of apoptosis
Vero cells were exposed to compounds 1 and 2 at the concentration of 0.55 µM for 24 hours. Annexin V-FITC (MedSystems Diagnostics, Vienna, Austria) (fluorescein isothiocyanate) was used in conjunction with a vital dye, propidium iodide (PI) (Sigma-Aldrich), to distinguish apoptotic (Annexin V-FITC positive, PI negative; or Annexin V-FITC positive, PI positive) from necrotic (Annexin V-FITC negative, PI positive) cells. Briefly, cells were incubated with Annexin-V-FITC and in a binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂) for 10 minutes at room temperature, washed, and resuspended in the same buffer. Analysis of apoptotic cells was performed by flow cytometry (FACScan; BD, Franklin Lakes, NJ, USA). For each sample, 2 × 10⁴ events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

Results
Peptide analysis
The literature describes the potential of fusion peptides and/or membranotropic peptides of orthomyxoviruses, paramyxoviruses, and HIV as virus entry inhibitors. The accepted view is that the inhibition of infectivity may be due to the formation of inactive aggregates between the fusogenic stretches present in both the viral protein and the synthetic peptides. These aggregates are formed as a consequence of their nature for intrinsic oligomerization or their ability to mimic the modes of binding of their original domains in their partner protein; they are thus predicted to stabilize a pre-fusion intermediate and prevent merging of the bilayers. The Winley–White interfacial hydrophobicity scale was used to identify the sequence gh625 that proved to effectively interact with the membrane bilayer and possess some antiviral activity, therefore, gh625 has the potential of being developed into an inhibitor to prevent viral entry and viral infectivity. This sequence was selected for coupling to the dendrimer to investigate whether a dendrimer-gH625 conjugate can yield an HSV-1 inhibitor. The sequence alignment of HSV-1 and HSV-2 gH(625-644) (Table 1) shows that the two segments have a high degree of sequence identity with the substitution of the histidine with the arginine at the N-terminus and two other substitutions in the rest of the molecule (L627V and T630V). While His625 (HSV-1) was proven to be fundamental for the interaction with the membrane bilayer and for translocation across the membrane, the absence of this residue induced similar levels of viral inhibition when compared with the full peptide. This indicates a direct correlation between the α-helical potential and the inhibitory activity and suggests that the substitution of His625 with an arginine residue, as in HSV-2, may not exhibit a significant difference in viral inhibitory activity. The substitution of Leu627 with a valine residue does not alter the hydrophobicity of the peptide, and thus should not influence its infectivity inhibition properties. The authors of this present paper have demonstrated previously

Table 1 Peptide amino acid sequence of gH625-644 in HSV-1 and HSV-2

<table>
<thead>
<tr>
<th>Virus</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>GelasLTRWHYNALIRAF</td>
</tr>
<tr>
<td>HSV-2</td>
<td>GvasLTRWHYNALIRAF</td>
</tr>
</tbody>
</table>

Abbreviation: HSV, herpes simplex virus.
that the substitution of leucine with a polar residue (serine) substantially reduces the peptide’s inhibitory activity.\(^\text{29}\) There is no evidence from previous works on the substitution of Thr630 with a hydrophobic residue, but the current authors hypothesize this substitution does not alter the aggregation properties of gH625, as the valine is a small hydrophobic residue.

**Synthesis of peptidodendrimers**

The starting point was the poly(amide)-based dendrimer 1 (Figure 1A) that is based on the 1→3 connectivity scheme first reported by Newkome et al.\(^\text{42}\) Poly(amide)-based dendrimers have been reported to exhibit high biocompatibility due to the peptide-like backbone,\(^\text{43}\) and thus have been shown to be suitable delivery vehicles.\(^\text{44}\) The synthesis of I was performed according to a previous report.\(^\text{34}\)

To obtain compound 2, compound 1 was functionalized via the CuAAC using a modified gH625 peptide containing a PrA residue at the C-terminus (NH\(_2\)-HGLASTL-TRWAHYNALIRAFX-CONH\(_2\), X = PrA) (Figure 1B). The reaction was run in a water/methanol solution using CuSO\(_4\)/sodium ascorbate as catalyst. After purification,

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**Figure 1** (A) Structures of the poly(amide)-based azidodendrimer 1 and cartoon of the peptidodendrimer 2. The helices on the structure represent the gH625 peptide sequence. (B) Schematic representation of the CuAAC reaction to functionalize 1 with peptides. Shown is the C-terminal PrA residue.

**Abbreviations:** CuAAC, copper catalyzed 1,3-dipolar alkyne/azide cycloaddition; PrA, propargylglycine.

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**Figure 2** Cell viability measured by the MTT assay for 3 and 24 hours for the dendrimer (1) and peptidodendrimer (2).

**Abbreviation:** MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
quantitative functionalization was determined by UV (ε_{peptide} = 7000 M^{-1} cm^{-1} at λ = 280 nm) and infrared (disappearance of signal at 2100 cm^{-1}) analyses. See also Supplementary materials.

**Cytotoxicity studies**

To confirm that dendrimer 1 and peptidodendrimer 2 do not exert toxic effects on cells, monolayers of Vero cells were exposed to different concentrations (0.28, 0.55, 1.1, 2.8, 5.5 μM) of each compound for 3 and 24 hours, and cell viability was quantified by the MTT assay. No statistical difference was observed between the viability of control (untreated) cells and that of cells exposed to the peptidodendrimer (Figure 2) up to the concentration used in antiviral testing. Minimal toxicity was observed for the dendrimer without the peptides linked to its termini, but only at concentrations that were considerably higher than those required for antiviral activity.

Thereafter, the effects of treatments with 1 and 2 in inducing apoptosis, assessed by FACS analysis after double labeling with FITC-Annexin V and PI, were evaluated. It was found that neither of the treatments induced apoptosis of Vero cell populations (Figure 3 and Table 2). Over 86% of cells treated with 1 and over 89% of cells treated with 2 were alive with only 3% of cells treated with 1 showing markers for early apoptosis.

**Antiviral studies**

To test whether the peptidodendrimer can affect HSV infectivity in vitro, a virus yield reduction assay in which the peptidodendrimer of interest was present in the cell culture during and after viral adsorption was used. The extent of HSV-1 and HSV-2 replication, assessed by titration of harvested viruses, showed a consistent decrease in replication efficiency for both viruses with more than 80% inhibition at a peptidodendrimer concentration of 280 nM; inhibition of HSV-1 replication was able to reach 90% under these conditions. Dendrimer 1, without any peptide functionalization, was able to produce an inhibition close to 35% at a concentration of 280 nM, suggesting that the dendrimer structure itself confers an antiviral activity which is enhanced by the specific amino acid sequence added to its termini. The IC_{50} of the peptidodendrimer was 100 and 300 nM against HSV-1 and HSV-2 respectively, while the IC_{50} of 1 was over 550 nM for both viruses (Figure 4).

To elucidate the mechanism of inhibition, the compounds were tested under different conditions to identify the step in the entry process that was being inhibited by the dendrimer. Since inhibition of HSV infectivity could be due to either an interference during the early penetration phase or as a consequence of a peptidodendrimer action inside the cell at a post-entry event, a “posttreatment” test was performed by adding the compounds at different concentrations 2 hours after virus infection (Figure 5). None of the concentrations used significantly reduced HSV-1 and HSV-2 replication. Similarly, a cytopathic effect inhibition assay proved that both the dendrimer and the peptidodendrimer were ineffective once the viruses had already gained their access inside the cell. These results strongly suggest that both the dendrimer and the peptidodendrimer target an early step of the HSV infection cycle.

To investigate whether the inhibitory activities of 1 and the peptidodendrimer 2 are due to the inhibition of HSV entry into cells, prechilled Vero monolayers were infected with HSV-1 and HSV-2 for 2 hours at 4°C. The incubation at 4°C

**Table 2** Study of apoptosis in Vero cells after treatment with 1 and 2

<table>
<thead>
<tr>
<th>24 hour</th>
<th>Necrosis</th>
<th>Late apoptosis</th>
<th>Alive</th>
<th>Early apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>11.6%</td>
<td>0.86%</td>
<td>85.4%</td>
<td>2.1%</td>
</tr>
<tr>
<td>1</td>
<td>9.2%</td>
<td>1.14%</td>
<td>86.5%</td>
<td>3.1%</td>
</tr>
<tr>
<td>2</td>
<td>8.99%</td>
<td>0.6%</td>
<td>89.5%</td>
<td>0.88%</td>
</tr>
</tbody>
</table>
blocks the virus’ penetration but allows its attachment to the cell surface via its cellular receptors, thereby synchronizing cell penetration. Dendrimer 1 and 2 were then added to the infected cells which were shifted to 37°C. After 45 minutes at 37°C, unpenetrated viruses were inactivated by an acid wash and overlaid with CMC to measure infectivity of penetrated viruses. As shown in Figure 6A, none of the concentrations of 1 or 2 investigated was able to affect HSV-1 or HSV-2 entry and replication. When prechilled Vero monolayers were infected with HSV-1 and HSV-2 in the presence of 1 or 2 for 1 hour at 4°C, however, an inhibitory effect was observed (Figure 6B). For this experiment, a modification of the procedure was introduced, in fact, the same attachment test was also performed adding heparin as a control, and as expected,
heparin was completely able to block the ability of HSV-1 and HSV-2 to attach to Vero cells, preventing the interaction of the virions with cell surface heparin sulfate.

The possibility of 2 to interfere with an early penetration step was further explored. Vero cells were pretreated with 1 or 2 for 1 hour before infection. No significant reduction of infectivity with 2 was observed (Figure 7). In contrast, a clear activity of the dendrimer itself was observed. Since the toxicity of the dendrimer is marginal, it is assumed that it may exert an antiviral activity by blocking the cell surface. The lack of activity of the peptidodendrimer is probably due to the fact that this molecule is able to penetrate inside the cell efficiently in virtue of its functionalization with the membranotropic HSV-1 gH derived sequence, therefore, during the 1 hour of cell pretreatment, the majority of 2 may have already translocated inside the cell where it is

Figure 6 Effect of 1 and 2 on viral attachment. (A) Vero cell monolayers were infected with HSV-1 (blue lines) or HSV-2 (red lines) at an MOI of 0.1 pfu/cell for 2 hours at 4°C. After unattached virus removal, plates were treated with various concentrations of 1 (triangles), 2 (squares), or heparin (circles) for 3 hours at 37°C. Finally, cells were overlaid with CMC and incubated for 2 days at 37°C. (B) Vero cell monolayers were treated with inocula containing both the viruses and the antiviral compounds precooled at 4°C. Notes: Plaque numbers were scored, and the percentage of inhibition was calculated with respect to “no-compound” control experiments. Data are reported in triplicate, and error bars represent standard deviations.

Abbreviations: HSV, herpes simplex virus; MOI, multiplicity of infection; pfu, plaque-forming unit; CMC, carboxymethyl cellulose.
The amount of peptidodendrimer left on the cell surface may be below the concentration needed to maintain an antiviral activity. Since inhibition of HSV penetration might result from an irreversible peptidodendrimer-induced inactivation of the virions, the possibility that the compounds could interfere with HSV particles and inactivate viral infectivity was explored. To do this, HSV-1 and HSV-2 aliquots were incubated with different concentrations of 1 and 2 at 4°C and 37°C for 2 hours. After incubation, the samples were diluted to reduce the antiviral agents’ concentrations well below the threshold for HSV replication inhibition (0.001 to 0.1), and the infectivity of preincubated virions was measured by titration on Vero cells. As shown in Figure 8, the incubation of virions with the peptidodendrimer reduced the infectivity in both HSV-1 and HSV-2. The pre-incubation of virions with 1 also produced a decrease of infectivity, albeit of lower intensity.

Discussion
The goal of the research reported herein is to investigate the antiviral properties of a dendrimer whose termini have been functionalized with the HSV-1 derived peptide gH625 that has been shown previously to possess antiviral activity.28,29,31 The key characteristics of gH625 are: (1) the ability to interact with model membranes, (2) penetration of the bilayer with its N-terminus, (3) high efficiency of inducing lipid mixing of model membranes, and (4) the ability to adopt a helical conformation with its hydrophobic residues on one face of the helix and polar or charged residues on the opposite face.29,31,33 The inhibitory effect of gH625 appeared conditioned by its ability to partition into membranes and aggregate within them. Since gH625 peptides self-associate in aqueous and lipidic solutions, it is possible that they bind to their counterparts in the HSV-1 gH fusion protein, suggesting that the inhibition of viral entry may occur via peptide association with their counterpart on wild-type gH. In this contribution, a peptidodendrimer carrying the gH625 peptide at its termini was constructed. The functionalization of the dendrimer with the gH625 peptide was quantitative as evidenced by UV and infrared analyses. Dynamic light-scattering and scanning electron microscopy showed the average particle size to be 12 nm. Circular dichroism showed that the terminal peptides fold into an α-helix in a membrane-mimetic environment.34 To analyze the antiviral activity of the peptidodendrimer, a virus yield assay on both HSV-1 and HSV-2 was first
performed, and a consistent decrease in replication efficiency for both viruses was obtained. Interestingly, the dendrimer itself was able to inhibit virus replication, albeit to a lesser extent compared with the peptidodendrimer, while the peptide on its own was unable to show any inhibition when tested at a concentration corresponding to the peptide present on the peptidodendrimer. The multivalent display of gH625 on the dendrimer scaffold results in an almost sixfold increase of antiviral activity for HSV-1 and twofold for HSV-2 in comparison to the activity of the dendrimer itself, and a more than 100-fold increase in the activity of the unsupported peptide. The IC_{50} of the peptidodendrimer was 100 and 300 nM against HSV-1 and HSV-2, respectively, while the IC_{50} of the unfunctionalized dendrimer was over 550 nM.

Figure 8 Virucidal assay. Different concentrations of 1 (triangles) and 2 (squares) were added to aliquots (10^4 pfu) of HSV-1 (blue lines) or HSV-2 (red lines), and incubated at either 4°C (A) or 37°C (B) for 2 hours. After incubation, the samples were diluted to reduce the compound concentrations below the threshold for inhibition of viral replication, and the virus was titrated on Vero cells.

Notes: Plaque numbers were scored and the percentage of inhibition was calculated with respect to “no-compound” control experiments. Data points represent an average of three experiments, and error bars represent standard deviations.

Abbreviations: HSV, herpes simplex virus; pfu, plaque-forming unit.
for both viruses. The cytotoxicity profile measured by the MTT assay showed that the peptidodendrimer was not toxic to Vero cells up to the highest concentration investigated in antiviral testing, while some toxicity was observed for the unfunctionalized dendrimer, especially at higher concentrations, demonstrating another advantage of the peptide functionalization. To further analyze intact cell activity, a FACS analysis was performed after double labeling with FITC-Annexin V and PI to evaluate the effects of 1 and 2 on induction of apoptosis and concluded that the majority of cells are still alive and functional after 24 hours at the active antiviral concentration.

Further experiments were carried out to identify which step in the entry process could be responsible for the observed infectivity inhibition. Any inhibitory activity can be excluded when the compounds were added at a post-entry step and also when cells were pretreated with the dendrimer derivatives. Both the peptidodendrimer and the dendrimer are not able to interfere with viral replication once the virus has gained access to the cellular milieu. When cells were pretreated with 1 or 2 for 1 hour before infection, the peptidodendrimer was unable to yield a significant reduction of infectivity, while a clear inhibition was obtained by the dendrimer itself. The lack of activity of the peptidodendrimer is probably due to the fact that this molecule is able to penetrate inside the cell efficiently in virtue of its functionalization with the membrandotropic HSV-1 gH derived sequence. Therefore, during the 1 hour of cell pretreatment, the majority of the peptidodendrimer may have already translocated inside the cell where it was probably inactive according to the posttreatment assay. On the other hand, 1 enters much more slowly and, more importantly, it partially remains on the cell surface. This allows the dendrimer to reduce membrane mobility and inhibit viral entry or to produce a consistent delay in viral entrance. After excluding also the attachment step (Figures 6 and 7) as being responsible for the antiviral activity, the possibility that the peptidodendrimer could directly inactivate the virions was explored. The results of virus-pretreatment assay showed a strong and consistent inhibition of viral infectivity for HSV-1 and a slightly lower inhibition of HSV-2. The pre-incubation of virions with the unfunctionalized dendrimer also produced a decrease of infectivity, albeit of lower intensity. The authors hypothesize that the peptidodendrimer might sterically hinder the gH relative domain, either in a pre-fusogenic or in an intermediate conformation, preventing a complete and functional interaction between gH and the membrane to fuse. Due to the high degree of complexity of the interaction of HSV surface glycoproteins during the fusion process, it was not able to unequivocally identify the path of antiviral action of the peptidodendrimer. It may act by binding to gH itself through oligomerization of the gH625-644 domain present on the glycoprotein, in a probable rearrangement of its structural domains. Alternatively, the gH sequence present on the peptidodendrimer may interact with other glycoproteins present on the virion envelope, such as gB or gD.

The results of this study indicate that the modification of a dendrimer scaffold with antiviral peptides represent an attractive strategy for the design of a new class of antiviral drugs that exert their effect, coupling the intrinsic antiviral properties of the dendrimer with the activity of antiviral peptides. These antiviral drugs possess several advantageous features, such as target specificity, low toxicity, and the possibility to modify surface characteristics easily. Moreover, peptidodendrimers have the potential of being developed as multifunctionalized scaffolds to provide a therapeutic molecule that could be directly delivered to its target.

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Disclosure
The authors report no conflicts of interest in this work.

References
Supplementary materials

Materials and methods

The analysis of the three molecules: crude peptidodendrimer, peptide and dendrimer was carried out on an analytical Agilent Technologies (Santa Clara, CA, USA) 1200 Series HPLC system with a Phenomenex (Torrance, CA, USA) analytical C₅ column (Jupiter 5 µ 300Å, 150 mm x 4.60 mm, 5 micron). The mobile phase used was: H₂O 0.1% trifluoroacetic acid as eluent A and CH₃CN 0.1% TFA as eluent B from 30% to 95% over 20 minutes at 1 mL min⁻¹ flow. The compounds were dissolved in a 30% CH₃CN 0.1% TFA and 70% H₂O 0.1% TFA solution before being loaded onto the column: the coupling was performed using one equivalent of dendrimer and two equivalents of peptide.

Discussion

The peptidodendrimer presents a different retention time from both the peptide and dendrimer alone. As we can see from the crude peptidodendrimer chromatogram, the peak relative to the dendrimer disappears, confirming that all the dendrimer is bound to peptide.

Figure S1 LC-MSQ spectra of gh625-PRA peptide. gh625-PRA (MW: 2392.7) purity and identity was assessed by analytical LC-MS analyses using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Thermo Electron, San Jose, CA, USA), column: C₁₈-Phenomenex eluted with H₂O/0.1% TFA (A) and CH₃CN/0.1% TFA (B) from 20% to 80% over 10 minutes at a flow rate of 0.8 mL min⁻¹.

Notes: The final yield of purified peptide was approximately 40%. NH₂-HGLASTLTRWAHYNALIRFX-CONH₂, X = PrA, Rt = 9.4 minutes; (MW = 2392.7) [M + 2H]⁺/2 = 1197 amu.

Abbreviations: LC-MS, liquid chromatography-mass spectrometry; LC-MSQ, liquid chromatography-mass spectrometry quadrupole; MW, molecular weight; TFA, trifluoroacetic acid.
Figure S2 LC-MSQ spectra of the crude peptidodendrimer.