Dendrimers functionalized with membrane-interacting peptides for viral inhibition

Rossella Tarallo¹
Tom P Carberry²
Annarita Falanga¹
Mariateresa Vitiello³
Stefania Galdiero¹
Massimiliano Galdiero³
Marcus Weck²

¹Dipartimento di Farmacia, Università di Napoli "Federico II," and DFM Scarl, Napoli, Italia; ²Molecular Design Institute and Department of Chemistry, New York University, New York, NY, USA; ³Dipartimento di Medicina Sperimentale, Seconda Università degli Studi di Napoli, Napoli, Italia

Correspondence: Massimiliano Galdiero
Dipartimento di Medicina Sperimentale,
Seconda Università degli Studi di Napoli,
Via de Crecchio 7, Napoli 80138, Italia
Tel +39 0815667646
Fax +39 0815667578
Email massimiliano.galdiero@unina2.it

Marcus Weck
Molecular Design Institute and
Department of Chemistry, New York
University, New York, NY 10003, USA
Tel +1 212 992 7968
Fax +1 212 995 4895
Email marcus.weck@nyu.edu

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 (HSVs) 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.¹¹ 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.⁵ 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 that 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*-methylbenzhydrylamine (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 $\times$ 18 cm Sephadex G-50 column. The octadecaazidedendrimer (**1**) was synthesized as described in the literature.³⁴

Solid-phase peptide synthesis

Peptides were synthesized on a Syro I Multisynthes GmbH (Witten, Germany) automatic synthesizer. Syntheses were performed using standard Fmoc solid-phase techniques on a 20 μ mol 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 (300Å, 250 mm $\times$ 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-HGLASTLTRWAHYNALIRAFX-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 μ g, 0.0146 μ mol) in 208 μ L of water/methanol (1:1) was added 660 μ L of a 1:1 methanol water solution of the peptide gH625-PrA (30.33 eq), 10 μ L of a water solution of CuSO₄ $\cdot$ 5H₂O (1.46 mM, 1 eq), and 50 μ L 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 ($\epsilon = 7000 \text{ M}^{-1} \text{ cm}^{-1}$ 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 *lacZ* gene driven by the CMV IE-1 promoter to express β -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 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 peptides on inhibition of HSV infectivity, cell monolayers were treated in different ways:

a. Virus yield reduction assay

Confluent Vero cell monolayers (12-well plates) were washed with phosphate-buffered saline (PBS) and infected with either HSV-1 or HSV-2 at multiplicity of infection (MOI) of 1 plaque-forming unit (pfu)/cell for 1 hour 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. The infected cells were washed with PBS, covered with fresh culture medium, and incubated for 48 hours. The infected cells were then scraped into culture medium and disrupted by sonication. The total virus yield in each well was titrated by plaque assay. Plaques were stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and microscopically counted. The mean plaque counts for each drug concentration were expressed as a percentage of the mean plaque count for the control virus. The number of plaques was plotted as a function of drug concentration; concentrations producing 50% reductions in plaque formation were determined as the IC_{50} .

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.

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 0.1 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 hours at 37°C prior to inactivation of extracellular virus with citrate buffer for 2 minutes at room temperature. Subsequently, the cells were washed three times with warm medium to return the pH to neutral and overlaid with CMC and incubated for 2 days at 37°C. Plates were then fixed, stained with X-gal, and plaques were counted. The second attachment experiment was performed as the former, except the prechilled Vero cell monolayers were treated with inocula containing both the viruses and the antiviral compounds precooled to 4°C. Following a 2 hour incubation at 4°C to allow viral attachment, the plates were treated as before and shifted at 37°C.

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.³⁵ 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 μ M 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 μ M 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 $MgCl_2$, 2.5 mM $CaCl_2$) 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, 2×10^4

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.^{28,29,31,36–38} 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.^{28,30,31} 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 Wimley–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;^{30,31} 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,^{31,33,39–41} 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

Virus	Peptide sequence
HSV-1	HGLASILTRWAHYNALIRAF
HSV-2	RGVASVILTRWAHYNALIRAF

Abbreviation: HSV, herpes simplex virus.

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.

that the substitution of leucine with a polar residue (serine) substantially reduces the peptide's inhibitory activity.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.⁴² Poly(amide)-based dendrimers have been reported to exhibit high biocompatibility due to the peptide-like backbone,⁴³ and thus have been shown to be suitable delivery vehicles.⁴⁴ The synthesis of **1** was performed according to a previous report.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 (NH₂-HGLASTL-TRWAHYNALIRAFX-CONH₂, X = PrA) (Figure 1B). The reaction was run in a water/methanol solution using CuSO₄/sodium ascorbate as catalyst. After purification,

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.

Figure 3 Apoptotic effects of **1** and **2** on Vero cells. FACS analysis after double labeling with propidium iodide and FITC-Annexin V of Vero cells: (A) untreated cells, (B) treated with **1**, and (C) treated with **2** for 24 hours at 0.55 μ M. The experiments were performed three times, and the results were always similar.

Note: Insets, percentage of positive cells.

Abbreviations: FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate.

quantitative functionalization was determined by UV ($\epsilon_{\text{peptide}} = 7000 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 280 \text{ nm}$) and infrared (disappearance of signal at 2100 cm^{-1}) analyses.³⁴ 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 μ M) 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**

24 hour Vero	Necrosis	Late apoptosis	Alive	Early apoptosis
Untreated	11.6%	0.86%	85.4%	2.1%
1	9.2%	1.14%	86.5%	3.1%
2	8.99%	0.6%	89.5%	0.88%

Figure 4 Virus yield reduction assay. Vero cells were infected with either HSV-1 (blue lines) or HSV-2 (red lines) in the presence of increasing concentrations of the dendrimer **1** (triangles) or the peptidodendrimer **2** (squares).

Notes: 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.

Abbreviation: HSV, herpes simplex virus.

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,

Figure 5 Posttreatment assay. HSV-1 (blue lines) and HSV-2 (red lines) infected Vero cells were treated with increasing concentrations of the dendrimer **1** (triangles) or the peptidodendrimer **2** (squares), then overlaid with CMC and incubated for 24 hours at 37°C.

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; CMC, carboxymethyl cellulose.

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.

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,^{34,45} therefore, during the 1 hour of cell pretreatment, the majority of **2** may have already translocated inside the cell where it is

Figure 7 Cell pretreatment assay. Vero cell monolayers were treated with various concentrations of **1** (triangles), **2** (squares), or heparin (circles) for 30 minutes at 4°C before being infected with precooled HSV-1 (blue lines) or HSV-2 (red lines) at 0.1 pfu/cell in the presence of the compounds for 2 hours at 4°C. After the removal of excess compounds, cells were overlaid with CMC, and incubated for 2 days at 37°C.

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; CMC, carboxymethyl cellulose.

probably inactive accordingly with the posttreatment assay. 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

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.

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

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 membranotropic HSV-1 gH derived sequence.³⁴ 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.³⁴ 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.

Acknowledgments

We thank the National Science Foundation (CHE-0234863, CHE-0958457, CHE-01162222) and New York University for support of the analytical equipment. SG and AF thank MIUR for financial support by PON01_02388 “Verso la medicina personalizzata: nuovi sistemi molecolari per la diagnosi e la terapia di patologie oncologiche ad alto impatto sociale - SALUTE DELL’UOMO E BIOTECNOLOGIE Responsabile Scientifico Prof. Carlo Pedone. Viral strains were kindly donated by Dr. Helena Browne, Department of Pathology of the University of Cambridge, UK.

Disclosure

The authors report no conflicts of interest in this work.

References

1. van Benthem BH, Spaargaren J, van Den Hoek JA, Merks J, Coutinho RA, Prins M. Prevalence and risk factors of HSV-1 and HSV-2 antibodies in European HIV infected women. *Sex Transm Infect.* 2001;77(2): 120–124.
2. Superti F, Ammendolia MG, Marchetti M. New advances in Anti-HSV chemotherapy. *Curr Med Chem.* 2008;15(9):900–911.
3. Greco A, Diaz JJ, Thouvenot D, Morfin F. Novel targets for the development of anti-herpes compounds. *Infect Disord Drug Targets.* 2007;7(1): 11–18.
4. Keller MJ, Tuyama A, Carlucci MJ, Herold BC. Topical microbicides for the prevention of genital herpes infection. *J Antimicrob Chemother.* 2005;55(4):420–423.
5. Lee CC, MacKay JA, Fréchet JMJ, Szoka FC. Designing dendrimers for biological applications. *Nat Biotech.* 2005;23(12):1517–1526.
6. Esfand R, Tomalia DA. Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. *Drug Discov Today.* 2001;6(8):427–436.

7. Sadler K, Tam JP. Peptide dendrimers: applications and synthesis. *Rev Mol Biotechnol*. 2002;90:195–229.
8. Cloninger MJ. Biological applications of dendrimers. *Curr Opin Chem Biol*. 2002;6:742–748.
9. Niederhaffner P, Šebestík J, Ježek J. Peptide dendrimers. *J Peptide Sci*. 2005;11:757–788.
10. Tekade RK, Kumar PV, Jain NK. Dendrimers in oncology: an expanding horizon. *Chem Rev*. 2009;109(1):49–87.
11. Newkome GR, Moorefield CN, Vögtle F. *Dendrimers and Dendrons: Concepts, Synthesis, Applications*. Weinheim: Wiley-VCH; 2001.
12. Fox ME, Szoka FC, Fréchet JMJ. Soluble polymer carriers of the treatment of cancer: the importance of molecular architecture. *Acc Chem Res*. 2009;42(8):1141–1151.
13. Kolb HC, Finn MG, Sharpless KB. Click chemistry: diverse chemical function from a few good reactions. *Angew Chem Int Ed*. 2001;40(11):2004–2021.
14. Franc G, Kakkar A. Dendrimer design using Cu-catalyzed alkyne-azide “click chemistry”. *Chem Commun*. 2008;44(42):5267–5276.
15. Astruc D, Liang L, Rapakousiou A, Ruiz J. Click dendrimers and triazole-related aspects: catalysts, mechanism, synthesis, and functions. A bridge between dendritic architectures and nanomaterials. *Acc Chem Res*. 2012;45(4):630–640.
16. Bourne N, Stanberry LR, Kern ER, Holan G, Matthews B, Bernstein DI. Dendrimers, a new class of candidate topical microbicides with activity against herpes simplex virus infection. *Antimicrob Agents Chemother*. 2000;44(9):2471–2474.
17. Gao H, Louche G, Sumerlin BS, Jahed N, Golas P, Matyjaszewski K. Gradient polymer elution chromatographic analysis of α,ω -dihydroxypolystyrene synthesized via ATRP and click chemistry. *Macromolecules*. 2005;38(22):8979–8982.
18. Vogt AP, Sumerlin BS. An efficient route to macromonomers via ATRP and click chemistry. *Macromolecules*. 2006;39(16):5286–5292.
19. Li H, Cheng F, Duft AM, Adronov A. Functionalization of single-walled carbon nanotubes with well-defined polystyrene by “click” coupling. *J Am Chem Soc*. 2005;127(41):14518–14524.
20. Luganini A, Nicoletto SF, Pizzuto L, et al. Inhibition of herpes simplex virus type I and type II infections by peptide-derivatized dendrimers. *Antimicrob Agents Chemother*. 2011;55(7):3231–3239.
21. Jiang Y-H, Emau P, Cairns JS, et al. SPL7013 gel as a topical microbicide for prevention of vaginal transmission of SHIV in macaques. *AIDS Res Hum Retrovir*. 2005;21(3):207–213.
22. Gong E, Matthews B, McCarthy TD, et al. Evaluation of dendrimer SPL7013, a lead microbicide candidate against herpes simplex viruses. *Antivir Res*. 2005;68(3):139–146.
23. O’Loughlin J, Millwood I, McDonald HM, Price CF, Kaldor JM, Paull JRA. Safety, tolerability, and pharmacokinetics of SPL7013 gel (VivaGel). *Sex Transm Dis*. 2010;37(2):100–104.
24. Telwate S, Moore K, Johnson A, et al. Virucidal activity of the dendrimer microbicide SPL7013 against HIV-1. *Antivir Res*. 2011;90(3):195–199.
25. Luganini A, Giuliani A, Pirri G, Pizzuto L, Landolfo S, Gribaudo G. Peptide-derivatized dendrimers inhibit human cytomegalovirus infection by blocking virus binding to cell surface heparan sulfate. *Antivir Res*. 2010;85(3):532–540.
26. Donalisio M, Rusnati M, Civra A, et al. Identification of a dendrimeric heparan sulfate-binding peptide that inhibits infectivity of genital types of human papillomaviruses. *Antimicrob Agents Chemother*. 2010;54(10):4290–4299.
27. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv Drug Deliv Rev*. 2006;58(14):1523–1531.
28. Galdiero S, Falanga A, Vitiello M, et al. Peptides containing membrane-interacting motifs inhibit herpes simplex virus type I infectivity. *Peptides*. 2008;29:1461–1471.
29. Galdiero S, Falanga A, Vitiello M, et al. Analysis of a membrane interacting region of herpes simplex virus type I glycoprotein H. *J Biol Chem*. 2008;283(44):29993–30009.
30. Galdiero S, Falanga A, Vitiello M, Browne H, Pedone C, Galdiero M. Fusogenic domains in herpes simplex virus type I glycoprotein H. *J Biol Chem*. 2005;280(31):28632–28643.
31. Galdiero S, Falanga A, Vitiello M, et al. The presence of a single N-terminal histidine residue enhances the fusogenic properties of a membranotropic peptide derived from herpes simplex virus type I glycoprotein H. *J Biol Chem*. 2010;285(22):17123–17136.
32. Galdiero S, Falanga A, Vitiello G, et al. Role of membranotropic sequences from herpes simplex virus type I glycoproteins B and H in the fusion process. *Biochim Biophys Acta*. 2010;1798(3):579–591.
33. Galdiero S, Russo L, Falanga A, et al. Structure and orientation of the gH625–644 membrane interacting region of herpes simplex virus type I in a membrane mimetic system. *Biochemistry*. 2012;51(14):3121–3128.
34. Carberry TP, Tarallo R, Falanga A, et al. Dendrimer functionalization with a membrane-interacting domain of herpes simplex virus type I: towards intracellular delivery. *Chem Eur J*. 2012;18(43):13678–13685.
35. Mosmann T. Rapid Colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65(1–2):55–63.
36. Vitiello M, Galdiero M. Inhibition of viral-induced membrane fusion by peptides. *Protein Pept Lett*. 2009;16(7):786–793.
37. Xu Y, Rahman NA, Othman RB, Hu P, Huang M. Computational identification of self-inhibitory peptides from envelope proteins. *Proteins*. 2012;80(9):2154–2168.
38. Galdiero S, Falanga A, Vitiello M, et al. Evidence for a role of the membrane-proximal region of herpes simplex virus type I glycoprotein H in membrane fusion and virus inhibition. *ChemBiochem*. 2007;8(8):885–895.
39. Tarallo R, Accardo A, Falanga A, et al. Clickable functionalization of liposomes with the gH625 peptide from herpes simplex virus type I for intracellular drug delivery. *Chem Eur J*. 2011;17(45):12659–12668.
40. Falanga A, Vitiello MT, Cantisani M, et al. A peptide derived from herpes simplex virus type I glycoprotein H: membrane translocation and applications to the delivery of quantum dots. *Nanomed Nanotechnol*. 2011;7(6):925–934.
41. Galdiero S, Vitiello M, Falanga A, Cantisani M, Incoronato N, Galdiero M. Intracellular delivery: exploiting viral membranotropic peptides. *Curr Drug Metab*. 2012;13(1):93–104.
42. Newkome GR, Kotta KK, Moorefield CN. Convenient synthesis of 1 $\rightarrow$ 3 C-branched dendrons. *J Org Chem*. 2005;70(12):4893–4896.
43. Roberts JC, Bhalgat MK, Zera RT. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst™ dendrimers. *Biomed Mater Res*. 1996;30(1):53–65.
44. Majoros IJ, Myc A, Thomas T, Mehta CB, Baker JR Jr. PAMAM dendrimer-based multifunctional conjugate for cancer therapy: synthesis, characterization, and functionality. *Biomacromolecules*. 2006;7(2):572–579.
45. Guarnieri D, Falanga A, Muscetti O, et al. Shuttle-mediated nanoparticle delivery to the blood-brain barrier. *Small*. Epub November 7, 2012. doi: 10.1002/smll.201201870

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 $\times$ 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-MS/MS 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-HGLASTLTRWAHYNALIRAFX-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.

International Journal of Nanomedicine

Dovepress

Publish your work in this journal

The International Journal of Nanomedicine is an international, peer-reviewed journal focusing on the application of nanotechnology in diagnostics, therapeutics, and drug delivery systems throughout the biomedical field. This journal is indexed on PubMed Central, MedLine, CAS, SciSearch®, Current Contents®/Clinical Medicine,

Journal Citation Reports/Science Edition, EMBase, Scopus and the Elsevier Bibliographic databases. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <http://www.dovepress.com/international-journal-of-nanomedicine-journal>