



Infectivity inhibition by overlapping synthetic peptides derived from the gH/gL heterodimer of herpes simplex virus type 1

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Herpes simplex virus (HSV) is a human pathogen that infects epithelial cells. The cutaneous lesions, caused by the virus, spread to the nervous system creating several complications. Fusion of host membranes with the viral envelope is mandatory and mediated by a group of glycoproteins conserved in all Herpesviridae subfamilies, such as the glycoproteins B (gB), H (gH), L (gL) and D (gD). We investigated the inhibitory activity mediated by synthetic overlapping peptides spanning the entire ectodomains of gH and gL glycoproteins. We have performed a brute analysis of the complete gH/gL heterodimer in order to explore the inhibitory activity of peptides modelled on these glycoproteins against HSV-1 infection. Twenty-four of the gH peptides at a concentration of 150 μ M reached the 50% of inhibition cut-off. Interestingly, they are mainly located in the gH carboxy-terminal domain. None of the gL peptides had a clear inhibiting effect. No peptide toxicity was observed by lactate dehydrogenase assay at the concentrations used in our experimental conditions. HSV-1 therapy is based on acyclovir treatment, but some resistant strains are emerging. In this scenario, innovative approaches for HSV-1 treatment are necessary. Our data support the direct involvement of the described domains in the process of virus penetration; therefore, these results are of relevance to the potential development of novel therapeutic compounds to prevent HSV-1 infections. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: overlapping peptides; herpes simplex virus; infectivity inhibition; viral glycoprotein

Introduction

Herpes simplex viruses (HSV) are human pathogens that infect epithelial cells [1]. They are dsDNA viruses divided in three sub-families: Alphaherpesvirinae, Betaherpesvirinae and Gamma herpesvirinae. The Alphaherpesvirinae are grouped together for their relative fast replicative cell cycle, their host range and the ability to generate latency in the sensitive ganglionic nerves [2]. Among this sub-family, the important species for human infections are represented by HSV-1, HSV-2 and Varicella-Zoster virus. The common HSV treatment is based on acyclovir, valacyclovir and famcyclovir. Resistant strains are emerging; therefore, new strategies are needed to deal with these infections [3].

Therapeutic peptides have become an attractive tool in drug discovery, and the best characterized therapeutic antiviral peptide inhibitor is Enfuvirtide (fusion inhibitor) which mimics the C-terminal repeat coil region of human immunodeficiency virus (HIV) fusion protein, gp41 [4–7]. Specific functional domains of the proteins involved in the mechanism of cell penetration of a number of viruses have been found able to inhibit infectivity [8–13].

HSV-1 infection is initiated by the binding of glycoprotein C (gC) to cell surface heparan sulfate [14,15]. The HSV-1 gB protein (gB) can also bind to heparan sulfate proteoglycans, but the binding is less efficient [16]. Following attachment, gD binds to one of a

number of co-receptors on the host cell membrane, including herpesvirus entry mediator, nectin 1, nectin 2 or 3-O-sulfated heparan sulfate, resulting in a conformational change in gD [17–22]. The conformational change in gD is believed to trigger the formation of a fusion complex, which enables fusion to ensue. The co-expression of gD, gB and gH-gL heterodimer in the same cell results in cell–cell fusion, indicating that these four proteins constitute the minimal fusion apparatus [23–26]. Although the entry pathways of other enveloped viruses share similar patterns, most systems for which molecular details have been gathered rely on a single fusion protein [27]. HSV-1 is singular because it uses both gB and gH/gL as its core fusion machinery [28,29]. The gH/gL complex and gB only

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interact with each other in response to receptor binding by gD [30,31] and this interaction is required for allowing fusion to occur [32]. The tridimensional structure of gH/gL of HSV-2 showed that the gH/gL heterodimer does not resemble any known viral fusogen, which may support different roles for gH including that of being a fusion regulator through its interaction with gB [33]. A broad literature describes gH as involved in the entry process, for example, certain mutations in the transmembrane (TM) region and cytoplasmic tail affect fusion [34–37], as do mutations in the region preceding the TM [38]. Furthermore, peptides matching a number of regions of the gH ectodomain have been shown to interact with membranes and proposed to play a role in the fusion process, probably through regulation of gB activity and/or direct membrane interactions at the fusion site [39–47]. In HSV-infected cells and on mature virions, gH and gL are always found together as a heterodimer, in a stable 1 : 1 complex [33,48] and rely on one another for proper folding, post-translational processing and transport to the cell and virion surface [49–52]. HSV-1 gH is a 838-residue type 1 membrane glycoprotein. The ectodomain contains seven N-glycosylation sites and eight cysteine residues forming at least two disulphide bonds between cysteines 5 and 6 (residue 554 and 589) and cysteines 7 and 8 (residues 652 and 706) [48,53,54]; gL is a 224-residue protein with a signal peptide but no TM region.

The crystal structure of the gH/gL complex has been solved [33] and shows an overall shape of a boot. gH can be divided in three distinct domains: (i) the N-terminal domain, which binds gL (H1), (ii) the central helical domain (H2), and (iii) the C-terminal β -sandwich domain (H3).

The N-terminal domain is located in the upper part of the gH–gL boot and consists of two subdomains, which are connected by a linker (residues Gly116–Pro136); the first subdomain (residues Arg49–Leu115) contains a β -hairpin that forms a six-stranded mixed β -sheet with four strands coming from gL plus three short helices, while the second subdomain (residues Ala137–Pro327) contains a six-stranded antiparallel β -sheet. It is interesting to note that the N-terminal domain does not have a folded core and is the most divergent in sequence. The central domain (residues Asn332–Phe644) is globular and mostly helical. The C-terminal domain (residues Val645–Pro797) is located at the toe end of the boot. It is a 10-stranded β -sandwich where each side is composed of a five-stranded β -sheet and leads to the TM region. The central and C-terminal domains are more conserved and probably have the same fold in different gH proteins.

Similarly to the N-terminal domain of gH, gL does not have a stable core. Only ~30% of gL residues adopt regular secondary structure, which include three helices and two β -sheets.

Peptide-based strategies have recently been used to study herpesvirus glycoprotein function. Several laboratories have analysed synthetic peptides homologous to regions from gC, gB and gH of different herpesviruses in order to inhibit infection [40,44,45,55–62].

We have previously shown that peptides modelled on gH domains with high interfacial hydrophobicity were able to inhibit HSV infectivity [45,63], as well as peptides corresponding to helical domains [64]. Recently, several studies have analysed viral glycoproteins with generalized peptide-based approaches to dissect the whole glycoproteins in order to search for functionally important regions besides the predictable bioinformatic motifs. The mechanism that underlies the ability of some synthetic peptides to inhibit fusion is currently unclear, but some regions of these glycoproteins are exposed on the surface, and they may be involved in interactions with other proteins, or they may become exposed to the surface after the conformational rearrangements that probably

take place during the fusion process; this could perhaps explain the ability of the corresponding synthetic peptides to inhibit cell fusion.

To date, few peptide molecules outside the well-known inhibitory regions of Class 1 viral fusion proteins, the heptad repeats, should be as fusion; therefore, a brute force approach to the identification of peptide entry inhibitors may help in the dissection of HSV-1 glycoproteins domains.

In the present study, we used a peptide scanning inhibition approach for the identification of functional domains and/or lead compounds as infectivity inhibitors. We generated overlapping peptide libraries of the complete sequences of gL and gH ectodomains. These peptides were screened for their ability to inhibit HSV-1 infection in Vero cells at a concentration of 150 μ M.

Materials and Methods

Peptide Synthesis

Peptides were prepared by standard 9-fluorenylmethoxycarbonyl polyamine solid-phase synthesis, using a PSSM8 multispecific peptide synthesizer (Shimadzu Corporation Biotechnology Instruments Department Kyoto JAPAN). The TGA resin (substitution 0.3 mmol g^{-1}) was used as the solid-phase support, and syntheses were performed on a scale of 100 μ mol. All amino acids, 4 equiv. relative to resin loading, were coupled according to the TBTU/HOBT/DIEA method: 1 equiv. of Fmoc-amino acid, 1 equiv. of TBTU, 1 equiv. of HOBT (1 M HOBT in DMF) and 2 equiv. of DIEA (2 M DIEA in DMF). The Fmoc protecting group was removed with 30% piperidine in DMF (v/v). Peptides were fully deprotected and cleaved from the resin by TFA solution (89% TFA, 5.5% thioanisole, 3.3% ethanedithiol and 2.2% anisole as scavengers); the crude peptides were precipitated with ice-cold ethyl ether, filtered, re-dissolved in water and lyophilized. The crude peptides were purified to homogeneity by preparative reverse-phase high-pressure liquid chromatography on a Waters Delta Prep 3000 chromatographic system, equipped with an UV Lambda Max Mod. 481 detector. The samples were injected on a Jupiter (Phenomenex) C18 column (21.20 mm \times 25 cm, 15 μ m) eluted with a H₂O/0.1% TFA (A) and CH₃CN/0.1% TFA (B) solvent mixture. A linear gradient from 20 to 75% of B over 55 min at a flow rate of 20 ml min^{-1} was employed. The collected fractions were lyophilized to dryness and analysed by analytical reverse-phase high-pressure liquid chromatography on a Shimadzu class-LC10 equipped with a diode array detector SPD-M10AV using a Phenomenex C18 analytical column (10 \times 250 mm, 10 μ m); a linear gradient from 20 to 75% of B over 55 min at a flow rate of 1 ml min^{-1} was used. The identity of purified peptides was confirmed by Maldi spectrometry. All purified peptides were obtained with high yields (50–60%).

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 bovine serum. HSV-1 (strain SC16), carrying a lacZ gene driven by the CMV IE-1 promoter to express β -galactosidase, was propagated on Vero cells monolayers.

Virus Entry Assays

Peptides were dissolved in DMEM without serum and used at final concentration of 150 μ M. Experiments were conducted in parallel with scrambled peptides and no-peptide controls.

To assess the effect of peptides on inhibition of HSV infectivity, a co-treatment assay was performed in triplicate. The statistical analysis was carried out via *t*-test and *P*-value. The cells were incubated with peptides at 150 μ M in the presence of serial dilutions of viral inoculum for 45 min at 37 °C.

After inactivation of nonpenetrated viruses by citrate buffer (pH 3.0), cell monolayers were incubated for 48 h at 37 °C in DMEM supplemented with carboxymethylcellulose. Finally, monolayers were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and plaque numbers were scored. Experiments were performed in triplicate, and the percentage of inhibition was calculated with respect to no-peptide control experiments.

The IC₅₀ and IC₉₀ values, peptide concentration that resulted in 50 and 90% of HSV infectivity reduction in a co-treatment assay, respectively, were extrapolated via Graphpad Prism software from the resulting sigmoidal dose–response curve.

Toxicity

Peptide cytotoxicity was measured by a lactate dehydrogenase (LDH) assay [65] and was carried out according to manufacturer's instructions using a cytotoxicity detection kit (Roche Diagnostic SpA, Milano, Italy). Release of LDH from the cytosol to culture is a marker of cell death. The increase of LDH activity in the supernatant was related with the percentage of necrotic cells. Vero cells were cultured in a 96-well plate at a density of 3×10^4 cells/well for 24 h, followed by treatment with investigated concentrations of each peptides for 24 h. Maximal LDH release was obtained after the treatment of control cells with 1% solution of Triton X-100 (Sigma Chemical Company) for 10 min at room temperature. One hundred microlitres of supernatant from the top of all the wells was mixed with the prepared detection kit reagent. After 30-min incubation, the absorbance was measured at 490 nm by Tecan spectrophotometer plate. For each experiment, at least three replicate wells were examined.

Results

To test the potential of using peptides as an antiviral agent and to identify functionally important regions of HSV-1 membrane glycoproteins, two peptide libraries were constructed on the sequences of gH and gL ectodomains. Considering the different size of the two glycoproteins under study, we decided to prepare libraries with different overlapping lengths: a library of 25-mer peptides extending from residues 21 to 805 (just at the beginning of the TM domain) of gH and a second library of 15-mer peptides from residues 20 to 224 corresponding to the whole sequence of gL. The first (gH) library was made of peptides overlapping each other by 20 residues (Supplementary Table S1), while the latter was designed with a 10 residue overlap (Supplementary Table S2), and both signal peptides were excluded from our analysis. In detail, we excluded the first 25 aa from gH sequence (including the peptide signal 1–18 aa) and the first 19 aa from gL sequence. The libraries were screened for peptides with inhibitory activity by using a plaque reduction assay in which both virus and cells were exposed to the peptides prior to infection and the peptides remained present throughout the assay. The peptide efficacy was measured by a plaque reduction assay as described in the Materials and Methods. The criterion for considering a peptide of potential interest was the inhibition of at least 50% of plaque formation at a concentration of 150 μ M. Figure 1 shows the gH-overlapping peptides and their antiviral activities in the screening. Of the 148 peptides in the gH-library, 21 peptides showed an inhibition activity more than 50% (Table 1), while the threshold was not reached by any of the gL peptides (Supplementary Table S2). The 21 gH peptides showing inhibitory activity corresponded to residues 346 to 375 (H65–H66), 381 to 405 (H72), 396 to 425 (H75–H76), 446 to 470 (H85), 456 to 485 (H87–H88), 491 to 520 (H94–H95), 506 to 530 (H97), 596 to 620 (H115), 606 to 630 (H117), 616 to 660 (H119–H123), 646 to 670 (H125), 746 to 775 (H145–H146). The locations of the inhibitory peptides on the gH crystal structure are shown in Figure 2, and we graphically subdivided their localization in four areas. It is

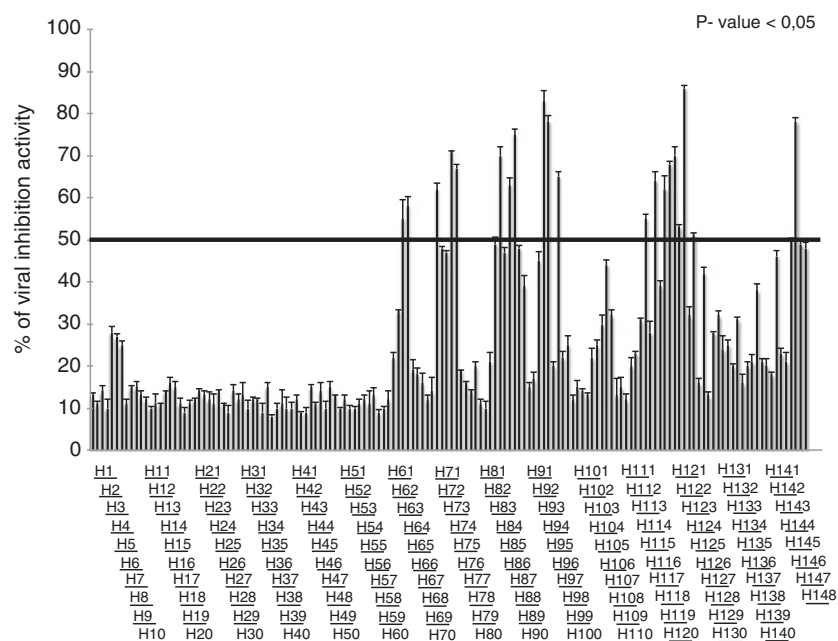
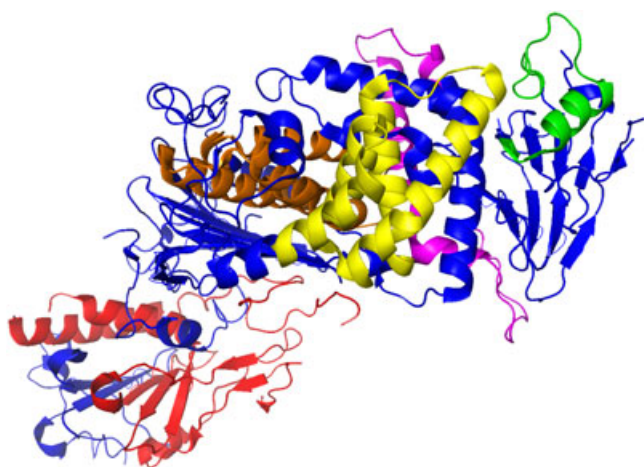


Figure 1. Inhibitory activity of gH peptides. The relative activity was normalized and reported as % of inhibition. The threshold lane was fixed at 50% of infection activity. The statistical analysis was carried out via *P*-value < 0.05.

Table 1. Active peptide details. Inhibitory gH peptides at 150 μM with the respective sequences, % of inhibition and protein position and the respective IC_{50} and IC_{90} . The highest peptide concentration used is 250 μM ; therefore, Graphpad Prism results above this concentration for IC_{90} calculations were reported as $>250 \mu\text{M}$

P.I.	Sequences	% inhibition at 150 μM	Position	IC_{50}	IC_{90}
H65	PEEGTNYAQFLSRAYAEFFSGDAGA	55	325–350	145 ± 5	>250
H66	NYAQFLSRAYAEFFSGDAGAEQGPR	58	330–355	147 ± 3	>250
H75	AAHANGAVCLSDLLGFLAHSRALAG	67	375–400	118 ± 6	>250
H76	GAVCLSDLLGFLAHSRALAGLAARG	71	380–405	124 ± 4	>250
H87	LVAEILEREQSLALHALGYQLAFVL	63	435–460	120 ± 6	>250
H88	LEREQSLALHALGYQLAFVLDSPSA	75	440–465	116 ± 2	>250
H94	PSAAHLIDALYAEFLGGRVLTPVW	83	470–495	85 ± 8	180 ± 7
H95	LIDALYAEFLGGRVLTPVWHRALF	78	475–500	104 ± 2	200 ± 11
H97	GGRVLTPVWHRALFYASAVLRQPF	65	485–510	138 ± 3	>250
H115	DLDESVIDDALAQATRSETPVEVL	55	575–600	146 ± 1	>250
H117	ALAQATRSETPVEVLAQQTHGLAST	64	485–610	121 ± 7	>250
H119	PVEVLAQQTHGLASTLTRWAHYNAL	62	595–620	124 ± 4	>250
H120	AQQTHGLASTLTRWAHYNALIRAFV	68	600–625	117 ± 9	>250
H121	GLASTLTRWAHYNALIRAFVPEASH	70	605–630	111 ± 2	>250
H122	LTRWAHYNALIRAFVPEASHRCGGQ	53	610–635	148 ± 3	>250
H123	HYNALIRAFVPEASHRCGGQSANVE	86	615–640	76 ± 5	165 ± 8
H125	PEASHRCGGQSANVEPRILVPITHN	51	625–650	148 ± 6	>250
H145	VLLVDTDNTQQQIAAGPTEGAPSVF	50	725–750	150 ± 2	>250
H146	TDNTQQQIAAGPTEGAPSVFSSDVP	78	730–755	102 ± 7	225 ± 7

**Figure 2.** Localization of antiviral gH region in the heterodimer gH/gL. The ribbon structure of HSV-1 gH/gL (PDB: 3M1C) and the locations of antiviral gH regions. In blue is reported gH structure, while in red gL one. Antiviral gH regions are indicated in orange, yellow, pink and green.

interesting to note that some of the best inhibitors were located at the domain of interaction between gH (aa 259–323) and gL.

Previous works using a physico-chemical algorithm, the Wimley–White Interfacial Hydrophobicity Scale (WWIHS), in combination with other structural data allowed us to predict regions in gH potentially involved in membrane interactions during the entry and fusion process, and some of them were found to possess HSV antiviral activity in dose-dependent inhibition assays [66].

In the present study, we have extended these studies applying a more generalized peptide-based approach to scan the entire gH ectodomain and its accompanying glycoprotein gL. The chosen threshold for including into the category of putative inhibitory peptides was dictated by the general high concentration needed for exerting an antiviral activity of HSV-derived peptides. Previous

works have shown that concentrations of peptide ranging from 10 to over 150 μM are needed to inhibit HSV infectivity. Nevertheless, the results obtained prove that peptide-based strategies targeting regions of the proteins with predictable structures or motifs represent a valid option when searching functional regions of a protein; in fact, our whole protein scan confirmed as regions of interest several domains which were already found to possess some antiviral activity based on their structural characteristics. Peptides H85, H87 and H88, spanning the region of gH from 446 to 485, partially overlap the helical peptide previously named H-HR1 (aa 444–479) able to reduce infectivity in a consistent manner, albeit at the high concentration of 250 μM [64,67]. Peptides H94, H95, H97 and H119, H120, H121, H122 and H123 overlap peptides gH493–537 and gH626–644, which were found able to inhibit HSV entry with approximately 60% of inhibition at 250 μM . Hydrophathy analysis based on the hydrophobicity-at-interface scale proposed by Wimley and White [66] enabled the detection of these two domains of HSV gH able to induce rapid membrane fusion and inhibit viral entry. A shorter peptide gH493–512, corresponding only to the N-terminus of gH493–537 and almost completely overlapping H94, H95 and H97 of the present work, was able to inhibit HSV entry even more effectively [45]. Finally, peptides H145 and H146 at the extreme C-terminus of the gH ectodomain (the TM helical domain starts at aa 804) have been previously individuated as being part of a larger pre-TM domain with the ability of inhibiting virus infectivity, and a peptide previously named pTM6 [44] exactly corresponds to peptide H146 of the present study. Nevertheless, the remaining peptides have been newly identified and presented an inhibitory activity above the set threshold. These are peptides H65–H66, H75–H76, H115, H117 and H125, which were found to exhibit inhibitory effects against HSV-1 plaque formation (Table 1).

In order to define if chemical as well as conformational properties could represent common and shareable characteristics of active peptide, we performed bioinformatics analysis based on Pepcalc (www.pepalc.com) and Pep-Fold (<http://bioserv.rpbs>).

univ-paris-diderot.fr/) databases, respectively (Figures 3 and 4). Chemical and physical properties highlight shared characteristic for some peptides. In detail, we reported in Figure 3 the 21 peptides subdivided for their isoelectric point grouped as acid (pH from 0 to 6), neutral (pH from 6 to 8) and basic (pH from 8 to 14). In second instance, the peptides were clustered based on their charge at pH 7. As shown in Figure 3, we found that some peptides are represented in specific clusters like H65, H145 and H146 for the first group as well as for H75, H95 and H123 in the second. Interestingly, most new inhibiting peptides are present in the acid (H65, H66, H115, H117) and basic (H125, H76) groups, while only one peptide (H75) is present in the neutral group. Moreover, we performed a primary and secondary structure analysis on the inhibiting gH peptides (Figure 4). Peptides clustered together by a sequence homology point of view (Figure 4A), and the clusters are directly associated with inhibition activity (Figure 4B). For instance, the cluster 5 had a percentage of viral inhibition close to 80%, while the clusters 3 and 6 just above 50%. Secondary structure bioinformatics peptide analysis is reported in Figure 4C, where we observed that in the cluster 5, a different distribution of helical and coil structure with low presence of extended regions is present compared to the others.

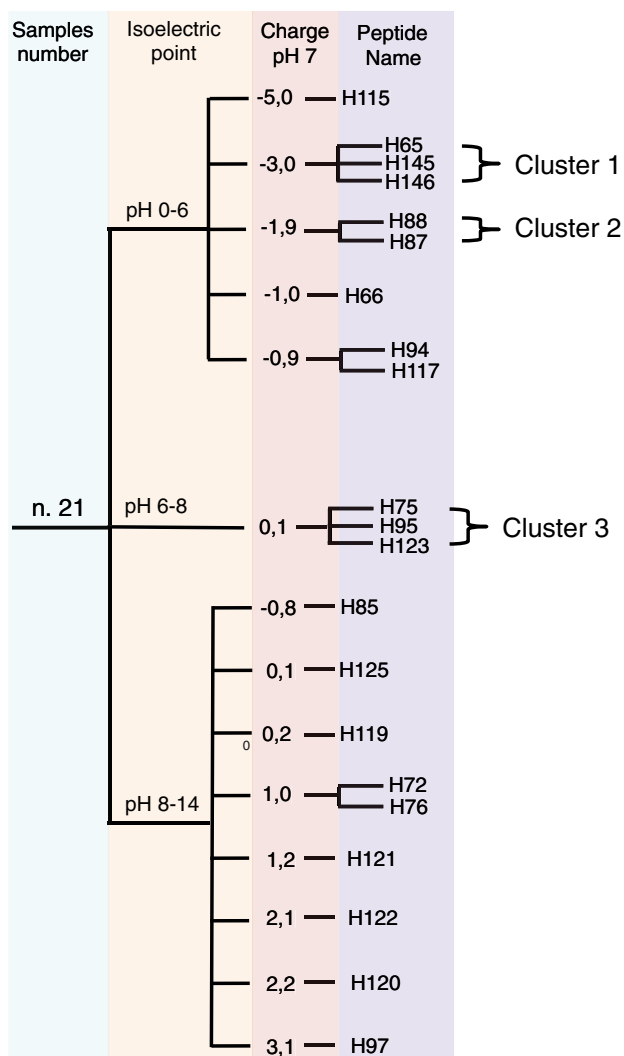


Figure 3. Chemical clusterization of gH active peptides. Tree clusterization of peptides based on isoelectric point and charge at pH 7.0.

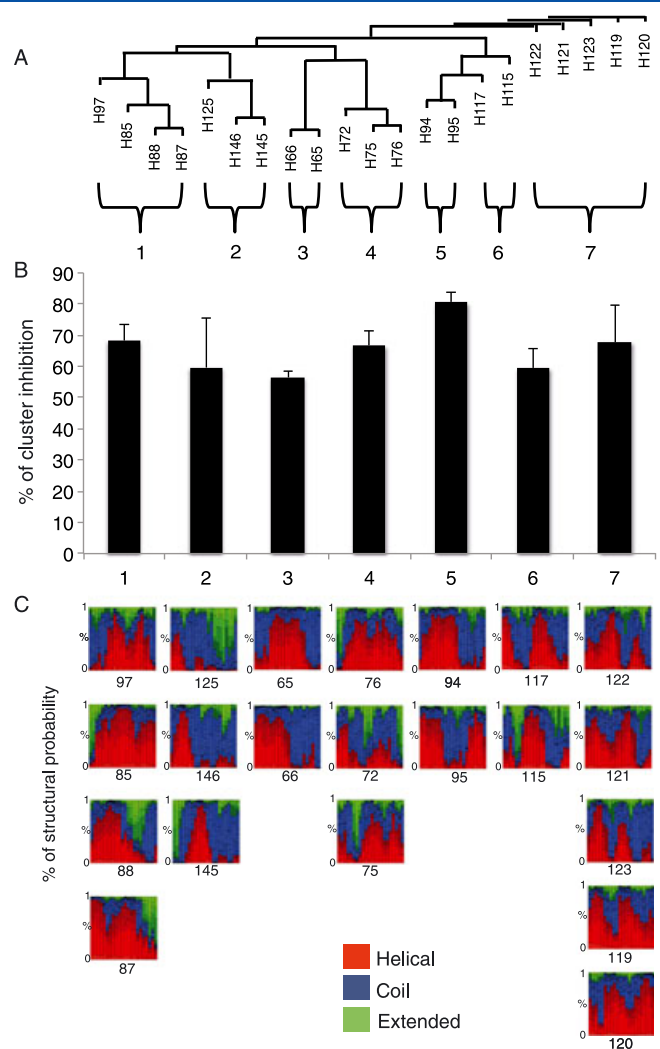


Figure 4. Sequence homology clusterization of gH active peptides. A. Sequence homology tree based of active gH peptides. B. Inhibition activity recorded based on sequence homology clusterization. C. Structural alphabet (SA) prediction profile. The probabilities, at each position of the sequence, of the 27 SA letters are sorted from helical (red), coil (blue) to extended (green). Note: SA letters are 4 residue length; therefore, the profile is of the size of the amino acid sequence minus 3.

Finally, to confirm that these peptides did not exert toxic effect on Vero cells, monolayers were exposed to a range of concentrations (10, 50, 150, 200 μ M) of each inhibitory peptide for 24 h, and cell viability was assayed by an LDH assay. No statistical difference was observed between the viability of control (untreated) cells and that of cells exposed to the peptides (data not shown); thus, none of the peptides exhibited cytotoxic effects at the concentrations tested.

Discussion

Most of the viral peptide entry inhibitors described so far were intentionally designed in order to inhibit viral entry by competing with intermolecular interactions using amino acid specific sequences mostly derived from viral fusion proteins. However, an activity without a specific molecular target can be envisaged for many entry inhibitor peptides. These peptides, albeit being derived from viral surface proteins, do not belong to well-known HR

domains, but are often scattered in exposed regions of surface proteins or in domains that may only become exposed following conformational modifications triggered by binding of viruses to receptors or by environmental changes inside endosomes. The current understanding of the mechanisms of action of these peptides finds a hallmark in their propensity for membrane binding due to their interfacial hydrophobicity or amphipaticity [44,45,64,68]. In view of this characteristic, peptides could hamper virus entry by establishing direct physical interactions at hydrophobic interfaces on membranes or fusion proteins. Essentially, peptides can bind membranes, membrane proteins or virus particles, thereby exert a steric hindering effect on the early stages of viral penetration such as viral binding and/or membrane fusion. Sometimes, they can interfere with fusion protein modifications by premature initiation of the fusion proteins, which become unable to promote fusion once the peptides are bound to the viral envelope membrane. In this study, we have described an alternative approach for identifying functionally important regions of HSV-1 gH and gL, showing for the first time the HSV-1 inhibition activity of unreported peptides derived from gH protein. The approach involved the synthesis of two libraries of overlapping peptides that encompassed the entire ectodomains of gH and gL.

Brute-force scanning approaches have proved to be of interest for analysing proteins of several viruses; in fact, potential antiviral peptides against hepatitis C virus (HCV) have been discovered by screening 441 overlapping peptides (18-mers) covering the entire HCV polyprotein [69,70]. Eleven peptides with strong antiviral activity were identified. The most active inhibitory peptide, called C5A, showed significant hydrophobic and membrane binding propensity and was suggested to directly interact with the HCV viral envelope blocking infection when treating viruses during or prior to the initial entry stage. Interestingly, C5A was also able to inhibit HIV by disrupting the integrity of the viral envelope [71]. Following this line, four proteins considered to play a role for promoting HCV entry (namely: CD81, scavenger receptor B1, claudin 1 and occluding) were analysed by an overlapping 18 amino acid peptide scanning strategy, and out of 113 peptides, two of them derived from claudin 1 showed potent inhibitory activity against HCV infectivity [72]. Furthermore, HCV has also been recently analysed by screening a peptide library covering the full-length E1 and E2 amino acids sequences where a peptide from the E2 stem domain was found to block cell entry of HCV pseudoparticles at nanomolar concentrations. This peptide is of sure potential interest because it proved to be efficient against the major HCV genotypes from 1 to 6 [73]. Another study assessed the activity of 95-overlapping peptides (15-mers) covering the entire VP1 capsid protein of Enterovirus 71, an important human pathogen which may cause severe neurological complications. In this case, the strategy proved to be also usable for searching potential peptide inhibitors in non-enveloped viruses, with the discovery of four peptides able to inhibit EV-71 infection by more than 80% [74]. A further study described a 10-mer overlapped peptide library synthesized by combinatorial approach where a peptide derived from the S1 subunit of the spike glycoprotein of the severe acute respiratory syndrome associated coronavirus (SARS-CoV) could block the binding to the cellular receptor and, thus, the entry into target cells [75]. Bai *et al.* [76] used a phage display methodology to identify a peptide (named P1) to inhibit West Nile virus (WNV) infectivity, possibly by binding to the envelope glycoprotein (E protein) necessary for membrane fusion. A modified version of P1, named P9, showed a strongly increased inhibitory activity against WNV at micrometre concentrations, and, more interestingly, entered the brain by crossing the blood brain barrier

to reduce infectivity in a mouse model of brain infection. Moreover, an interesting study sparkling from the observation that HIV patients co-infected with the common and asymptomatic GB virus C (a virus related to HCV) present lower HIV viremia suggested the analysis of overlapping peptides from the E2 protein from the GB virus C and allowed the identification of two peptides able to inhibit HIV *in vitro* by their competitive binding at the gp41–gp120 interface [77,78]. Also, HSV glycoproteins have been analysed by a peptide scanning strategy. A library of overlapping 15-mer peptides encompassing the ectodomain of gB was synthesized and tested for HSV infectivity inhibition [79]. Seven of the peptides inhibited infectivity by 50% or more when tested at 100- μ M concentrations.

Interestingly, many of the antiviral peptides identified by these brute force approaches overlap with many peptides discovered by analysing hydrophobicity at interface scales [68]. The example of HSV gB is remarkable, because all the peptides (at least the most active) had also been previously described as membrane-interacting [80] sequences using the WWIHS⁶⁶. Our results, scanning the entire sequences of HSV-1 gH and gL, confirm that the WWIHS is a powerful mean to identify potential antiviral peptides, but some regions of potential interest can remain underscored; therefore, a systematic analysis of the whole sequence by overlapping peptide libraries can add more detailed information on the regions involved in inhibition. In fact, we were able to detect four areas where peptides could be grouped for their antiviral activity. Some of the identified regions overlap with peptides already described which were discovered by bioinformatics tools, thereby strengthening this clear relationship between the function and the physicochemical character of peptides. In particular, the four identified inhibitory areas are mainly located in the regions of the glycoprotein named H2, while only a small area (S4) is located in the H3 region. The H2 domain of gH is mainly characterized by a bundle of helices and a few extended regions. In gL derived from Pseudorabies virus (PRV), a synthaxin like bundle (SLB) is present in this region [81], and this motif has proved to be of importance because its disruption can lead to impaired replication activity of the virus [82]. Interestingly, one of our inhibitory peptides is located in the HSV-1 gH corresponding region. Surprisingly, we did not detect any activity in inhibition assays when testing gL derived peptides. This may likely account for the negligible role of gL in membrane interactions. Nevertheless, also domain H1 of gH which is mainly devoted to interact with gL did not provide any inhibitory peptides. H1 sub-domains clamp gL like tongs and make extensive contacts between the interacting highly complementary surfaces. In fact, the two proteins need each other to fold correctly and gL is a powerful scaffolding protein for gH. The inability of gH peptides derived from the H1 domain to function as inhibitors of infectivity can be explained by the fact that the formation of the highly stable complex between the two glycoproteins happens during the maturation and egress from the infected cell; therefore, the structure is already definitive when the heterodimer becomes expressed on the mature virion envelope. Disruption of the gH:gL interaction is not likely to happen because the whole H1 domain would result in an unfolded structure in absence of gL. The four areas of active peptides are depicted in Figure 5 where the filled surface of the protein is shown. It is of interest to note that inhibitory areas S2, S3 and S4 are mainly occupying regions of gH domains H2 and H3 and are exposed to the external surface of the protein, while only area S1 is partially buried inside and only partially exposed to the outside. Therefore, this observation is consistent with the minor inhibitory activity of peptides belonging to area S1. Furthermore, inhibitory area S4 precedes the C-terminal TM domain of gH and is part of

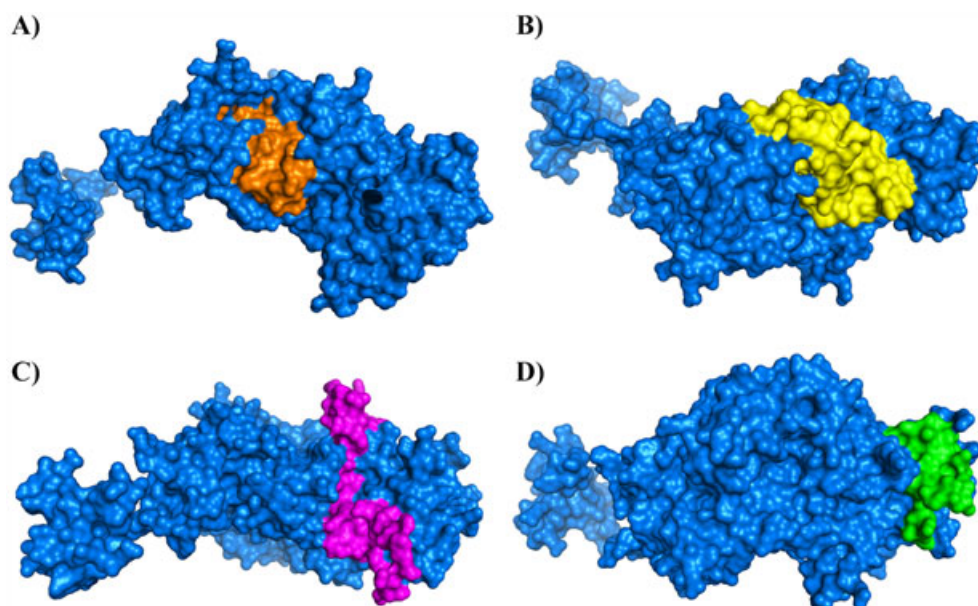


Figure 5. Filled surface representation of gH and the areas hosting inhibitory peptides. The target protein (gH) is rendered as a filled surface (light blue), and the locations of the four areas (from the N-terminal to the C-terminal of the protein) are shown. S1 is depicted in orange, S2 is in yellow, while S3 is in pink, and S4 is in green. gL is not shown, and the protein is rotated to show the best rendering for each of the inhibitory areas. Images generated from files from the PDB.

the C-terminal H3 ectodomain mainly composed of β -sheets which constitute a large patch of hydrophobic residues able to interact with the cell membrane during the fusion process or modifying the curvature of the viral envelope.

Membrane fusion is an important step in enveloped virus entry into host cells. The present study on the antiviral activity of HSV-1 derived peptides provides a large set of data obtained with overlapping peptides covering the whole ectodomains of gH and gL. These peptides may be useful for probing gH activity during membrane fusion, but their major use could be as direct antiviral molecules. Acting on early stages, the discovered peptides can be used as microbicide for topical administration following further analysis and appropriate modifications for improving their viability and efficiency. As a matter of fact, considering their molecular weight, these peptides can be categorized as mid-size drugs, which are, nowadays, receiving considerable attention in medicinal chemistry.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Table S1. gH peptide names with the respective sequences.

Table S2. gL peptide names with the respective sequences.