

Hexapeptides that interfere with HIV-1 fusion peptide activity in liposomes block GP41-mediated membrane fusion

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Abstract Upon receptor-mediated activation, the gp41 hydrophobic, conserved fusion peptide inserts into the target membrane and promotes the kind of perturbations required for the progression of the HIV-cell fusion reaction. Using a synthetic combinatorial library we have identified all D-amino acid hexapeptide sequences that inhibited the fusion peptide capacity of perturbing model membranes. Two hexapeptides that effectively inhibited the fusion peptide in these systems were subsequently shown to inhibit cell–cell fusion promoted by gp41 expressed at cell surfaces. These observations might be of importance for understanding the mechanisms underlying fusion peptide activity and suggest new strategies for screening compounds that target these viral sequences.

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1. Introduction

The human immunodeficiency virus-1 (HIV-1) relies on the fusogenic activity of the gp120/41 glycoprotein at the surface of the virion to enter and infect its CD4⁺ host cells [1]. When expressed at the surface of cells infected by HIV-1, this envelope protein may also induce cell–cell fusion and syncytia formation [2]. Induction of syncytia is a phenotype associated to the predominant T-tropic virus isolated from symptomatic individuals [3]. This process may contribute to the depletion of cells supporting viral replication and be related to the progression of the disease in humans [4]. Consequently, HIV-1 envelope glycoproteins constitute an important focus for the development of antiviral therapies [5–7].

Mutational analysis confirms the involvement of the conserved gp41 N-terminal hydrophobic fusion peptide (FP) seg-

ment in triggering the HIV-cell fusion process [8–12]. Comprehensive structure–function investigations of the complete glycoprotein have been restricted due to the difficulties of expressing gp41 constructs including this region. Consequently, HIV FP action mechanism analyses have been circumscribed to the study of representative synthetic sequences (reviewed in: [13]). Membrane interactions of these synthetic FP derivatives were first studied at a molecular level by Rafalski et al. [14] and extensively characterized afterwards by other groups (reviewed in: [15,16]; see also: [17–21]). The fact that lipid-bilayer destabilization is specifically promoted by functional, but not defective mutant FP analogs indicates that: (i) the activity of synthetic derivatives measured in model membranes is physiologically relevant; and (ii) FP sequence bears an intrinsic capacity to perturb membranes, i.e., it does not function as an inert integral anchor.

Given its high degree of residue conservation among the different HIV-1 clades and isolates [8], FP action might in principle constitute a suitable target for therapeutic intervention [8,22–25]. In the present study, we have utilized a biophysical assay: FP-induced liposome permeabilization, as a surrogate of FP activity for screening a synthetic peptide combinatorial library (SCL) [26,27]. We have developed an assay in which each peptide mixture making up an SCL can be tested for its ability to inhibit FP lytic activity on large unilamellar vesicles (LUVs). Several individual hexapeptides were identified that were found to inhibit FP activity in these model systems. Notably, two of these sequences were also inhibitory in gp41-induced cell–cell fusion assays. Our findings provide further support to the relevance of FP activity measured in model membrane systems, and also validate its use as a functional assay for SCL screening.

2. Materials and methods

2.1. Materials

NH₂-AVGIGALFLGFLGAAGSTMGARS-CONH₂ (FP) was synthesized and purified (estimated homogeneity >90%) at the Proteomics Unit of the University Pompeu-Fabra (Barcelona, Spain). Peptide stock solutions were prepared in dimethylsulfoxide (DMSO) (spectroscopy grade). AVGIGA hexapeptide was from Bachem (Bubendorf, Switzerland) and T-20 was a gift of Roche Palo Alto (Palo Alto, CA, USA). *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), *N*-(lissamine rhodamine B sulfonylethanolamine (*N*-Rh-PE), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) were

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Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid sodium salt; DMSO, dimethylsulfoxide; DPX, *p*-xylenebis(pyridinium)bromide; FP, fusion peptide; LUVs, large unilamellar vesicles; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; PS, positional scanning; SCL, synthetic combinatorial library

purchased from Avanti Polar Lipids (Birmingham, AL, USA). 8-Aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylenebis(pyridinium)bromide (DPX) were from Molecular Probes (Junction City, OR, USA). Triton X-100 was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Synthesis of peptide libraries and individual hexapeptides

The peptide library and individual hexapeptides were synthesized by solid-phase methods and simultaneous multiple peptide synthesis using *N*-(9-fluorenyl) methoxy-carbonyl (Fmoc) chemistry [28]. Peptides were assembled on polystyrene aminomethyl-RAM resin to yield peptide as C-terminal amide using diisopropylcarbodiimide/hydroxibenzotriazol-mediated couplings. The all-D-amino acid hexapeptide SCL was synthesized in the so-called positional scanning (PS) format described previously [29]. The mixture ("X") positions were incorporated by coupling a mixture of 19 D-amino acids (cysteine was omitted to avoid the formation of uncontrolled intermolecular disulphide bridges), with the relative ratios adjusted to yield near equimolar incorporation. After synthetic procedures [30] the peptide mixtures were solubilized in 5% DMSO/H₂O, to a final concentration of 3 mg/ml, and stored at -20°C .

Individual peptides were synthesized using the corresponding D-amino acids and purified by preparative reversed phase (RP) high-pressure liquid chromatography (HPLC) with a Waters Delta Pak C18 column. HPLC and MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry were used to characterize the purified peptide.

2.3. Vesicle preparation and permeability assay

LUVs were prepared following the extrusion method of Hope et al. [31] in 5 mM HEPES, 100 mM NaCl (pH 7.4) buffer. Lipid concentrations of liposome suspensions were determined by phosphate analysis. Mean diameter of vesicles in suspension, 95 nm, was estimated by quasielastic light scattering using a Malvern Zeta-Sizer instrument.

Release of vesicular contents to the medium was monitored by the ANTS/DPX assay [32]. LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 5 mM HEPES were obtained by separating the unencapsulated material by gel filtration in a Sephadex G-75 column eluted with 5 mM HEPES, 100 mM NaCl (pH 7.4). Osmolarities were adjusted to 200 mosm in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). ANTS fluorescence measurements were conducted in a Perkin-Elmer LS50-B spectrofluorimeter. ANTS emission was set at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. 0% leakage corresponded to the fluorescence of the vesicles at time zero, in the absence of peptide; 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5% v/v). For the screening of the SCL, the FP concentration was fixed at 1 μM and extents of leakage were measured at the FP-to-lipid mole ratio of 1:50. Each hexapeptide mixture was incubated for 30 min in DMSO with the FP (FP-to-hexapeptide mole ratio was 1:100) prior to addition to the vesicles.

2.4. Cell-cell fusion assay

Syncytium formation assays were carried out using CHO-Env cells (NIH AIDS Research and Reference Reagent Program, contributed by C. Weiss and J. White). CHO-WT expressing HIV-1 HXB2 Env were grown and assayed according to contributors' instructions. CHO-WT cells (effector cells) were co-seeded with HeLaT4⁺ cells (target cells) expressing CD4 and CXCR4 (ARRRP-NIH, contributed by R. Axel) and incubated overnight. Syncytia were subsequently scored under phase contrast or after fixing and staining, using an Olympus CKX41 inverted microscope equipped with a Camedia C-5050 digital camera (Olympus-Europe, Hamburg, Germany). Syncytium inhibition assay was performed incubating peptides in the culture medium for 60 min with effector CHO-WT cells prior to co-culturing with target HeLaT4⁺ cells.

3. Results

FP sequence is absolutely required for gp41 fusogenic activity [10–12]. Previous studies have demonstrated that viral FP-

induced pore-formation in liposomes is sensitive to physiologically relevant factors such as single blocking mutations [33–36]. In fact, pore-formation activity of functional FPs occurs well below the membrane rupture tensions caused by simple transfer of mass to the bilayers (insertion), indicating that this phenomenon reflects an intrinsic property of these viral sequences [13,37]. Thus, we have used pore-formation in model vesicles as a functional assay to carry out the isolation of all D-amino acid hexapeptides potentially inhibitory of FP activity (Fig. 1).

Fig. 1A illustrates the ability of the gp41 FP 23-mer analog to permeabilize POPG LUV. This is a well-documented phenomenon that reflects the assembly of permeating pores in the vesicles [14,16,34,38,39]. A peptide dose that approximately induced half of the total leakage (i.e., 1:50 peptide-to-lipid mole ratio) was selected for the hexapeptide SCL screening (Fig. 1B). The PS-SCL consisted of six sub-libraries; in each sub-library, a single position of the hexapeptide was defined with each one of 19 D-amino acids (i.e., 19 peptide mixtures/sub-library, each one corresponding to one of the natural amino acids except cysteine), while the remaining positions were composed of mixtures of all D-amino acids except cysteine. Each sub-library was pre-mixed with FP in DMSO prior to addition to the POPG LUV suspension. As an example, panel B in Fig. 1 displays the effects of representative mixtures in

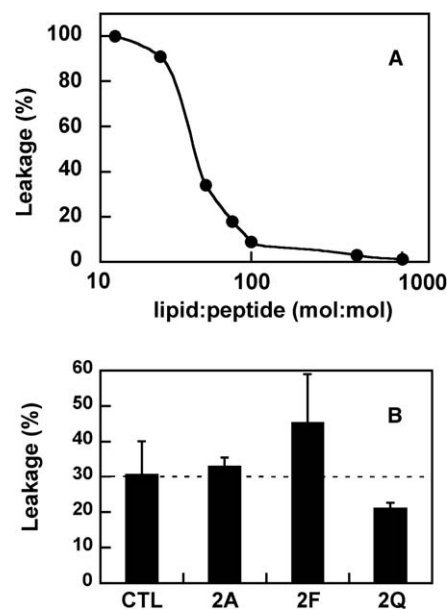


Fig. 1. (A) Leakage of vesicular contents (ANTS/DPX assay) induced by the HIV-1 FP in POPG vesicles. Increasing doses of peptide were incubated with POPG LUV (50 μM phospholipid) for 1 h at 37°C . Extent of leakage (means of two independent measurements) was subsequently plotted as a function of the lipid-to-peptide mole ratio. (B) Effects on FP-induced leakage of all D-amino acid hexapeptide mixtures derived from a sub-library with fixed positions 2. The FP concentration was 1 μM and extents of leakage were measured at the FP-to-lipid mole ratio of 1:50. Each mixture was incubated for 30 min in DMSO with the FP (FP-to-hexapeptide mole ratio was 1:100) prior to addition to the vesicles. No effect, stimulation and inhibition are illustrated by D-Ala, D-Phe, and D-Gln, respectively. CTL corresponds to the value in the absence of hexapeptides but with the same amount of DMSO. The sub-libraries alone did not induce any leakage. The bars represent means of four independent measurements plus standard deviations. Conditions otherwise as in the previous panel.

a sub-library (defined amino acids at position 2) on the FP-induced leakage.

Thus, the library used in our study (Fig. 2) was made up of 114 peptide mixtures and is represented as: Ac-o₁xxxxx-NH₂, Ac-xo₂xxxx-NH₂, Ac-xxo₃xxx-NH₂, Ac-xxxo₄xx-NH₂, Ac-xxxxo₅x-NH₂, and Ac-xxxxxo₆-NH₂ (o: defined by one of 19 D-amino acids; x: near equimolar mixture of 19 D-amino acids). Each of the 114 peptide mixtures contained approximately 2.5×10^6 (19^5) different peptides. Each sub-library addressed a separate position of the hexapeptide and, when used in concert, the data provided information about the most important amino acid residue at each position. Such segregation

permits information to be obtained in a single assay, which is then used to synthesize series of individual peptides directly from the screening results.

Fig. 2A shows the effects of the complete SCL on FP-induced POPG LUV permeability. The increase or decrease in leakage over that observed in absence of the hexapeptide mixtures (0 level) was plotted for each defined position. For most positions the intrinsic lytic effect of the sub-library alone was null or negligible, but in certain instances (indicated by the stars) the value of the sub-library-induced leakage oscillated between 10% and 15%. Thus, leakage induced by the sub-library alone was subtracted from the observed leakage when required. Clear

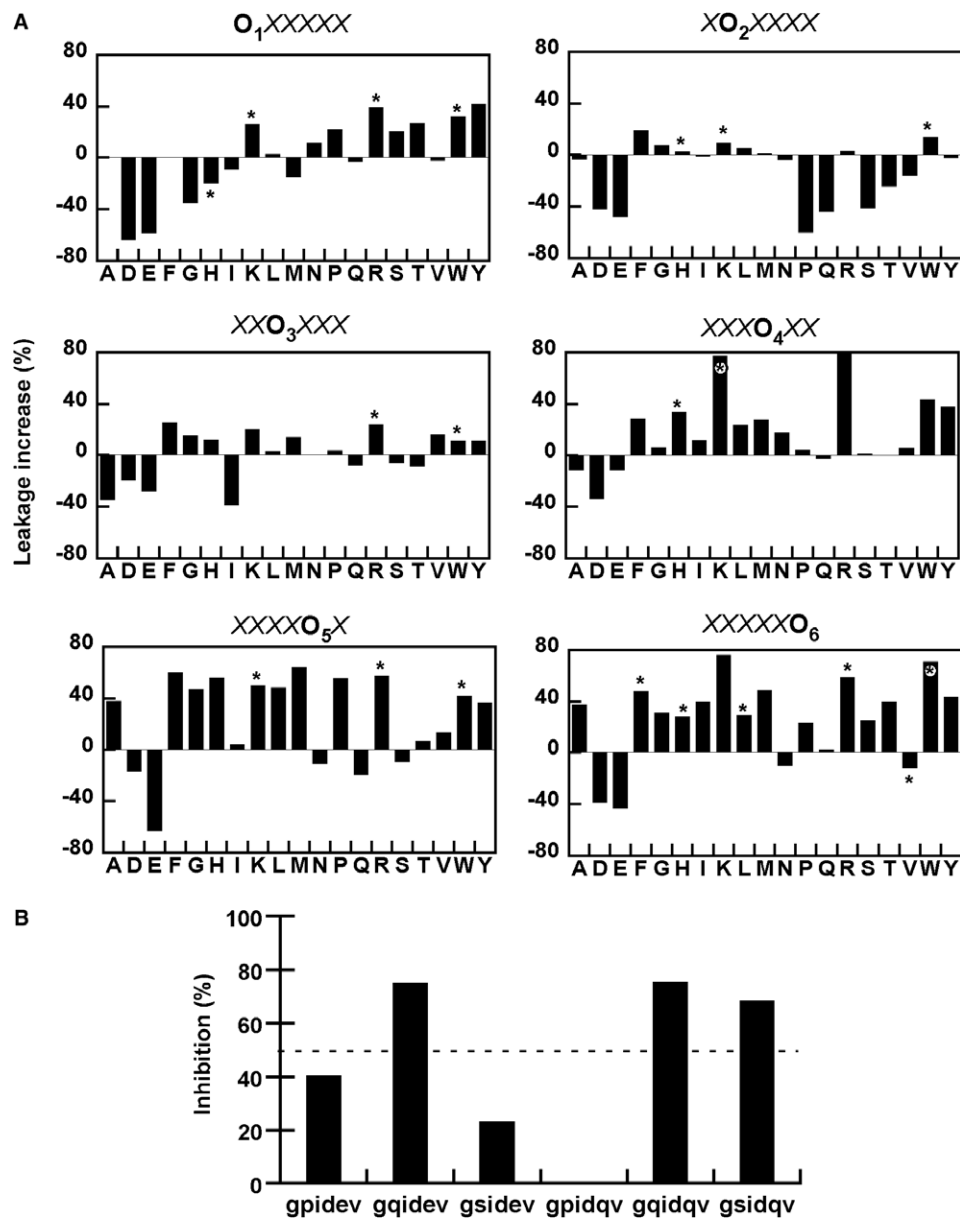


Fig. 2. (A) Screening of Ac-o₁xxxxx-NH₂, Ac-xo₂xxxx-NH₂, Ac-xxo₃xxx-NH₂, Ac-xxxo₄xx-NH₂, Ac-xxxxo₅x-NH₂, and Ac-xxxxxo₆-NH₂ for inhibition of FP permeabilizing activity. Each peptide mixture was screened at FP-to-hexapeptide 1:100 mole ratio under the experimental conditions described in the previous figure. Each panel represents a set of 19 peptide mixtures having a common residue at a defined position (X axis). The bars represent percentages of leakage increase (i.e., negative increase reflects inhibition). Means of four independent measurements are plotted. (B) Inhibition of FP-induced POPG LUV permeabilization by all D-amino acid hexapeptides derived from the previous SCL screening. The sequences were acetylated at N-terminus and amidated at C-terminus. In these assays the FP concentration was 1 μ M and the FP-to-hexapeptide mole ratio 1:40.

differences were observed between the most and least active mixtures, suggesting that inhibition or promotion were due to individual peptide(s) present in the mixture. The so-called deconvolution process of the library consisted, first, in selecting the most active mixtures as inhibitors, and identifying the relevant amino acid residues for each position. The greatest differences in the inhibitory effect (values below 0 level) between the most active mixtures and the others were observed within positions 2 and 5. Since target vesicles were made of anionic phospholipids, as an additional criterion, Asp and Glu effects were neglected when these residues exerted at the same position comparable inhibition (e.g., at positions 1, 2, 3 or 6).

As a result of our focused deconvolution process, we selected the following mixtures at each position: g(1)-p/q/s(2)-i(3)-d(4)-e/q(5)-v(6). The six possible D-amino acid hexapeptide sequences with putative anti-FP activity were synthesized and subsequently assayed against FP (Fig. 2B). Three sequences resulted clearly inhibitory (i.e., they induced >50% reduction in observed leakage): Ac-gqidev-NH₂, Ac-gqidqv-NH₂ and Ac-gsidqv-NH₂. These sequences were further selected as potential inhibitors of gp41 activity to be tested in gp41-induced cell–cell fusion assays (Fig. 3).

In accordance with their capacity to inhibit gp41 activity, Ac-gqidev-NH₂ and Ac-gqidqv-NH₂ significantly reduced syncytium formation at a concentration of 5 μM (Fig. 3A). The Ac-gsidqv-NH₂ hexapeptide, which also inhibited FP-induced pore formation in liposomes, did not appreciably suppress gp41 activity under these conditions. Finally, a negative control sequence, Ac-gpidqv-NH₂, which did not affect FP activity in model membranes (Fig. 2B), was not inhibitory in the cell–cell fusion assays either. All D-amino acid hexapeptides tested in these assays inhibited cell growth only when used at ×10³ higher concentrations (not shown). Thus, cell–cell fusion results appear to be consistent with specific gp41 inhibition induced by Ac-gqid(e/q)v-NH₂ sequences.

The inhibitory potency of the isolated Ac-gqidev-NH₂ and Ac-gqidqv-NH₂ hexapeptides was subsequently compared with those of the specific inhibitors T-20 and all L-amino acid AVGIGA hexapeptide (panels B and C). The former has been shown to inhibit gp41-induced fusion in the nM range [40,41], while the latter is inhibitory in the mM range [22]. Co-incubation of the effector and target cells at 37 °C in the absence of peptides resulted in formation of large syncytia (control in panel B). When T-20 (20 nM), Ac-gqidev-NH₂ (50 μM) or AVGIGA (1 mM) were present in the external solution, they inhibited fusion as evidenced by the large decrease in multinucleated cells in the culture (Fig. 3B). These compounds inhibited fusion in a dose-dependent manner showing apparent IC₅₀ values of ca. 4 nM, 20 μM and 150 μM, respectively (Fig. 3C). Thus, when compared with isolated Ac-gqidev-NH₂ hexapeptide, T-20 showed a ca. 5000× inhibitory potency (or ≈500–1000×, when considered the differences in molecular weight). In contrast, AVGIGA hexapeptide required higher doses than Ac-gqidev-NH₂ to suppress fusion. The sequence Ac-gqidqv-NH₂ (apparent IC₅₀ value of ca. 100 μM) was also less effective than Ac-gqidev-NH₂, but still more inhibitory than AVGIGA. Neither of the peptides showed a lytic activity on the cells within the concentration range tested here. Thus, the isolated D-amino acid sequences seem to represent optimized versions of the previously described inhibitory hexapeptides [22].

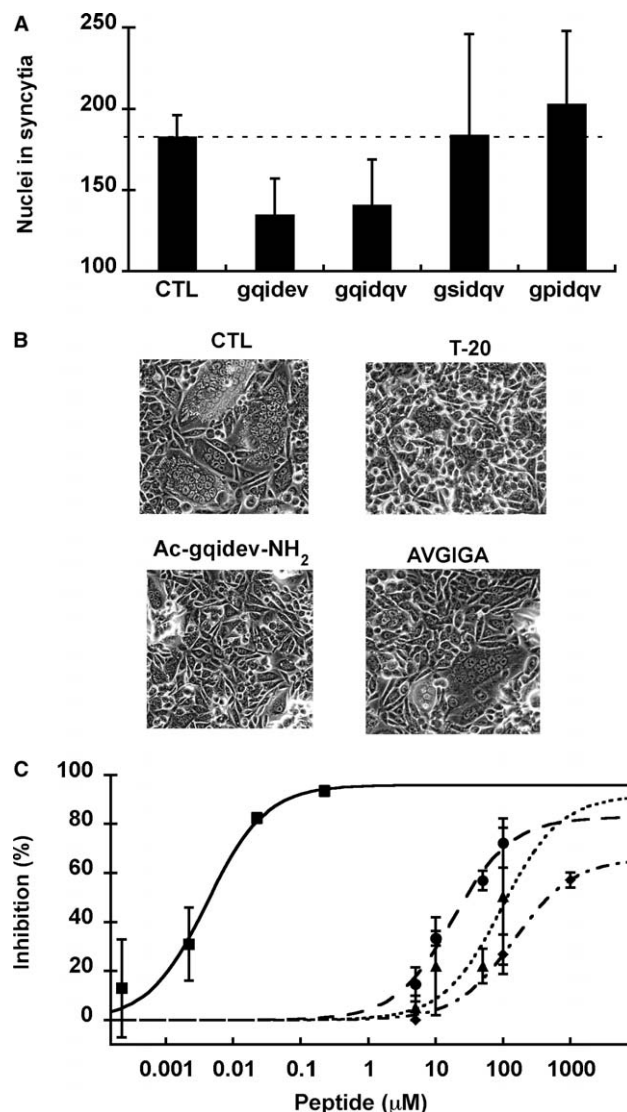


Fig. 3. Peptide inhibition of gp41-induced cell–cell fusion. (A) Effect of all D-amino acid hexapeptides on syncytium formation (hexapeptide concentration: 5 μM). Bars represent the number of nuclei present in syncytial plaques (fused cells containing 4 or more nuclei) per field. Values correspond to means of four independent experiments plus standard deviations. Depicted sequences were acetylated at N-terminus and amidated at C-terminus. (B) Inhibition of gp41 fusion activity by incubation with peptides. Target-effector cells grown in 96-well plates were treated with T-20 (20 nM), Ac-gqidev-NH₂ (50 μM) or AVGIGA (1 mM). CTL corresponds to untreated control. (C) Quantitation of inhibition by pre-incubation in solution with T-20 (squares and solid lines), Ac-gqidev-NH₂ (circles and dashed lines), Ac-gqidqv-NH₂ (triangles and dotted line) and AVGIGA (diamonds and dot-dashed line). Means of four independent measurements plus standard deviations are displayed. Lines correspond to best fits to hyperbolic functions.

4. Discussion

The growth in worldwide AIDS epidemic together with the emergence and spread of drug-resistant HIV variants reinforces the need of implementing new therapies with unprecedented mechanisms of action [5–7]. Rescue therapies based on the fusion inhibitor T-20 (enfuvirtide, fuzeon) have been recently proven useful in the treatment of multidrug-resistant virus in-

fecting AIDS patients [7]. Our results support the suggestion that the repertoire of potential anti-HIV fusion-inhibitors might be improved with the addition of agents showing ability to interfere with conserved gp41 FP activity [8,22–25]. Owens and coworkers [22] demonstrated the capacity of AVGIGA hexapeptide, identical in amino acid sequence to the gp41 N-terminus, to completely inhibit syncytium formation mediated by HIV-1 envelope glycoproteins expressed at the surface of HeLa CD4⁺ cells, an observation that has been reproduced in the present work (Fig. 3). The inhibitory effect was sequence-specific and dose-dependent. These authors suggested that the inhibitors might interact with its homologous FP sequence at gp41 thereby preventing its normal fusogenic function. This suggestion was supported by later studies in model membranes by Pereira et al. [42], which revealed that N-terminal AVGIGA hexapeptide also interfered with FP-vesicle interaction.

Based on those findings, we decided to make use of the membrane-permeabilizing FP activity for screening an all D-amino acid hexapeptide PS-SCL (Figs. 1 and 2). The inhibitory Ac-gqidv-NH₂ and Ac-gqidqv-NH₂ hexapeptides identified using the liposome-based screening also inhibited gp41-induced fusion in a dose-dependent manner (Fig. 3). Both sequences showed an increased potency in comparison to AVGIGA (Fig. 3). A substitution of polar D-Gln by hydrophobic D-Pro in position 2 rendered a sequence inactive against FP (Fig. 2) or gp41 (Fig. 3). Moreover, Ac-gqidv-NH sequence inhibited cell–cell fusion more potently than less polar Ac-gqidqv-NH₂ (Fig. 3). Thus, the inhibitory effect of these compounds seemed to be sequence-specific, and not merely based on hydrophobic interactions with either FP or membranes.

HIV-envelope-mediated membrane fusion is a multi-step process that offers several targets for intervention. According to Blumenthal and coworkers [43], it is presumed that the FP inserts into the target membrane upon engagement of the co-receptor by the envelope, a process that is preceded by at least three distinct steps: CD4 receptor engagement, pre-hairpin formation and co-receptor–receptor co-localization. The fact that anti-gp41 hexapeptides blocked FP-induced LUV permeabilization suggests that these compounds interfere with FP fusogenic function. Thus, we surmise that hexapeptides interact with target FP along the fusion pathway at the stages that precede insertion and alter its capacity to interact with membranes. In line with this prediction, we have observed that the hexapeptides that were inhibitory for leakage and gp41-induced cell–cell fusion, enhanced peptide-induced cation-assisted vesicle fusion (not shown). This is consistent with our previous work indicating that leakage and fusion are conflicting processes sustained by different peptide structures [16,38,39]. Thus, our results indicate that the inhibition mechanism might be based on the capacity of inhibitory hexapeptides to promote the extended FP conformations that reduce leakage and stimulate vesicle fusion. Structural analyses are under way to sustain that hypothesis.

Therefore, the inhibitory hexapeptides and T-20 are postulated to inhibit gp41-mediated fusion according to different mechanisms [5–7,22,40,41]. Supporting this view, when Ac-gqidv-NH₂ or Ac-gqidqv-NH₂ was mixed with T-20 a slightly negative synergy was observed in fusion inhibition assays (not shown). Thus, we anticipate that optimized versions of the fusion-inhibitors described here might be used in combination with, and/or as an alternative to T-20 in order to reduce the probability of resistant virus emergence during rescue therapies.

5. Concluding remarks

The use of a biophysical assay that included isolated synthetic FP derivatives and model membranes has been proven useful in the screening and identification of compounds with specific anti-gp41 activity. This novel approach obviates potential unspecific side effects that SCLs might cause on complex cellular systems during the screening procedure. Consequently, a similar methodology could be applied to the identification of compounds active against various viral fusion domains, such as influenza hemagglutinin or Ebola-GP2 FP sequences, whose abilities to form permeating pores in model membranes have been shown to be sensitive to mutations that block glycoprotein fusogenic activity [33,35–37]. Finally, we caution that the approach described here might serve to establish potential inhibitory compounds, which might turn out not being the most active sequences. However, these compounds might comprise useful leads for rational optimization once the inhibition mechanism is firmly established.

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