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Potent Strategy To Inhibit HIV-1 by Binding both gp120 and gp41▽†

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The development of an anti-HIV microbicide is critical in the fight against the spread of HIV. It is shown here that the covalent linking of compounds that bind gp120 with compounds that bind gp41 can inhibit HIV entry even more potently than individual inhibitors or noncovalent combinations. The most striking example involves griffithsin, a potent HIV inhibitor that binds to the surface of HIV gp120. While griffithsin inhibits HIV Env-mediated fusion in a CCR5-tropic cell-cell fusion assay with a 50% inhibitory concentration (IC_{50}) of 1.31 ± 0.87 nM and the gp41-binding peptide C37 shows an IC_{50} of 18.2 ± 7.6 nM, the covalently linked combination of griffithsin with C37 (Griff37) has an IC_{50} of 0.15 ± 0.05 nM, exhibiting a potency 8.7-fold greater than that of griffithsin alone. Similarly, in CXCR4-tropic cell-cell fusion assays, Griff37 is 5.2-fold more potent than griffithsin alone. In viral assays, both griffithsin and Griff37 inhibit HIV replication at midpicomolar levels, but the linked compound Griff37 is severalfold more potent than griffithsin alone against both CCR5- and CXCR4-tropic virus strains. Another example of this strategy is the covalently linked combination of peptide C37 with a variant of the gp120-binding peptide CD4M33 (L. Martin et al., Nat. Biotechnol. 21:71–76, 2003). Also, nuclear magnetic resonance (NMR) spectra for several of these compounds are shown, including, to our knowledge, the first published NMR spectrum for griffithsin.

About 2.7 million people are infected with HIV each year, and women constitute 50% of the 33 million people living with AIDS (57). In the developing world, effective prevention strategies are lacking, often because women have limited freedom to choose sexual situations or to insist on condom use. Therefore, the development of an anti-HIV microbicide is extremely important. Properties that are desirable in a microbicide include the ability to inhibit HIV infection effectively at low concentrations, the ability to be applied topically on a regular basis without causing inflammation, stability to fluctuating temperatures, and inexpensive production.

The early events in an HIV infection in T cells can be described as follows. The HIV envelope protein gp120 first makes contact with the human cell surface protein CD4, which causes conformational changes in gp120. The gp120–CD4 interaction facilitates the formation and exposure of the binding site on gp120 for its coreceptor on the human cell, the chemokine receptor CCR5 (R5) (or CXCR4, or both for some strains) (2, 31, 52, 55). These HIV-cell interactions lead to the exposure of the HIV protein gp41, which mediates cell fusion. gp41 exists as a trimer with three major segments: the N-terminal fusion peptide (FP), which is inserted into the cell; the so-called N-terminal heptad repeat; and the C-terminal heptad repeat. After the fusion peptide has been inserted into the cell membrane, the N-terminal and C-terminal segments come together to form a 6-helix bundle, a "trimer of hairpins" (reviewed in references 15 and 51). This action has the effect of pulling the viral membrane surface close to the cellular surface, facilitating the formation or stabilization of a viral pore. It has been reported recently that these events may occur partly in the endosome: early binding events in cell fusion may occur at the cell surface, after which the entire complex is internalized into an endosome for the final fusion process (39).

Several compounds have been shown to be successful in the inhibition of early events in HIV infection (entry inhibition) (23, 24, 58). Griffithsin is an alga-derived entry inhibitor that is a leading candidate for a protein microbicide, having been shown to inhibit HIV infection potently (16, 40), to be stable at warm temperatures and in the low-pH environment of cervical fluid (16), and, recently, to be able to be produced in gram quantities by overexpression in plants (41). The mechanism of action of griffithsin is likely based on its ability to bind the saccharides (particularly mannose) that cover the surfaces of both HIV gp120 and gp41 (40). As evidence that the mechanism of this inhibition involves binding of griffithsin to the glycosylated surface of gp120/gp41, exogenous addition of several types of individual saccharides has been shown to block the ability of griffithsin to inhibit HIV (40). Also, griffithsin crystallizes in the presence of mannose (62), glucose, and *N*-acetylglucosamine (64), in each case revealing three saccharide binding sites per monomer. A smaller molecule that is targeted to the gp120–CD4 interaction is the designed peptide CD4M33. This 27-amino-acid peptide has been shown to bind gp120 tightly, although micromolar concentrations are required for inhibition in cell-cell fusion assays using vaccinia virus technology (38).

Another very effective anti-HIV strategy has been to inhibit the formation of the gp41 "trimer of hairpins". This has been accomplished effectively by peptides that bind to the helical

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regions of gp41, disallowing the formation of the full 6-helix bundle (51). The T-20 molecule is such a peptide that is derived from the C-terminal heptad repeat of gp41 (a "C-peptide") and effectively binds the N-terminal region of the protein (4, 33, 47). T-20 is currently in clinical use, and further work has also focused on the similar C-peptides C34 and C37, which are nearly identical to each other (differing by three extra amino acids at the N terminus for C37) and which also bind to the N-terminal region of gp41 and prevent the formation of the 6-helix bundle that is critical for viral fusion (5, 36, 50, 51).

We hypothesized that a single compound with the capability of interrupting both the gp120–CD4 interaction and the formation of the gp41 6-helix bundle could have several advantages. From a structural standpoint, the physical distance between target binding areas for linked compounds is not large, since gp120 and gp41 are part of the same complex in the viral spike. Functionally, a linked compound may improve binding affinity and/or specificity over that of its individual counterparts. Such a compound may have advantages in terms of the ability to target the virus under conditions of blood flow or in a physiological environment. Finally, an inhibitor encompassing two separate strategies may provide comprehensive protection against multiple clades of virus even in the event of viral mutation.

In the present study, we tested several linked compounds that carry out the strategy of binding both gp120 and gp41. It was found that one compound, griffithsin covalently linked via a 16-amino-acid peptide linker with the gp41-binding peptide C37 (Griff37), is effective at lower concentrations than griffithsin alone or griffithsin in combination with C37 (1:1) in cell-cell fusion assays and in viral assays. Griff37 also exhibits a great deal of specificity under conditions of washout and competition. Overall, this and the other HIV inhibitors reported here may be useful as general anti-HIV therapeutics or, more specifically, as anti-HIV microbicides.

MATERIALS AND METHODS

Protein production and purification. Two peptide fusion inhibitors, N-acetylated, C-terminally amidated C37 and N-acetylated, C-terminally amidated C37(Q652L), were obtained from Genescript (Piscataway, NJ). The synthesized peptide CD4M33 was kindly provided by Loïc Martin. The genes for GriffC37 and C37CD4M33$_{C1F23}$ (the gp41-binding peptide C37 covalently linked via a 16-amino-acid peptide linker with CD4M33$_{C1F23}$) DNA were synthesized by Genscript. All the constructs were placed in pET-15b (Novagen, Madison, WI). The amino acid sequence of CD4M33$_{C1F23}$ (defined in reference 36 with Cys at position 1 and Phe at position 23) is CNLHF CQLRC KSLGL LGKCA GSFCA CV. The amino acid sequence of the linker used for Griff37 as well as for C37CD4M33$_{C1F23}$ is SSSGG GGSGG GSSSG S, with minor variations between the different constructs due to cloning procedures. The gene for griffithsin and Griff37 includes a 6-histidine affinity tag as well as a thrombin cleavage site, similar to that previously described (22). The sequence for Griff37 is MGGSS HHHHH HSSGL VPRGS LTHRK FGGSG GSPFS GLSSI AVRSG SYLDA IIIDG VHHGG SGGNL SPTFT FGSGE YISNM TIRSG DYIDN ISFET NMGRR FGPYG GSGGS ANTLS NVKVI QINGS AGDYL DSLDI YYEQY SSSGG GGSGG GSSSG SHTTW MEWDR EINNY TSLIH SLIEE SQNQQ EKNEQ ELL. The sequence for C37CD4M33$_{C1F23}$ is MHHHH HHIEG RHTTW MEWDR EINNY TSLIH SLIEE SQNQQ EKNEQ ELLSS SGGGG SGGGG SSSSC NLHFC QLRCK SLGLL GKCAG SFCAC V.

Griffithsin was expressed in *Escherichia coli* BL21(DE3) (Novagen) cells in Luria-Bertani (LB) broth. Protein production was induced upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, followed by incubation for 4 h at 37°C. Pellets from these cells were resuspended in a 30-ml solution (500 mM NaCl, 20 mM Tris [pH 8], 10 mM benzamidine) and were then French pressed twice at 16,000 lb/in^2. After centrifugation for 1 h at 17,000 × *g*, the supernatant was loaded onto a Ni chelating column (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris (pH 8.0)–500 mM NaCl and was eluted with a gradient using 500 mM imidazole, 50 mM Tris (pH 8.0), and 500 mM NaCl. The fractions containing purified protein were dialyzed in a buffer (20 mM Tris [pH 8.0]) at 4°C overnight. The concentrated protein was further purified on a C4 reversed-phase chromatography column (Vydac, Hesperia, CA) and was then lyophilized in a Labconco freeze-dry system (Labconco Corporation). For nuclear magnetic resonance (NMR) and some functional studies, protein was produced in minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source, and the same purification procedure was used after production. In early preparations, cleavage of the N-terminal histidine tag at the thrombin site was attempted by incubation at room temperature in the presence of thrombin. This attempt was unsuccessful, so experiments proceeded in the presence of the His tag for griffithsin, Griff37, Griff37(Q652L), Griff37-shortlink, Griff37(I642D), and griffithsin covalently linked via a 16-amino-acid peptide linker with CD4M33$_{C1F23}$ (GriffCD4M33$_{C1F23}$). Others have shown that the presence of an N-terminal His tag does not affect the activity of griffithsin (22, 40).

Griff37, Griff37(Q652L), Griff37-shortlink, Griff37(I642D), GriffCD4M33$_{C1F23}$, and C37CD4M33$_{C1F23}$ were expressed in BL21(DE3) either in LB medium or in minimal medium as indicated above, although these proteins were found in the inclusion body. Therefore, after induction for 4 h upon addition of 1 mM IPTG at 37°C, the cells were resuspended in 30 ml of 5 M guanidinium chloride–500 mM NaCl–20 mM Tris (pH 8.0) and were then French pressed at 16,000 lb/in^2. After centrifugation for 1 h at 17,000 × *g* to remove undissolved material, the supernatant was loaded onto a Ni chelating column (Amersham Pharmacia Biotech) equilibrated with 5 M guanidinium, 50 mM Tris (pH 8.0), and 500 mM NaCl. Elution was carried out with 5 M guanidinium, 500 mM imidazole, 50 mM Tris (pH 8.0), and 500 mM NaCl. Fractions containing purified protein were combined; β-mercaptoethanol was added to a final concentration of 10 mM; and the mixture was incubated for 2 h with slow stirring. The protein was then dialyzed in 20 mM Tris (pH 8.0) at 4°C overnight, and it was further purified as described above, by C4 reversed-phase chromatography followed by lyophilization. For C37CD4M33$_{C1F23}$, a further step of proteolytic cleavage of the His tag by factor Xa was added (the reaction buffer consisted of 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1 mM $CaCl_2$). Cleaved protein was finally purified by C4 reversed-phase chromatography and was then lyophilized in a Labconco freeze-dry system.

The proteins were analyzed by mass spectrometry on an Agilent 1100 high-performance liquid chromatograph (HPLC) and a Thermo Fisher LCQ ion trap mass spectrometer (Stanford University). Experimentally obtained M_rs (with expected values based on amino acid sequences shown in parentheses) are as follows: for griffithsin, 14,552 (14,554, assuming N-terminal Met cleavage); for Griff37, a broad peak at 20,544 (20,519); for Griff37(Q652L), 20,259 (20,504); and for C37CD4M33$_{C1F23}$, 8,696 (8,709).

Cell culture. Seven cell lines were used. (i) HeLa-ADA cells, which stably express HIV-1 Env from the CCR5-tropic strain ADA, were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) plus 2 μM methotrexate (Sigma) as a selective reagent. This cell line was a kind gift from M. Alizon and Anne Brelot (Cochin Institute, Paris, France) (43). (ii) HeLa-P5L cells, which stably express the human receptors CD4 and CCR5, were maintained in RPMI-1640 supplemented with 10% FBS plus 0.5 mg/ml zeocin (Invitrogen) for selection of CCR5 expression. They were a kind gift from M. Alizon and Anne Brelot (Cochin Institute, Paris, France) (43). (iii) HeLa-TZM-bl cells, which stably express the human receptors CD4, CCR5, and CXCR4, were maintained in DMEM supplemented with 10% FBS. This cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and was a gift to that program from John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc. (13, 42, 56, 59). (iv) HL2/3 cells, which stably express HIV-1 Env and Tat from the CXCR4-tropic strain HXB2, were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and were a gift to that program from Barbara K. Felber and George N. Pavlakis (8). The cells were maintained in DMEM supplemented with 10% FBS and 500 μg/ml G418. (v) MAGI-CCR5 cells, which stably express human CD4 and CCR5, naturally express CXCR4, and contain an HIV-1 long terminal repeat (LTR)–β-galactosidase reporter gene construct, were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine, 0.2 mg/ml G418, 0.1 mg/ml hygromycin B, and 1 μg/ml puromycin. These cells were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, and were a gift to that program from Julie Overbaugh (3). (vi) 293FT cells were maintained in DMEM supplemented with 10% FBS and were a kind gift from Jennifer Manilay; they were originally obtained from Invitrogen (Carlsbad, CA). (vii) Mouse 3T3 cells (a kind gift from

Jean Phillipe Pellois; originally obtained from the American Type Culture Collection [ATCC]) were maintained in DMEM supplemented with 10% FBS.

In addition, fresh human peripheral blood mononuclear cells (PBMCs) were isolated and used in antiviral assays as previously described (32, 48).

Virus isolates. HIV-1 isolates were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, as follows: HIV-1 Ba-L from Suzanne Gartner, Mikulas Popovic, and Robert Gallo (18, 44); HIV-1 Ada-M (referred to below as HIV-1 ADA) from Howard Gendelman (19–21, 60); HIV-1 92HT599 from Neal Halsey and the DAIDS, NIAID; HIV-1 IIIB from Robert C. Gallo (45, 46, 49); HIV-1 91US005 from Beatrice Hahn and the DAIDS, NIAID; and NL4-3 from Malcolm Martin (1). HIV-1 Ba-L, 92HT599, NL4-3, and 91US005 were used for PBMC assays, while HIV-1 Ba-L, ADA, and IIIB were used for antiviral assays using MAGI cells (multinuclear-activation galactosidase indicator cells).

Cell-cell fusion assay. HIV-1 envelope-mediated cell-cell fusion assays have been described elsewhere (43). Briefly, for the CCR5-tropic fusion assay, 10^4 HeLa-P5L cells (target) per well were seeded in a 96-well plate. After ~12 h, the medium was replaced with 50 μl RPMI-1640. Serial dilutions of inhibitor were added to the wells of the plate: 20 μl of protein or peptide was added to the first well and mixed; then 20 μl was removed and added to the next well; and so on. A total of 10^4 HeLa-ADA cells (effector) in 50 μl RPMI-1640 were then added to each well (total, 100 μl medium per well). The cells were allowed to fuse for 24 h at 37°C. Cells were then lysed by the addition of 0.5% NP-40 (US Biological) in phosphate-buffered saline (PBS) for 30 min, after which 8 mM substrate (chlorophenol red-β-D-galactopyranoside [CPRG]; Calbiochem) in PBS with 20 mM KCl and 10 mM β-mercaptoethanol (Sigma) was added, and the cells were assayed for β-galactosidase activity. The absorbance at 570 nm (signal) divided by the absorbance at 630 nm (background) was measured. The percentage of cell-cell fusion was calculated as [100 × (mean absorbance of the treated well − mean absorbance of the HeLa-P5L-only well)]/(mean absorbance of the untreated well − mean absorbance of the HeLa-P5L-only well). The "treated" wells contained an inhibitor, while "untreated" wells contained effector cells plus target cells in the absence of inhibitors. KaleidaGraph (Synergy Software, Reading, PA) was used to fit the data into a four-parameter logistic equation. Some combinations of compounds were assessed for possible synergistic effects by calculating the combination index (CI) (7). *P* values were obtained using an unpaired, 2-tailed Student *t* test in Microsoft Excel. The tables in the supplemental material show more comparisons by this method.

In the CXCR4-tropic fusion assay, HeLa-TZM-bl cells were used as target cells and HL2/3 cells were used as effector cells. The rest of the procedure was identical to that for the CCR5-tropic fusion assay.

In the competition CCR5-tropic fusion assay, 5×10^3 HeLa-P5L cells were seeded together with 5×10^3 3T3 cells per well. The rest of the procedure was identical to the normal CCR5-tropic fusion assay.

In the low-temperature CCR5-tropic fusion assay, the addition of effector cells to target cells was immediately followed by 2 h of incubation at 16°C. Afterwards, the plate was returned to the 37°C cell incubator for 24 h.

For the washout assay, HeLa-ADA or HeLa-P5L cells (10^4 cells) were seeded in a 96-well plate the day before the assay. After the formation of the inhibitor gradient by serial dilution, as described above, the cells remained at room temperature for 30 min. Afterwards, the medium containing the inhibitor was removed, and every well was washed with PBS twice. A new medium without inhibitor was added, and the complementary cells (HeLa-P5L cells or HeLa-ADA cells) were seeded in the plate.

Single-round infection assay. HIV virions in which *vpr* and *env* had been deleted were used to make single-round infectious particles as follows. Plasmids pNL-luc3-R^-E^-, containing the firefly luciferase gene, pSV-ADA(R5), and pSV-JRFL(R5) were kind gifts from Nathaniel Landau (10). For virus production, 293FT cells were doubly transfected with pNL-luc3-R^-E^- and either the pSV-ADA or the pSV-JRFL plasmid according to the product manual (47a). Forty-eight hours posttransfection, the supernatant was harvested, centrifuged at low speed, and filtered with a 0.45-μm-pore-size syringe filter. This viral stock was stored at −80°C. For the assay, 10^4 TZM-bl cells per well were seeded in a 96-well plate. The next day, the medium was removed and replaced with 50 μl of new medium. Serial dilutions of the inhibitor were carried out in the wells of the plate: 20 μl of protein or peptide was added to the first well and mixed; then 20 μl was removed and added to the next well; and so on. Virus was added in an amount sufficient to produce a luciferase signal between 60,000 and 80,000 arbitrary units for the ADA-env pseudovirus (while the noninfected-cell control gave ~600 arbitrary units) and between 40,000 and 60,000 arbitrary units for the JR-FL-env pseudovirus. The total volume of medium per well after pseudovirus addition was 100 μl. After 24 h, the old medium was removed and replaced with new medium. Another 24 h later, the medium was removed, and the cells were lysed using Glo lysis buffer (Promega) according to the manual. The luciferase substrate was then added (Luciferase assay system; Promega), and the plate was read using an Orion II microplate luminometer (Berthold Techniques, Germany). For Griff37(I642D) and Griff37-shortlink, the CPRG substrate was used as described for the cell fusion assay. The percentage of viral infection was calculated as [100 × (mean absorbance of the treated well − mean absorbance of the TZM-only well)]/(mean absorbance of viral infection in the absence of inhibitor − mean absorbance of the TZM-only well). The results were plotted using Microsoft Excel, and the 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) were calculated using a linear equation fitted between two experimental points surrounding the IC_{50} or IC_{90}.

MAGI antiviral assays. The CCR5- and CXCR4-tropic MAGI antiviral assays were both performed using MAGI-CCR5 cells in a manner identical to that for the CCR5-tropic HIV-1 entry assay described previously (32), except that the virus and test compounds were left in the culture for the entire 48-h incubation period, in contrast to the washing out of the virus and test compound 3 h postinfection for the HIV-1 entry assay.

HIV-1 strains Ba-L and ADA were used for the CCR5-tropic assays, and HIV-1 strain IIIB was used for the CXCR4-tropic assays. Coreceptor dependence for each of the viruses used to infect MAGI-CCR5 cells, which express both CCR5 and CXCR4, was verified by the use of AMD3100 (CXCR4 inhibitor; positive-control inhibitor for IIIB and negative-control inhibitor for Ba-L and ADA) and TAK779 (CCR5 inhibitor; positive-control inhibitor for Ba-L and ADA and negative-control inhibitor for IIIB) as control compounds (data not shown). Data were processed in a manner similar to that described above for the single-round infection assay. In these cell-based assays, cell viability and 50% toxic concentrations (TC_{50}s) were derived with a commercially available soluble tetrazolium-based MTS reagent (CellTiter 96 AQueous One Solution cell proliferation assay) by using a colorimetric method for determining the number of viable cells in proliferation, cytotoxicity, or chemosensitivity assays. All compounds tested showed no toxicity at the highest tested concentration (100 nM for C37; 10 nM for griffithsin and Griff37) (32).

NMR spectroscopy. Samples that were isotopically labeled with ^{15}N were prepared by growing BL21(DE3) containing the expression vector pET-15b in the presence of minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source as described above. Samples were dissolved in 20 mM sodium phosphate buffer (pH 7.0) with 5% D_2O and a small amount of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for spectral referencing (61). The samples were placed in Shigemi tubes (Shigemi, Inc., Allison Park, PA). Spectra were measured at 25°C on a four-channel 600-MHz Bruker Avance III spectrometer equipped with a GRASP II gradient accessory and a TCI cryoprobe. ^1H-^{15}N correlation spectra were measured with 760 complex points in the ^1H dimension and 64 complex points in the ^{15}N dimension, were processed using the nmrPipe program, and were visualized using nmrDraw (11).

RESULTS

Strategically linked compounds are potent in R5-tropic cell-cell fusion assays. We hypothesized that linking a gp120-binding protein with a gp41-binding protein could lead to an even more potent compound, because potentially both gp120 and gp41 could be blocked from mediating the viral entry process. As a test of this hypothesis, the potent HIV entry inhibitor griffithsin was covalently linked via a 16-amino-acid peptide linker with the gp41-binding peptide C37 to form "Griff37." Cell-cell fusion assays represent a common method of determining the antiviral potencies of many compounds. In this technique, HeLa cells presenting the human proteins CD4, CCR5, and/or CXCR4 on their surfaces (target cells) are combined with HeLa cells presenting the HIV-1 Env proteins gp120 and gp41 on their surfaces and HIV-1 Tat in their cytoplasm (effector cells). The cells fuse by interaction of their respective surface proteins in an event that generally mimics the HIV entry process. The extent of cell-cell fusion can be measured using a reporter assay, because β-galactosidase in the target cells is expressed under the control of the HIV-1 LTR promoter, which is activated by Tat from the effector cell upon fusion (43).

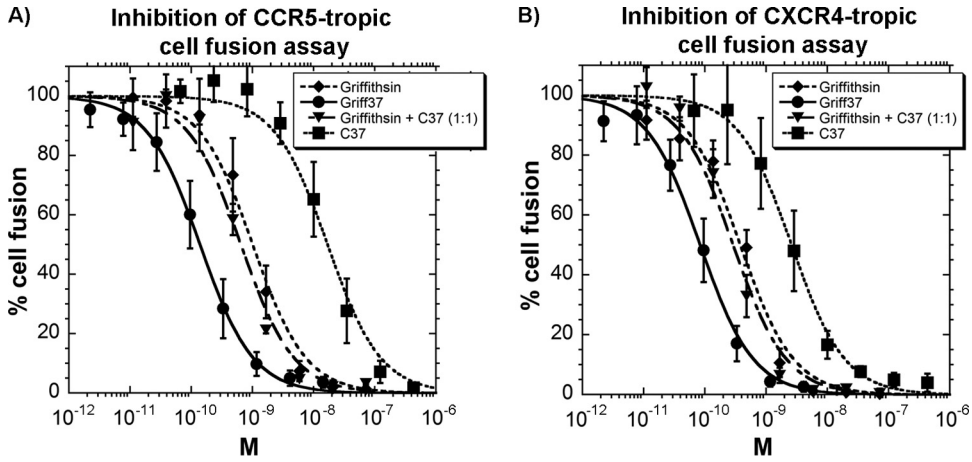

FIG. 1. Cell-cell fusion assays. IC_{50}s determined by various numbers (n) of independent experiments carried out in triplicate were averaged. (A) CCR5-tropic fusion assay using HeLa-ADA effector cells and HeLa-P5L CCR5-bearing target cells. The average IC_{50}s of the compounds tested are as follows: griffithsin, 1.31 ± 0.87 nM (n = 22); Griff37, 0.148 ± 0.05 nM (n = 11); C37, 28.2 ± 8.3 nM (n = 7); griffithsin plus C37 (1:1), 0.911 ± 0.37 nM (n = 4). (B) CXCR4-tropic fusion assay using HL2/3 effector cells and TZM-bl CXCR4-bearing target cells. The average IC_{50}s of the compounds tested are as follows: griffithsin, 0.468 ± 0.27 nM (n = 21); Griff37, 0.088 ± 0.03 nM (n = 5); C37, 2.70 ± 1.3 nM (n = 6); griffithsin plus C37 (1:1), 0.542 ± 0.39 nM (n = 5).

The results of CCR5-tropic cell-cell fusion assays are shown in Fig. 1 and Table 1. Griffithsin alone performs well, with an IC_{50} of 1.31 ± 0.87 nM, and the C-peptide C37 has an IC_{50} of 18.2 ± 7.5 nM in this assay. The two proteins in combination without being linked inhibit quite well, exhibiting an IC_{50} of 0.46 nM (for each protein, for a total concentration of 0.91 nM at 50% inhibition). However, when griffithsin and C37 are covalently joined by a 16-amino-acid linker to form Grft-linker-C37 (referred to here as Griff37), the effectiveness of the compound in the fusion assay increases dramatically, giving an IC_{50} of 0.15 ± 0.05 nM (Fig. 1A; Table 1), which is 8.7-fold greater than that of griffithsin alone. It has been reported that the activity of C-peptides can be enhanced by making the point substitution Q652L (53), so this substitution was made in the linked compound. Griff37(Q652L) was produced and purified, but in the CCR5-tropic fusion assay, this compound had approximately the same effectiveness as Griff37 (Table 1). Conversely, substitution of Asp for Ile at position 642 in C37 has been reported to diminish the activity of C37 (28), so this I642D substitution was made in Griff37, and the resulting protein showed 2.4-fold worse inhibition than that of Griff37 in the fusion assay, but still 2-fold better than that of griffithsin alone (data not shown). When the linker between griffithsin and C37 was shortened to 4 amino acids, the resulting protein was 1.6-fold less potent than Griff37 but still 3.1-fold more potent than griffithsin alone (data not shown).

As a further test of the overall strategy to link gp120-binding molecules with gp41-binding molecules, peptide C37 was linked with a 16-amino-acid linker to a modified version of peptide CD4M33, an HIV inhibitor that was designed to bind gp120 in a manner similar to the CD4 protein (38). While the published peptide contains unnatural amino

TABLE 1. Inhibition of HIV Env-mediated cell-cell fusion

Compound[a]	IC_{50} (nM)[b]			
	CCR5-tropic fusion assay	CXCR4-tropic fusion assay	CCR5-tropic fusion assay with competition	CCR5-tropic fusion assay with washout on HeLa-ADA cells
Griffithsin	1.31 ± 0.87	0.468 ± 0.27	1.73 ± 0.60	4.28 ± 1.50
Griff37	0.148 ± 0.05 (2.98×10^{-5})	0.088 ± 0.03 (0.046)	0.369 ± 0.03 (0.000067)	3.39 ± 0.56 (0.0588)
Griffithsin + C37 (1:1)	0.911 ± 0.37	0.542 ± 0.39	1.04 ± 0.23	6.23 ± 1.7
Griff37(Q652L)	0.121 ± 0.06 (7.70×10^{-6})	0.167 ± 0.09 (0.063)	ND	ND
Griffithsin + C37(Q652L) (1:1)	2.01 ± 0.66	1.35 ± 0.65	ND	ND
$C37CD4M33_{C1F23}$[c]	6.84 ± 2.9	2.30 ± 0.98	7.78 ± 1.6	77.8 ± 1.2
$GriffCD4M33_{C1F23}$[c]	3.47 ± 0.97	1.19 ± 0.44	12.6 ± 1.4[d]	ND
C37	18.2 ± 7.5	2.70 ± 1.3	61.4 ± 7.9	No inhibition
C37(Q652L)	28.2 ± 8.3	ND	ND	ND
CD4M33	>1,000	>1,000	ND	ND

[a] A 1:1 ratio indicates equal molar amounts.
[b] IC_{50}s for all combinations are reported as total protein concentrations. Each experiment was carried out in triplicate and was repeated at least 3 times except where otherwise noted. The IC_{50}s were calculated as the averages of the values obtained for the independent experiments ± standard deviations. Numbers in parentheses are the P values resulting from a t test comparing the indicated compound with griffithsin plus C37. A P value of <0.05 is considered statistically significant. ND, not determined. The peptide C37 is a product of chemical synthesis and is capped at both ends, with N-terminal acetylation and C-terminal amidation.
[c] $CD4M33_{C1F23}$ is a recombinantly produced protein based on the synthetically produced peptide CD4M33 designed by Martin et al. (38).
[d] This experiment was carried out in triplicate and was repeated twice.

acids in positions 1 and 23, we replaced these with the natural amino acids Cys and Phe, respectively, in order to allow expression in *E. coli*. We refer to this peptide as $CD4M33_{C1F23}$. In CCR5-tropic fusion assays, C37-linker-$CD4M33_{C1F23}$ ($C37CD4M33_{C1F23}$) exhibited an IC_{50} of 6.84 ± 2.9 nM (Table 1). This value is significantly lower than that for either component alone in cell-cell fusion assays, since C37 has an IC_{50} of 18.2 nM, and His tag-containing $CD4M33_{C1F23}$ was not an effective inhibitor even at our highest tested concentration of 1.4 μM (data not shown). Synthetically produced CD4M33 (38) had an IC_{50} higher than 1,000 nM in our hands in this fusion assay (Table 1). We also produced the compound griffithsin-linker-$CD4M33_{C1F23}$ ($GriffCD4M33_{C1F23}$), which could provide two molecules that bind to gp120. This compound performed reasonably well in CCR5-tropic cell-cell fusion assays, with an IC_{50} of 3.47 ± 0.97 nM, but did not appear to have enhanced activity compared to the individual components of the molecule.

These results suggest the possibility that combining a gp120-binding protein (such as griffithsin or CD4M33) with a gp41-binding protein (such as C37) is a potent strategy for the inhibition of CCR5-tropic HIV.

Strategically linked compounds are potent in CXCR4-tropic cell-cell fusion assays. The linked combination of griffithsin and C37 was also quite potent in CXCR4-tropic cell-cell fusion assays. As shown in Fig. 1 and Table 1, while griffithsin alone exhibited an IC_{50} of 0.47 ± 0.27 nM, the linked Griff37 was 5.3-fold more potent, with an IC_{50} similar to that of the point mutant of the linked compound, Griff37(Q652L) (Table 1). When unlinked, the combination of griffithsin and C37 was only slightly more potent than either compound alone (Fig. 1B and Table 1).

When C37 was linked with the gp120-binding peptide $CD4M33_{C1F23}$, the resulting compound, $C37CD4M33_{C1F23}$, had an IC_{50} of 2.30 ± 0.98 nM. Since this is close to the value for C37 alone, and CD4M33 has been shown to inhibit in a CXCR4-tropic fusion assay in the micromolar range (IC_{50}, 0.8 ± 0.09 μM) (38), it appears that linking the two inhibitors in this case does not improve inhibition. Interestingly, these results differ from those for the CCR5-tropic cell-cell fusion assay, where the $C37CD4M33_{C1F23}$ compound had an IC_{50} lower than that of either compound alone (Table 1).

Strategically linked compounds perform well in viral and pseudoviral assays. To confirm that the strategy of linking a gp120-binding protein with a gp41-binding peptide is successful in inhibiting HIV replication, a series of assays were performed using several types of target cells and viral strains. In replication-competent HIV assays, the linked compound Griff37 consistently performs better than griffithsin alone. For example, when the CCR5-tropic HIV-1 strain Ba-L is used to infect MAGI cells, griffithsin alone exhibits an IC_{50} of 0.04 ± 0.01 nM, while Griff37 is 2.7-fold more potent, with an IC_{50} of 0.015 ± 0.005 nM (Table 2; Fig. 2), and the unlinked combination of griffithsin and C37 has an IC_{50} of 0.05 nM for each protein. Similarly, when the CCR5-tropic HIV-1 strain ADA infects MAGI cells, Griff37 is 2-fold more potent than griffithsin alone and 6.3-fold more potent than the unlinked combination of griffithsin and C37 (total-protein concentration) (Table 2). The effect in most cases is even more dramatic when the IC_{90}s of these compounds against viral strains are

TABLE 2. Inhibition of HIV-1 replication in MAGI cells

Compound[a]	Inhibition of replication[b] of:					
	Ba-L (CCR5 tropic)		ADA (CCR5 tropic)		IIIB (CXCR4 tropic)	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Griffithsin	0.040 ± 0.0100.0.040 ± 0.01	0.515 ± 0.06	0.030 ± 0.01	0.450 ± 0.04	0.145 ± 0.07	0.890 ± 0.11
C37	7.56 ± 1.1	79.7 ± 2.5	6.15 ± 1.4	68.9 ± 4.2	10.3 ± 1.9	>100
Griffithsin + C37 (1:1)	0.100 ± 0.03	0.750 ± 0.02	0.095 ± 0.02	0.705 ± 0.005	0.230 ± 0.02	2.21 ± 0.57
Griff37	0.015 ± 0.005 (0.1077)	0.080 ± 0.01 (0.0011)	0.015 ± 0.005 (0.037)	0.085 ± 0.005 (0.00013)	0.035 ± 0.005 (0.011)	0.325 ± 0.07 (0.081)

[a] A 1:1 ratio indicates equal molar amounts.

[b] IC_{50} and IC_{90} values (nanomolar concentrations) were determined for viral replication assays. Inhibitory concentrations for all combinations are reported as total-protein concentrations. Numbers in parentheses are *P* values resulting from a *t* test comparing the indicated compound with griffithsin plus C37. A *P* value of <0.05 is considered statistically significant. Each experiment was carried out in triplicate and repeated twice. The IC_{50} and IC_{90} values were calculated as the averages of values for independent experiments ± standard deviations.

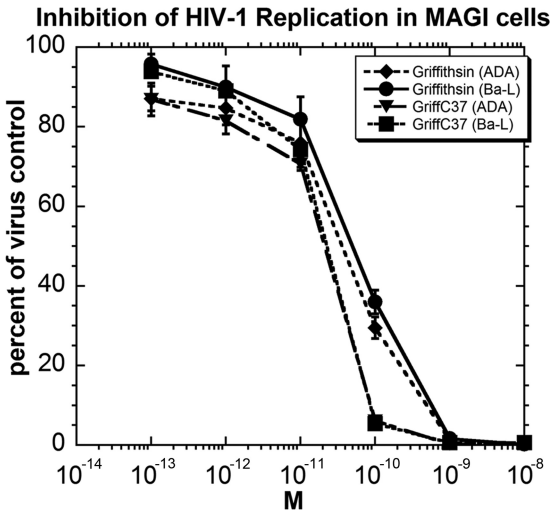

FIG. 2. HIV assays. Both griffithsin and the linked compound Griff37 were tested for inhibition of ADA and Ba-L virus strains in infection assays against MAGI cells. Griff37 (inverted triangle, against strain ADA; square, against strain Ba-L) performs better than griffithsin (diamond, against strain ADA; circle, against strain Ba-L) in both cases. For Griff37, the two lines are nearly overlapping.

compared. For Ba-L, Griff37 is 6.4-fold more potent than griffithsin and 9.4-fold more effective than the unlinked combination (Table 2), and similar results are observed for the inhibition of strain ADA.

As described for fusion assays, these compounds are also highly effective against CXCR4-tropic HIV strains. When MAGI cells are infected by the CXCR4-tropic strain HIV-1 IIIB, Griff37 again shows severalfold higher potency than its components (Table 2).

Similar results are observed when human PBMCs are infected with HIV-1 Ba-L. In this case, griffithsin alone has an IC_{50} of 0.28 ± 0.17 nM, while Griff37 is 4.7-fold more potent, with an IC_{50} of 0.059 ± 0.0004 nM, and the unlinked components griffithsin plus C37 are less than 2-fold more potent than griffithsin alone (Table 3). When the CCR5-tropic HIV-1 clinical isolate 91US005 is used to infect PBMCs, the result is about a 4-fold improvement for Griff37 over griffithsin alone (Table 3). Griff37 also shows a 3.8-fold increase in anti-HIV potency over griffithsin alone for the CXCR4-tropic strains NL4-3 and 92HT599 (primary strain; Table 3). Since the griffithsin-containing compounds were clearly more potent than the $CD4M33_{C1F23}$-containing compounds, the $CD4M33_{C1F23}$ compounds were not tested in replication-competent viral assays.

In single-round infection assays, griffithsin and its analogs all perform quite well. HIV virions pseudotyped with Env from the CCR5-tropic HIV-1 strain JR-FL or ADA were used in inhibition assays. While Griff37 performed marginally better (as determined by the IC_{50}) than griffithsin alone with strain JR-FL, it did not perform better with strain ADA (Table 4). Indeed, the use of strain ADA in a single-round infection assay was the only instance we identified in which Griff37 was not superior to griffithsin (Tables 1, 2, 3, and 4). Mutation to disrupt the activity of the C37 component of Griff37 [Griff37(I642D)] showed a loss of activity against the JR-FL strain, while mu-

TABLE 3. Inhibition of HIV-1 replication in PBMCs

Compound[a]	Inhibition of replication[b] of:									
	Ba-L (CCR5 tropic)		91US005 (CCR5 tropic)		NL4-3 (CXCR4 tropic)		92HT599 (CXCR4 tropic)			
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}		
Griffithsin	0.280 ± 0.17	0.760 ± 0.15	0.280 ± 0.17	0.760 ± 0.15	0.170 ± 0.04	0.725 ± 0.13	0.74 ± 0.37	1.86 ± 0.89		
C37	2.39 ± 0.64	7.40 ± 1.2	2.39 ± 0.64	7.40 ± 1.2	2.89 ± 0.39	8.33 ± 0.25	14.5 ± 1.4	27.2 ± 0.60		
Griffithsin + C37 (1:1)	0.33 ± 0.05	0.97 ± 0.33	0.33 ± 0.05	0.97 ± 0.33	0.220 ± 0.04	0.58 ± 0.02	0.76 ± 0.24	1.77 ± 0.03		
Griff37	0.059 ± 0.0004 (0.044)	0.155 ± 0.07 (0.20)	0.059 ± 0.0004 (0.077)	0.155 ± 0.07 (0.16)	0.045 ± 0.005 (0.049)	0.205 ± 0.04 (0.011)	0.195 ± 0.03 (0.14)	0.630 ± 0.07 (0.004)		

[a] A 1:1 ratio indicates equal molar amounts.

[b] IC_{50} and IC_{90} values were determined for viral replication assays. Inhibitory concentrations for all combinations are reported as total-protein concentrations. Numbers in parentheses are *P* values resulting from a *t* test comparing that compound with griffithsin plus C37. A *P* value of <0.05 is considered statistically significant. Each experiment was carried out in triplicate and repeated twice. The IC_{50} and IC_{90} values were calculated as the averages of values for independent experiments ± standard deviations.

TABLE 4. Inhibition of HIV single-round pseudovirus in TZM-bl cells

Compound	Inhibition[a] of:			
	ADA (CCR5 tropic)		JR-FL (CCR5 tropic)	
	IC_{50} (nM)	IC_{90} (nM)	IC_{50} (nM)	IC_{90} (nM)
Griffithsin	0.022 ± 0.01	0.428 ± 0.23	0.035 ± 0.01	1.25 ± 0.19
Griff37	0.042 ± 0.02 (0.25)	0.240 ± 0.04 (0.81)	0.025 ± 0.02 (0.63)	0.260 ± 0.10 (0.0003)
Griffithsin + C37 (1:1)	0.021 ± 0.02	0.277 ± 0.18	0.032 ± 0.02	1.38 ± 0.39
Griff37(Q652L)	0.023 ± 0.01 (0.70)	0.274 ± 0.07 (0.52)	ND	ND
Griff37-shortlink	0.078 ± 0.07 (0.13)	0.352 ± 0.28 (0.70)	0.046 ± 0.01 (0.25)	0.315 ± 0.01 (0.06)
Griff37(I642D)	0.061 ± 0.02 (0.04)	0.298 ± 0.03 (0.89)	0.082 ± 0.01 (0.005)	0.919 ± 0.02 (0.49)
Griffithsin + C37(Q652L) (1:1)	0.021 ± 0.01	0.374 ± 0.19	ND	ND
$C37CD4M33_{C1F23}$	2.33 ± 2.8	17.3 ± 17	2.25 ± 1.4	24.3 ± 20
$GriffCD4M33_{C1F23}$	0.222 ± 0.02	2.69 ± 0.23	0.270 ± 0.15	2.89 ± 0.25
C37	27.2 ± 17	637 ± 290	15.2 ± 4.2	462 ± 360
C37(Q652L)	114 ± 5	ND	ND	ND

[a] IC_{50} and IC_{90} values (nanomolar concentrations) were determined for single-round pseudovirus assays. Numbers in parentheses are *P* values resulting from a *t* test comparing the indicated compound with griffithsin plus C37. A *P* value of <0.05 is considered statistically significant. Each experiment was carried out in triplicate and repeated at least three times. The IC_{50} and IC_{90} values were calculated as the averages of values for independent experiments ± standard deviations. ND, not determined.

tation to shorten the linker (Griff37-shortlink) resulted in a protein that was still quite potent, with an activity close to that of Griff37 (Table 4). In addition, the combination of the gp41-binding peptide C37 with gp120-binding $CD4M33_{C1F23}$, designated $C37CD4M33_{C1F23}$, also appears to perform moderately better in CCR5-tropic pseudoviral assays than the component C37 alone (~13-fold), recombinant $CD4M33_{C1F23}$ (not active), or synthetic CD4M33, which Martin et al. showed to exert nanomolar inhibition in other strains, although in our hands inhibition by CD4M33 alone was less effective (Table 4) (38). Again, this indicates that the combination of a gp120-binding molecule with a gp41-binding molecule can be a potent HIV inhibition strategy, particularly since the CD4M33 component of our $C37CD4M33_{C1F23}$ was not optimized for amino acid usage or foldedness during preparation.

Strategically linked compounds maintain effectiveness in competition assays and in washing assays. For a potential therapeutic to be beneficial in an organism, it needs to be able to find its target effectively in the milieu of other cell types and potential binding partners. As a test of this ability, the CCR5-tropic cell-cell fusion assay was modified by the addition of mouse 3T3 fibroblasts, which serve as a connective tissue surrogate, supplying proteins and carbohydrates. These cells cannot be infected by HIV and do not have human coreceptors on their surfaces, but they present a myriad of proteins and carbohydrates that could potentially bind an anti-HIV therapeutic and confound its ability to inhibit in the assay.

As we previously reported, in a CCR5-tropic fusion assay, the peptide C37 performs significantly worse in the presence of unrelated competitor cells than in the absence of such cells, with an IC_{50} of 61.4 ± 7.9 nM (27) (Table 1). This leads to the possibility that Griff37 also performs worse in the presence of unrelated cells due to the presence of C37. However, the linked compounds perform quite well under these conditions. The presence of competitor cells does not greatly affect griffithsin-containing compounds, since griffithsin alone still performs well. But Griff37 again performs significantly better than griffithsin alone (Table 1). When C37 is linked to the gp120-binding peptide CD4M33 to make $C37CD4M33_{C1F23}$, the resulting IC_{50} is 7.78 ± 1.6 nM, which is close to the value in the absence of competition. Similar results were also observed for Griff37 in single-round infection assays using virus pseudotyped with Env from the CCR5-tropic HIV-1 strain JR-FL in the presence of competitor cells. In these experiments, all griffithsin-containing compounds performed well, but Griff37 performed better than griffithsin alone (data not shown).

To determine whether the linked inhibitors maintain some of their activity under washout conditions, modified R5 fusion assays were carried out. In these assays, either the target cells or the effector cells were placed in a well in the presence of an inhibitor. The supernatant was then removed; cells were washed twice with PBS; and inhibitor-free medium was added, followed by the addition of the other cell type to allow fusion to proceed. Therefore, inhibition would be observed only for inhibitors that can maintain their presence rather than be washed away. In these assays, the IC_{50} of C37 could not be determined, because it was apparently fully washed out. However, both griffithsin and Griff37 maintained nanomolar effectiveness when they first bound to the ADA "HIV effector cells" before the washing step. It is worth mentioning that when the inhibitors were incubated with the target cell before the washing step, vigorous washing was needed to wash away griffithsin and Griff37, indicating a low level of nonspecific binding of griffithsin to the cell surface (data not shown). This low level of nonspecific binding does not affect the anti-HIV potency of griffithsin, as indicated both by a competition assay (Table 1) and by a control experiment utilizing single-round HIV virions pseudotyped with SVA-murine leukemia virus (MuLV), which would not be expected to be inhibited by griffithsin. (Griffithsin inhibits SVA-MuLV with an IC_{50} of >6.8 μM [>100 μg/ml] [41].) Overall, these experiments indicate that under physiological conditions where many different cell types and fluids may be present, these inhibitors maintain very high effectiveness, in contrast to C37 alone.

NMR experiments on anti-HIV compounds. Nuclear magnetic resonance (NMR) is a very effective technique for determining the structural details of proteins, including the extent of their foldedness and the possibilities for their oligomeric state (9, 17, 34). Figure 3A shows the ^{15}N-^{1}H correlation spectrum (^{15}N heteronuclear single quantum coherence [HSQC] spec-

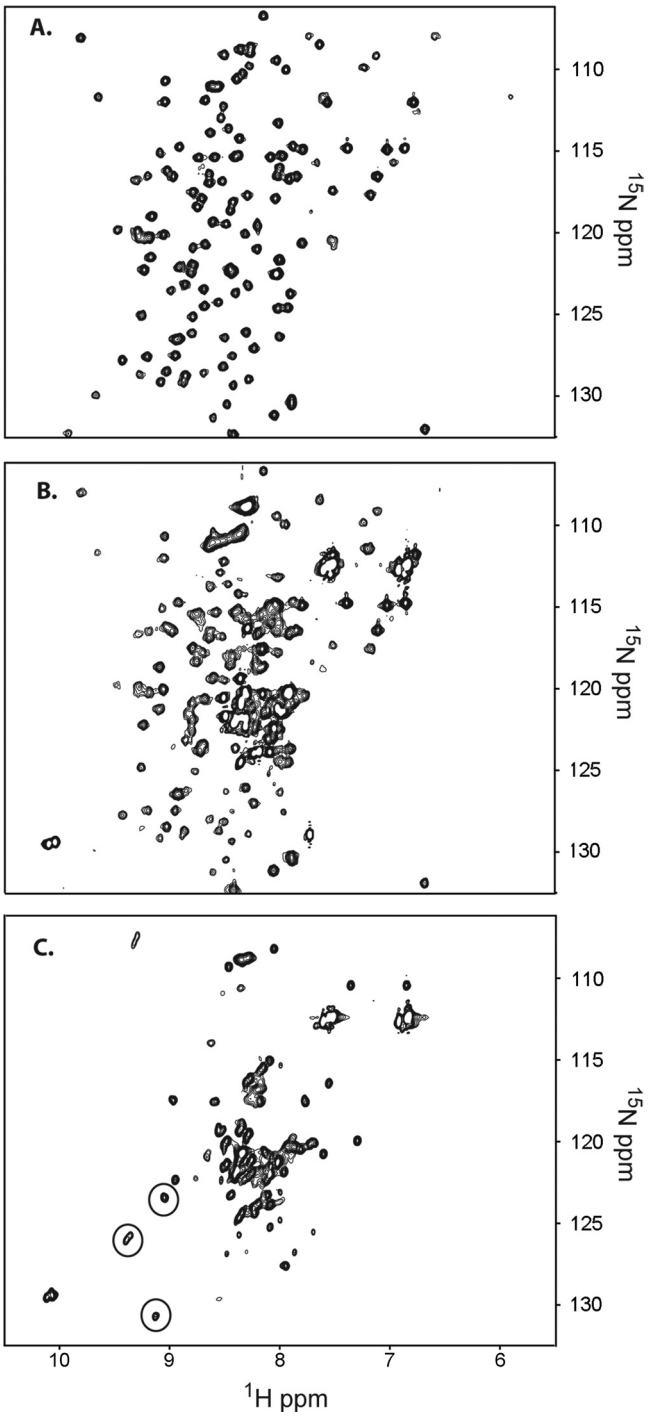

FIG. 3. ^{15}N-^{1}H correlation spectra of some of the HIV inhibitors used in this study. (A) Wild-type griffithsin; (B) Griff37 (griffithsin linked to C37); (C) C37CD4M33$_{C1F23}$. Circled peaks on this spectrum indicate resonances that are consistent with folded protein.

trum) of griffithsin alone, which is a 121-amino-acid protein that crystallizes as a dimer (62). This type of spectrum shows one peak for every N-H pair in the protein and therefore provides a fingerprint that is unique for each protein. The spectrum of griffithsin shows good peak dispersion in the ^{1}H dimension with well-resolved peaks, strongly suggesting a nicely folded protein, as would be expected for a stable, wild-type, relatively low molecular weight protein.

Figure 3B shows the spectrum of the linked compound Griff37. Although this protein exhibits potent anti-HIV activity in numerous assays, the spectrum shows a strong signal around 8.2 ppm in the ^{1}H dimension, indicative of unfolded or random coil protein in the sample. There are also peaks (of lower intensity) in the "folded" region of the spectrum (particularly above 9 ppm), and an overlay of this spectrum with griffithsin alone indicates that these peaks arise from folded griffithsin (overlay not shown).

NMR experiments were also carried out on the C37CD4M33$_{C1F23}$ peptide combination. Since this compound is a fusion of two peptides (albeit functionally active peptides), the unfolded peaks that are observed in the 8.2-ppm region of Fig. 3C were expected. However, there were also peaks indicative of folded protein (several of which are circled in Fig. 3C). Since C34 (nearly identical to C37) has been shown to be unfolded in the absence of its gp41-binding partner (36), it is likely that the peaks in this region of the spectrum result from folded CD4M33$_{C1F23}$. The parent compound, CD4M33, was designed by Martin et al. to be a folded peptide containing unnatural amino acids and was produced for their work by chemical synthesis (38). Therefore, it is notable that although we made multiple changes to CD4M33, including the use of "natural" amino acids to replace the designed unnatural amino acids, the use of recombinant expression rather than synthesis, and linkage with another peptide, the resulting protein still is an active anti-HIV compound that is at least partly folded.

DISCUSSION

Many binding targets, both on the HIV-1 virion and on the human cell, have been established as effective sites for HIV inhibition. But an underexplored area for HIV inhibition is to elucidate an overall strategy, possibly combining multiple inhibitors, that optimizes the effectiveness of inhibition under a number of different conditions and with different viral strains. The present work examines the possible benefits of combining a gp120-binding and a gp41-binding moiety in a single compound. It was found that such a strategy consistently performs well both in cell-cell fusion assays and in viral assays, using both CCR5- and CXCR4-tropic strains, as well as under conditions of competition and washing out. In most cases, the linked compounds performed better than their parent compounds, most strikingly in the improvement of the already highly potent protein griffithsin.

Griffithsin has shown a great deal of promise in tests of its anti-HIV microbicidal characteristics. In addition to its remarkably high potency, it is stable upon incubation at 37°C, a necessary property if it must remain active in the human body for hours or be stored without refrigeration (16). It also retains its activity in cervical/vaginal lavage fluid (16), is noninflammatory in human cervical explants, is nonirritating in a rabbit vaginal model, and is active against multiple clades of HIV, indicating likely usefulness in many or all of the areas hardest hit by this disease (41).

The most effective inhibitor in our experiments was Griff37, or possibly its slight variant Griff37(Q652L), both compounds that covalently link griffithsin with the C-peptide C37. This

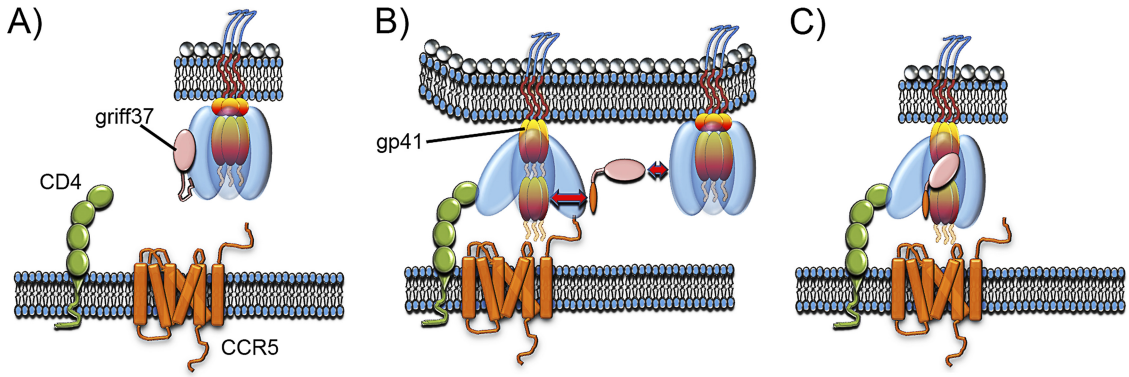

FIG. 4. Possible models of action of the linked compound Griff37. Note that griffithsin may act as a dimer, which is not depicted in this simplified figure. (A) The inhibitory action of the molecule is due to the binding of griffithsin to gp120, but extra steric blocking is provided by C37. (B) Inhibition is provided by either griffithsin or C37, but only one is properly bound to its target at any given time. (C) Inhibition is provided by griffithsin and C37, both of which are bound simultaneously to their respective targets (gp120 and gp41).

linked inhibitor performed at subnanomolar levels in both CCR5- and CXCR4-tropic fusion assays and at midpicomolar levels in viral assays. These values are in almost every case better than those for griffithsin alone and better than those for an unlinked combination of griffithsin and C37 (Tables 1, 2, 3, and 4). This indicates that the linked combination of inhibitors is indeed more potent than the individual components and is worthy of strong consideration as a microbicide or therapeutic. These effects are not likely due to synergistic action of the two inhibitors, because an examination of the combination index (an indicator of synergy [7]) showed essentially no synergy of a 1:1 combination in fusion or viral assays and, in fusion assays, no synergy for 1:10, 1:30, or 1:300 ratios of griffithsin to C37 (data not shown).

In the replication-competent viral assays, the improvement in the IC_{90} for the linked compound Griff37 was more statistically significant than the improvement in the IC_{50}; this is a positive indicator, since 90% inhibition is perhaps more critical for truly stopping infection. Statistical significance is judged by a comparison of the *P* values derived from the Student *t* test as described in Materials and Methods. The tables in the supplemental material also contain *P* values for a comparison of the linked compounds with their unlinked controls for many of the types of assay presented here. The greater effectiveness of Griff37 at the higher concentrations needed for 90% inhibition in the viral assay could be explained partly by the increasing importance of the C37 component at these concentrations, because C37 inhibits at nanomolar levels. As the concentration of the inhibitor rises from picomolar levels (where griffithsin functions, based on its IC_{50}), to nanomolar levels, the C-peptide may contribute more to the overall inhibition. As a further test of the strategy of covalently linking a gp120-binding compound with a gp41-binding compound, the linked peptide $C37CD4M33_{C1F23}$ was produced. Again, this compound consistently showed more-potent inhibition than either component alone in the CCR5-tropic cell-cell fusion assay (Tables 1 and 4).

In deriving a set of possible models to explain the effectiveness of Griff37, each model must account for the fact that this linked molecule is a significantly better inhibitor than the unlinked combination of the two components. In keeping with this observation, there are at least three plausible models for the mechanism of action of Griff37. The first model is that the griffithsin moiety binds to gp120 with high affinity, and the role of the linked C37 is simply to provide a more sizeable protein on gp120 (Fig. 4A), making a more effective blockade against binding to CD4 or CCR5. However, arguing against this simple steric mechanism is the evidence that griffithsin linked to the peptide CD4M33 ($GriffCD4M33_{C1F23}$) would also be expected to be able to provide a similar binding blockade, yet this molecule does not show enhanced inhibition compared to griffithsin alone. Also, Griff37(I642D), which carries a substitution that negatively affects the binding of C37 to gp41, shows decreased activity. This indicates that C37 function is important to the activity of the linked compound. A second model is one in which the griffithsin component binds to gp120, thereby delivering C37 close to its binding site on gp41. By being in physical proximity to gp41, C37 is able to bind gp41 and to inhibit fusion if and when gp41 is exposed, possibly dislodging the linked griffithsin at that time. In this scenario, it is assumed that only one component of the linked pair is binding to its site at any given time, although they may both be in an on-off equilibrium with their respective binding sites (Fig. 4B). Note that griffithsin is a tight dimer (62, 63), so Griff37 likely brings two molecules of C37 to a single viral spike, although for the sake of simplicity, the dimer structure is not shown in Fig. 4.

In the third model of action for Griff37, both components (i.e., griffithsin and C37) are bound to their respective targets simultaneously. The 16-amino-acid linker used in Griff37 was designed to be flexible and to allow simultaneous binding of the two components even if the binding sites are fairly distant. For example, an 8-amino-acid extended chain in griffithsin spans about 24 Å, so a 16-amino-acid linker could potentially allow binding at sites tens of angstroms apart. In the structure and the structural model provided by Chen et al. in their work on unliganded simian immunodeficiency virus (SIV) gp120 (6), much of the surface of gp120 is close enough to gp41 to allow simultaneous binding of griffithsin and C37.

In order to address whether the model presented in Fig. 4B or that presented in Fig. 4C is more correct, a shortened linker was constructed for Griff37, and the compound was called Griff37-shortlink. We reasoned that if both griffithsin and C37

bound simultaneously to their targets (as in Fig. 4C), a shortened linker would significantly reduce activity (down to the level of griffithsin), since this length is likely not sufficient for simultaneous binding. We found that in cell fusion assays, Grif37-shortlink did inhibit less well than Griff37, indicating that linker length is important in this type of assay. In contrast, Griff37-shortlink inhibited similarly to Griff37 in single-round viral assays (Table 4). Since a single-round infection assay is more representative of a replication-competent viral infection, we believe that the model in Fig. 4B, showing nonsimultaneous binding of griffithsin and C37, is more likely. Experiments are ongoing in this regard.

Like the results for Griff37, the experiments reported here with other linked peptides also indicate the beneficial effects of binding both gp120 and gp41, although the production in *E. coli* without optimization leaves open the possibility that the effect could be stronger after refolding conditions are improved. The compound C37CD4M33$_{C1F23}$ inhibits in CCR5-tropic fusion assays better than either component alone and is particularly effective in washing assays, indicating the advantage of a bifunctional compound. Small differences in IC_{50}s between C37 and the linked compound C37CD4M33$_{C1F23}$ in the CXCR4-tropic cell-cell fusion assay may be explained by a lower affinity of CD4M33$_{C1F23}$ for HXB2 Env. It should be noted that the values for our version of CD4M33$_{C1F23}$ are expected to be significantly less favorable than the values reported for the CD4M33 peptide, since our version is fully recombinant without the benefit of selectively designed unnatural components. Another important point is that the high number of cysteines (6 total) in C37CD4M33$_{C1F23}$ could allow for potentially different combinations of S-S bonds, leading to unfolded protein. Even if C37CD4M33$_{C1F23}$ shows several folded peaks in the NMR spectrum, it is not clear that any or all of the proper disulfide bonds are formed. Therefore, it is possible that CD4M33$_{C1F23}$ does not have the proper folding to bind as tightly to gp120 as the chemically synthesized peptide CD4M33. Even with these drawbacks, it is fairly easy and economical to produce C37CD4M33$_{C1F23}$, and its relatively small size makes it an attractive candidate for further improvement.

It is possible that the linked compounds offer additional advantages aside from improved potency and their potential as microbicides. The C-peptide T-20 is clinically approved but requires frequent dosing due to a short lifetime of only hours *in vivo* (54). It has been shown that linking the similar peptide C34 with albumin (a larger protein) allows for high detectable concentrations of the linked compound in the plasma of rats for days, suggesting that linking a C-peptide with a larger protein may delay renal clearance and/or susceptibility to protease degradation (54). Our experiments involve the protein C37, which, like T-20, is derived from the sequence of gp41 and which is identical to C34 except for an additional 3 amino acids on the N terminus. It is possible that Griff37 has an *in vivo* lifetime of several days due to its larger size, like albumin-linked C34 (54). In addition, an advantage of a drug with two different sites of action is the difficulty of viral escape, since viral mutations to escape from one of the linked inhibitors would be expected to have little or no effect on the other inhibitor.

Other groups have also investigated the strategy of linking HIV inhibitors in order to increase effectiveness. One very effective linked compound is an anti-CD4 antibody having each heavy chain extended with a gp41-binding C-peptide. This chimeric protein showed HIV inhibition with an IC_{50} as low as 14 pM in single-round HIV assays (25, 26). This group also produced another excellent compound by linking an anti-CCR5 antibody with a C-peptide (30). In both cases, the antibody was expressed with one C-peptide at the C terminus of each heavy chain of the antibody, resulting in a C-peptide/antibody ratio of 2:1. While both of these chimeric compounds would likely be highly effective microbicides, they have the disadvantage of being produced in a mammalian cell system (HEK cells), which would make it difficult to produce gram quantities of the inhibitor. In our system, Griff37 was produced from *E. coli* in shaker flasks and could likely be produced in large quantities by fermentation or in a plant-based expression system, both processes that were recently shown to be useful in making large quantities of functional griffithsin (22, 41). Another effective HIV inhibitor was produced by linking two domains from CD4 with a single-chain variable region from antibody 17b, which is known to interact with gp120 on a site near its CCR5-binding region. This inhibitor was shown to be effective at nanomolar levels and was expressed by recombinant vaccinia virus (14). A recent study of an anti-gp120 antibody in various monomeric/dimeric covalent configurations and an anti-gp41 antibody in these configurations also showed the importance of multivalency in HIV inhibition (29).

Structurally, the ^{1}H, ^{15}N correlation spectrum of griffithsin alone shows a nicely dispersed set of peaks indicative of a fully folded protein (Fig. 3A). To our knowledge, this is the first NMR spectrum reported for griffithsin, although several high-quality X-ray structures have been determined (62–64). The benefit of NMR spectra is that comparisons between variants of a protein can be made quickly and easily without the need for a full structural determination if sequence assignments have been completed. Such work for griffithsin is ongoing.

The NMR spectra of the strategically linked compounds show some resonances to indicate that both Griff37 and C37CD4M33$_{C1F23}$ are folded, although it is not clear what percentage of each sample is folded. For Griff37, there are several likely explanations for the presence of strong peaks in the unfolded region of the spectrum even though the protein is quite active. First, the 16-amino-acid linker between griffithsin and C37 was designed to be structurally flexible, so the signal from this region is expected to resonate in the "unfolded" region of the spectrum. Second, the C37 peptide is likely unfolded in the absence of a binding partner (35), which would lead to peaks in the unfolded region of the spectrum due to this peptide, even if the peptide would be fully active when presented with a binding partner in an assay. Finally, if the Griff37 protein forms oligomers or loose aggregates, line broadening would occur, which would have the effect of decreasing the intensities of all the peaks, particularly those from residues that are not free to move quickly in solution. Therefore, a globular protein such as griffithsin would be expected to lose a great deal of signal intensity upon oligomerization. Overall, the spectrum of Griff37 suggests a sample that is at least partially folded (due to the clear presence of peaks that overlap with those in the griffithsin sample) but that may have a portion unfolded and/or in an oligomerized state. In this case, small

improvements to the refolding procedure may lead to significantly more-potent inhibitors.

The strategically linked compounds reported here show picomolar levels of activity in many assays, but further improvements may be possible. For example, it may be possible to improve the C-peptide component by capping the charged ends that occur naturally when a protein is expressed rather than synthesized (53). In our hands, N-acetylated, C-amidated C37 inhibits in the CCR5-tropic fusion assay with an IC_{50} of 18.2 nM, as reported above. However, C37 without the terminal modifications inhibits with an IC_{50} of >100 nM. Therefore, it is possible that capping the C-terminal ends of Griff37 and $C37CD4M33_{C1F23}$ could increase the potencies of these compounds. In addition, others have reported further improvements in C-peptides that increase their potencies (12); it may be possible to incorporate such improvements into the strategy presented here.

This paper presents evidence that the strategy of linking a gp120-binding molecule with a gp41-binding molecule can lead to a compound that has greater anti-HIV activity than its individual components alone or in combination. It was demonstrated that the highly potent microbicidal candidate griffithsin could be made even more potent by using this strategy.

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