A Covalent Inhibitor Targeting an Intermediate Conformation of the Fusogenic Subunit of the HIV-1 Envelope Complex*

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Peptide inhibitors corresponding to sequences in the six helix bundle structure of the fusogenic portion (gp41) of the HIV envelope glycoprotein have been successfully implemented in preventing HIV entry. These peptides bind to regions in HIV gp41 transiently exposed during the fusion reaction. In an effort to improve upon these entry inhibitors, we have successfully designed and tested peptide analogs composed of chemical spacers and reactive moieties positioned strategically to facilitate covalent attachment. Using a temperature-arrested state prime wash in vitro assay we show evidence for the trapping of a pre-six helix bundle fusion intermediate by a covalent reaction with the specific anti-HIV-1 peptide. This is the first demonstration of the trapping of an intermediate conformation of a viral envelope glycoprotein during the fusion process that occurs in live cells. The permanent specific attachment of the covalent inhibitor is projected to improve the pharmacokinetics of administration in vivo and thereby improve the long-term sustainability of peptide entry inhibitor therapy and help to expand its applicability beyond salvage therapy.

The human immunodeficiency virus (HIV)3 envelope protein complex consisting of gp41 and gp120 are non-covalently associated on the virus surface (1–4). The first step of HIV entry is gp120 association with the CD4 receptor on the target cell surface, which initiates conformational changes that reveal a second site on gp120 that binds to a co-receptor (CXCR4 or CCR5) (5–8). Concomitantly, the ectodomain of HIV-1 gp41 undergoes conformational change(s) resulting in the six helix bundle formation that drives membrane fusion (9, 10). The post-fusion six helix bundle is a trimer (Fig. 1). Each monomer has an N-terminal helical region (NHR) followed by a more disordered loop and a C-terminal helical region (CHR). The N-terminal helices pack tightly together to form a three-helical bundle while the C-terminal helices pack around the outside of this bundle into hydrophobic grooves (10–16).

Synthetic peptides based upon NHR and CHR sequences of HIV gp41 inhibit HIV entry (17–20). The most successful of these peptides have turned out to be those derived from the CHR of gp41. During the fusion process, CHR peptides bind to the hydrophobic grooves of the NHR as a result of their transient exposure, inhibiting the formation of the six helix bundle (21–27). The challenge in developing peptide-based therapeutics is complicated primarily by their relatively short half-lives and poor distribution in vivo because of their susceptibility to enzymatic degradation and rapid renal clearance. Effective anti-HIV activity in humans taking Fuzeon® (T20, Trimeris/Roche Applied Sciences) requires two subcutaneous injections daily totaling 180 mg of drug (28). We postulated that an anti-fusogenic peptide chemically modified to react covalently with the surface of HIV, and more specifically with gp41, would produce a more effective anti-viral such that (i) the peptide renders the gp41 to which it is attached fusion incompetent on a permanent basis; (ii) the covalently attached peptide is sheltered from proteolysis by plasma or lymph-derived enzymes and (iii) permanent specific attachment of the covalent inhibitor is projected to counteract rapid clearance of the compound after in vivo administration.

Using a temperature-arrested state prime wash in vitro assay (29–31), we report a novel inhibition strategy of HIV that is based upon the design of compounds composed of two parts: a binding element responsible for conferring selectivity and high affinity for the HIV-1 envelope glycoprotein, and a reactive group responsible for the formation of a covalent bond between the affinity probe and the virus. We used CHR peptides composed of chemical spacers and labile groups strategically designed to form a covalent bond with the ε-NH of Lys574 (Fig. 1). Based upon x-ray crystal and NMR structures reported to date (11, 12, 32), the addition of the reactive groups was hypoth-
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Peptide Synthesis—Synthesis of the corresponding reactive peptides, CHR peptide analogs, and the scrambled peptides corresponding to maleimido-glucagon-like peptide 1 (GLP-1), and maleimido-growth hormone releasing factor (GRF) peptides, were performed using an automated solid-phase procedure on a Symphony Peptide Synthesizer with manual intervention during the generation of the peptides. The synthesis was performed on Fmoc-protected Ramage amide linker resin, using Fmoc-protected amino acids. Coupling was achieved by using O-benzotriazol-1-yl-N,N,N',N''-tetramethyl-uronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIEA) as the activator mixture in N,N-dimethylformamide (DMF) solution. The Fmoc protective group was removed using 20% piperidine/DMF. A Boc-protected amino acid was used at the N terminus to generate the free α-N terminus following cleavage of the peptides from the resin. Simgacotted glass reaction vessels were used during the synthesis.

When the maleimide is positioned at the C terminus portion of the molecule (Compounds E and F, see Table 1), the solid-phase synthesis of the peptide was initiated by the addition of Fmoc-Lys(Aloc). Aloc is a specific orthogonal protective group stable to acidic medium. The peptide chain was then elongated on solid support via the sequential addition of amino acids having their side chains protected with groups labile to acidic medium. When the peptide chain was completed, the Aloc protective group on the C-terminal lysine was removed selectively using tetrakis(triphenylphosphine) palladium. The Fmoc-aminoethoxy ethoxy acetic acid (AEEA) linker was then chemically coupled to the unprotected lysine. Following classical Fmoc deprotection protocols, maleimide propionic acid (MPA) was then chemically coupled to the AEEA spacer. Finally, the acid labile protecting groups were removed from the peptide and the peptide was cleaved from the solid support using a strong acidic mixture.

Because the C34 peptide is composed of only two free amino groups, the free N terminus and the ε-NH of Lys655 of gp41, insertion of the chemical spacers and reactive moieties onto the intended sites within the C34 peptide was facilitated by substitution of Lys655 with an arginine residue, followed by direct chemical coupling either onto the free N terminus of the peptide (Compound A) or by insertion of a new Fmoc-Lys(Aloc) residue at the site normally occupied by Asp632 (Compound C). Therefore, a negative control (i.e., unreactive) C34 peptide analog is also composed of Lys655 substituted with an arginine residue (Compound B).

Peptide Purification—Each product was purified by preparative reverse-phase HPLC, using a Varian (Dynamax) binary HPLC system. Purification of all DAC peptides were performed using a Phenomenex Luna phenyl-hexyl (10 micron, 50 mm × 250 mm) column equilibrated with a water/trifluoroacetic acid mixture (0.1% trifluoroacetic acid in H₂O; Solvent A) and acetonitrile/trifluoroacetic acid (0.1% trifluoroacetic acid in CH₃CN; Solvent B). Elution was achieved at 50 ml/min by running various gradients of Solvent B over 180 min. Fractions containing peptide were detected by UV absorbance (Varian Dynamax UVD II) at 214 and 254 nm.

Fractions containing the desired product were identified by mass after direct injection onto LC/MS. The selected fractions were subsequently analyzed by analytical HPLC (20–60% B over 20 min; Phenomenex Luna 5 micron phenyl-hexyl, 10 mm × 250 mm column, 0.5 ml/min) to identify fractions with ≥90% purity for pooling. The pool was then freeze-dried using liquid nitrogen and subsequently lyophilized yielding a white powder.
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| TABLE 1 | Covalent HIV Entry Inhibitor |
|----------------|-----------------------------|
| The peptides were synthesized using an automated solid phase procedure and purified by HPLC as explained in the supporting information. The HIV peptides are numbered as appropriate for HIV-1 HXB2. The letters stand for the one-letter amino acid code unless otherwise designated. | |
| C34 | Ac<sup>a</sup>(628)WM EW DREI NNTYSLHSLIESQNQQEKNQE(661)-CONH<sub>b</sub> |
| A | MPA-AEEA-(628)WM EW DRE I NNTYSLHSLIESQNQQEKNQE(661)-CONH<sub>b</sub> |
| B | Ac<sup>a</sup>(628)WM EW DREI NNTYSLHSLIESQNQQEKNQE(661)-CONH<sub>b</sub> |
| C | Ac<sup>a</sup>(628)WM EW DREI NNTYSLHSLIESQNQQEKNQE(661)-CONH<sub>b</sub> |
| D | Ac<sup>a</sup>(628)WM EW DREI NNTYSLHSLIESQNQQEKNQE(661)-CONH<sub>b</sub> |
| E | H(A)EGFTTSVSYLEQKA KEF(628)WLVGK(661)-CONH<sub>b</sub> |
| F | Y(A)DAIFTSQSYKRA QKSLR(549)DILUSRK(MPA)-CONH<sub>b</sub> |
| N36 | Ac<sup>a</sup>(546)SGIVQQQNLLRA(549)AEQ(628)HLLQTVG(661)-CONH<sub>b</sub> |
| N36(K574R) | Ac<sup>a</sup>(546)SGIVQQQNLLRA(549)AEQ(628)HLLQTVG(661)-CONH<sub>b</sub> |

<sup>a</sup>Ac acetyl.  
<sup>b</sup>CONH<sub>2</sub>, carboxamide.

In Vitro Complex Formation between NHR and CHR Peptide—N36 peptide derived from the sequence of the N-terminal helical region of HIV gp41 was allowed to react with the covalent and non-covalent entry inhibitors in vitro and analyzed by HPLC. 2 mm of each CHR peptide analog was prepared in 50 mm sodium phosphate buffer pH 7 and N36 peptide was prepared at 2 mm in 95% ethanol. The peptides were then diluted to 0.2 mm in 50% ethanol/50% sodium phosphate buffer pH 7 and allowed to incubate at 37 °C for 30 min. Reverse-phase HPLC assays were performed on a Waters<sup>®</sup> model 2695 system in line with a UV absorbance detector (model 2487) set at 214 nm, and fitted with a Zorbax 300 SB-C18 column (Agilent Technologies; 150 X 4.6 mm; 3.5 μm, 300 Å) equilibrated with a mixture of H<sub>2</sub>O/0.1% trifluoroacetic acid (Solvent A) and acetonitrile/0.1% trifluoroacetic acid (Solvent B) at 35% solvent B at a flow rate of 0.5 ml/min. For each sample injection, ~10 μg of each peptide was loaded onto HPLC and the various compounds (i.e. CHR peptide analogs, N36 peptides, dimers of CHR peptide analog/N36) were resolved by applying a gradient from 35–37% solvent B over 2 min followed by a linear gradient from 37–55% solvent B over 20 min.

Cell-Cell Fusion Assay—HL2/3 cells were obtained through the AIDS Reference and Reagent Program, NIH from Dr. Barbara Felber and Dr. George Pavlakis (34). HL2/3 cells stably expressing HIV Gag, Env, Tat, Rev, and Nef proteins were co-incubated with TZM-bl cells which stably express large amounts of CD4 and CCR5 obtained through the AIDS Reference and Reagent Program, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc (35, 36). Co-cultures were incubated with TZM-bl cells which stably express large amounts of CD4 and CCR5 obtained through the AIDS Reference and Reagent Program (39). The mutation was verified by DNA sequence analysis by the DNA sequencing facility in the Research Technology Program at the NCI-Frederick. HeLa cells were cotransfected with EnvK574R, Rev, and Tat (pBsTAT and pCMVsREV were gifts of George Pavlakis and Margherita Rosati, National Cancer Institute, Vaccine Branch, Frederick, MD) in a 1:1:1 ratio using ExGen500 (Fermentas). Transfected cells were incubated 48 h at 37 °C and TAS fusion assays with TZM cells were performed as described above.

Mutagenesis and Transfection—The mutant K574R was made in the plasmid pHXB2-env from the NIH AIDS Research and Reference Reagent Program (39). The mutation was verified by DNA sequence analysis by the DNA sequencing facility in the Research Technology Program at the NCI-Frederick. HeLa cells were cotransfected with EnvK574R, Rev, and Tat (pBsTAT and pCMVsREV were gifts of George Pavlakis and Margherita Rosati, National Cancer Institute, Vaccine Branch, Frederick, MD) in a 1:1:1 ratio using ExGen500 (Fermentas). Transfected cells were incubated 48 h at 37 °C and TAS fusion assays with TZM cells were performed as described above.

RESULTS

HPLC Analysis of Covalent Entry Inhibitors Added to N36—The CHR peptide analogs were designed to bind with high affinity to the N-terminal helix of gp41 (Table 1). Compounds A, C, and D are CHR peptide analogs designed for specific alignment with Lys<sup>574</sup> of gp41 yet expected to have differing reaction rates (i.e. reactive group location more distant or maleimide ring versus phenyl ester). Compounds E and F are negative controls made up of a GLP-1 and a GRF peptide respectively, both of which were not expected to associate with the NHR. Compound B was a control peptide that is identical to C34 except for an acetylated N terminus and Lys<sup>574</sup> substituted with an arginine; necessary to have only one reactive lysine in the experimental peptides. Following purification by HPLC, the compounds were tested for their ability to link covalently to the N-terminal helix of gp41. A small NHR peptide, N36, corresponding to the amino acid sequence from positions 546–581 within the N-terminal helix of gp41, was incubated with the covalent entry inhibitors and controls and the binding was studied by reverse-phase HPLC. It is important to note that the N36 peptides used in these experiments were capped at the free
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**TABLE 2**

IC₅₀ values for HIV entry inhibitors

Cell-to-cell fusion measured by luciferase reporter gene assay. The IC₅₀ is the concentration of peptide inhibitor reducing the fusion levels by 50% in comparison with untreated control. IC₅₀ values were computed by fitting a four-parameter nonlinear regression model (37, 38) with R statistical software.

|      | PWᵃ | PNWᵇ | NPWᶜ |
|------|------|------|------|
| C34  | NIᵈ  |      |      |
| A    | 465 ± 102.7 | 2.09 ± 0.20 | NIF⁹ |
| B    | NIᵈ  | 1.01 ± 0.07 | NIF⁹ |
| C    | 679.0 ± 77.5 | 1.99 ± 0.22 | NIF⁹ |
| D    | NIᵈ  | 1.05 ± 0.08 | NIF⁹ |
| E    | NIᵈ  | NIᵈ  | NIF⁹ |
| F    | NIᵈ  | NIᵈ  | NIF⁹ |

ᵃ Prime wash.
ᵇ Prime-no wash.
ᶜ No prime wash.
ᵈ Little or no inhibition at [inhibitor] ≤ 5 μM.

N terminus with an acetyl group (Table 1) resulting in a compound containing only one free amine group on the peptide, that being the lysine representing the structural equivalent to Lys⁵⁷⁴ of gp41. Following incubation of N36 with Compound A or Compound C shown in Fig. 2, A and C, a new species with a retention time of ~18 min is observed that does not correspond to any of the peaks that appear when the compounds are analyzed prior to co-incubation. Furthermore, LC/MS analysis confirms that the identities of these new species correspond to one copy each of N36 and the C-peptide (data not shown). Conversely, the co-incubation of compound B with N36 did not show the formation of any additional peak (Fig. 2B) that would have indicated formation of a new species. This panel also demonstrates that the reverse-phase HPLC conditions used in these studies abrogated non-covalent interactions between N36 and the C-peptide. The overall yield and rate of reaction in forming this new species appears greatest when using compound C in these experiments. Furthermore, the precise location of covalent attachment within N36 was identified by preparing a mutant N36 peptide wherein Lys⁵⁷⁴ was substituted with an arginine, N36(K574R) (Fig. 2D). Co-incubation of compound C with the N36(K574R) peptide yields results that are similar to the co-incubation of Compound B with N36; no formation of an additional peak representing a covalent dimer (Fig. 2, B and D).

**IC₅₀ Determination**—The compounds and controls were first analyzed for their anti-fusogenic activity using a standard fusion inhibition assay. IC₅₀ values were computed by fitting a four-parameter nonlinear regression model (38, 40) with R statistical software. The IC₅₀ values for the experimental compounds (A, B, C, and D) and the C₃₄ control without washing were very similar ranging from 1.1–2.2 nM (Table 2). Hence, the chemical modifications found in compounds A and C had no impact upon the affinity of the C₃₄ peptide for the NHR region of gp41. Compounds E and F correspond to maleimido peptides composed of the peptide sequences of human GLP-1 and a mutated form of GRF. As expected, compounds E and F were found to be inactive in all assays performed.

**Prime Wash Inhibition**—The prime wash assay has been a useful method for dissecting the steps of viral membrane fusion and for determining the mechanism of action of various inhibitors at different steps in the fusion process (29–31, 41, 42). This assay is based upon the temperature sensitivity of envelope-mediated membrane fusion and functions by using low temperature to arrest the fusion process at an intermediate state. Fusion is a multi-step mechanism activated by the addition of cells bearing the CD4 receptor and the CXCR4/CCR5 co-receptors to a cell line which has the HIV envelope proteins expressed on the surface. Unlike traditional HIV replication...
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and fusion assays, in vitro incubation at 24 °C (priming) was found to establish a conformationally arrested state which exhibits no evidence of fusion pore formation until the temperature is shifted to 37 °C (29), thus allowing a homogeneous and prolonged exposure of the normally camouflaged NHR and CHR regions of gp41 (see Fig. 5). Because of the ability to arrest the intermediate conformational state of gp41 using lowered temperature, the temperature-arrested state prime wash assay was used to test the panel of experimental compounds designed to covalently cross-link the inhibitor to gp41 for permanent inhibition (Table 2 and Fig. 3).

The control compounds B and C34 were inactive in the prime wash assay indicating that they were being removed by washing. Conversely, the anti-fusogenic activity (IC_{50}) of Compounds A and C after the cells have been primed and washed is 0.5 μM and 0.7 μM, respectively (Table 2 and Fig. 3). The inhibition is lower, however, if the cells are not primed when Compounds A and C are presented (NPW) indicating that the binding affinity for Compounds A and C is specific for regions of gp41 that are camouflaged prior to the priming step and supporting the mechanism of action already described for CHR peptide analogs (29, 30). It is important to note that all compounds were tested in the presence of serum in the surrounding medium in order to best mimic the native cellular environment (i.e., serum composed of molecules such as the thiol of cysteine, glutathione, and the reactive Cys\(^{34}\) residue of serum albumin and the base medium containing free amino acids including lysine and cysteine). These conditions may contribute to a certain level of chemical instability for the maleimide group prior to any reaction with gp41, and consequently, may impact upon the IC_{50} values for these compounds in the PW experiments detailed in Table 2 and Fig. 3. To test this phenomenon, we measured the half-life of the maleimide activity in medium both with the presence and absence of serum (Table 3). After incubating Compounds A and C under these conditions for various periods of time the residual inhibitory activity was measured. Indeed, the maleimide activity is lessened with increasing time. The half-life of Compound A in serum is 55 min and without serum, it is 79 min. The half-life for Compound C is 19 min with serum and 61 min with no serum present. However, it is interesting to note that the measured maximum of inhibitor activity occurs well before the half-lives at 5–10 min for both Compounds A and C. This suggests that the specific reaction is strongly favored.

Compounds E and F were found to be inactive, thus confirming that covalent attachments derived from the activity of the maleimide in Compounds A and C in the PW assays are specific because of its incorporation into a peptide sequence possessing a specific affinity for the intended target. Although reaction of maleimide with lysine is usually favored at high pH because of the pK\(_a\) of 9–10 for the ε-NH\(_2\) reaction between maleimide and Lys\(^{574}\) is observed in these situations even under neutral pH conditions. Therefore, despite the reported selectivity of maleimide reacting with free thiols over free amines, particularly at physiologic pH, the overall combination (a) insertion of the maleimide ring onto the C-peptide which acts as an efficient binding element, and (b) modeling the positioning of the maleimide ring within the C-peptide at either the N terminus or position 632 such that it comes into close proximity to the ε-NH of Lys\(^{574}\), causes the maleimide to react with at least sufficient efficacy to this lysine residue so as to observe inhibition because of covalent attachment.

**Mutation K574R**—To confirm our original hypothesis that Lys\(^{574}\) is indeed the specific site of covalent attachment on gp41 of HIV (Fig. 1), Lys\(^{574}\) was mutated to an arginine residue. The substitution of the ε-NH of lysine for the guanidine group of arginine was intended to eliminate or reduce the nucleophilicity at this specific site without significantly altering (or abrogating) the fusion efficiency of the mutant strain. The K574R mutant was found to be 4-fold less fusion competent than the wild-type level. As shown in Fig. 4, Compound C failed to inhibit the K574R mutant in a dose-dependent manner in the temperature-arrested state prime wash assays, thus corroborating our original hypothesis that the specific site of covalent attachment onto gp41 is Lys\(^{574}\), and our results using the N36 (K574R) mutant peptide, which fails to attach covalently to compound C (Fig. 2D, Fig. 4). There was partial inhibition at 5 μM, which could be due to nonspecific reaction with either alternate lysine residues nearby in the envelope sequence or reactive components of the medium at saturating concentrations of peptide.

**DISCUSSION**

Past studies in vitro with the peptide inhibitors C34 and T20 and more recently in the clinic with Fuzeon® (T20 from Trimeris/Roche Applied Sciences), have shown that peptide inhibitors derived from the sequence of the CHR of gp41 are success-

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**TABLE 3**

Full inhibition and half-life of maleimide activity

| Compound   | Full permanent inhibition time point | Half-life with serum | Half-life without serum |
|------------|------------------------------------|----------------------|------------------------|
| Compound A | 5–10 min                           | 55.26 min            | 78.63 min              |
| Compound C | 5–10 min                           | 28.84 min            | 61.21 min              |
ful in preventing the formation of the final six helix bundle structure and thereby preventing viral entry. These compounds bind to an intermediate conformation of gp41 that is transiently present during the fusion process (29, 43, 44). However, these peptide inhibitors associate non-covalently and hence are subject to the limitations of binding equilibria, such as susceptibility to proteolytic digestion and renal clearance. By adding a covalent cross-linking mechanism to the peptide inhibitor, our goal was to make the association permanent and targeted to a specific site on HIV-1 gp41 (Figs. 1 and 5).

To demonstrate the covalent attachment of an anti-fusogenic peptide inhibitor onto HIV in vitro, we have utilized a condition called the temperature-arrested state of HIV fusion (29–31). This method allows for the separation of the initial conformational changes that the envelope protein complex undergoes from the final fusion pore formation that signals full membrane fusion. The peptide inhibitors bind to transiently exposed NHR sequences between these two stages. When there is significant washing at the temperature-arrested state, the non-covalently associated peptide inhibitors are diluted to levels far below their intrinsic IC50 values and ultimately this leads to full restoration of fusion activity. For example, this is apparent when testing control C34 for which inhibition was abolished following the washing steps (Table 2; PW and NPW and Fig. 3). Conversely, despite excessive washings of the two test compounds A and C, a dramatic dose-dependence was apparent and IC50 values were measured to be 0.5 μM and 0.7 μM, respectively. Hence, this was a clear indication that there had been covalent cross-linking that had occurred between the modified inhibitors and the gp41 molecule. Compound D was designed to be similar to compound C yet with a far less reactive chemical moiety (i.e. a phenyl ester) than maleimide under the current experimental conditions thus explaining why it inhibited like a non-reactive compound.

It was very intriguing to learn that Compound A had a lower IC50, indicating better efficacy, than Compound C (Table 2 and Fig. 3) considering both that the reasoning behind the initial design and the HPLC analyses suggested otherwise. Compound C was designed so that the chemical moiety would be directly placed at the salt bridge Asp632/Lys574. As such, it would have been predicted to be the optimal placement. Compound A instead has the moiety shifted to the terminus of the C34 peptide (Table 1) farther away from this salt bridge toward the loop region. Also, the HPLC analyses of the binding of isolated NHR and CHR peptides showed greater covalent dimer formation for Compound C (Fig. 2). It is possible that the derivatized lysine of Compound C is not a good steric match to come into close proximity with the target lysine 574 in the context of the entire gp41 protein. However, it is also likely that there are larger conformational
changes occurring just prior to six helix bundle formation that are affecting the efficacy of placement of the reactive groups and this will be the subject of further study.

To prove specificity of inhibition is due more to the binding affinity and specificity of the C-peptide rather than to the reactive group, compounds E and F were also tested corresponding to GLP-1 and GRF peptides, respectively (Table 1). These peptides lack any affinity for the gp41 NHR region and were hypothesized to be non-inhibitory (covalent or non-covalent) despite being composed of a maleimide ring. Indeed, compounds E and F were found to be inactive in all of the fusion reactions (Table 2), indicating the covalent attachment onto compounds lack any affinity for the gp41 NHR region and were affinity and specificity of the C-peptide rather than to the reaction event of the C-peptide rather than to nonspecific conjugation of the maleimide.

Furthermore, to show the level of specificity for the intended target, ε-NH of the amino acid residue Lys^574, we substituted this residue with an arginine using site-directed mutagenesis and tested this mutant, K574R, in the TAS prime wash assay (Fig. 4). There was no dose-dependence of inhibition of fusion after prime wash when the targeted residue was mutated to a less reactive amino acid. There was, however, partial inhibition at 5 μM. Based upon the lack of dose-dependence, we hypothesize that at this concentration, we have saturated the binding sites for the CHR peptide. Because of this, the reactive peptide is allowed to circulate freely for the three hour incubation period during which time, the excess inhibitor is susceptible to reaction with alternate targets such as nearby lysine residues in the envelope sequence and to the reactive components in the cell culture medium. This hypothesis is supported by a comparison of the timing of the loss of activity (29–79 min.) from preincubation in medium and the timing of maximum covalent modification (5–10 min).

Herein, we report the design and testing of analogs of the C34 peptide capable of forming a covalent bond with the side chain of Lys^574 of gp41. An inhibitor that covalently cross-links to a gp41 intermediate will permanently disable HIV gp41 six helix bundle formation (Figs. 1 and 5). Although maleimido-containing compounds, A and C, are the most efficient in performing a covalent attachment to gp41, maleimido peptides are expected to bioconjugate preferentially to free thiols (such as the cysteine 34 of human serum albumin present in excess) following their administration to patients intravenously or subcutaneously (45–48). This would chemically quench the reactive moiety and render the anti-fusogenic peptide less capable of forming a covalent bond to its intended target. Therefore, the in vitro experiments presented herein were not expected to be viable in the presence of serum. With this caveat in mind, it was surprising that permanent inhibition levels in the low micromolar range were achievable in the presence of serum and that the time point of maximum permanent inhibition occurs ~5–10 times earlier than the loss of activity. This is encouraging information that further design improvements will be promising. These results may also be applied to other viruses, which operate by similar mechanisms. Finally, as is depicted in the model in Fig. 5, our results prove that an intermediate can be permanently trapped and provide definitive supporting evidence for reports that have suggested that gp41 intermediates exist transiently during the HIV fusion process.

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