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Synthesis and analysis of the membrane proximal external region epitopes of HIV-1‡

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The membrane proximal external region (MPER) of gp41 abuts the viral membrane at the base of HIV-1 envelope glycoprotein spikes. The MPER is highly conserved and is rich in Trp and other lipophilic residues. The MPER is also required for the infection of host cells by HIV-1 and is the target of the broadly neutralizing antibodies, 4E10, 2F5, and Z13e1. These neutralizing antibodies are valuable tools for understanding relevant conformations of the MPER and for studying HIV-1 neutralization, but multiple approaches used to elicit MPER binding antibodies with similar neutralization properties have failed. Here we report our efforts to mimic the MPER using linear as well as constrained peptides. Unnatural amino acids were also introduced into the core epitope of 4E10 to probe requirements of antibody binding. Peptide analogs with C-terminal Api or Aib residues designed to be helical transmembrane (TM) domain surrogates exhibit enhanced binding to the 4E10 and Z13e1 antibodies. However, we find that placement of constrained amino acids at nonbinding sites within the core epitope significantly reduce binding. These results are relevant to an understanding of native MPER structure on HIV-1, and form a basis for a chemical synthesis approach to mimic MPER stricture and to construct an MPER-based vaccine. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: HIV-1; gp41; MPER; 4E10; Z13e1; α, α-disubstituted amino acid

Introduction

An effective vaccine against HIV-1 may depend on the ability of the envelope glycoprotein component (Env) to elicit broadly neutralizing antibodies (bNAbs) [1–6]. The Env spike consists of a heterotrimer of two noncovalently linked glycoproteins, gp120 and gp41 anchored through the gp41 transmembrane domain [5,6]. It has been proposed that the conserved functional regions of Env provide a short window of opportunity in the receptor-activated state, which could be used to induce bNAbs [7–9]. Detailed structural information about the native conformation of Env is not available, and human monoclonal antibodies that are broadly neutralizing have only rarely been isolated. One class of bNAbs to HIV-1 targets the membrane proximal external region (MPER) [10,11] of gp41 (Figure 1). This class contains the well-characterized bNAbs 2F5 [12], 4E10 [13–15], and Z13e1 [16,17], which can be used as conformational probes to assess immunogens developed to mimic immunogenic or neutralizing conformations of the MPER of gp41. By designing peptidomimetics based on MPER-4E10 interactions, we expect to provide important structural and conformational information about the MPER of HIV-1.

Crystal structures of MPER-derived peptides bound to 4E10 suggest that the MPER adopts a highly helical conformation toward the C-terminus (near the transmembrane (TM) domain) that abruptly unwinds toward its N-terminus as the peptide exits the antibody-binding pocket (Figure 2A) [13,18].

In an earlier work, we mapped the 4E10 epitope through truncation and Ala scanning, as well as physically constrained the helical C-terminus of the epitope, using either lactam and thioether tethers [19] or the α, α-disubstituted amino acid (2-aminoisobutyric acid, Aib) [20]. Two peptidomimetics were co-crystallized with 4E10 Fab, which illuminated the compatibility of the nonbinding face of the helix with unnatural amino acid constraints. Here, we report efforts to introduce unnatural amino acids into the N-terminal core of the 4E10 epitope ($W^{672}FDIT^{676}$) designed to restrict the conformational heterogeneity of the peptide and to introduce unnatural elements into the binding face that could increase the immunogenicity of this epitope. In addition, incorporation of the charged cyclic α, α- disubstituted amino acid (4-aminopiperidine-4-carboxylic acid, Api) [21–23] was explored with the aim of increasing peptide solubility while simultaneously promoting helical structure.

Materials and Methods

All reagents were purchased from commercial sources and used without further purification: dimethylformamide (DMF) from OmniSolV, dichloromethane (DCM) from Fisher, trifluoroacetic acid, biograde (TFA) from EMD Biosciences, acetonitrile (CH_3CN) from J. T. Baker. Water was purified using a Millipore MilliQ water purification system. Triisopropylsilane (TIS), phenol, N, *N*-diisopropylethylamine (DIPEA), anisole,

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Figure 1. Schematic representation of MPER epitope.

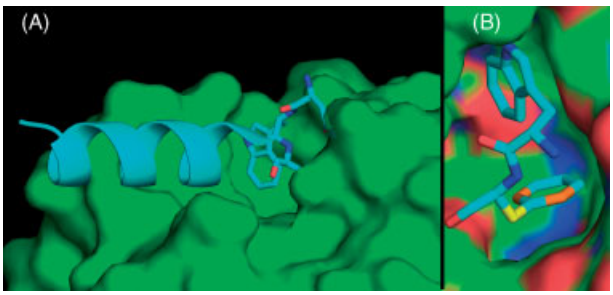

Figure 2. Structure of 4E10 antibody bound to native gp41 MPER peptide (pdb ID: 2FX7); (A) full epitope with lysine tail and (B) binding pocket near residue Trp672 and Phe673.

piperidine (Pip), and 4-methyl piperidine were acquired from Sigma-Aldrich, Acros, and Alfa Aesar. 2-(1H-benzotriazole-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were from ChemPep Inc., 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 1-hydroxybenzotriazole (HOBt) were purchased from Peptides International and N^{α}-Boc/Fmoc-amino acids from Peptides International, Chem-Impex, Anaspec, Bachem, and EMD Biosciences. Hydrofluoric acid (HF) was obtained from Matheson Trigas. Ninhydrin reagents were from Applied Biosystems.

Peptide Synthesis

The peptides were synthesized manually using solid-phase peptide synthesis on a C-terminal amide yielding MBHA resin, using *in situ* neutralization cycles for Boc-SPPS method. Except where otherwise noted, side-chain protecting groups used were Api(Fmoc), Asn(Xan), Asp(OcHex), Lys(ClZ), Ser(Bzl), Thr(Bzl), and Trp(formyl). Aib and Api was activated using 0.5 mmol Boc-Aib-OH or Boc-Api(Fmoc)-OH, 0.5 mmol HATU, 0.7 mmol DIEA in 1.5 ml of DMF for 2 min, 25 °C. The activated amino acid was added to the deprotected polypeptide resin without prior neutralization and coupled for 45 min. When necessary, double couplings were performed. The N-terminus of the peptides was left unprotected, and after the final step the resin was washed with DCM and dried *in vacuo* before cleavage.

HF Cleavage

Following the chain assembly, peptides were cleaved from resin with HF. In a typical reaction, 300 mg of resin were stirred in a vessel containing 1 ml of scavenger (anisole) in 10 ml of HF. The reaction is allowed to proceed for 1 h at 0 °C, and then HF was distilled off under vacuum. The peptide resin mixture was washed 2–3 times with ice-cold diethyl ether (~30 ml) and filtered. The crude peptide was then dissolved in 30% B (50 ml, ~30% CH_3CN in H_2O containing 0.1%TFA) and lyophilized. The peptides were purified by HPLC.

High-performance Liquid Chromatography

Analytical HPLC was carried out using a Ranin HPLC system equipped with a Ranin detector. Analytical injections were monitored at 220 nm. Separations were performed using a Phenomenex Jupiter 4 μ proteo 90 Å column (150 × 4.60 mm) at a flow rate of 1 ml/min with a gradient from 0 to 100% B in 30 min (eluent A: 0.1% TFA in H_2O, eluent B: 0.1% TFA, 90% CH_3CN, and 9.9% H_2O). Preparative HPLC was carried out using a Waters Delta Prep 4000 equipped with a UV detector and Phenomenex Jupiter 10 μ C-18 300 Å column (250 × 21.2 mm) at a flow rate of 15 ml/min employing linear gradient. Preparative injections were monitored at 220 nm.

Mass Spectrometry

Peptides were characterized using electrospray ionization MS on an LC/MS API I plus quadrupole mass spectrometer (Sciex). Peptide masses were calculated from the experimental mass to charge (*m/z*) ratios from all of the observed protonation states of a peptide by using the MacSpec software (Sciex).

Circular Dichroism (CD)

Data was collected on an Aviv Spectrophotometer Model 202 from 260 to 190 nm at 25 °C with a cell path length of 0.1 cm. Spectra were collected as an average of one to three scans, with a step of 1.0 nm. The control CD spectra were subtracted to eliminate background effects. The data were recorded in millidegrees and converted to mean residue molar ellipticity [Θ] in unit as follows: degree.cm^2.$dmol^{-1}$. The exact concentrations of peptide were determined by UV measurements at 280 nm on a Genesys 6 UV detector.

Enzyme-linked Immunosorbent Assay

The in-solution competition ELISA was performed as previously described by Brunel *et al.* [20]. In brief, 96-well plates (Corning) were coated overnight with 200 ng/well neutravidin (Pierce) in PBS at 4 °C. Plates were washed and blocked with 4% nonfat dry milk (NFDM) in TPBS (PBS containing 0.05% Tween 20) for 1 h at room temperature. In the meantime, different competing MPER peptide analogs (serially diluted 1 : 3 in 1% NFDM in TPBS at a starting concentration of 40 μM) were mixed with a constant concentration of biotinylated peptide 0705-bio [NWFDITNWLWYIKKKK(biotin)-NH_2, 0.1 μg/ml] or bio-29 [SLWNWFDITNWLWRRK(biotin)-NH_2, 0.3 μg/ml] and the corresponding human monoclonal antibody 4E10 (0.5 μg/ml) or Z13e1 (0.4 μg/ml), respectively. The antibody–peptide mixture was incubated for 2 h at 37 °C, subsequently transferred to the washed neutravidin plates and incubated at 37 °C for 20 min. Finally, the plates were incubated with a 1 : 1,000 dilution of goat antihuman F(ab)$_2$-horseradish peroxidase conjugate (Pierce). After 1 h at RT, wells were washed, developed (TMB solution (Pierce)) and stopped according to the manufacturer's instructions. The optical density at 450 nm was read on a microplate reader (molecular devices). The concentration of competitor peptide corresponding to a half-maximal signal (IC_{50}) was determined by interpolation of the resulting binding curve. Each peptide competitor was tested in duplicate in at least two separate experiments.

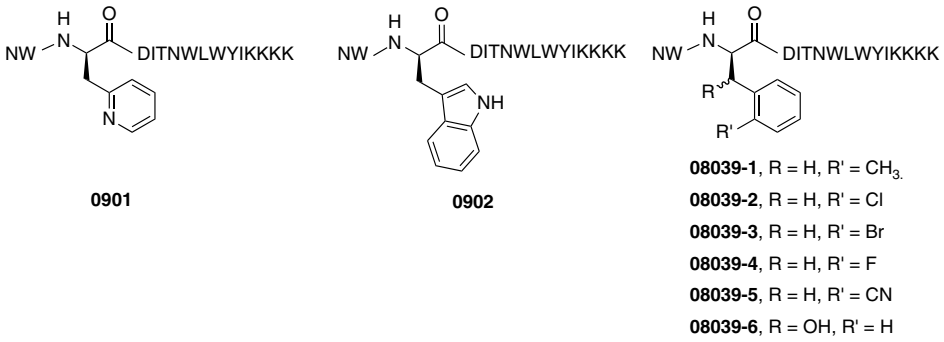

Figure 3. Amino acid analogs substituted at position Phe673.

Peptide Virus Neutralization Assay

JR-FL and VSV-G pseudotyped virus were generated as previously described [20,24] by co-transfection of 293 T cells using the corresponding HIV-1 envelope plasmids and the pSG3ΔEnv backbone vector. Pseudotyped virus was added at a 1 : 1 ratio to serially diluted (1 : 3) MPER peptide variants (10 μM final concentration) and incubated at 37 °C, for 1 h. TZM-bl cells were then seeded (1 : 1 by volume) at 1×10^4 cells/well in a final concentration of 10 μg/ml DEAE dextran. After 48-h incubation at 37 °C, the cells were washed, lysed and finally developed using the luciferase assay reagent according to the manufacturer's instructions (Promega). Luminescence in relative light units (RLUs) was measured using an Orion microplate luminometer (Berthold Detection Systems). All MPER peptides were tested in duplicate.

Results

Peptides Containing Unnatural Amino Acids

The linear epitope of 4E10 spans gp41 residues 671–683 including the core residues NWFDIT (671–676) in which the 'WF' are significantly buried in the antibody-binding pocket (Figure 2B) [18,25,26]. To address the importance of the WF motif, we introduced unnatural aromatic amino acids using Boc-solid-phase peptide synthesis (SPPS). The apparent binding affinities of the resulting peptide analogs to 4E10 were measured using an in-solution competition ELISA approach [20]. Peptides increasing both the size and rigidity of Phe673 were selected in the context of the peptide, NWF[673]DITNWLWYIK-KKK where F673 is replaced by L-pentafluoro-Phe-OH, Phe(4-nitro)-OH, naphthylalanine, or diphenyl alanine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic-OH). Unfortunately, none of these peptides showed significant binding affinity to 4E10 (>1 μM). Interestingly, peptide analogs with 2-pyridyl alanine **0901** and tryptophan at Phe673 **0902** showed moderate binding affinities of 1.0 and 0.35 μM, respectively (Figure 3). Encouraged by this result, we focused our attention on peptide analogs modified at the 2-position in the phenyl ring of Phe673 residue (Figure 3).

As the size of the substitution increases, the binding affinity to 4E10 decreases compared to the native epitope (**0708**, Table 1). Interestingly, analog Phe(2-F)-OH **08039-4**, showed only a threefold decrease in affinity, suggesting that the fluoro group is reasonably compatible with the 4E10 binding pocket. Inspection of the 4E10- peptide complex suggested that a polar substitution at the beta-position of the Phe673 would be accommodated. Correspondingly, the (*R*,*S*)-Phe(β-OH) derivative **08039-6** (Table 1) bound with an IC_{50} of 160 nM.

Table 1. 4E10 binding affinities (IC_{50}) as well as antiviral peptide activity

Analog	IC_{50} (nM)[a] 4E10	JRFL-IC_{50} (μM)[b]	VSV-G IC_{50} (μM)
0708	34	>10	>10
08039-1	2300	>10	>10
08039-2	1200	>10	>10
08039-3	8900	>10	>10
08039-4	100	>10	>10
08039-5	1600	>10	>10
08039-6	160	>10	>10

[a] The peptide concentration needed to inhibit the half-maximal binding signal of the mAb and the biotinylated peptide, NWFDITNWLWYIK-KKK(biotin)-NH_2 in the absence of any competing peptide.
[b] The neutralization titers are expressed as the peptide concentration that inhibited virus infection by 50% (IC_{50}).

MPER-derived peptides have been reported to display modest antiviral activity [27,28]. Accordingly, the antiviral activity of our peptide analogs was determined against the HIV-1 pseudotyped HIV-1 using Env from the JR-FL strain and a virus control, which was pseudotyped using the G glycoprotein from vesicular stomatitis virus (VSV-G), in a TZM-bl cell single-round infectivity assay [24]. As shown in Table 1, none of the peptides reached an IC_{50} at the highest concentration tested (10 μM). We also synthesized other Phe673 analogs, where Phe673 was replaced with D-Phe-OH (IC_{50} = 8.5 μM), D-Phe(2-F)-OH (>10 μM), (*R*, *S*)-Phe(β-methyl)-OH (2.0 μM), or γ-Phe-OH (>10 μM).

Peptides Containing 'Aib' and 'Api'

In our earlier report [20], we designed a 4E10 epitope peptide that constrained the existing α-helical conformation by using Aib (B) substitution or the addition of thioether side-chain constraints in the C-terminal region of the epitope [19]. To further these studies, we synthesized a series of peptide analogs modified C-terminally to the extended sequence, S[668]LWNWFDITNWLWYIK[683] that encompasses the epitopes of both 4E10 and Z13e1. (Figure 1) (Note: the native peptide **09122** has a > twofold lower affinity for 4E10 than the shorter peptide **0708**, Table 1.) In particular, the achiral Api (U) residue was of interest, having a γ-nitrogen at the side chain and being known to stabilize a helical structure [21]. Moreover, the secondary amine of Api will be protonated at neutral pH increasing the solubility of the peptide. Peptide analogs with Api residues displayed better binding to 4E10 (**09028**, Table 2)

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Table 2. Amino acid sequences, 4E10 and Z13e1 binding data (IC_{50}), and antiviral peptide activity

Analog	Sequence[a]	'X'	IC_{50} (nM)[b] 4E10	IC_{50} (nM)[b] Z13e1	JR-FL IC_{50} (μM)[c]	VSV-G IC_{50} (μM)
09122	**SLWNWFDITNWLWYIK**KKK-NH_2	Native	80	5500	>10	>10
09016	**SLWNWFDITNWLWYIK**BKBKK-NH_2	–	65	1600	5.0	>10
09018	**SLWNW**X**DITNWLWYIK**BKBKK-NH_2	Phe(2-F)	220	1900	5.8	>10
09019	**SLWNW**X**DITNWLWYIK**BKBKK-NH_2	Phe(β-OH)	240	9000	9.5	>10
09028	**SLWNWFDITNWLWYIK**UKUKK-NH_2	–	19	340	8.5	>10
09030	**SLWNW**X**DITNWLWYIK**UKUKK-NH_2	Phe(2-F)	70	1000	7.7	>10
09029	**SLWNW**X**DITNWLWYIK**UKUKK-NH_2	Phe(β-OH)	100	7800	>10	>10

[a] The amino acids shown in bold belong to the native sequence of gp41 MPER. B indicates Aib, U indicates, Api (4-aminopiperidine-4-carboxylic acid).
[b] The peptide concentration needed to inhibit the half-maximal binding signal of the mAb and the biotinylated peptide, SLWNWFDITNWLWRRK(biotin)-NH_2 in the absence of any competing peptide.
[c] The neutralization titers are expressed as the peptide concentration that inhibited virus infection by 50% (IC_{50}).

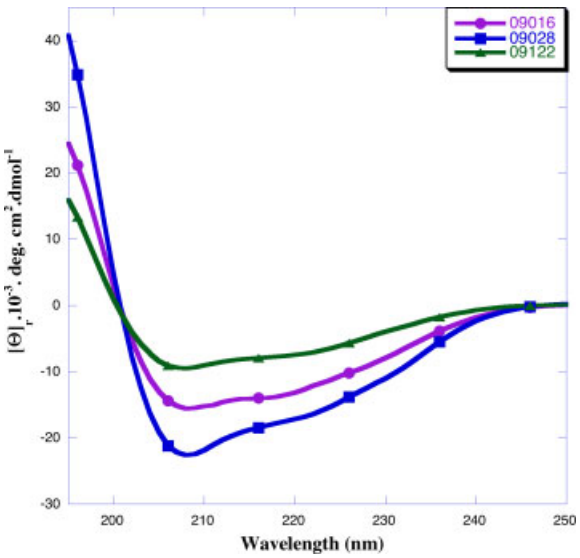

Figure 4. CD spectra of Aib (**09016**, Magenta), Api (**09028**, blue) and Lys tail (**09122**, blue) containing peptide analogs. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

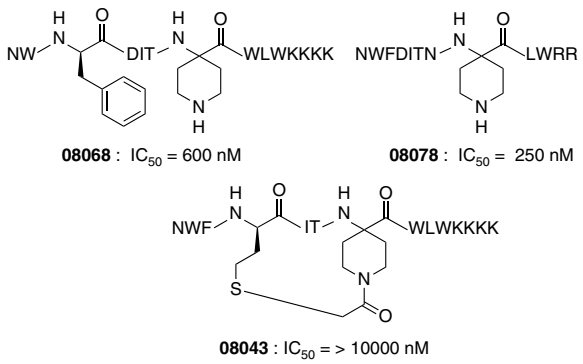

Figure 5. Peptide analogs containing 'Api' residue.

compared with Aib analogs (**09016**, Table 2). Significantly, the Api peptide **09028,** binds both 4E10 and Z13e1 with higher affinity (4- and 16-fold) than the native peptide sequence **09122**.

Combining the two approaches, replacement of Phe673 by Phe(2-F)-OH and Phe(β-OH)-OH in the context of either Aib or Api yielded MPER analogs with tight binding to 4E10 (Table 2). With the addition of the helical C-terminus, many of these analogs blocked viral entry of HIV-1_{JR-FL} at low micromolar concentration. In contrast no antiviral activity was seen against VSV-G (>10 μM). No correlation was found between the 4E10 binding data for peptides containing Aib, **09016** or Api, **09028** in the flanking C-terminus and the ability of peptide to inhibit HIV-1_{JR-FL}.

To assess the relative helical content of these peptide analogs, CD experiments were performed at 25 °C in 10 mM phosphate buffer pH = 7.2, with 20–50% CH_3CN to maintain full peptide solubility, while having limited affect on peptide helicity [22]. (#2) CD spectra of both Aib and Api containing peptide analogs were consistent with a highly helical conformation, characterized by strong minima in the 206–208-nm region accompanied by a pronounced negative shoulder at approximately 222 nm (Figure 4) [22]. In this set of peptide analogs, increased helicity indeed correlated to tighter binding to 4E10: **09028** is more helical than **09016** and has a lower IC_{50} (19 vs 65 nM; Table 2).

Thioether Constraints in 4E10 Epitope Peptides

To evaluate the general utility of Api in enhancing peptide helicity and/or 4E10 binding, it was substituted at internal positions in the core-binding epitope. The peptides NWFDITN**U**LWRR, **08078** and NWFDITUWLWKKKK, **08068** were synthesized by standard Boc-SPPS (Api incorporated using HATU) in high yield. In addition, an i to i + 3 tethered peptide NWF (XITU)WLWKKKK (X= homocysteine) was synthesized using an intramolecular thioether ligation strategy [19] in which the side-chain Api residue was bromoacetylated on resin and cleaved to form the linear NWFXITU(BrAc)WLWKKKK peptide following cleavage. Without purification, this peptide was cyclized in 6 M GdmCl pH 7.2 to yield the thioether-linked macrocycle **08043** in nearly quantitative conversion.

This peptide failed to bind 4E10 (IC_{50} = >10 000 nM; Figure 5). Despite the poor binding affinity, the side-chain tethered peptide, **08043**, and its unconstrained linear counterpart **08043-Lin** exhibit typical right-handed α–helix, determined by the appearance of two minima in the CD spectrum at 208 and 222 nm (Figure 6).

Discussion

The MPER of gp41 encompasses epitopes for three bNAbs (4E10, Z13e1, and 2F5). Multiple approaches to design immunogens

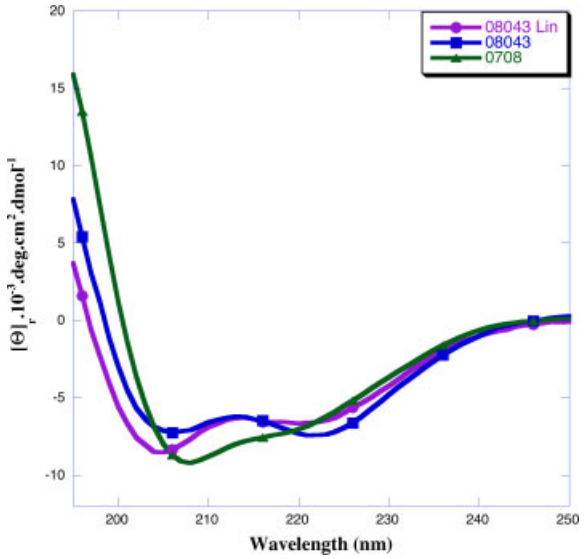

Figure 6. CD spectra of thioether tether peptide **08043**. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci.

based on these epitopes have failed to elicit similar antibodies [11]. One explanation has been that following CD4 receptor activation, gp41 in native Env transitions from its native conformation to a postfusion state, via a prehairpin intermediate structure [11]. It has been suggested that there is a short window of opportunity in which the MPER on the prehairpin intermediate is exposed to the immune system, thus limiting the elicitation of NAbs that recognize the MPER [15,25].

It has also been suggested that 4E10 interacts with hydrophobic MPER residues that are normally embedded in the membrane [14]. Such a mechanism of epitope 'extraction' is proposed to be facilitated by initial interaction of 4E10 with exposed MPER residues that results in a conformational rearrangement involving buried hydrophobic side-chain resides, and subsequent insertion of these residues into the binding pocket of 4E10. The effects of the membrane and the transient exposure of particular MPER conformations may be why such antibodies are rare, and suggests that stable mimics of relevant structures could make for more effective immunogens. The focus of this study has been to identify peptide antigens that will help to not only characterize binding specificity of these antibodies but also to inform the design of more stable MPER peptidomimetics that might elicit 4E10 or Z13e1 like NAbs [16,20].

The optimal length of the peptide epitope that binds to 4E10 has been identified as N^{671}WFDITNWLWYIK^{683}; with a binding IC_{50} of 34 nM (**0708**, Table 1) [20]. Initial immunizations with this peptide conjugated to carrier protein BSA in rabbits have elicited antibodies that bind to MPER antigens but do not neutralize HIV-1 (our unpublished observations) consistent with studies involving unmodified MPER peptides or MPER fusion proteins [11,29].

In order to mimic the 4E10-bound conformation in the kinked region of the epitope, amino acid substitutions were made at Phe673. A substitution at this position by bulky amino acids results in loss of binding to 4E10 (over 1000-fold decrease, data not shown) indicating that the flat aromatic structure is required. In contrast, the substitution at position 2-on phenyl ring of Phe673 **08039-4** and at the beta-position of Phe673 **08039-6**, resulted in analogs that bind to 4E10 with only a three- to fivefold weaker affinity (Table 1), indicating that these substitutions are compatible with the binding pocket of 4E10. The size of the substituent is clearly important as it showed positive correlation with increasing van der Waals radii and IC_{50} values for fluoro, chloro, and bromo substitution [30].

Since MPER-related peptides have been shown to exhibit antiviral activity, the ability of these peptide analogs to inhibit HIV-1_{JR-FL} was tested in a single-round infectivity assay using a VSV-G pseudotyped virus control [24,27,28]. The micromolar neutralization activity (IC_{50}) observed with some of the peptide analogs (Table 1) is much weaker than that of two known HIV inhibitors, C34 and T20, which we used as controls (IC_{50} of 0.2 and 0.7 nM, respectively) [28]. The latter two peptides bind to the gp41 N-terminal heptad repeat region to block gp41 conformational changes, which are essential for the viral entry process [31]. Interestingly, none of our MPER analog peptides that span residues 671–683 showed any antiviral activity, whereas the N-terminally extended peptides (668–683) did not inhibit and unnatural Phe analogs showed slightly diminished activity (Tables 1 and 2). Thus, a specific interaction with Env or the viral membrane at Phe673 might be important in the mechanism of peptide inhibition.

After establishing compatible substitutions at Phe673, the peptide sequence was extended at the N-terminus to incorporate the overlapping epitope recognized by a neutralizing antibody, Z13e1. Consistent with previous results, this extended sequence has lower affinity to the neutralizing antibodies but may better represent the full-length MPER domain. Native peptide sequence **09122,** SLWNWFDITNWLWYIK-*KKK* (italic correspond to a solubilizing tail) has an $IC_{50} = 80$ nM for 4E10 and 5500 nM for Z13e1.

The MPER sequence used in this study is thought to be solvent exposed and is immediately followed by the TM domain. Since the TM domain is expected to be helical (a typical 20 amino acid hydrophobic sequence) [32], we postulated that incorporation of a highly helical C-terminal tail would mimic the TM domain in a more soluble context and provide a 'running start' to the helical region found in the 4E10 co-crystal structure. Accordingly, the helix-inducing residue Aib (B) was incorporated into a – *BKBKK* tail, peptide **09016,** Table 2. With the helical tail, the IC_{50} dropped to 65 nM, for 4E10 and 1600 nM for Z13e1, a modest improvement from the original solubility tag **09122**.

Encouraged by this result, we investigated a cyclic amino acid analog that combines the helix-inducing α, α disubstitution of Aib with the charged amino side chain of Lysine. Api (U), 4-aminopiperidine-4-carboxylic acid, was developed by Hammer and coworkers for stabilizing 3_{10} helices in model peptides [23]. We reasoned that a – *UKUKK* tag **09028** would provide added charge to improve the solubility of the hydrophobic MPER sequence while simultaneously inducing the desired helical structure. Indeed, the resulting peptide was highly helical and bound tightly to both 4E10 and Z13e1, 19 nM and 340 nM, respectively. Importantly, the UKUKK tag that provides better peptide solubility has the lowest IC_{50} observed for longer (SLW containing) MPER sequences. Indeed, a series of conservative Phe673 analogs bearing the UKUKKK tag had IC_{50} values with both 4E10 and Z13e1 that are significantly lower than with the – KKK tag. The UKUKKK tagged MPER peptides may make for superior immunogens and/or 'bait' molecules in neutralizing antibody-screening efforts.

The CD spectra of the MPER region fused to the Lys, Aib, and Api C-terminal tails result in a progressively larger negative mean residue molar ellipticity (Θ). Since Aib helices are generally fully helical, this suggests that the Api helix may induce more helicity in the MPER region, consistent with the observed IC_{50}.

In a previous study, we observed that Aib replacement in the core epitope could increase the affinity of a truncated 4E10 epitope peptide by approximately fourfold. Incorporation of Api

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(U) at the same position, NWFDITN**U**LWRR (W678U) resulted in an IC_{50} of 250 nM, which is comparable to the 150 nM observed using Aib [13]. In addition, we explored the use of the cyclic secondary amine in the Api residue to facilitate macrocyclization of the peptide. In principle, this approach would combine α,α disubstitution and tethering in a manner analogous to peptide 'stapling' [33]. Peptide **08043** (Figure 3) was synthesized with the same number of atoms in the macrocycle (18 atoms) as a previously synthesized i to $i+3$ tethered peptide (104-2, $IC_{50} = 10$ nM) [13]. In contrast to the simple tether, the Api-tether had no measurable affinity to 4E10. In addition, the control peptide substituted with just Api (U), NWFDITUWLWKKKK, **08068** displayed a significantly reduced affinity ($IC_{50} = 600$ nM) compared to the control peptide (**0708**, 34 nM). Interestingly, the Api-tethered analog **08043** has a higher molar ellipticity value at a minimum around 222 nm, indicating an increase in helicity, compared to the native peptide **0708** (Figure 4). Hence, the tether did not induce a large change in backbone conformation. Clearly then, the exact location of the substitution of Api is very important for antibody recognition, and the larger cyclic side chain is more likely to perturb binding interactions. In addition, the cyclic 6-membered side chain results in a fused ring system with the 18-membered, side-chain macrocycle, which will significantly affect the conformation of the side-chain tether. Future studies will focus on the positioning and size of the Api side-chain tether.

Conclusions

In summary, we have identified peptide analogs with a charged, helical Api or Aib tail that display enhanced binding to the 4E10 and Z13e1 HIV neutralizing antibodies. We suggest that these C-terminal tags provide a soluble mimic of the gp41 TM domain and may find utility in the development of soluble MPER-based antigens. The use of the Api residue not only increases the helicity of the peptide but also provides a handle to incorporate side-chain functionality. A series of unnatural Phe673 analogs were analyzed and residues Phe(2F) and Phe(β-OH) were found to be compatible with 4E10 binding. When combined into the helical Api tail, these peptide analogs bind with high affinity to 4E10, suggesting generality of the Api tag to induce favorable interactions between 4E10 and the MPER epitope.

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