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Design of a Novel Cyclotide-Based CXCR4 Antagonist with Anti-Human Immunodeficiency Virus (HIV)-1 Activity

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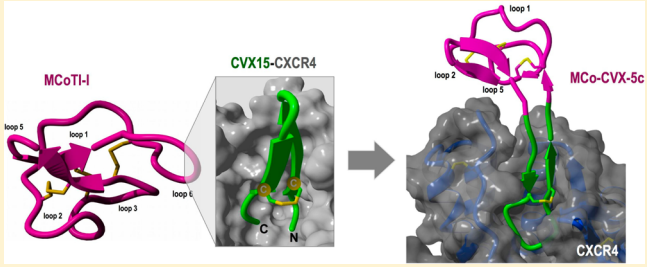
Supporting Information

ABSTRACT: Herein, we report for the first time the design and synthesis of a novel cyclotide able to efficiently inhibit HIV-1 viral replication by selectively targeting cytokine receptor CXCR4. This was accomplished by grafting a series of topologically modified CVX15 based peptides onto the loop 6 of cyclotide MCoTI-I. The most active compound produced in this study was a potent CXCR4 antagonist ($EC_{50} \approx 20$ nM) and an efficient HIV-1 cell-entry blocker ($EC_{50} \approx 2$ nM). This cyclotide also showed high stability in human serum, thereby providing a promising lead compound for the design of a novel type of peptide-based anticancer and anti-HIV-1 therapeutics.



INTRODUCTION

Chemokine receptors are G-protein-coupled receptors (GPCRs) that play a key regulatory role in embryonic development and controlling leukocyte functions during inflammation and immunity.[1−3] The CXCR4 receptor is one of the 19 chemokine receptors known so far. This receptor is activated exclusively by the cytokine CXCL12, also known as stromal cell-derived factor-1α (SDF1α). Activation of CXCR4 promotes chemotaxis in leukocytes,[4] progenitor cell migration,[5] and embryonic development of the cardiovascular, hemaotopoietic, and central nervous systems.[6−9] CXCR4 has also been associated with multiple types of cancers where its overexpression/activation promotes metastasis, angiogenesis, and tumor growth and/or survival.[10,11] Furthermore, CXCR4 is involved in HIV replication, as it is a co-receptor for viral entry into host cells.[12,13] Altogether, these features make CXCR4 a very attractive target for drug discovery.[14−16] Hence, several small molecules and small peptides have been developed to antagonize CXCR4 for anticancer and anti-HIV activity.[15] CXCR4 antagonists have also been shown to induce the mobilization of hematopoietic stem cells (HSCs) by disrupting the CXCR4−CXCL12 interaction, which is required for retaining HSCs in the bone marrow,[17−19] and therefore have been used to facilitate the mobilization of HSCs to the periphery for their isolation.[20]

Cyclotides are small globular microproteins (ranging from 28 to 37 amino acids) with a unique head-to-tail cyclized backbone, which is stabilized by three disulfide bonds forming a cystine-knot motif[21−23] (Figure 1A). This cyclic cystine-knot (CCK) framework provides a rigid molecular platform[24,25] with exceptional stability toward physical, chemical, and biological degradation.[22,23] These microproteins can be considered natural combinatorial peptide libraries structurally constrained by the cystine-knot scaffold and head-to-tail cyclization but in which hypermutation of essentially all residues is permitted with the exception of the strictly conserved cysteines that comprise the knot.[26−28] Furthermore, naturally occurring cyclotides have been shown to possess various pharmacologically relevant activities[22,29] and have been reported to cross cell membranes.[30,31] Altogether, these features make the cyclotide scaffold an excellent molecular framework for the design of novel peptide-based therapeutics,[23,32] making them ideal substrates for molecular grafting of biological peptide epitopes.[33−36]

Several small disulfide cyclic peptides derived from the horseshoe crab peptides polyphemusin-I/II have recently been reported to be efficient CXCR4 antagonists and effective as anti-HIV-1 and antimetastatic agents.[37−39] Some of these peptides, however, have shown limited proteolytic stability and/or poor bioavailability.[38] By using the crystal structure of CXCR4 bound to the polyphemusin-derived peptide CVX15,[40] we report here for the first time the design and synthesis of an engineered cyclotide able to effectively antagonize CXCR4 and inhibit CXCR4-tropic HIV-1 entry in human lymphocytes.

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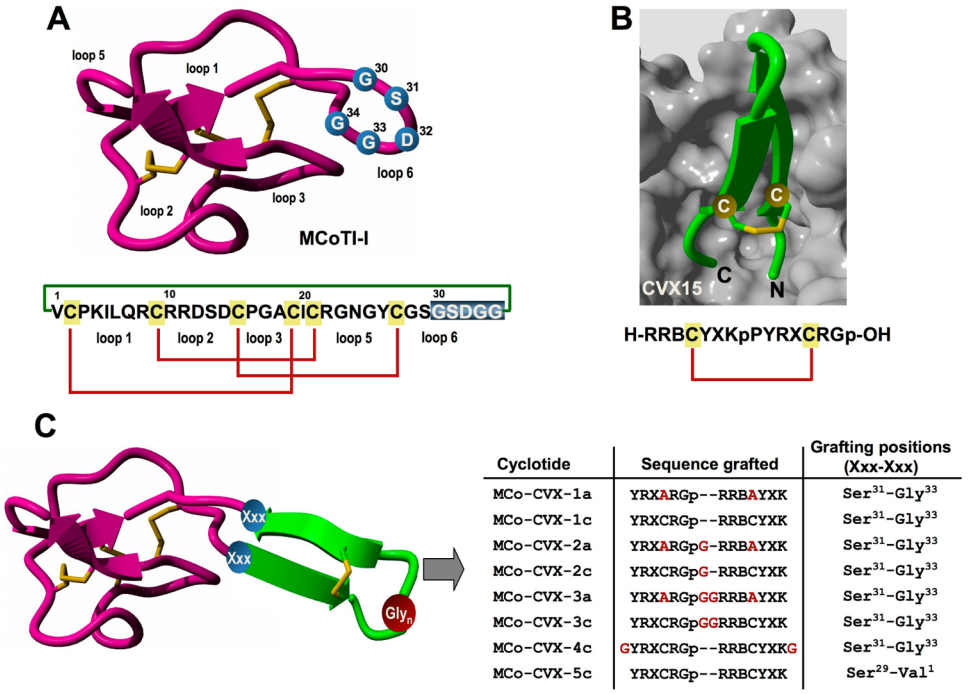

Cyclotide	Sequence grafted	Grafting positions (Xxx-Xxx)
MCo-CVX-1a	YRXARGp--RRBAYXK	Ser^{31}-Gly^{33}
MCo-CVX-1c	YRXCRGp--RRBCYXK	Ser^{31}-Gly^{33}
MCo-CVX-2a	YRXARGpG-RRBAYXK	Ser^{31}-Gly^{33}
MCo-CVX-2c	YRXCRGpG-RRBCYXK	Ser^{31}-Gly^{33}
MCo-CVX-3a	YRXARGpGGRRBAYXK	Ser^{31}-Gly^{33}
MCo-CVX-3c	YRXCRGpGGRRBCYXK	Ser^{31}-Gly^{33}
MCo-CVX-4c	GYRXCRGp--RRBCYXKG	Ser^{31}-Gly^{33}
MCo-CVX-5c	YRXCRGp--RRBCYXK	Ser^{29}-Val^{1}

Figure 1. Design of MCoTI-based cyclotides to target the cytokine receptor CXCR4. (A) Primary and tertiary structures of cyclotide MCoTI-I. Structure is based on a homology model using the solution structure of MCoTI-II as template (PDB code 1IB9).[45] The backbone cyclized peptide (connecting bond shown in green) is stabilized by the three disulfide bonds (shown in red). The residues used for the grafting of a CVX15-based peptide are shown in blue on the structure and sequence of MCoTI-I. (B) Sequence and cocrystal structure of peptide CVX15 bound to cytokine receptor CXCR4 (PDB: code 3OE0).[40] Peptide CVX15 is shown as a ribbon representation in green with the side chains of the Cys residues involved in the disulfide bond in ball-and-stick form. The solvent accessible surface of the binding site of CXCR4 is shown in gray. (C) Scheme depicting the approach used to design the different MCo-CVX cyclotides. A circularly permuted version of CVX15 was grafted onto loop 6 of MCoTI-I at different residues. The CVX15-based insert was created by joining the C- and N-terminus directly through a flexible Gly$_n$ linker and opening the new sequence at the D-Pro-Pro segment. Residues in red denote mutations or extra Gly residues introduced to increase flexibility. Single letter codes B, X, and p represent the amino acids 2-naphthylalanine, citruline, and D-proline, respectively. Molecular graphics were built with Yasara (www.yasara.org).

RESULTS AND DISCUSSION

To produce a novel cyclotide with CXCR4 antagonistic activity, we used MCoTI-I as a molecular scaffold (Figure 1A). MCoTI-cyclotides have been recently isolated from the dormant seeds of *Momordica cochinchinensis*, a plant member of the *cucurbitaceae* family, and are potent trypsin inhibitors ($K_i \approx$ 20−30 pM).[41] MCoTI-cyclotides show very low toxicity in human cells[30] and represent a desirable molecular scaffold for engineering new compounds with unique biological properties.[33−35]

According to the X-ray crystal structure of CVX15 bound to CXCR4, the N- and C-termini of the CVX15 peptide are deeply buried in the CXCR4 binding pocket (Figure 1B). Therefore, a circularly permuted version of the CVX15 peptide was grafted into loop 6 of the cyclotide MCoTI-I in order to preserve the biological activity of the grafted peptide. The CVX15 sequence was designed by linking the original N- and C-termini directly or through a flexible Gly$_n$ (n = 1, 2) linker, removing residues D-Pro^8 and Pro^9 and leaving the new N- and C-terminal groups on residues Tyr^{10} and Lys^7, respectively (Figures 1B and Figure 1C). Residue Gln^6 was also replaced by citruline, which has been shown to increase the affinity of CVX15 for CXCR4.[42] We also explored the effect of replacing the original Cys residues in the CVX15-based sequence, which are involved in a disulfide bond, by Ala residues to see the effect on the biological activity of the resulting cyclotides. The different sequences were grafted onto loop 6 by replacing residue Asp^{32} or the peptide segment Gly^{30}-Gly^{34} (Figure 1C). Loop 6 of MCoTI-cyclotides has been shown to be less rigid in solution[24,25] and quite tolerant to sequence grafting.[33−36]

All grafted MCo-CVX cyclotides were chemically synthesized using Fmoc-based solid-phase peptide synthesis on a sulfonamide resin.[30] Activation of the sulfonamide linker with iodoacetonitrile, followed by cleavage with ethyl mercaptoacetate and acidolytic deprotection, provided the fully deprotected linear peptide α-thioester (Table S1 of Supporting Information). The corresponding peptide thioester precursors were efficiently cyclized and folded in a one-pot reaction using sodium phosphate buffer at pH 7.2 in the presence of 1 mM GSH. The cyclization/folding reactions were complete in 24−96 h (Figures 2A and S1, Table 1). The cyclization/folding yields ranged from around 20% (MCoTI-CVX-4c) to 80% (MCo-CVX-5c) (Table 1). Folded MCo-CVX cyclotides were purified by reverse-phase HPLC and characterized by ES-MS confirming ≥95% purity (Figures 2B and S1, and Table 1). Grafted MCo-CVX-5c cyclotide was also characterized by ^{1}H NMR indicating that it adopts a native cyclotide fold (Figures 2C and S2).

Next, we tested the ability of the CVX15-grafted cyclotides to inhibit SDF1α-mediated CXCR4 activation using a CXCR4-β-lactamase U2OS cell-based fluorescence assay (Figure 3A). All grafted cyclotides were able to block SDF1α-mediated CXCR4 activation in a dose dependent manner with EC_{50} values ranging from 23.8 ± 0.3 μM (MCo-CVX-3a) to 19 ± 3 nM (MCo-CVX-5c). Intriguingly, the peptide CVX15 Gln6Cit

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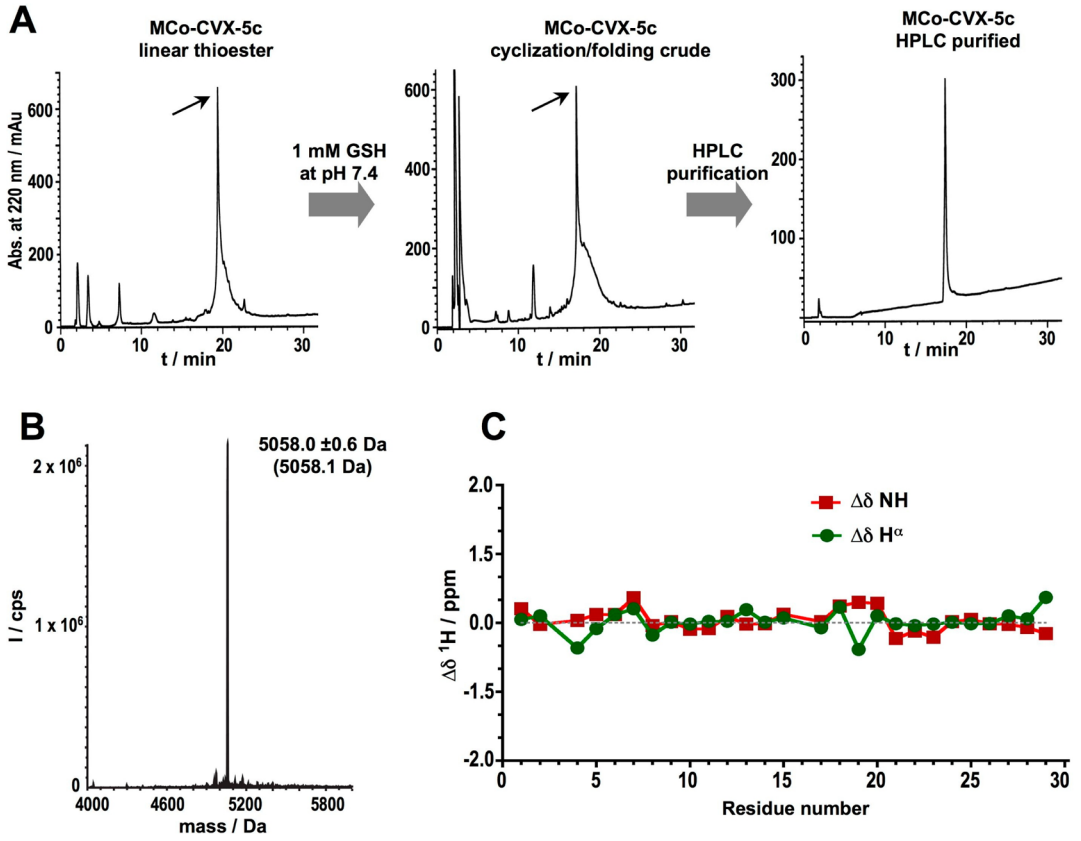

Figure 2. Chemical synthesis and characterization of cyclotide MCo-CVX-5c. (A) Analytical HPLC traces of the linear thioester precursor, GSH-induced cyclization/folding crude after 96 h, and purified cyclotide. An arrow indicates the desired peptide. (B) ES-MS characterization of pure MCo-CVX-5c. The expected average molecular weight is shown in parentheses. (C) Chemical shift differences of the backbone, NH, and H^{α} protons between the common sequence (residues 1−29) of MCoTI-I[24,25] and MCo-CVX-5c (Table S2).

Table 1. Molecular Weight, Cyclization/Folding Yield, and Biological Activity Summary for the MCo-CVX Grafted Cyclotides Produced in This Work

	molecular weight (Da)		cyclization/folding		EC_{50} (nM)	
peptide	linear thioester	cyclized/folded	yield (%)	time (h)[a]	CXCR4 inhibition	HIV-1 inhibition
MCo-CVX-1a	5380.0 ± 1.0 (5380.3)	5254.0 ± 0.4 (5252.2)	43	24	1040 ± 45	ND[b]
MCo-CVX-1c	5444.4 ± 0.2 (5444.4)	5317.1 ± 0.8 (5316.4)	61	24	102 ± 12	ND[b]
MCo-CVX-2a	5437.7 ± 0.5 (5437.3)	5311.1 ± 0.5 (5311.3)	41	24	4900 ± 600	ND[b]
MCo-CVX-2c	5501.4 ± 0.4 (5501.4)	5373.2 ± 0.3 (5373.4)	43	24	2140 ± 300	ND[b]
MCo-CVX-3a	5494.7 ± 0.7 (5494.3)	5368.2 ± 0.5 (5368.3)	26	24	23800 ± 3000	ND[b]
MCo-CVX-3c	5559.1 ± 0.5 (5558.4)	5430.2 ± 0.6 (5430.4)	47	24	3110 ± 480	ND[b]
MCo-CVX-4c	5559.2 ± 0.7 (5558.4)	5430.2 ± 0.2 (5430.4)	17	24	39 ± 1	ND[b]
MCo-CVX-5c	5185.1 ± 0.5 (5186.1)	5058.0 ± 0.6 (5058.1)	81	96	19 ± 3	2.0 ± 0.3

[a]Time for efficient cyclization. [b]Not determined.

alone showed an EC_{50} of 71 ± 13 nM, which is around 3 times weaker than that of the best cyclotide inhibitor (MCo-CVX-5c). As expected, the naturally occurring cyclotide MCoTI-I did not show any inhibitory activity in this assay (Figure 3A), indicating that the biological activity of grafted MCo-CVX cyclotides is specific and comes from the grafted sequence. The small molecule AMD3100[20] was also used as positive control. The importance of the original Cys residues in peptide CVX15 is highlighted by comparing the EC_{50} values of the cyclotides grafted onto Asp[32]. Mutation of the Cys residues to Ala significantly reduced the biological activity of the corresponding cyclotides. For example, cyclotides MCo-CVX-1c and MCo-CVX-3c were around 10 times more potent than the corresponding mutants MCo-CVX-1a and MCo-CVX-3a, respectively. The decrease in potency was less pronounced in cyclotide MCo-CVX-2a, where this mutation resulted only in a ~2-fold decrease in EC_{50} (Figure 3A). The length of Gly linker used to build the CVX15-based insert, which was grafted onto the cyclotide scaffold, was also critical to the biological activity of the resulting grafted MCo-CVX cyclotides. The most active cyclotide in this series was MCo-CVX-1c (EC_{50} = 0.10 ± 0.01 μM), which was designed by linking directly the original N- and C-termini of the CVX15 peptide. Addition of extra Gly residues on MCo-CVX-2c and MCo-CVX-3c had a detrimental effect on their potencies, yielding EC_{50} values around 2 and 3 μM, respectively (Figure 3A and Table 1). These results are likely due to the increase in flexibility provided by the extra Gly residues, which may reduce the binding energy. Interestingly,

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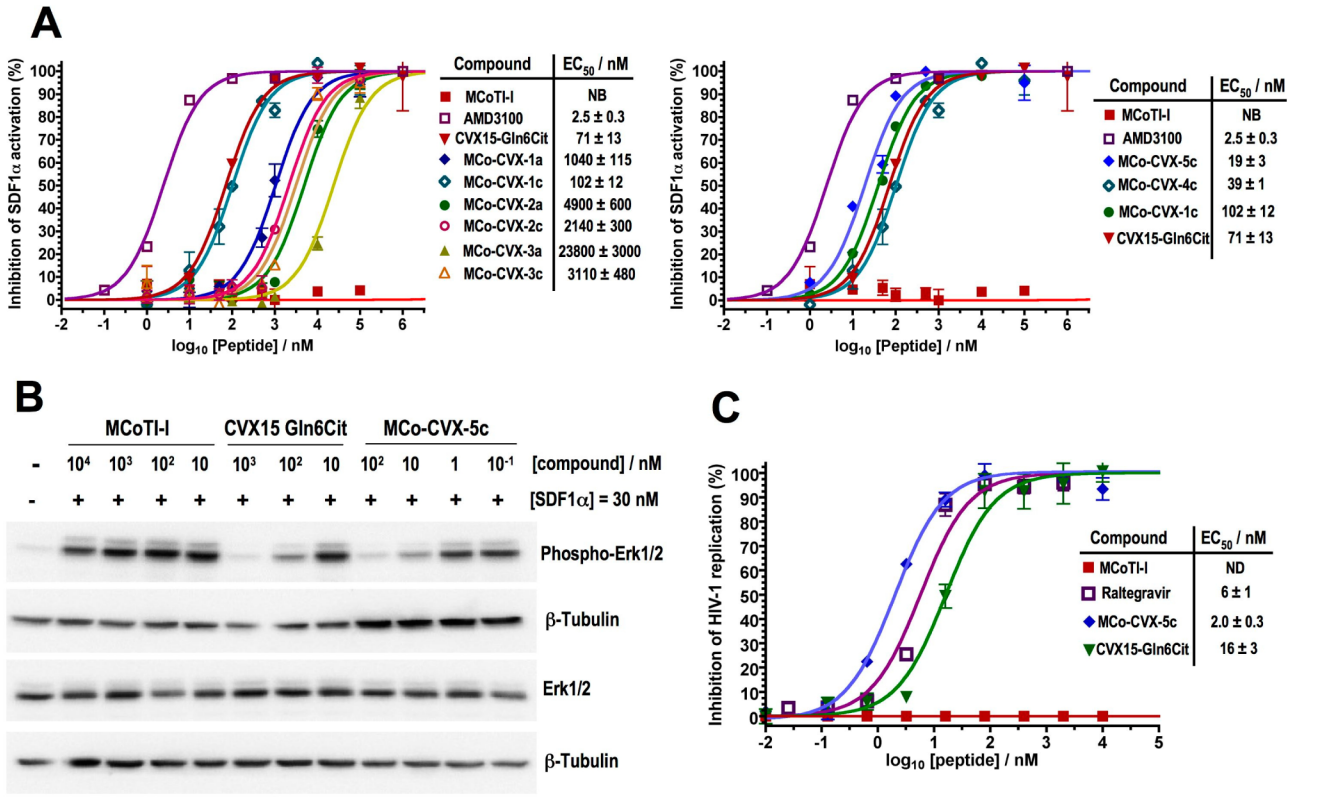

Figure 3. Biological characterization of MCo-CVX cyclotides. (A) Competitive inhibition of SDF1α-mediated CXCR4 activation by different cyclotides. The peptide CVX15 Gln6Cit and the small molecule CXCR4 antagonist AMD3100 were used as controls. The assay was performed using CXCR4-bla U2OS cells. (B) Inhibition of Erk phosphorylation (residues Thr^{202} and Tyr^{204}) by cyclotide MCo-CVX-5c. Cyclotide MCoTI-I and peptide CVX15 Gln6Cit were used as negative and positive controls, respectively. Erk phosphorylation was visualized by Western blot using CaOV3 cells treated with increasing amounts of CXCR4 inhibitor in the presence of SDF1α. (C) Dose response inhibition of HIV-1 replication in MT-4 cells by cyclotides MCoTI-I and MCo-CVX-5c. The peptide CVX15 Gln6Cit and the small molecule HIV-1 integrase inhibitor, raltegravir, were used as positive controls. Cyclotide MCoTI-I was used as negative control. The average of standard deviation of three experiments is shown. NB and ND stand for not bound and not determined, respectively.

the position on loop 6 where the CVX15-based peptide was grafted was also important for the biological activity of the resulting grafted cyclotides. The most active cyclotide was MCo-CVX-5c (EC_{50} = 19 ± 3 nM), where the CVX15-based peptide is grafted between residues Gly^{30} and Gly^{34}. Grafting the bioactive peptide farther away from the cyclotide core resulted in less active cyclotides. Thus, cyclotides MCo-CVX-1c (graft at residue Asp^{32}) and MCo-CVX-4c (graft at residue Asp^{32} but with extra Gly residues at both termini of the peptide graft) showed EC_{50} values of 102 ± 12 and 39 ± 1 nM, respectively (Figure 3A and Table 1).

Cyclotide MCo-CVX-5c was also able to inhibit SDF1α-induced Erk phosphorylation and internalization of CXCR4 in a dose dependent manner, confirming that this cyclotide is an efficient CXCR4 antagonist (Figures 3B and S3). In these experiments, cyclotide MCo-CVX-5c was around 10 times more active than the peptide CVX15 Gln6Cit. More importantly, cyclotide MCo-CVX-5c also inhibited the entry and replication of CXCR4-tropic HIV-1 in human lymphocyte MT4 cells in a dose dependent manner with an EC_{50} of 2.0 ± 0.3 nM (Figure 3C). The EC_{50} for peptide CVX15 Gln6Cit was around 8 times higher (Figure 3C), which is in agreement with the data obtained in the inhibition of Erk phosphorylation and CXCR4 internalization. More notably, cyclotide MCo-CVX-5c showed a CC_{50} (cytoxic concentration to reduce 50% cell viability) in MT4 cells greater than 10 μM (data not shown), therefore providing a selectivity index of more than 4000. It is also worth noting that cyclotide MCo-CVX-5c was 3 times more potent than raltegravir,[43] an integrase inhibitor recently approved by the FDA to treat HIV infection (Figure 3C).

We also studied the biological stability of MCo-CVX-5c and compared it to that of the empty scaffold (MCoTI-I) and the grafted peptide (CVX15 Gln6Cit) (Figure S4). This was accomplished by incubating the corresponding peptides in human serum at 37 °C. The quantitative analysis of undigested polypeptides was performed using liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS). Naturally occurring MCoTI-cyclotides present a very rigid structure,[24,25] which makes them extremely stable to proteolytic degradation. Remarkably, cyclotide MCo-CVX-5c showed greater stability in human serum ($\tau_{1/2}$ = 62 ± 3 h) than the parent cyclotide MCoTI-I ($\tau_{1/2}$ = 52 ± 3 h, Figure S4). In contrast, peptide CVX15 Gln6Cit was degraded considerably faster under the same conditions ($\tau_{1/2}$ = 21 ± 4 h, Figure S4). A linearized, reduced, and alkylated version of MCo-CVX-5c was also rapidly degraded ($\tau_{1/2}$ = 21 ± 3 min) indicating the importance of the circular Cys-knot topology for proteolytic stability. We also investigated the fraction of cyclotide bound to serum proteins. Serum binding has been recently used to extend serum half-life of bioactive peptides.[44] The binding, however, has to be reversible in order to be pharmacologically useful. Cyclotides MCoTI-I and MCo-CVX-5c were both found to be more than 99% bound to serum proteins under the conditions employed in the serum stability assay. The fact that

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these cyclotides are almost completely degraded after 120 h of treatment (Figure S4) suggests that their binding to serum proteins may be reversible. To further explore this possibility, we studied the association and dissociation rate constants of MCo-CVX-5c to human serum proteins. This was accomplished by biolayer interferometry analysis using the commercially available platform Blitz from FortéBio. The results indicated that the cyclotide MCo-CVX-5c is able to bind serum proteins with association and dissociation rate constants of $(3.6 \pm 0.7) \times 10^3$ M^{-1} s^{-1} and $(1.4 \pm 0.2) \times 10^{-2}$ s^{-1}, respectively (Figure S5), which provide a relatively weak dissociation constant of ~4 μM when compared to the low nanomolar affinity of MCo-CVX-5c for the CXCR4. These results are in agreement with the biological activities found for MCo-CVX-5c, which were obtained in the presence of serum, 1% and 10% for the CXCR4 translocation and HIV-1 cell entry inhibition assays, respectively.

CONCLUSIONS

In summary we report here for the first time the design and synthesis of a novel cyclotide able to efficiently inhibit the GPCR CXCR4. This was successfully accomplished by grafting a series of topologically modified CVX15 based peptides onto loop 6 of the cyclotide MCoTI-I. ^{1}H NMR studies also revealed that the grafting of CVX15 based peptides onto this loop did not affect the native cyclotide scaffold, indicating the tolerance of this loop for the grafting of long peptide sequences.[29,35] The most active compound produced in this study, MCo-CVX-5c, is a potent CXCR4 antagonist (EC_{50} = 19 ± 3 nM) and an efficient HIV-1 cell-entry blocker (EC_{50} = 2.0 ± 0.3 nM). Intriguingly, cyclotide MCo-CVX-5c was significantly more active than the cyclic peptide CVX15 Gln6Cit used in the design of the grafted cyclotide. Although more detailed structural studies are required to analyze the interaction between the cyclotide MCo-CVX-5c and CXCR4, altogether these results suggest that some of the residues from the neighboring loops in the cyclotide may contribute positively to the interaction with CXCR4. To further explore this possibility, we built a model of MCo-CVX-5c bound to CXCR4 using the crystal structure of CVX15-CXCR4 (PDB code 3OE0)[40] and the solution structure of MCoTI-II (PDB code 1IB9)[45] (Figure S6). According to this model, loops 2 and 5 may be in proximity to the extracellular receptor surface facilitating new interactions. This should make possible the design of even more potent antagonists based on MCo-CVX-5c by the introduction of appropriate mutations in these loops to improve the molecular complementarity between the cyclotide and receptor surfaces. It is also worth noting that the cyclotide MCo-CVX-5c showed a remarkable resistance to biological degradation in human serum, with a $\tau_{1/2}$ of 62 ± 3 h. This value is similar to that of the cyclotide MCoTI-I and significantly higher than the half-life of the peptide CVX15 ($\tau_{1/2}$ = 21 ± 4 h). In addition, the binding affinity of cyclotide MCo-CVX-5c to serum proteins was significantly weaker than for CXCR4, which should be able to decrease the renal clearance of this cyclotide without affecting its activity. Although further analysis will be required to evaluate the therapeutic value of these compounds in vivo, altogether our results show that engineered cyclotides hold great promise for the development of a novel type of peptide-based therapeutic able to efficiently target extracellular protein−protein interactions. Our results demonstrate for the first time the design of an engineered cyclotide able to target the GPCR CXCR4 with low nanomolar affinity and significant serum stability, thereby providing a promising lead compound for the design of anticancer and anti-HIV-1 compounds.

ASSOCIATED CONTENT

Supporting Information

Experimental details for the synthesis, purification, and characterization of MCo-CVX peptides, cell-based CXCR4 competitive binding assays, SDF1α-mediated Erk phosphorylation and CXCR4 internalization assays, HIV-1 replication and MT-4 cytotoxicity assays, NMR spectroscopy, serum stability, binding kinetics of cyclotide MCo-CVX-5c to human serum proteins, and modeling of the CXCR4-MCo-CVX-5c complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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