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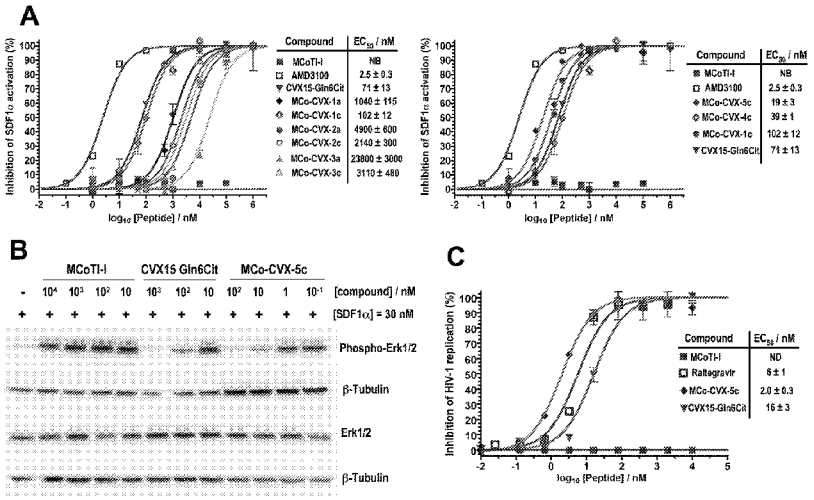

FIG 3

(57) Abstract: Disclosed is an isolated peptide comprising a CXCR4 antagonist peptide grafted to a cyclotide. Also described are compositions and methods for treating cancer and HIV infections using the isolated peptides.

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CYCLOTIDE-BASED CXCR4 ANTAGONISTS WITH ANTI-HIV ACTIVITY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/703,117, filed September 19, 2012, the contents of which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant No. R01 GM090323 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] 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. The crystal structure of CXCR4 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, progenitor cell migration, and embryonic development of the cardiovascular, hemaotopoietic and central nervous system. CXCR4 has also been associated with multiple types of cancers where its overexpression/activation promotes metastasis, angiogenesis and tumor growth and/or survival. Furthermore, CXCR4 is involved in HIV replication, as it is a co-receptor for viral entry into host cells. Altogether, these features make CXCR4 a very attractive target for drug discovery. Accordingly, there is a need in the art for effective therapies that antagonize the CXCR4 receptor and exhibit anti-cancer and anti-HIV properties.

SUMMARY

[0004] This disclosure relates to anti-cancer and anti-HIV therapeutics comprising CXCR4 antagonists peptides grafted to a cyclotide. One aspect relates to an isolated peptide comprising a CXCR4 antagonist peptide grafted to a cyclotide. Cyclotides are micro-proteins (≈30 aa long) present in plants from the *Violaceae, Rubiaceae, Cucurbitaceae* and more recently *Fabaceae* and featuring various biological actions such as protease inhibitory, anti-microbial, insecticidal, cytotoxic, anti-HIV or hormone-like activity. They share a unique head-to-tail circular knotted topology of three disulfide bridges, with one disulfide penetrating through a macrocycle formed by the two other disulfides and inter-connecting peptide backbones, forming what is called a

cystine knot topology. Cyclotides belong to the family of knottins, a group of microproteins that also includes conotoxins (389 sequences) and spider toxins (257 sequences). Cyclotides can be considered as 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. The main features of cyclotides are therefore a remarkable stability due to the cystine knot, a small size making them readily accessible to chemical synthesis, and an excellent tolerance to sequence variations. Applicants have previously utilized cyclotides for the screening and design of biologically relevant peptides (see WO 2011/005598, incorporated herein by reference).

[0005] Further aspects relate to polynucleotides encoding the polypetides described herein, host cells comprising the isolated peptides or polynucleotides described herein, and therapeutic methods for treating cancer and HIV using the isolated peptides described herein.

BRIEF DESCRIPTION OF THE FIGURES

[0006] **FIG. 1** shows the 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: 1IB9). 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. The sequence of the cyclotide represents SEQ ID NO. 1. **B.** Sequence and co-crystal structure of peptide CVX15 bound to cytokine receptor CXCR4 (PDB: 3OE0). 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 grey. **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. Molecular graphics were built with Yasara (www.yasara.org). The sequences in FIG 1C represent SEQ ID NOs: 3-10, in order of appearance from top to bottom.

[0007] **FIG. 2** shows the 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 parenthesis. **C.** Chemical shifts differences of the backbone, H′ and H^{α} protons between the common sequence (residues 1 through 28) of MCoTI-I and MCo-CVX-5c (Table 2). The large $\Delta\delta$ values for the H' protons of residues Arg^{10} and Arg^{11} were attributed to the interaction of these residues with the grafted sequence.

[0008] **FIG. 3** shows the 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 Tango™ 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..

[0009] **FIG. 4** shows the analytical reverse-phase C18-HPLC traces and ESI mass spectra (deconvoluted) of MCo-CVX linear thioesters, cyclization/folding crudes and purified folded cyclotides. HPLC analysis was performed using a linear gradient of 0-70% solvent B over 30 minutes.

[0010] **FIG. 5** shows $^{1}H\{^{1}H\}$-NOESY spectrum of cyclotide MCo-CVX-5c (red) and MCoTI-I (blue) at pH 6.5.

[0011] **FIG. 6** shows the SDF1α-induced internalization of CXCR4 Tango™ CXCR4-bla U2OS cells is inhibited by MCo-CVX-5c and small molecule AMD3100 in a dose dependent manner.

[0012] **FIG. 7** shows the stability of cyclotides MCo-CVX-5c and MCoTI-I, and peptide CVX15 Gln6Cit to human serum at 37° C. Undigested peptide was quantified by HPLC-MS/MS.

[0013] **FIG. 8** shows the binding kinetics of MCo-CVX-5c to human serum proteins.

[0014] **FIG. 9** shows a model of MCo-CVX-5c bound to CXCR4. Cyclotide MCo-CVX5c is shown as a ribbon representation in magenta and green (grafted fragment) with the side-chains of the Cys residues in ball-and-stick form. The solvent accessible surface of the binding site of CXCR4 is shown in grey. Graphic was generated using Yasara (www.yasara.org).

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0015] Before the compositions and methods are described, it is to be understood that the invention is not limited to the particular methodologies, protocols, cell lines, assays, and reagents described, as these may vary. It is also to be understood that the terminology used herein is intended to describe particular embodiments of the present invention, and is in no way intended to limit the scope of the present invention as set forth in the appended claims.

[0016] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of tissue culture, immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook and Russell eds. (2001) Molecular Cloning: A Laboratory Manual, 3rd edition; the series Ausubel et al. eds. (2007) Current Protocols in Molecular Biology; the series Methods in Enzymology (Academic Press, Inc., N.Y.); MacPherson et al. (1991) PCR 1: A Practical Approach (IRL Press at Oxford University Press); MacPherson et al. (1995) PCR 2: A Practical Approach; Harlow and Lane eds. (1999) Antibodies, A Laboratory Manual; Freshney (2005) Culture of Animal Cells: A Manual of Basic Technique, 5th edition; Gait ed. (1984) Oligonucleotide Synthesis; U.S. Patent No. 4,683,195; Hames and Higgins eds. (1984) Nucleic Acid Hybridization; Anderson (1999) Nucleic Acid Hybridization; Hames and Higgins eds. (1984) Transcription and Translation; Immobilized Cells and Enzymes (IRL Press (1986)); Perbal (1984) A Practical Guide to Molecular Cloning; Miller and Calos eds. (1987) Gene Transfer Vectors for Mammalian Cells (Cold Spring Harbor Laboratory); Makrides ed. (2003) Gene Transfer and Expression in Mammalian Cells; Mayer and Walker eds. (1987) Immunochemical Methods in Cell and Molecular Biology (Academic Press, London); Herzenberg et al. eds (1996) Weir's Handbook of Experimental Immunology; Manipulating the Mouse Embryo: A Laboratory Manual, 3rd edition (Cold Spring Harbor Laboratory Press (2002)); Current Protocols In Molecular Biology (F. M. Ausubel, et al. eds., (1987)); the series Methods in Enzymology (Academic Press, Inc.): PCR 2: A Practical Approach (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)); Harlow and Lane, eds. (1988) Antibodies, A Laboratory Manual; Harlow and Lane, eds. (1999) Using Antibodies, A Laboratory Manual; Animal Cell Culture (R.I. Freshney, ed. (1987)); Zigova, Sanberg and Sanchez-Ramos, eds. (2002) Neural Stem Cells.

[0017] All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1 or 1 where appropriate. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". The term "about" also includes the exact value "X" in addition to minor increments of "X" such as "X + 0.1 or 1" or "X – 0.1 or 1," where appropriate. It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0018] As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges disclosed herein also encompass any and all possible subranges and combinations of subranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," and the like include the number recited and refer to ranges which can be subsequently broken down into subranges as discussed above.

[0019] Throughout and within this disclosure the patent and technical literature is identified by a bibliographic citation or by a Arabic number. The bibliographic citations for these references are found in this disclosure immediately preceding the claims. All references disclosed here are incorporated by reference to more fully describe the state of the art to which this invention pertains.

Definitions

[0020] As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

[0021] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination for the stated purpose. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate

buffered saline, preservatives and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention or process steps to produce a composition or achieve an intended result. Embodiments defined by each of these transition terms are within the scope of this invention.

[0022] The term "isolated" as used herein with respect to cells, nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. The term "isolated" as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to cells or polypeptides which are isolated from other cellular proteins or tissues. Isolated polypeptides is meant to encompass both purified and recombinant polypeptides.

[0023] The term "isolated" as used with respect to cells, refers to cells separated from other cells or tissue that are present in the natural tissue in the body.

[0024] As used herein, the term "recombinant" as it pertains to polypeptides or polynucleotides intends a form of the polypeptide or polynucleotide that does not exist naturally, a non-limiting example of which can be created by combining polynucleotides or polypeptides that would not normally occur together.

[0025] A "subject," "individual" or "patient" is used interchangeably herein and refers to a vertebrate, for example a primate, a mammal or preferably a human. Mammals include, but are not limited to equines, canines, bovines, ovines, murines, rats, simians, humans, farm animals, sport animals and pets.

[0026] "Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0027] "Amplify" "amplifying" or "amplification" of a polynucleotide sequence includes methods such as traditional cloning methodologies, PCR, ligation amplification (or ligase chain reaction, LCR) or other amplification methods. These methods are known and practiced in the art. See, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202 and Innis et al. (1990) Mol. Cell Biol. 10(11):5977-5982 (for PCR); and Wu et al. (1989) Genomics 4:560-569 (for LCR). In general, the PCR procedure describes a method of gene amplification which is comprised of (i) sequence-specific hybridization of primers to specific genes within a DNA sample (or library), (ii) subsequent amplification involving multiple rounds of annealing, elongation, and denaturation using a DNA polymerase, and (iii) screening the PCR products for a band of the correct size. The primers used are oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization, i.e. each primer is specifically designed to be complementary to each strand of the genomic locus to be amplified.

[0028] Reagents and hardware for conducting PCR are commercially available. Primers useful to amplify sequences from a particular region are preferably complementary to, and hybridize specifically to sequences in the target region or in its flanking regions. Nucleic acid sequences generated by amplification may be sequenced directly. Alternatively the amplified sequence(s) may be cloned prior to sequence analysis. A method for the direct cloning and sequence analysis of enzymatically amplified genomic segments is known in the art.

[0029] The term "genotype" refers to the specific allelic composition of an entire cell, a certain gene or a specific polynucleotide region of a genome, whereas the term "phenotype' refers to the detectable outward manifestations of a specific genotype.

[0030] As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. A gene may also refer to a polymorphic or a mutant form or allele of a gene.

[0031] "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, though preferably less than 25% identity, with one of the sequences of the present invention.

[0032] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 98 % or 99 %) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel et al. eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, last accessed on May 21, 2008. Biologically equivalent polynucleotides are those having the above-noted specified percent homology and encoding a polypeptide having the same or similar biological activity.

[0033] The term "a biological equivalent nucleic acid or polynucleotide" refers to a nucleic acid having a nucleotide sequence having a certain degree of homology with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof.

[0034] Hybridization reactions can be performed under conditions of different "stringency". In general, a low stringency hybridization reaction is carried out at about 40°C in about 10 x SSC or a solution of equivalent ionic strength/temperature. A moderate stringency hybridization is typically performed at about 50°C in about 6 x SSC, and a high stringency hybridization reaction is generally performed at about 60°C in about 1 x SSC. Hybridization reactions can also be performed under "physiological conditions" which is well known to one of skill in the art. A non-limiting example of a physiological condition is the temperature, ionic strength, pH and concentration of Mg^{2+} normally found in a cell.

[0035] As used herein, the term "oligonucleotide" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being

described, single (sense or antisense) and double-stranded polynucleotides. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine, and deoxythymidine. For purposes of clarity, when referring herein to a nucleotide of a nucleic acid, which can be DNA or an RNA, the terms "adenosine", "cytidine", "guanosine", and "thymidine" are used. It is understood that if the nucleic acid is RNA, a nucleotide having a uracil base is uridine.

[0036] The terms "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, dsRNA, siRNA, miRNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this invention that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0037] A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term "polynucleotide sequence" is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching. The term "polymorphism" refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a "polymorphic region of a gene". A polymorphic region can be a single nucleotide, the identity of which differs in different alleles.

[0038] As used herein, the term "carrier" encompasses any of the standard carriers, such as a phosphate buffered saline solution, buffers, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Sambrook and Russell (2001), supra. Those skilled in the art will know many other suitable carriers for binding polynucleotides, or will be able to ascertain the same by use of routine experimentation. In one aspect of the invention, the carrier is a buffered solution such as, but not limited to, a PCR buffer solution.

[0039] A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, or viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

[0040] "Gene delivery," "gene transfer," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection, sometimes called transduction), transfection, transformation or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). Unless otherwise specified, the term transfected, transduced or transformed may be used interchangeably herein to indicate the presence of exogenous polynucleotides or the expressed polypeptide therefrom in a cell. The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

[0041] A cell that "stably expresses" an exogenous polypeptide is one that continues to express a polypeptide encoded by an exogenous gene introduced into the cell either after replication if the cell is dividing or for longer than a day, up to about a week, up to about two weeks, up to three weeks, up to four weeks, for several weeks, up to a month, up to two months, up to three months, for several months, up to a year or more.

[0042] The term "express" refers to the production of a gene product.

[0043] As used herein, the term "grafted" intends replaced or inserted, e.g., the phrase "grafted between" means that the CXCR4 antagonist peptide replaces the amino acid residues between the two indicated amino acids. For example, in one embodiment, the CXCR4 antagonist peptide is grafted or inserted between Ser^{31} and Gly^{33} of SEQ ID NO: 1.

[0044] As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in an eukaryotic cell.

[0045] A "gene product" or alternatively a "gene expression product" refers to the amino acid (e.g., peptide or polypeptide) generated when a gene is transcribed and translated.

[0046] "Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operatively linked to an element which contributes to the initiation of, or promotes, transcription. "Operatively linked" intends the polynucleotides are arranged in a manner that allows them to function in a cell.

[0047] The term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

[0048] As used herein, a "vector" is a vehicle for transferring genetic material into a cell. Examples of such include, but are not limited to plasmids and viral vectors. A viral vector is a virus that has been modified to transduct genetic material into a cell. A plasmid vector is made by splicing a DNA construct into a plasmid. As is apparent to those of skill in the art, the

appropriate regulatory elements are included in the vectors to guide replication and/or expression of the genetic material in the selected host cell.

[0049] A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either in vivo, ex vivo or in vitro. Examples of viral vectors include retroviral vectors, lentiviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like. Alphavirus vectors, such as Semliki Forest virus-based vectors and Sindbis virus-based vectors, have also been developed for use in gene therapy and immunotherapy. See, Schlesinger and Dubensky (1999) Curr. Opin. Biotechnol. 5:434-439 and Ying et al. (1999) Nat. Med. 5(7):823-827.

[0050] In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism. A "lentiviral vector" is a type of retroviral vector well-known in the art that has certain advantages in transducing nondividing cells as compared to other retroviral vectors. See, Trono D. (2002) Lentiviral Vectors, New York: Spring-Verlag Berlin Heidelberg.

[0051] In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. See, e.g., International PCT Application No. WO 95/27071. Ads do not require integration into the host cell genome. Recombinant Ad derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. See, International PCT Application Nos. WO 95/00655 and WO 95/11984. Wild-type AAV has high infectivity and specificity integrating into the host cell's genome. See,

Hermonat and Muzyczka (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470 and Lebkowski et al. (1988) Mol. Cell. Biol. 8:3988-3996.

[0052] Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

[0053] Gene delivery vehicles also include several non-viral vectors, including DNA/liposome complexes, and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., a cell surface marker found on stem cells.

[0054] A "plasmid" is an extra-chromosomal DNA molecule separate from the chromosomal DNA which is capable of replicating independently of the chromosomal DNA. In many cases, it is circular and double-stranded. Plasmids provide a mechanism for horizontal gene transfer within a population of microbes and typically provide a selective advantage under a given environmental state. Plasmids may carry genes that provide resistance to naturally occurring antibiotics in a competitive environmental niche, or alternatively the proteins produced may act as toxins under similar circumstances.

[0055] "Plasmids" used in genetic engineering are called "plasmic vectors". Many plasmids are commercially available for such uses. The gene to be replicated is inserted into copies of a plasmid containing genes that make cells resistant to particular antibiotics and a multiple cloning site (MCS, or polylinker), which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments at this location. Another major use of plasmids is to make large amounts of proteins. In this case, researchers grow bacteria containing a plasmid harboring the gene of interest. Just as the bacteria produces proteins to confer its antibiotic resistance, it can also be induced to produce large amounts of proteins from the

inserted gene. This is a cheap and easy way of mass-producing a gene or the protein it then codes for.

[0056] "Eukaryotic cells" comprise all of the life kingdoms except monera. They can be easily distinguished through a membrane-bound nucleus. Animals, plants, fungi, and protists are eukaryotes or organisms whose cells are organized into complex structures by internal membranes and a cytoskeleton. The most characteristic membrane-bound structure is the nucleus. A eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells. Non-limiting examples include simian, bovine, ovine, porcine, murine, rats, canine, equine, feline, avian, reptilian and human.

[0057] "Prokaryotic cells" that usually lack a nucleus or any other membrane-bound organelles and are divided into two domains, bacteria and archaea. Additionally, instead of having chromosomal DNA, these cells' genetic information is in a circular loop called a plasmid. Bacterial cells are very small, roughly the size of an animal mitochondrion (about 1-2 μm in diameter and 10 μm long). Prokaryotic cells feature three major shapes: rod shaped, spherical, and spiral. Instead of going through elaborate replication processes like eukaryotes, bacterial cells divide by binary fission. Examples include but are not limited to prokaryotic Cyanobacteria, *bacillus* bacteria, *E. coli* bacterium, and *Salmonella* bacterium.

[0058] The term "propagate" means to grow a cell or population of cells. The term "growing" also refers to the proliferation of cells in the presence of supporting media, nutrients, growth factors, support cells, or any chemical or biological compound necessary for obtaining the desired number of cells or cell type.

[0059] The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (i.e., morphologically, genetically, or phenotypically) to the parent cell.

[0060] A "probe" when used in the context of polynucleotide manipulation refers to an oligonucleotide that is provided as a reagent to detect a target potentially present in a sample of interest by hybridizing with the target. Usually, a probe will comprise a label or a means by which a label can be attached, either before or subsequent to the hybridization reaction. Suitable labels are described and exemplified herein.

[0061] A "primer" is a short polynucleotide, generally with a free 3' -OH group that binds to a target or "template" potentially present in a sample of interest by hybridizing with the target, and

thereafter promoting polymerization of a polynucleotide complementary to the target. A "polymerase chain reaction" ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a "pair of primers" or a "set of primers" consisting of an "upstream" and a "downstream" primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme. Methods for PCR are well known in the art, and taught, for example in MacPherson et al. (1991) PCR: A Practical Approach, IRL Press at Oxford University Press. All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as "replication." A primer can also be used as a probe in hybridization reactions, such as Southern or Northern blot analyses. Sambrook et al., supra. The primers may optionall contain detectable labels and are exemplified and described herein.

[0062] As used herein, the term "detectable label" intends a directly or indirectly detectable compound or composition that is conjugated directly or indirectly to the composition to be detected, e.g., polynucleotide or protein such as an antibody so as to generate a "labeled" composition. The term also includes sequences conjugated to the polynucleotide that will provide a signal upon expression of the inserted sequences, such as green fluorescent protein (GFP) and the like. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. The labels can be suitable for small scale detection or more suitable for high-throughput screening. As such, suitable labels include, but are not limited to radioisotopes, fluorochromes, chemiluminescent compounds, dyes, and proteins, including enzymes. The label may be simply detected or it may be quantified. A response that is simply detected generally comprises a response whose existence merely is confirmed, whereas a response that is quantified generally comprises a response having a quantifiable (e.g., numerically reportable) value such as an intensity, polarization, and/or other property. In luminescence or fluoresecence assays, the detectable response may be generated directly using a luminophore or fluorophore associated with an assay component actually involved in binding, or indirectly using a luminophore or fluorophore associated with another (e.g., reporter or indicator) component.

[0063] Examples of luminescent labels that produce signals include, but are not limited to bioluminescence and chemiluminescence. Detectable luminescence response generally comprises a change in, or an occurrence of, a luminescence signal. Suitable methods and luminophores for luminescently labeling assay components are known in the art and described for example in Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research

Chemicals (6th ed.). Examples of luminescent probes include, but are not limited to, aequorin and luciferases.

[0064] Examples of suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue.TM., and Texas Red. Other suitable optical dyes are described in the Haugland, Richard P. (1996) Handbook of Fluorescent Probes and Research Chemicals (6th ed.).

[0065] In another aspect, the fluorescent label is functionalized to facilitate covalent attachment to a cellular component present in or on the surface of the cell or tissue such as a cell surface marker. Suitable functional groups, including, but not are limited to, isothiocyanate groups, amino groups, haloacetyl groups, maleimides, succinimidyl esters, and sulfonyl halides, all of which may be used to attach the fluorescent label to a second molecule. The choice of the functional group of the fluorescent label will depend on the site of attachment to either a linker, the agent, the marker, or the second labeling agent.

[0066] Attachment of the fluorescent label may be either directly to the cellular component or compound or alternatively, can by via a linker. Suitable binding pairs for use in indirectly linking the fluorescent label to the intermediate include, but are not limited to, antigens/antibodies, e.g., rhodamine/anti-rhodamine, biotin/avidin and biotin/strepavidin.

[0067] The phrase "solid support" refers to non-aqueous surfaces such as "culture plates" "gene chips" or "microarrays." Such gene chips or microarrays can be used for diagnostic and therapeutic purposes by a number of techniques known to one of skill in the art. In one technique, oligonucleotides are attached and arrayed on a gene chip for determining the DNA sequence by the hybridization approach, such as that outlined in U.S. Patent Nos.: 6,025,136 and 6,018,041. The polynucleotides of this invention can be modified to probes, which in turn can be used for detection of a genetic sequence. Such techniques have been described, for example, in U.S. Patent Nos.: 5,968,740 and 5,858,659. A probe also can be attached or affixed to an electrode surface for the electrochemical detection of nucleic acid sequences such as described by Kayem et al. U.S. Patent No. 5,952,172 and by Kelley et al. (1999) Nucleic Acids Res. 27:4830-4837.

[0068] Various "gene chips" or "microarrays" and similar technologies are known in the art. Examples of such include, but are not limited to, LabCard (ACLARA Bio Sciences Inc.); GeneChip (Affymetric, Inc); LabChip (Caliper Technologies Corp); a low-density array with

electrochemical sensing (Clinical Micro Sensors); LabCD System (Gamera Bioscience Corp.); Omni Grid (Gene Machines); Q Array (Genetix Ltd.); a high-throughput, automated mass spectrometry systems with liquid-phase expression technology (Gene Trace Systems, Inc.); a thermal jet spotting system (Hewlett Packard Company); Hyseq HyChip (Hyseq, Inc.); BeadArray (Illumina, Inc.); GEM (Incyte Microarray Systems); a high-throughput microarry system that can dispense from 12 to 64 spots onto multiple glass slides (Intelligent Bio-Instruments); Molecular Biology Workstation and NanoChip (Nanogen, Inc.); a microfluidic glass chip (Orchid Biosciences, Inc.); BioChip Arrayer with four PiezoTip piezoelectric drop-on-demand tips (Packard Instruments, Inc.); FlexJet (Rosetta Inpharmatic, Inc.); MALDI-TOF mass spectrometer (Sequnome); ChipMaker 2 and ChipMaker 3 (TeleChem International, Inc.); and GenoSensor (Vysis, Inc.) as identified and described in Heller (2002) Annu. Rev. Biomed. Eng. 4:129-153. Examples of "gene chips" or a "microarrays" are also described in U.S. Patent Publication Nos.: 2007/0111322; 2007/0099198; 2007/0084997; 2007/0059769 and 2007/0059765 and U.S. Patent Nos.: 7,138,506; 7,070,740 and 6,989,267.

[0069] In one aspect, "gene chips" or "microarrays" containing probes or primers homologous to a polynucleotide described herein are prepared. A suitable sample is obtained from the patient, extraction of genomic DNA, RNA, protein or any combination thereof is conducted and amplified if necessary. The sample is contacted to the gene chip or microarray panel under conditions suitable for hybridization of the gene(s) or gene product(s) of interest to the probe(s) or primer(s) contained on the gene chip or microarray. The probes or primers may be detectably labeled thereby identifying the sequence(s) of interest. Alternatively, a chemical or biological reaction may be used to identify the probes or primers which hybridized with the DNA or RNA of the gene(s) of interest. The genotypes or phenotype of the patient is then determined with the aid of the aforementioned apparatus and methods.

[0070] A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

[0071] A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use in vitro, in vivo or ex vivo.

[0072] As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The

compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin (1975) Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton).

[0073] An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages. Such delivery is dependent on a number of variables including the time period for which the individual dosage unit is to be used, the bioavailability of the therapeutic agent, the route of administration, etc. It is understood, however, that specific dose levels of the therapeutic agents of the present invention for any particular subject depends upon a variety of factors including the activity of the specific compound employed, bioavailability of the compound, the route of administration, the age of the animal and its body weight, general health, sex, the diet of the animal, the time of administration, the rate of excretion, the drug combination, and the severity of the particular disorder being treated and form of administration. Treatment dosages generally may be titrated to optimize safety and efficacy. Typically, dosage-effect relationships from in vitro and/or in vivo tests initially can provide useful guidance on the proper doses for patient administration. Studies in animal models generally may be used for guidance regarding effective dosages for treatment of diseases. In general, one will desire to administer an amount of the compound that is effective to achieve a serum level commensurate with the concentrations found to be effective in vitro. Thus, where a compound is found to demonstrate in vitro activity, for example as noted in the Tables discussed below one can extrapolate to an effective dosage for administration in vivo. These considerations, as well as effective formulations and administration procedures are well known in the art and are described in standard textbooks. Consistent with this definition and as used herein, the term "therapeutically effective amount" is an amount sufficient to treat a specified disorder or disease or alternatively to obtain a pharmacological response treating a glioblastoma.

[0074] As used herein, "treating" or "treatment" of a disease in a patient refers to (1) preventing the symptoms or disease from occurring in an animal that is predisposed or does not yet display symptoms of the disease; (2) inhibiting the disease or arresting its development; or (3) ameliorating or causing regression of the disease or the symptoms of the disease. As understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For the purposes of this invention, beneficial or desired results can include one or more, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of a condition (including a disease), stabilized (i.e., not worsening) state of a condition (including disease), delay or slowing of condition (including disease), progression, amelioration or palliation of the condition (including disease), states and remission

(whether partial or total), whether detectable or undetectable. Preferred are compounds that are potent and can be administered locally at very low doses, thus minimizing systemic adverse effects.

[0075] "Suppressing" or "inhibiting" tumor growth indicates a growth state that is curtailed compared to growth without any therapy. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and "suppressing" tumor growth indicates a growth state that is curtailed when stopping tumor growth, as well as tumor shrinkage.

[0076] A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the purpose of the experiment is to determine a correlation of a mutated allele with a particular phenotype, it is generally preferable to use a positive control (a sample from a subject, carrying such mutation and exhibiting the desired phenotype), and a negative control (a subject or a sample from a subject lacking the mutated allele and lacking the phenotype).

[0077] The term "CXCR4" refers to C-X-C chemokine receptor type 4 also known as fusin or CD184 (cluster of differentiation 184). CXCR4 is a protein that in humans is encoded by the *CXCR4* gene. The GenBank accession Nos. NM_001008540.1 and NP_001008540.1 represent the human mRNA and protein sequence, respectively. The sequence of these GenBank Accession Nos. is herein incorporated by reference in their entirety.

Descriptive Embodiments

[0078] This disclosure provides an isolated peptide comprising, or alternatively consisting essentially of, or yet further consisting of a CXCR4 antagonist peptide grafted to a cyclotide. 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. This cyclic cystine-knot (CCK) framework provides a rigid molecular platform with exceptional stability towards physical, chemical and biological degradation. These micro-proteins 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. Furthermore, naturally-occurring cyclotides have shown to posses various

pharmacologically-relevant activities, and have been reported to cross cell membranes. Altogether, these features make the cyclotide scaffold an excellent molecular framework for the design of novel peptide-based therapeutics, making them ideal substrates for molecular grafting of biological peptide epitopes.

[0079] The construction of the cyclotide is known in the art and has been described previously (see WO 2011/005598, which is incorporated herein for all purposes). Synthesis of peptides useful in the methods and compositions of the disclosure are also described in the Examples that follow.

[0080] The preparation of a cyclotide may also entail the generation of a linear peptide that contains the desired cyclotide in a linear form, flanked by two peptide fragments that have affinity to each other so as to be capable of bringing two ends of the linear cyclotide together, facilitating cyclization. In one aspect, the two peptide fragments are the C-terminus and N-terminus domains of a split intein. Accordingly, the present disclosure provides a polypeptide precursor for generating a cyclotide. In one embodiment, the polypeptide comprises a linear cyclotide fused to a C-terminal fragment and an N-terminal fragment of a split intein, at the N-terminus and C-terminus of the cyclotide, respectively.

[0081] A "split intein" is an interin of a precursor protein that comes from two separate genes. For example, in cyanobacteria, DnaE, the catalytic subunit α of DNA polymerase III, is encoded by two separate genes, dnaE-n and dnaE-c. The dnaE-n product consists of an N-extein sequence followed by a 123-AA intein sequence, whereas the dnaE-c product consists of a 36-AA intein sequence followed by a C-extein sequence.

[0082] In one embodiment, the split intein comprises a DnaE split intein. In one embodiment, the DnaE split intein comprises a Nostoc punitiforme PCC73102 DnaE split intein.

[0083] In one embodiment, the C-terminal fragment comprises an amino acid sequence of SEQ ID NO: 292 (MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASN). In one embodiment, the N-terminal fragment comprises an amino acid sequence of SEQ ID NO: 293 (CLSYETEILTVEYGLLPIGKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYC LEDGSLIRATKDHKFMTVDGQMLPIDEIFERELDLMRVDNLPN

[0084] In one embodiment, the CXCR4 antagonist peptide is grafted into loop 6 of the cyclotide. The cyclotide comprises a molecular framework comprising a sequence of amino acids forming a cyclic backbone wherein the cyclic backbone comprises sufficient disulfide

bonds to confer knotted topology on the molecular frameword or part therof. The cyclic backbone comprises the structure:
$C[X^{I}_{1}...X_a]C[X^{II}_{1}...X_b]C[X^{III}_{1}...X_c]C[X^{IV}_{1}...X_d]C[X^{V}_{1}...X_e]C[X^{VI}_{1}...X_f]$ wherein C is cysteine; and each of each of $[X^{I}_{1}...X_a]$, $[X^{II}_{1}...X_b]$, $[X^{III}_{1}...X_c]$, $[X^{IV}_{1}...X_d]$, $[X^{V}_{1}...X_e]$, and $[X^{VI}_{1}...X_f]$, represents one or more amino acid residues wherein each one or more amino acid residues within or between the sequence residues may be the same or different; and wherein a, b, c, d, e and f represent the number of amino acid residues in each respective sequence and each of a to f may be the same or different and range from 1 to about 20. When the CXCR4 antagonist peptide is grafted into loop 6 of the cyclotide, the amino acid residues corresponding to $[X^{VI}_{1}...X_f]$ in the cyclotide comprise the CXCR4 antagonist peptide. In some embodiments, the CXCR4 antagonist peptide is grafted into loop 1 of the cyclotide. In this embodiment, the amino acid residues corresponding to $[X^{I}_{1}...X_a]$ in the cyclotide comprise the CXCR4 antagonist peptide. In another embodiment, the CXCR4 antagonist peptide is grafted into loop 2 of the cyclotide. In this embodiment, the amino acid residues corresponding to $[X^{II}_{1}...X_b]$ in the cyclotide comprise the CXCR4 antagonist peptide. In a further embodiment, the CXCR4 antagonist peptide is grafted into loop 3 of the cyclotide. In this embodiment, the amino acid residues corresponding to $[X^{III}_{1}...X_c]$ in the cyclotide comprise the CXCR4 antagonist peptide. In yet a further embodiment, the CXCR4 antagonist peptide is grafted into loop 4 of the cyclotide. In this embodiment, the amino acid residues corresponding to $[X^{IV}_{1}...X_c]$ in the cyclotide comprise the CXCR4 antagonist peptide. In another embodiment, the CXCR4 antagonist peptide is grafted into loop 5 of the cyclotide. In this embodiment, the amino acid residues corresponding to $[X^{V}_{1}...X_e]$ in the cyclotide comprise the CXCR4 antagonist peptide.

[0085] Reference herein to a "cyclic backbone" includes a molecule comprising a sequence of amino acid residues or analogues thereof without free amino and carboxy termini. The cyclic backbone of the disclosure comprises sufficient disulfide bonds, or chemical equivalents thereof, to confer a knotted topology on the three-dimensional structure of the cyclic backbone. The term "cyclotide" as used herein refers to a peptide comprising a cyclic cystine knot motif defined by a cyclic backbone, at least two but preferably at least three disulfide bonds and associated beta strands in a particular knotted topology. The knotted topology involves an embedded ring formed by at least two backbone disulfide bonds and their connecting backbone segments being threaded by a third disulfide bond. However, a disulfide bond may be replaced or substituted by another form of bonding such as a covalent bond.

[0086] In one embodiment, the cyclotide backbone MCoTI-I. The sequence of MCoTI-I is described as SEQ ID NO: 1 in FIG. 1A. In one embodiment, the cyclotide comprises a peptide

with the amino acid sequence of SEQ ID NO: 1 or a biological equivalent thereof. In a related embodiment, the CXCR4 antagonist peptide is grafted between the Ser^{29} and Val^{1} amino acids of SEQ ID NO: 1. As used herein, the phrase "grafted between" in this context means that the CXCR4 antagonist peptide replaces the amino acid residues between the two indicated amino acids. In another related embodiment, the CXCR4 antagonist peptide is grafted between Ser^{31} and Gly^{33} of SEQ ID NO: 1.

[0087] Additional cyclotides useful in the peptides, methods, and compositions described herein are known in the art and non-limiting examples include, the cyclotides tabulated below:

Cylcotide	**Protein Sequence**	**SEQ ID NO.**
kalata_B1	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	12
cycloviolacin_O1	GIPCAESCVYIPCTVTALLGCSCSNRVCYN	13
kalata_B2	GLPVCGETCFGGTCNTPGCSCTWPICTRD	14
palicourein	GDPTFCGETCRVIPVCTYSAALGCTCDDRSDGLCKRN	15
vhr1	GIPCAESCVWIPCTVTALLGCSCSNKVCYN	16
tricyclon_A	GGTIFDCGESCFLGTCYTKGCSCGEWKLCYGTN	17
circulin_A	GIPCGESCVWIPCISAALGCSCKNKVCYRN	18
N-KB1-C	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	19
Ac-KB1-C	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	20
N-KB1-Am	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	21
Ac-KB1-Am	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	22
Ac-[desGly]-KB1-Am	LPVCGETCVGGTCNTPGCTCSWPVCTRN	23
kalata_b1-1	TCVGGTCNTPGCTCSWPVCTRNLPVCG	24
kalata_b1-2	GTCNTPGCTCSWPVCTRNGLPVCGETCVG	25
kalata_b1-3	GCTCSWPVCTRNGLPVCGETCVGGTCN	26
kalata_b1-4	CSWPVCTRNGLPVCGETCVGGTCNTPGC	27
kalata_b1-5	VCTRNGLPVCGETCVGGTCNTPGCTCS	28
kalata_b1-6a	VCGETCVGGTCNTPGCTCSWPVCT	29
kalata_b1-6b	RNGLPVCGETCVGGTCNTPGCTCSWPVCT	30
cycloviolacin_O2	GIPCGESCVWIPCISSAIGCSCKSKVCYRN	31
des(24-28)kB1	VCGETCVGGTCNTPGCTCSWPVCT	32
[Ala1,15]kB1	GLPVAGETCVGGTCNTPGATCSWPVCTRN	33
kalata_B6	GLPTCGETCFGGTCNTPGCSCSSWPICTRN	34
kalata_B3	GLPTCGETCFGGTCNTPGCTCDPWPICTRD	35

Cylcotide	Protein Sequence	SEQ ID NO.
kalata_B7	GLPVCGETCTLGTCYTQGCTCSWPICKRN	36
cycloviolacin_O8	GTLPCGESCVWIPCISSVVGCSCKSKVCYKN	37
cycloviolacin_O11	GTLPCGESCVWIPCISAVVGCSCKSKVCYKN	38
kalata_B4	GLPVCGETCVGGTCNTPGCTCSWPVCTRD	39
vodo_M	GAPICGESCFTGKCYTVQCSCSWPVCTRN	40
cyclopsychotride_A	SIPCGESCVFIPCTVTALLGCSCKSKVCYKN	41
cycloviolacin_H1	GIPCGESCVYIPCLTSAIGCSCKSKVCYRN	42
cycloviolacin_O9	GIPCGESCVWIPCLTSAVGCSCKSKVCYRN	43
vico_A	GSIPCAESCVYIPCFTGIAGCSCKNKVCYYN	44
vitri_A	GIPCGESCVWIPCITSAIGCSCKSKVCYRN	45
kalata_S	GLPVCGETCVGGTCNTPGCSCSWPVCTRN	46
cycloviolacin_O12	GLPICGETCVGGTCNTPGCSCSWPVCTRN	47
vodo_N	GLPVCGETCTLGKCYTAGCSCSWPVCYRN	48
vico_B	GSIPCAESCVYIPCITGIAGCSCKNKVCYYN	49
kalata_B1_IIa	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	50
Hypa_A	GIPCAESCVYIPCTITALLGCSCKNKVCYN	51
circulin_B	GVIPCGESCVFIPCISTLLGCSCKNKVCYRN	52
circulin_C	GIPCGESCVFIPCITSVAGCSCKSKVCYRN	53
circulin_D	KIPCGESCVWIPCVTSIFNCKCENKVCYHD	54
circulin_E	KIPCGESCVWIPCLTSVFNCKCENKVCYHD	55
circulin_F	AIPCGESCVWIPCISAAIGCSCKNKVCYR	56
cycloviolacin_O4	GIPCGESCVWIPCISSAIGCSCKNKVCYRN	57
cycloviolacin_O3	GIPCGESCVWIPCLTSAIGCSCKSKVCYRN	58
cycloviolacin_O5	GTPCGESCVWIPCISSAVGCSCKNKVCYKN	59
cycloviolacin_O6	GTLPCGESCVWIPCISAAVGCSCKSKVCYKN	60
cycloviolacin_O7	SIPCGESCVWIPCTITALAGCKCKSKVCYN	61
cycloviolacin_O10	GIPCGESCVYIPCLTSAVGCSCKSKVCYRN	62
kalata_B5	GTPCGESCVYIPCISGVIGCSCTDKVCYLN	63
varv_peptide_B	GLPVCGETCFGGTCNTPGCSCDPWPMCSRN	64
varv_peptide_C	GVPICGETCVGGTCNTPGCSCSWPVCTRN	65
varv_peptide_D	GLPICGETCVGGSCNTPGCSCSWPVCTRN	66
varv_peptide_F	GVPICGETCTLGTCYTAGCSCSWPVCTRN	67
varv_peptide_G	GVPVCGETCFGGTCNTPGCSCDPWPVCSRN	68
varv_peptide_H	GLPVCGETCFGGTCNTPGCSCETWPVCSRN	69

Cylcotide	Protein Sequence	SEQ ID NO.
cycloviolin_A	GVIPCGESCVFIPCISAAIGCSCKNKVCYRN	70
cycloviolin_B	GTACGESCYVLPCFTVGCTCTSSQCFKN	71
cycloviolin_C	GIPCGESCVFIPCLTTVAGCSCKNKVCYRN	72
cycloviolin_D	GFPCGESCVFIPCISAAIGCSCKNKVCYRN	73
violapeptide_1	GLPVCGETCVGGTCNTPGCSCSRPVCTXN	74
vhl-1	SISCGESCAMISFCFTEVIGCSCKNKVCYLN	75
Vontr_Protein	ALETQKPNHLEEALVAFAKKGNLGGLP	76
hcf-1	GIPCGESCHYIPCVTSAIGCSCRNRSCMRN	77
htf-1	GIPCGDSCHYIPCVTSTIGCSCTNGSCMRN	78
Oantr_protein	GVKSSETTLMFLKEMQLKLP	79
vhl-2	GLPVCGETCFTGTCYTNGCTCDPWPVCTRN	80
cycloviolacin_H3	GLPVCGETCFGGTCNTPGCICDPWPVCTRN	81
cycloviolacin_H2	SAIACGESCVYIPCFIPGCSCRNRVCYLN	82
Hyfl_A	SISCGESCVYIPCTVTALVGCTCKDKVCYLN	83
Hyfl_B	GSPIQCAETCFIGKCYTEELGCTCTAFLCMKN	84
Hyfl_C	GSPRQCAETCFIGKCYTEELGCTCTAFLCMKN	85
Hyfl_D	GSVPCGESCVYIPCFTGIAGCSCKSKVCYYN	86
Hyfl_E	GEIPCGESCVYLPCFLPNCYCRNHVCYLN	87
Hyfl_F	SISCGETCTTFNCWIPNCKCNHHDKVCYWN	88
Hyfl_G_(partial)	CAETCVVLPCFIVPGCSCKSSVCYFN	89
Hyfl_H_(partial)	CAETCIYIPCFTEAVGCKCKDKVCYKN	90
Hyfl_I	GIPCGESCVFIPCISGVIGCSCKSKVCYRN	91
Hyfl_J	GIACGESCAYFGCWIPGCSCRNKVCYFN	92
Hyfl_K	GTPCGESCVYIPCFTAVVGCTCKDKVCYLN	93
Hyfl_L	GTPCAESCVYLPCFTGVIGCTCKDKVCYLN	94
Hyfl_M	GNIPCGESCIFFPCFNPGCSCKDNLCYYN	95
Hyfl_N_(partial)	CGETCVILPCISAALGCSCKDTVCYKN	96
Hyfl_O_(partial)	CGETCVIFPCISAAFGCSCKDTVCYKN	97
Hyfl_P	GSVPCGESCVWIPCISGIAGCSCKNKVCYLN	98
Hymo_A_(partial)	CGETCLFIPCIFSVVGCSCSSKVCYRN	99
Hymo_B_(partial)	CGETCVTGTCYTPGCACDWPVCKRD	100
Hyst_A_(partial)	CGETCIWGRCYSENIGCHCGFGICTLN	101
Hyve_A_(partial)	CGETCLFIPCLTSVFGCSCKNRGCYKI	102
Hyca_A_(partial)	CGETCVVDTRCYTKKCSCAWPVCMRN	103

Cylcotide	Protein Sequence	SEQ ID NO.
Hyde_A_(partial)	CVWIPCISAAIGCSCKSKVCYRN	104
Hyen_A_(partial)	CGESCVYIPCTVTALLGCSCKDKVCYKN	105
Hyen_B_(partial)	CGETCKVTKRCSGQGCSCLKGRSCYD	106
Hyep_A_(partial)	CGETCVVLPCFIVPGCSCKSSVCYFN	107
Hyep_B_(partial)	CGETCIYIPCFTEAVGCKCKDKVCYKN	108
tricyclon_B	GGTIFDCGESCFLGTCYTKGCSCGEWKLCYGEN	109
kalata_B8	GSVLNCGETCLLGTCYTTGCTCNKYRVCTKD	110
cycloviolacin_H4	GIPCAESCVWIPCTVTALLGCSCSNNVCYN	111
cycloviolacin_O13	GIPCGESCVWIPCISAAIGCSCKSKVCYRN	112
violacin_A	SAISCGETCFKFKCYTPRCSCSYPVCK	113
cycloviolacin_O14	GSIPACGESCFKGKCYTPGCSCSKYPLCAKN	114
cycloviolacin_O15	GLVPCGETCFTGKCYTPGCSCSYPICKKN	115
cycloviolacin_O16	GLPCGETCFTGKCYTPGCSCSYPICKKIN	116
cycloviolacin_O17	GIPCGESCVWIPCISAAIGCSCKNKVCYRN	117
cycloviolacin_O18	GIPCGESCVYIPCTVTALAGCKCKSKVCYN	118
cycloviolacin_O19	GTLPCGESCVWIPCISSVVGCSCKSKVCYKD	119
cycloviolacin_O20	GIPCGESCVWIPCLTSAIGCSCKSKVCYRD	120
cycloviolacin_O21	GLPVCGETCVTGSCYTPGCTCSWPVCTRN	121
cycloviolacin_O22	GLPICGETCVGGTCNTPGCTCSWPVCTRN	122
cycloviolacin_O23	GLPTCGETCFGGTCNTPGCTCDSSWPICTHN	123
cycloviolacin_O24	GLPTCGETCFGGTCNTPGCTCDPWPVCTHN	124
cycloviolacin_O25	DIFCGETCAFIPCITHVPGTCSCKSKVCYFN	125
[P20D,V21K]-kalata_B1	GLPVCGETCVGGTCNTPGCTCSWDKCTRN	126
[W19K,_P20N,_V21K]-kalata_B1	GLPVCGETCVGGTCNTPGCTCSKNKCTRN	127
[Glu(Me)]cyO2	GIPCGXSCVWIPCISSAIGCSCKSKVCYRN	128
[Lys(Ac)]2cyO2	GIPCGESCVWIPCISSAIGCSCXSXVCYRN	129
[Arg(CHD)]cyO2	GIPCGESCVWIPCISSAIGCSCKSKVCYXN	130
([Lys(Ac)]2[Arg(CHD)])cyO2	GIPCGESCVWIPCISSAIGCSCXSXVCYXN	131
kalata_B1_oia	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	132
kalata_B1_nfk	GLPVCGETCVGGTCNTPGCTCSWPVCTRN	133
kalata_B2_nfk	GLPVCGETCFGGTCNTPGCSCTWPICTRD	134
kalata_B2_kyn	GLPVCGETCFGGTCNTPGCSCTWPICTRD	135
kalata_B9	GSVFNCGETCVLGTCYTPGCTCNTYRVCTKD	136

Cylcotide	Protein Sequence	SEQ ID NO.
kalata_B10	GLPTCGETCFGGTCNTPGCSCSSWPICTRD	137
kalata_B10_oia	GLPTCGETCFGGTCNTPGCSCSSWPICTRD	138
kalata_B11	GLPVCGETCFGGTCNTPGCSCTDPICTRD	139
kalata_B12	GSLCGDTCFVLGCNDSSCSCNYPICVKD	140
kalata_B13	GLPVCGETCFGGTCNTPGCACDPWPVCTRD	141
kalata_B14	GLPVCGESCFGGTCNTPGCACDPWPVCTRD	142
kalata_B15	GLPVCGESCFGGSCYTPGCSCTWPICTRD	143
kalata_B16	GIPCAESCVYIPCTITALLGCKCQDKVCYD	144
kalata_B17	GIPCAESCVYIPCTITALLGCKCKDQVCYN	145
Amrad_5	CGETCVGGTCNTPGCTCSWPVCRRKRRR	146
Amrad_9	CGETCRRKRRRCNTPGCTCSWPVCTRNGLPV	147
Amrad_11	CGETCVGGTCNTRRKRRRGCTCSWPVCTRNGLPV	148
Amrad_17	CGETCVGGTCNTPGCTCRRKRRRVCTRNGLPV	149
Amrad_7	CGETCVGGTCNTPGCTCRRKRRRCTRNGLPV	150
Amrad_8	CGETCVGGTCRRKRRRCTCSWPVCTRNGLPV	151
kalata_B18	GVPCAESCVYIPCISTVLGCSCSNQVCYRN	152
PS-1	GFIPCGETCIWDKTCHAAGCSCSVANICVRN	153
CD-1	GADGFCGESCYVIPCISYLVGCSCDTIEKVCKRN	154
cycloviolacin_Y1	GGTIFDCGETCFLGTCYTPGCSCGNYGFCYGTN	155
cycloviolacin_Y2	GGTIFDCGESCFLGTCYTAGCSCGNWGLCYGTN	156
cycloviolacin_Y3	GGTIFDCGETCFLGTCYTAGCSCGNWGLCYGTN	157
cycloviolacin_Y4	GVPCGESCVFIPCITGVIGCSCSSNVCYLN	158
cycloviolacin_Y5	GIPCAESCVWIPCTVTALVGCSCSDKVCYN	159
vibi_A	GLPVCGETCFGGTCNTPGCSCSYPICTRN	160
vibi_B	GLPVCGETCFGGTCNTPGCTCSYPICTRN	161
vibi_C	GLPVCGETCAFGSCYTPGCSCSWPVCTRN	162
vibi_D	GLPVCGETCFGGRCNTPGCTCSYPICTRN	163
vibi_E	GIPCAESCVWIPCTVTALIGCGCSNKVCYN	164
vibi_F	GTIPCGESCVFIPCLTSALGCSCKSKVCYKN	165
vibi_G	GTFPCGESCVFIPCLTSAIGCSCKSKVCYKN	166
vibi_H	GLLPCAESCVYIPCLTTVIGCSCKSKVCYKN	167
vibi_I	GIPCGESCVWIPCLTSTVGCSCKSKVCYRN	168
vibi_J	GTFPCGESCVWIPCISKVIGCACKSKVCYKN	169
vibi_K	GIPCGESCVWIPCLTSAVGCPCKSKVCYRN	170

Cylcotide	Protein Sequence	SEQ ID NO.
Viba_2	GIPCGESCVYLPCFTAPLGCSCSSKVCYRN	171
Viba_5	GIPCGESCVWIPCLTATIGCSCKSKVCYRN	172
Viba_10	GIPCAESCVYLPCVTIVIGCSCKDKVCYN	173
Viba_12	GIPCAESCVWIPCTVTALLGCSCKDKVCYN	174
Viba_14	GRLCGERCVIERTRAWCRTVGCICSLHTLECVRN	175
Viba_17	GLPVCGETCVGGTCNTPGCGCSWPVCTRN	176
Viba_15	GLPVCGETCVGGTCNTPGCACSWPVCTRN	177
mram_1	GSIPCGESCVYIPCISSLLGCSCKSKVCYKN	178
mram_2	GIPCAESCVYIPCLTSAIGCSCKSKVCYRN	179
mram_3	GIPCGESCVYLPCFTTIIGCKCQGKVCYH	180
mram_4	GSIPCGESCVFIPCISSVVGCSCKNKVCYKN	181
mram_5	GTIPCGESCVFIPCLTSAIGCSCKSKVCYKN	182
mram_6	GSIPCGESCVYIPCISSLLGCSCESKVCYKN	183
mram_7	GSIPCGESCVFIPCISSIVGCSCKSKVCYKN	184
mram_8	GIPCGESCVFIPCLTSAIGCSCKSKVCYRN	185
mram_9	GVPCGESCVWIPCLTSIVGCSCKNNVCTLN	186
mram_10	GVIPCGESCVFIPCISSVLGCSCKNKVCYRN	187
mram_11	GHPTCGETCLLGTCYTPGCTCKRPVCYKN	188
mram_12	GSAILCGESCTLGECYTPGCTCSWPICTKN	189
mram_13	GHPICGETCVGNKCYTPGCTCTWPVCYRN	190
mram_14	GSIPCGEGCVFIPCISSIVGCSCKSKVCYKN	191
Viba_1	GIPCGEGCVYLPCFTAPLGCSCSSKVCYRN	192
Viba_3	GIPCGESCVWIPCLTAAIGCSCSSKVCYRN	193
Viba_4	GVPCGESCVWIPCLTSAIGCSCKSSVCYRN	194
Viba_6	GIPCGESCVLIPCISSVIGCSCKSKVCYRN	195
Viba_7	GVIPCGESCVFIPCISSVIGCSCKSKVCYRN	196
Viba_8	GAGCIETCYTFPCISEMINCSCKNSRCQKN	197
Viba_9	GIPCGESCVWIPCISSAIGCSCKNKVCYRK	198
Viba_11	GIPCGESCVWIPCISGAIGCSCKSKVCYRN	199
Viba_13	TIPCAESCVWIPCTVTALLGCSCKDKVCYN	200
Viba_16	GLPICGETCTLGTCYTVGCTCSWPICTRN	201
[G1A]kalata_B1	ALPVCGETCVGGTCNTPGCTCSWPVCTRN	202
[L2A]kalata_B1	GAPVCGETCVGGTCNTPGCTCSWPVCTRN	203
[P3A]kalata_B1	GLAVCGETCVGGTCNTPGCTCSWPVCTRN	204

Cylcotide	Protein Sequence	SEQ ID NO.
[V4A]kalata_B1	GLPACGETCVGGTCNTPGCTCSWPVCTRN	205
[G6A]kalata_B1	GLPVCAETCVGGTCNTPGCTCSWPVCTRN	206
[E7A]kalata_B1	GLPVCGATCVGGTCNTPGCTCSWPVCTRN	207
[T8A]kalata_B1	GLPVCGEACVGGTCNTPGCTCSWPVCTRN	208
[V10A]kalata_B1	GLPVCGETCAGGTCNTPGCTCSWPVCTRN	209
[G11A]kalata_B1	GLPVCGETCVAGTCNTPGCTCSWPVCTRN	210
[G12A]kalata_B1	GLPVCGETCVGATCNTPGCTCSWPVCTRN	211
[T13A]kalata_B1	GLPVCGETCVGGACNTPGCTCSWPVCTRN	212
[N15A]kalata_B1	GLPVCGETCVGGTCATPGCTCSWPVCTRN	213
[T16A]kalata_B1	GLPVCGETCVGGTCNAPGCTCSWPVCTRN	214
[P17A]kalata_B1	GLPVCGETCVGGTCNTAGCTCSWPVCTRN	215
[G18A]kalata_B1	GLPVCGETCVGGTCNTPACTCSWPVCTRN	216
[T20A]kalata_B1	GLPVCGETCVGGTCNTPGCACSWPVCTRN	217
[S22A]kalata_B1	GLPVCGETCVGGTCNTPGCTCAWPVCTRN	218
[W23A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSAPVCTRN	219
[P24A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSWAVCTRN	220
[V25A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSWPACTRN	221
[T27A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSWPVCARN	222
[R28A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSWPVCTAN	223
[N29A]kalata_B1	GLPVCGETCVGGTCNTPGCTCSWPVCTRA	224
Cter_A	GVIPCGESCVFIPCISTVIGCSCKNKVCYRN	225
Cter_B	GVPCAESCVWIPCTVTALLGCSCKDKVCYLN	226
hcf-1_variant	GIPCGESCHIPCVTSAIGCSCRNRSCMRN	227
Vpl-1	GSQSCGESCVLIPCISGVIGCSCSSMICYFN	228
Vpf-1	GIPCGESCVFIPCLTAAIGCSCRSKVCYRN	229
cO31	GLPVCGETCVGGTCNTPGCSCSIPVCTRN	230
cO28	GLPVCGETCVGGTCNTPGCSCSWPVCFRD	231
cO32	GAPVCGETCFGGTCNTPGCTCDPWPVCTND	232
cO33	GLPVCGETCVGGTCNTPYCTCSWPVCTRD	233
cO34	GLPVCGETCVGGTCNTEYCTCSWPVCTRD	234
cO35	GLPVCGETCVGGTCNTPYCFCSWPVCTRD	235
cO29	GIPCGESCVWIPCISGAIGCSCKSKVCYKN	236
cO30	GIPCGESCVWIPCISSAIGCSCKNKVCFKN	237
cO26	GSIPACGESCFRGKCYTPGCSCSKYPLCAKD	238

Cylcotide	Protein Sequence	SEQ ID NO.
cO27	GSIPACGESCFKGWCYTPGCSCSKYPLCAKD	239
Globa_F	GSFPCGESCVFIPCISAIAGCSCKNKVCYKN	240
Globa_A	GIPCGESCVFIPCITAAIGCSCKTKVCYRN	241
Globa_B	GVIPCGESCVFIPCISAVLGCSCKSKVCYRN	242
Globa_D	GIPCGETCVFMPCISGPMGCSCKHMVCYRN	243
Globa_G	GVIPCGESCVFIPCISSVLGCSCKNKVCYRN	244
Globa_E	GSAFGCGETCVKGKCNTPGCVCSWPVCKKN	245
Globa_C	APCGESCVFIPCISAVLGCSCKSKVCYRN	246
Glopa_D	GVPCGESCVWVPCTVTALMGCSCVREVCRKD	247
Glopa_E	GIPCAESCVWIPCTVTKMLGCSCKDKVCYN	248
Glopa_A	GGSIPCIETCVWTGCFLVPGCSCKSDKKCYLN	249
Glopa_B	GGSVPCIETCVWTGCFLVPGCSCKSDKKCYLN	250
Glopa_C	GDIPLCGETCFEGGNCRIPGCTCVWPFCSKN	251
Co36	GLPTCGETCFGGTCNTPGCTCDPFPVCTHD	252
cycloviolacin_T1	GIPVCGETCVGGTCNTPGCSCSWPVCTRN	253
cycloviolacin_T2	GLPICGETCVGGTCNTPGCSCSWPVCTRN	254
psyle_A	GIACGESCVFLGCFIPGCSCKSKVCYFN	255
psyle_B	GIPCGETCVAFGCWIPGCSCKDKLCYYD	256
psyle_C	KLCGETCFKFKCYTPGCSCSYFPCK	257
psyle_D	GIPCGESCVFIPCTVTALLGCSCQNKVCYRD	258
psyle_E	GVIPCGESCVFIPCISSVLGCSCKNKVCYRD	259
psyle_F	GVIPCGESCVFIPCITAAVGCSCKNKVCYRD	260
vaby_A	GLPVCGETCAGGTCNTPGCSCSWPICTRN	261
vaby_B	GLPVCGETCAGGTCNTPGCSCTWPICTRN	262
vaby_C	GLPVCGETCAGGRCNTPGCSCSWPVCTRN	263
vaby_D	GLPVCGETCFGGTCNTPGCTCDPWPVCTRN	264
vaby_E	GLPVCGETCFGGTCNTPGCSCDPWPVCTRN	265
Oak6_cyclotide_2	GLPICGETCFGGTCNTPGCICDPWPVCTRD	266
Oak7_cyclotide	GSHCGETCFFFGCYKPGCSCDELRQCYKN	267
Oak8_cyclotide	GVPCGESCVFIPCLTAVVGCSCSNKVCYLN	268
Oak6_cyclotide_1	GLPVCGETCFGGTCNTPGCACDPWPVCTRN	269
Cter_C	GVPCAESCVWIPCTVTALLGCSCKDKVCYLD	270
Cter_D	GIPCAESCVWIPCTVTALLGCSCKDKVCYLN	271
Cter_E	GIPCAESCVWIPCTVTALLGCSCKDKVCYLD	272

Cylcotide	Protein Sequence	SEQ ID NO.
Cter_F	GIPCGESCVFIPCISSVVGCSCKSKVCYLD	273
Cter_G	GLPCGESCVFIPCITTVVGCSCKNKVCYNN	274
Cter_H	GLPCGESCVFIPCITTVVGCSCKNKVCYND	275
Cter_I	GTVPCGESCVFIPCITGIAGCSCKNKVCYIN	276
Cter_J	GTVPCGESCVFIPCITGIAGCSCKNKVCYID	277
Cter_K	HEPCGESCVFIPCITTVVGCSCKNKVCYN	278
Cter_L	HEPCGESCVFIPCITTVVGCSCKNKVCYD	279
Cter_M	GLPTCGETCTLGTCYVPDCSCSWPICMKN	280
Cter_N	GSAFCGETCVLGTCYTPDCSCTALVCLKN	281
Cter_O	GIPCGESCVFIPCITGIAGCSCKSKVCYRN	282
Cter_P	GIPCGESCVFIPCITAAIGCSCKSKVCYRN	283
Cter_Q	GIPCGESCVFIPCISTVIGCSCKNKVCYRN	284
Cter_R	GIPCGESCVFIPCTVTALLGCSCKDKVCYKN	285
vitri_B	GVPICGESCVGGTCNTPGCSCSWPVCTTN	286
vitri_C	GLPICGETCVGGTCNTPGCFCTWPVCTRN	287
vitri_D	GLPVCGETCFTGSCYTPGCSCNWPVCNRN	288
vitri_E	GLPVCGETCVGGTCNTPGCSCSWPVCFRN	289
vitri_F	GLTPCGESCVWIPCISSVVGCACKSKVCYKD	290
hedyotide_B1	GTRCGETCFVLPCWSAKFGCYCQKGFCYRN	291

[0088] The CXCR4 antagonist may be any peptide known to act as an antagonist to the CXCR4 receptor. One example of a CXCR4 antagonist is CVX15. 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 (see, for example, Tamamura, H. et al., *Biochem Biophys Res Commun* **1998,** 253, (3), 877-82; DeMarco, S. J.; et al., *Bioorg Med Chem* **2006,** 14, (24), 8396-404; and Moncunill, G.; et al., *Mol Pharmacol* **2008,** 73, (4), 1264-73, which are incorporated by reference). Some of these peptides, however, have shown limited proteolytic stability and/or poor bioavailability. By using the crystal structure of CXCR4 bound to the polyphemusin-derived peptide CVX15 engineered cyclotide that effectively antagonize CXCR4 and inhibit CXCR4-tropic HIV-1 entry in human lymphocytes have been designed and are described herein. An example of the CVX15 peptide is a peptide with the sequence, RRBCYXKpPYRXCRGp (SEQ ID NO: 2) where B is the amino acid, 2-naphtylalanine, X is the amino acid citruline, and p is the amino acid D-PRO.

Another example sequence of CVX15 is RRBCYQpPYRXCRGp (SEQ ID NO: 11). CVX15 is also described in Wu et al., *Science* 2010, 330, (6007): 1066-1071, which is herein incorporated by reference. In further embodiments, the CVX15 peptide comprises a peptide with an amino acid sequence of the group: SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, and 10 shown in FIG. 1C. In a specific embodiment, the CVX15 peptide comprises a peptide with the amino acid sequence of SEQ ID NO: 10 from FIG. 1C.

[0089] In one embodiment, the cyclotide incorporates one or more unnatural amino acids. "Unnatural amino acids" are amino acids not in the standard 20-amino acid list but can be incorporated into a protein sequence. Non-limiting examples of unnatural amino acids include p-methyxyphenylalanine, p-azidophenylalanine, L-(7-hydroxycoumarin-4-yl)ethylglycine, acetyl-2-naphthyl alanine, 2-naphthyl alanine, 3-pyridyl alanine, 4-chloro phenyl alanine, alloisoleucine, Z-alloisoleucine dcha salt, allothreonine, , 4-Iodo- phenylalanine, L-benzothienyl-D-alanine OH.

[0090] In some aspects, the cyclotide comprises at least an unnatural amino acid residue but retains six cysteine residues that form three disulfide bonds in a cyclized cyclotide. In one aspect, the unnatural amino acid comprises one or more selected from p-methyxyphenylalanine, p-azidophenylalanine or L-(7-hydroxycoumarin-4-yl)ethylglycine.

[0091] In one embodiment, the unnatural amino acid is located in loop 2 of the cyclotide. In alternative embodiments, the unnatural amino acid is located in loop 1, 3, 4, 5 or 6. In some embodiments, the cyclotide contains two, three, four or more unnatural amino acids.

[0092] In another aspect, this disclosure provides a isolated polynucleotide encoding one or more of the isolated peptides described above, alone or in a replication or expression vector, e.g., a viral vector or a plasmid. The polynucleotide units further contain the necessary regulatory element operatively linked to the coding sequences for expression of the polynucleotide in a host cell. Thus, this disclosure also provides an isolated host cell comprising the recombinant peptide as described above or the recombinant polynucleotide, or vector containing same, also as described above. The isolated host cell is a prokaryotic or a eukaryotic cell. In one particular aspect, the host cell is an E. coli cell. The polynucleotides or peptide can also be chemically synthesized using methods known in the art and described herein.

[0093] Further provided s a method for recobinantly producing the peptides of this disclosure by growing an isolated host cell as described above under conditions that favor the expression fo

the polynucleotide. In one aspect, the peptides are isolated from the host cells. The peptides and polynucleotide can also be chemically synthesized.

[0094] "Host cell" refers not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0095] Examples of such include, prokaryotic cells such as E. coli cells. Examples of eukaryotic cells include, but are not limited to cells from animals, e.g., murines, rats, rabbit, simians, bovines, ovine, porcine, canines, feline, farm animals, sport animals, pets, equine, and primate, particularly human. The cells can be cultured cells or they can be primary cells. Cultured cell lines can be purchased from vendors such as the American Type Culture Collection (ATCC), U.S.A.

[0096] Method aspects of the disclosure relate to a method for inhibiting CXCR4 signaling in a cell or tissue comprising contacting the cell or tissue with an effective amount of one or more of: the polypeptide as described herein, the isolated polynucleotide described herein or the host cell described herein. The inhibition of CXCR4 signaling can be detected by methods known in the art and described herein. The CXCR4 antagonist peptide may function by interrupting the binding of CXCR4 with its biological ligand, CXCL12. An assay to test for inhibition of CXCL12 (also called SDF1α) is described in the examples that follow. The activation of downstream targets of CXCR4 may also be tested to determine the inhibition of CXCR4 signaling. For example, activation of CXCR4 by CXCL12 leads to ERK phosphorylation. Accordingly, a reduction in phospho-ERK indicates an inhibition of CXCR4 signaling. Also provided is a method for inhibiting CXCR4 signaling in a cell or tissue expressing the CXCR4 receptor comprising contacting the cell or tissue with an effective amount of one or more of: the polypeptide, polynucleotide, or host cells as described herein. The inhibition of the binding of the ligand to the receptor can be measured by binding assays known in the art. The contacting of the cell or tissue may be in vitro in tissue culture or in vivo in a subject.

[0097] A further method aspect relates to a method for reducing or inhibiting metastasis, angiogenesis, and/or tumor growth in a subject in need thereof comprising administering an effective amount of one or more of: the the polypeptide as described herein, the isolated polynucleotide described herein or the host cell described herein to the subject. Inhibition or metastasis, angiogenesis, and tumor growth may be demonstrated by assays known in the art. For example, the inhibition may be demonstrated by the reduction of pro-angiogenic or pro-

metastatic markers, the increase in anti-angiogenic or anti-metastatic factors, the reduction in tumor size, or the lack of new tumor growth.

[0098] Also provided is a method for promoting tumor cell death in a mammal in need thereof comprising administering an effective amount of one or more of: the polypeptide as described herein, the isolated polynucleotide described herein or the host cell described herein to the subject. Cell death of a tumor can be measured by a reduction in tumor growth or an increase in markers for cell death, necrosis, or apoptosis. In one embodiment, the subject suffers from a CXCR4 positive cancer or tumor. In a related embodiment, CXCR4 is overexpressed in the tumor cells. In another embodiment, CXCR4 is activated in the tumor cells. The status of CXCR4 may be established by, for example, the analysis of biopsied materials from the tumor itself. The analysis may include immunohistochemical staining for CXCR4 expression, analysis of CXCR4-activated gene expression, and mRNA or protein analysis of CXCR4 mRNA and/or protein levels.

[0099] A further method aspect relates to a method for inhibiting HIV replication or inhibiting viral entry into host cells comprising administering an effective amount of one or more of: the polypeptide as described herein, the isolated polynucleotide described herein or the host cell described herein to the subject. In one embodiment, HIV replication and/or viral entry into host cells is inhibitited *in vivo*. The phrase inhibiting "HIV replication" as used herein may refer to inhibition of entry of the HIV viral particle into the cell, decrease of the rate of infection, inhibition of replication of the virus in the cell, or inhibition of assembly and relase of new viral particles. In one embodiment, the host cell is a lymphocyte. Also provided is a method for treating HIV-related disorders in a subject comprising administering an effective amount of one or more of: the polypeptide, the isolated polynucleotide, or the host cell as described herein.

[0100] The compositions can be administered to an animal or mammal by a treating veterinarian or to a human patient by a treating physician.

[0101] Having described the general concepts of this invention, the following illustrative examples are provided.

Experimental

Design of a novel cyclotide-based CXCR4 antagonist with anti-HIV-1 activity

[0102] Herein, the design and synthesis of a novel cyclotide able to efficiently inhibit HIV-1 viral replication by selectively targeting cytokine receptor CXCR4 is described. 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 anti-cancer and anti-HIV-1 therapeutics.

[0103] 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 crystal structure of CXCR4 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 system.[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]

[0104] 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, 22] (FIG. 1A). This cyclic cystine-knot (CCK) framework provides a rigid molecular platform[23, 24] with exceptional stability towards physical, chemical and biological degradation.[21, 22] These micro-proteins 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.[25-27] Furthermore, naturally-occurring cyclotides have shown to posses various pharmacologically-relevant activities,[21, 28] and have been reported to cross cell membranes.[29, 30] Altogether, these features make the cyclotide scaffold an excellent molecular framework for the design of novel peptide-based therapeutics,[22, 31] making them ideal substrates for molecular grafting of biological peptide epitopes.[32-35]

[0105] 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.[36-38] Some of these peptides, however, have shown limited proteolytic stability and/or poor bioavailability.[37] By using the crystal structure of

CXCR4 bound to the polyphemusin-derived peptide CVX15[39] the design and synthesis of an engineered cyclotide able to effectively antagonize CXCR4 and inhibit CXCR4-tropic HIV-1 entry in human lymphocytes is reported and described herein.

Results and Discussion

[0106] To produce a novel cyclotide with CXCR4 antagonistic activity, the cyclotide MCoTI-I was used as molecular scaffold (FIG. 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).[40] MCoTI-cyclotides show very low toxicity in human cells[29] and represent a desirable molecular scaffold for engineering new compounds with unique biological properties.[32-34]

[0107] According to the X-ray crystal structure of CVX15 bound to CXCR4, the N- and C-termini of the CVX15 peptide are deeply buried into the CXCR4 binding pocket (FIG. 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 (FIGS. 1B and 1C). Residue Gln[6] was also replaced by citruline, which has been shown to increase the affinity of CVX15 for CXCR4.[41] 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] (FIG. 1C). Loop 6 of MCoTI-cyclotides has been shown to be less rigid in solution[23, 24] and quite tolerant to sequence grafting.[32-35]

[0108] All grafted MCo-CVX cyclotides were chemically synthesized using Fmoc-based solid-phase peptide synthesis on a sulfonamide resin.[29] Activation of the sulfonamide linker with iodoacetonitrile, followed by cleavage with ethyl mercaptoacetate and acidolytic deprotection, provided the fully deprotected linear peptide α-thioester. 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 (FIGS. 2A and 4, Table 1).

Table 1. Molecular weight, cyclization/folding yield and biological activity summary for the MCo-CVX grafted cyclotides produced in this work.

Peptide Name	Molecular weight (Da)		Cyclization/folding		EC_{50} (nM)	
	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

[0109] 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 (Fig. 2B and Table 1). Grafted MCo-CVX-5c cyclotide was also characterized by ^{1}H-NMR indicating that adopts a native cyclotide fold (FIGS. 2C and 5). The large $\Delta\delta$ values for the H' protons of residues Arg^{10} and Arg^{11} were attributed to the interaction of these residues with the grafted sequence. The $\Delta\delta$ values for the H^{α} protons of these two residues were smaller than 0.1 ppm (FIG. 2C and Table 2).

Table 2. Tabulation of chemical shifts of backbone amide protons (δ^1H' and δ^1H^{α}) protons of MCo-CVX-5c and their respective differences from cyclotide MCoTI-I.

Residue	δ^1H' (ppm)	δ^1H^{α} (ppm)	$\Delta\delta^1H'$ (ppm)	$\Delta\delta^1H^{\alpha}$ (ppm)
V1	8.176	3.826	0.208	0.049
C2	8.603	4.963	-0.021	0.103
P3	-	-	-	-
K4	8.106	4.495	0.036	-0.364
I5	7.504	4.342	0.122	-0.084
L6	8.467	4.288	0.122	0.123
Q7	8.423	4.255	0.366	0.207
R8	8.564	4.375	-0.038	-0.177
C9	8.285	4.743	0.02	0.01

R10	7.734	4.337	1.537	-0.015
R11	9.36	4.622	-1.401	0.023
D12	9.039	3.961	0.097	0.024
S13	8.072	3.979	-0.014	0.192
D14	7.649	4.495	-0.013	0.011
C15	7.883	4.849	0.126	0.074
P16	-	-	-	-
G17	8.37	3.69	0.022	-0.068
A18	8.075	4.154	0.248	0.231
C19	7.88	4.828	0.298	-0.386
I20	8.655	4.206	0.286	0.104
C21	9.261	4.833	-0.223	-0.01
R22	8.066	4.226	-0.117	-0.037
G23	9.017	3.818	-0.207	-0.016
N24	7.68	4.569	0.019	0.011
G25	8.27	3.869	0.052	-0.01
Y26	7.187	5.149	-0.013	-0.005

[0110] Next, the ability of the CVX15-grafted cyclotides to inhibit SDF1$\tilde{\alpha}$-mediated CXCR4 activation was tested by using a CXCR4-β-lactamase U2OS cell-based fluorescence assay (Life Technologies, FIG 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 alone showed an EC_{50} value of 71 ± 13 nM, which is around 3 times stronger 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 (FIG. 3A), indicating that the biological activity of grafted MCo-CVX cyclotides is specific and comes from the grafted sequence. The small molecule CXCR4 antagonist AMD3100 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} value (FIG. 3A).

The length of Gly linker used to build the CVX15-based insert, that 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 μM and 3 μM, respectively (FIG. 3A and Table 1). These results are likely due to the increase in flexibility provided by the extra Gly residues, which reduces the binding energy. Interestingly, 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 nM and 39 ± 1 nM, respectively (FIG. 3A and Table 1).

[0111] 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 (FIGS. 3B and 6). 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} value of 2.0 ± 0.3 nM (FIG. 3C). The EC_{50} value for peptide CVX15 Gln6Cit was around 8-times higher (FIG. 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) value in MT4 cells greater than 10 μM (data not shown), therefore providing a selectivity index of more than 4,000. It is also worth noting that cyclotide MCo-CVX-5c was 3-times more potent than Raltegravir, an integrase inhibitor recently approved by the FDA to treat HIV infection (FIG. 3C).

[0112] The biological stability of MCo-CVX-5c was also studied and compared to that of the empty scaffold (MCoTI-I) and the grafted peptide (CVX15 Gln6Cit) (FIG. 7). 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,[23, 24] 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, Fig. S4). In contrast, peptide CVX15 Gln6Cit was degraded considerably faster under the same conditions ($\tau_{1/2}$ = 21 ± 4 h, Fig. 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. The fraction of cyclotide bound to serum proteins was also investigated. Serum binding has been recently used to extend serum half-life of bioactive peptides.[42] 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 these cyclotides are almost completely degraded after 120 h of treatment (FIG. 7) suggests that their binding to serum proteins may be reversible. To further explore this possibility, the association and dissociation rate constants of MCo-CVX-5c to human serum proteins was studied. 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 an association and dissociation constant rates of 3.6 ± 0.7 x 10^3 $M^{-1}s^{-1}$ and 1.4 ± 0.2 x 10^{-2} s^{-1}, respectively (FIG. 8), 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 human serum, 2% and 12% for the CXCR4 translocation and HIV-1 cell entry inhibition assays, respectively.

Conclusions

[0113] In summary it is reported 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.[28, 34] 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 a model of MCo-CVX-5c bound to CXCR4 was built using the crystal structure of CVX15-CXCR4 (PDB: 3OE0)[39] and the solution structure of MCoTI-II (PDB: 1IB9)[43] (FIG. 9). According to this model, loops 2 and 5 may be in close 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 $\tau_{1/2}$ value of 62 ± 3 h. This value is similar to that of the cyclotide MCoTI-I and significantly higher that 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 renal clearance without affecting their activity. Although further analysis will be required to evaluate the therapeutic value of these compounds *in vivo*, altogether, these results show that engineered cyclotides are useful as a therapeutic for the development of a novel type of peptide-based therapeutic able to efficiently target extracellular protein/protein interactions. These results demonstrate for the first time the design of an engineered cyclotide able to target the GPCR CXR4 with low nanomolar affinity and significant serum stability, thereby providing a promising lead compound for the design of anti-cancer and anti-HIV-1 compounds.

Materials and instrumentation

[0114] Analytical HPLC was performed on a HP1100 series instrument with 220 nm and 280 nm detection using a Vydac C18 column (5 mm, 4.6 x 150 mm) at a flow rate of 1 mL/min. Semipreparative HPLC was performed on a Waters Delta Prep system fitted with a Waters 2487 Ultraviolet-Visible (UV-vis) detector using a Vydac C18 column (15-20 μm, 10 x 250 mm) at a flow rate of 5 mL/min. All runs used linear gradients of 0.1% aqueous trifluoroacetic acid (TFA, solvent A) vs. 0.1% TFA, 90% acetonitrile in H_2O (solvent B). UV-vis spectroscopy was carried out on an Agilent 8453 diode array spectrophotometer, and fluorescence analysis on a Jobin Yvon Flurolog-3 spectroflurometer. Electrospray mass spectrometry (ES-MS) analysis was routinely applied to all cyclized peptides. ES-MS was performed on a Sciex API-150EX single quadrupole electrospray mass spectrometer, MS/MS was performed on an Applied Biosystems API 3000 triple quadrupole mass spectrometer. Calculated masses were obtained by using ProMac v1.5.3. Protein samples were analyzed by SDS-PAGE. Samples were run on Invitrogen (Carlsbad, CA) 4-20% Tris-Glycine Gels. The gels were then stained with Pierce (Rockford, IL) Gelcode Blue, photographed/digitized using a Kodak (Rochester, NY) EDAS

290, and quantified using NIH Image-J software (http://rsb.info.nih.gov/ij All chemicals were obtained from Sigma-Aldrich (Milwaukee, WI) unless otherwise indicated.

[0115] Preparation of Fmoc-Tyr(tBu)-F. Fmoc-Tyr(tBu)-F was prepared using diethylaminosulfur trifluoride DAST as previously described (see, for example, Contreras, et al., *J Control Release*, **2011**, *155*, 134-143) and quickly used afterwards. Briefly, DAST (160 μL, 1.2 mmol) was added drop wise at 25° C under nitrogen current to a stirred solution of Fmoc-Tyr(tBu)-OH (459.6 mg, 1 mmol) in 10 mL of dry dichloromethane (DCM), containing dry pyridine (81 μL, 1 mmol). After 20 minutes, the mixture was washed with ice-cold water (3 x 20 mL). The organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure to give the corresponding Fmoc-amino acyl fluoride as white solid that was immediately used.

[0116] Loading of 4-sulfamylbutyryl AM resin with Fmoc-Tyr(tBu)-F. Loading of the first residue was accomplished using Fmoc-Tyr(tBu)-F as previously described (see, for example, Contreras, et al., *J Control Release*, **2011**, *155*, 134-143). Briefly, 4-sulfamylbutyryl AM resin (420 mg, 0.33 mmol) (Novabiochem) was swollen for 20 minutes with dry DCM and then drained. A solution of Fmoc-Tyr(tBu)-F (≈461 mg, 1 mmol) in dry DCM (2 mL) and di-isopropylethylamine (DIEA) (180 μL, 1 mmol) was added to the drained resin and reacted at 25° C for 1 h. The resin was washed with dry DCM (5 x 5 mL), dried and kept at –20°C until use.

[0117] Chemical synthesis of MCo-CVX cyclotides. Solid-phase synthesis of all grafted peptides was carried out on an automatic peptide synthesizer ABI433A (Applied Biosystems) using the Fast-Fmoc chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine (DIEA) activation protocol at 0.1 mmole scale on a Fmoc-Tyr(tBu)-sulfamylbutyryl AM resin. Side-chain protection compatible with Fmoc-chemistry was employed as previously described for the synthesis of peptide α-thiesters by the Fmoc-protocol (see, for example, Camarero, J.A. et al., *Protein Pept Lett*, **2005**, *12*, 723-728), except for the N-terminal Cys residue, which was introduced as Boc-Cys(Trt)-OH. Following chain assembly, the alkylation, thiolytic cleavage and side chain deprotection were performed as previously described (see, for example, Contreras, et al., *J Control Release*, **2011**, *155*, 134-143). Briefly, ≈100 mg of protected peptide resin were first alkylated two times with ICH_2CN (174 μL, 2.4 mmol; previously filtered through basic alumina) and DIEA (82 μL, 0.46 mmol) in N-methylpyrrolidone (NMP) (2.2 mL) for 24 h. The resin was then washed with NMP (3 x 5 mL) and DCM (3 x 5 mL). The alkylated peptide resin was cleaved from the resin with $HSCH_2CO_2Et$ (200 μL, 1.8 mmol) in the presence of a catalytic amount of sodium thiophenolate

(NaSPh, 3 mg, 22 μmol) in dimethylformamide (DMF):DCM (1:2 v/v, 1.2 mL) for 24 h. The resin was then dried at reduced pressure. The side-chain protecting groups were removed by treating the dried resin with trifluoroacetic acid (TFA):H_2O:tri-isopropylsilane (TIS) (95:3:2 v/v, 10 mL) for 3-4 h at room temperature. The resin was filtered and the linear peptide thioester was precipitated in cold Et_2O. The crude material was dissolved in the minimal amount of H_2O:MeCN (4:1) containing 0.1% TFA and characterized by HPLC and ES-MS as the desired grafted MCoTI-I linear precursor α-thioester (FIG. 4). Cyclization and folding was accomplished by flash dilution of the linear α-thioester TFA crude to a final concentration of ≈50 μM into freshly degassed 1 mM reduced glutathione (GSH), 0.1 M sodium phosphate buffer at pH 7.2 for 24 h (FIG. 4). Folded peptides were purified by semi-preparative HPLC using a linear gradient of 15-35% solvent B over 30 min. Purified peptides were characterized by HPLC and ES-MS (Fig. S1).

[0118] Cell-based CXCR4 competitive binding assays. Briefly, Tango™ CXCR4-bla U2OS cells (Life Technologies) were seeded at 10,000/well in 384-well tissue culture plate for 24 h in DMEM supplemented with 1% FBS. Cells were pre-treated with various concentrations of inhibitors for 30 minutes prior to the addition of 30 nM of SDF-1α and incubated for 5 h at 37°C. β-Lactamase substrate LiveBLAzer™-FRET B/G Substrate (Invitrogen) was incubated with treated cells for 2 h and fluorescence signal was measured by a Envision plate reader (Perkin Elmer) at 508/460 nm (substrate cleaved) and 508/540nm (substrate uncleaved).

[0119] SDF1α-mediated Erk phosphorylation assay. Western blotting was used to detect inhibition of SDF-1α induced Erk phosphoryaltion in CXCR4-expressing CaOV3 cells. Briefly, CaOV3 cells were seeded at 400,000 cells/well in 6-well tissue culture plate for 24 h in DMEM supplemented with 10% FBS. Cells were serum starved overnight and pre-treated with CXCR4 antagonists for 10 minutes prior to stimulation with 30 nM SDF1α for 5 minutes. Cells were lysed with RIPA buffer and analyzed by Western blot.

[0120] SDF1α-mediated CXCR4 internalization assay. Briefly, Tango™ CXCR4-bla U2OS cells (Life Technologies) were seeded at 10,000/well in 384-well tissue culture plate for 24 h in DMEM supplemented with 1% FBS. Cells were pre-treated with various concentrations of inhibitors for 30 min prior to the addition of 200 nM of SDF1α and incubated for 6 h at 37°C. Cells were fixed with 4% formaldehyde for 30 min at room temperature, washed with PBS and stored at 4° C. Wells were blocked with 20 μL blocking buffer (Li-Cor) for 2 h at room temperature and then incubated with CXCR4 antibody (Santa Cruz) for 2 h at 1:250 dilution. Cells were washed 6 times with PBS and incubated with mouse 700-IRDye goat anti-*mouse*

IgG1 (1:500 dilution, Li-Cor) for 2 h at room temperature. Plates were imaged and quantified on the Odyssey Imaging System (Li-Cor).

[0121] HIV-1 replication and MT-4 cytotoxicity assays. MT-4 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The cells were grown in RPMI 1640 supplemented with 10% FCS and 20 μg/ml gentamicin (RPMI-complete). The origins of the HIV-1 strain III_B have been previously described (Adachi, A. et al., *J Virol*, **1986**, *59*, 284-291). The inhibitory effect of antiviral drugs on the HIV-induced CPE in MT-4 cell culture was determined by the MTT-assay. This assay is based on the reduction of the yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotometrically. The 50% cell culture infective dose of the HIV strains was determined by titration of the virus stock using MT-4 cells. For the drug susceptibility assays, MT-4 cells were infected with 100 to 300 50% cell culture infective doses (CCID50) of the HIV strains in the presence of five-fold serial dilutions of the antiviral drugs. The concentration of the compound achieving 50% protection against the CPE of HIV, which is defined as the 50% effective concentration (IC_{50}), was determined. The concentration of the compound killing 50% of the MT-4 cells, which is defined as the 50% cytotoxic concentration (CC_{50}), was determined as well.

[0122] **NMR spectroscopy.** NMR samples were prepared by dissolving cyclotides into 80 mM potassium phosphate pH 6.0 in 90% H_2O/10% ^2H_2O (v/v) to a concentration of approximately 0.5 mM. All ^1H NMR data were recorded on either Bruker Avance III 500 MHz or Bruker Avance II 700 MHz spectrometers equipped with TCI cryoprobes. Data were acquired at 298 K, and 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, was used as an internal reference. The carrier frequency was centered on the water signal, and the solvent was suppressed by using WATERGATE pulse sequence. ^1H, ^1H-TOCSY (spin lock time 80 ms) and ^1H, ^1H-NOESY (mixing time 150 ms) spectra were collected using 4096 t_2 points and 256 t_1 of 64 transients. Spectra were processed using Topspin 2.1 (Bruker). Each 2D-data set was apodized by 90^0-shifted sinebell-squared in all dimensions, and zero filled to 4096 x 512 points prior to Fourier transformation. Assignments for H^{α} ($H\text{-}C^{\alpha}$) and H' ($H\text{-}N^{\alpha}$) protons of folded MCo-CVX-5c (Table S1) were obtained using standard procedures (Cavanagh, J. et al., *J. Magn. Res.*, **1992**, *96*, 670-678 and Wuthrich, K., *NMR of Proteins and Nucleic Acids.* 1986).

[0123] Serum stability. Peptides (≈150 μg dissolved in 50 μl PBS) were mixed with 500 μl human serum and incubated in a 37° C water bath. Aliquot samples (10 μL) were taken at

different time points (0 - 120 h) and precipitated with 20% trichloroacetic acid. After centrifugation the pellet was dissolved in 100 µL of 6 M guanidinium chloride. Both the supernatant and solubilized pellet fractions were analyzed by HPLC-MS/MS.

[0124] Binding kinetics of cyclotide MCo-CVX-5c to human serum proteins. MCo-CVX-5c was biotinylated using EZ-Link NHS-PEG4-Biotin (Thermo Scientific). Briefly, MCoTI-CVX-5c (1 mg, ≈200 nmol) was conjugated with three-fold molar excess of NHS-PEG4-Biotin in 0.1 M sodium phosphate buffer (1.9 mL) at pH 7.4 for 1 h. The reaction was quenched with 2% TFA at pH 4. The purification and desalting was carried out by using Zeba spin desalting columns (Thermo Scientific). Binding kinetics were carried out at 25° C on a BLItz™ instrument, using biotinylated MCoTI-CVX-5c immobilized onto a streptavidin-coated biosensor tip. Briefly, 4 µL of biotinylaed MCoTI-CVX-5c (35µM) in 0.1 M sodium phosphate buffer at pH 7.4 was first immobilized onto a streptavidin-coated biosensor. The biosensor was washed with PBS (20 mM sodium phosphate, 100 mM NaCl buffer at pH 7.4) and probed with different human serum dilutions for 2 minutes (binding) and with PBS for another 2 minutes (desorption). Nonlinear regression analysis was performed using GraphPad Prism (GraphPad) to provide the k_{on} and k_{off} rates, and K_D value (FIG. 8).

[0125] Model of the CXCR4-MCo-CVX-5c complex. The model of the CXCR4-MCo-CVX-5c complex was built on Yasara (www.yasara.org) using the crystal structure of CVX-15-CXCR4 (PDB: 3OE0) (Wu, B.;et al., *Science*, **2010**, *330*, 1066-1071) and the solution structure of MCoTI-II (PDB: 1IB9) (Felizmenio-Quimio, M.E. et al., *J Biol Chem*, **2001**, *276*, 22875-22882). The final model was minimized using an AMBER99 molecular force field.

[0126] It should be understood that although the present disclosure has been specifically disclosed by preferred embodiments and optional features, modification, improvement and variation of the disclosure embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications, improvements and variations are considered to be within the scope of this disclosure. The materials, methods, and examples provided here are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the disclosure.

[0127] The disclosure has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the disclosure with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0128] In addition, where features or aspects of the disclosure are described in terms of Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0129] All publications, patent applications, patents, and other references mentioned herein are expressly incorporated by reference in their entirety, to the same extent as if each were incorporated by reference individually. In case of conflict, the present specification, including definitions, will control. Several publications are identified by an Arabic numeral. The completed bibliographic citation of the referenced publications follow, immediately preceding the claims. The references also are expressly incorporated by reference in their entirety.

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CLAIMS:

1. An isolated peptide comprising a CXCR4 antagonist peptide grafted to a cyclotide.

2. The isolated peptide of claim 1, wherein the CXCR4 antagonist peptide is grafted into loop 6 of the cyclotide.

3. The isolated peptide of claim 1 or 2, wherein the cyclotide is selected from the group consisting of MCoTI-I, MCoTI-II, and kalata B1 or a biological equivalent thereof.

4. The isolated peptide of claim 3, wherein the cyclotide is MCoTI-I or a biological equivalent thereof.

5. The isolated peptide of claim 5, wherein the cyclotide comprises a peptide with the amino acid sequence of SEQ ID NO: 1 or a biological equivalent thereof.

6. The isolated peptide of claim 5, wherein the CXCR4 antagonist peptide is grafted between the Ser^{29} and Val^{1} amino acids of SEQ ID NO: 1.

7. The isolated peptide of claim 4, wherein the CXCR4 antagonist peptide is grafted between Ser^{31} and Gly^{33} of SEQ ID NO: 1.

8. The isolated peptide of any one of claims 1 to 7, wherein the CXCR4 antagonist peptide comprises the CVX15 peptide or a biological equivalent thereof.

9. The isolated peptide of claim 8, wherein the CVX15 peptide comprises a peptide with the amino acid sequence of SEQ ID NO: 2 or a biological equivalent thereof.

10. The isolated peptide of 8, wherein the CVX15 peptide comprises a peptide with an amino acid sequence of the group: SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, and 10.

11. The isolated peptide of claim 10, wherein the CVX15 peptide comprises a peptide with the amino acid sequence of SEQ ID NO: 10.

12. An isolated polynucleotide encoding the peptide of any of claims 1 to 11 or a biological equivalent thereof or an isolated polynucleotide that hybridizes under conditions of high stringency to the isolated polynucleotide or its complement.

13. A vector or an isolated host cell comprising the isolated peptide of any of claims 1 to 10 or the isolated polynucleotide of claim 12.

14. The isolated host cell of claim 13, wherein the host cell is a prokaryotic or a eukaryotic cell.

15. The isolated host cell of claim 13 or 14, wherein the host cell is an E. coli cell.

16. A composition comprising a carrier and one or more of the group of the isolated polypeptide of any of claims 1 to 10, the isolated polynucleotide of claim 12, or the vector or host cell of any one of claims 13 to 15.

17. The composition of claim 15, wherein the carrier is a pharmaceutically acceptable carrier.

18. A method for inhibiting CXCR4 signaling in a cell or tissue expressing the CXCR4 receptor comprising contacting the cell or tissue with an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15.

19. A method for inhibiting CXCL12 binding to CXCR4 in a cell or tissue expressing the CXCR4 receptor comprising contacting the cell or tissue with an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15.

20. A method for inhibiting HIV replication or inhibiting viral entry into host cells comprising administering an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15.

21. The method of any one of claims 18 to 20, wherein the contacting is in vitro or in vivo.

22. A method for reducing or inhibiting metastasis, angiogenesis, and/or tumor growth in a subject in need thereof comprising administering an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15 to the subject.

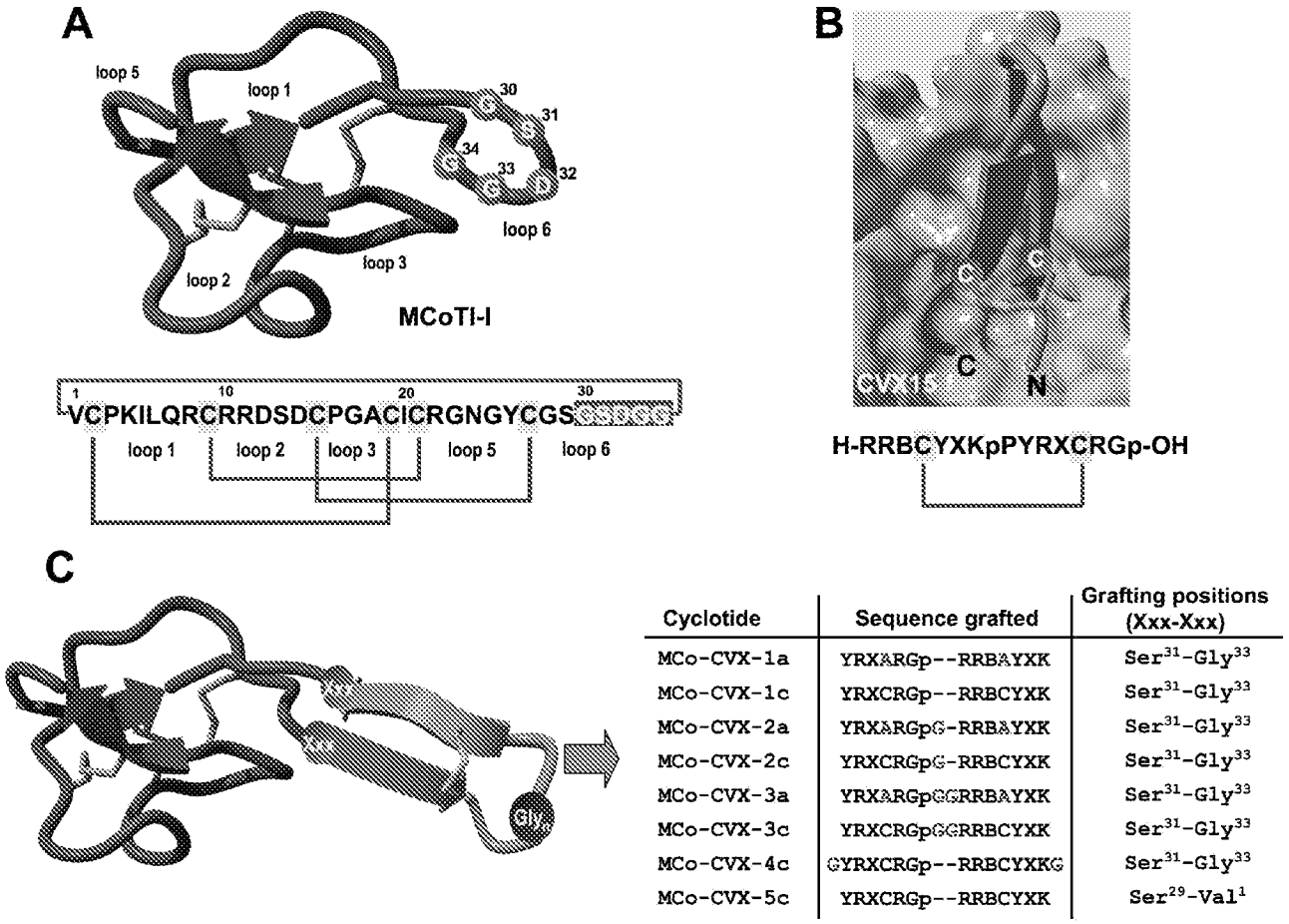
23. A method for promoting tumor cell death in a subject in need thereof comprising administering an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15 to the subject.

24. The method of claim 22 or 23, wherein the subject suffers from a CXCR4 positive cancer.

25. The method of claim 22 or 23, wherein CXCR4 is overexpressed in the tumor cells.

26. The method of claim 22 or 23, wherein CXCR4 is activated in the tumor cells.

27. A method for treating HIV-related disorders in a subject comprising administering an effective amount of one or more of: the polypeptide of any of claims 1 to 11, the isolated polynucleotide of claim 12, or the host cell of any one of claims 13 to 15.



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}

FIG 1

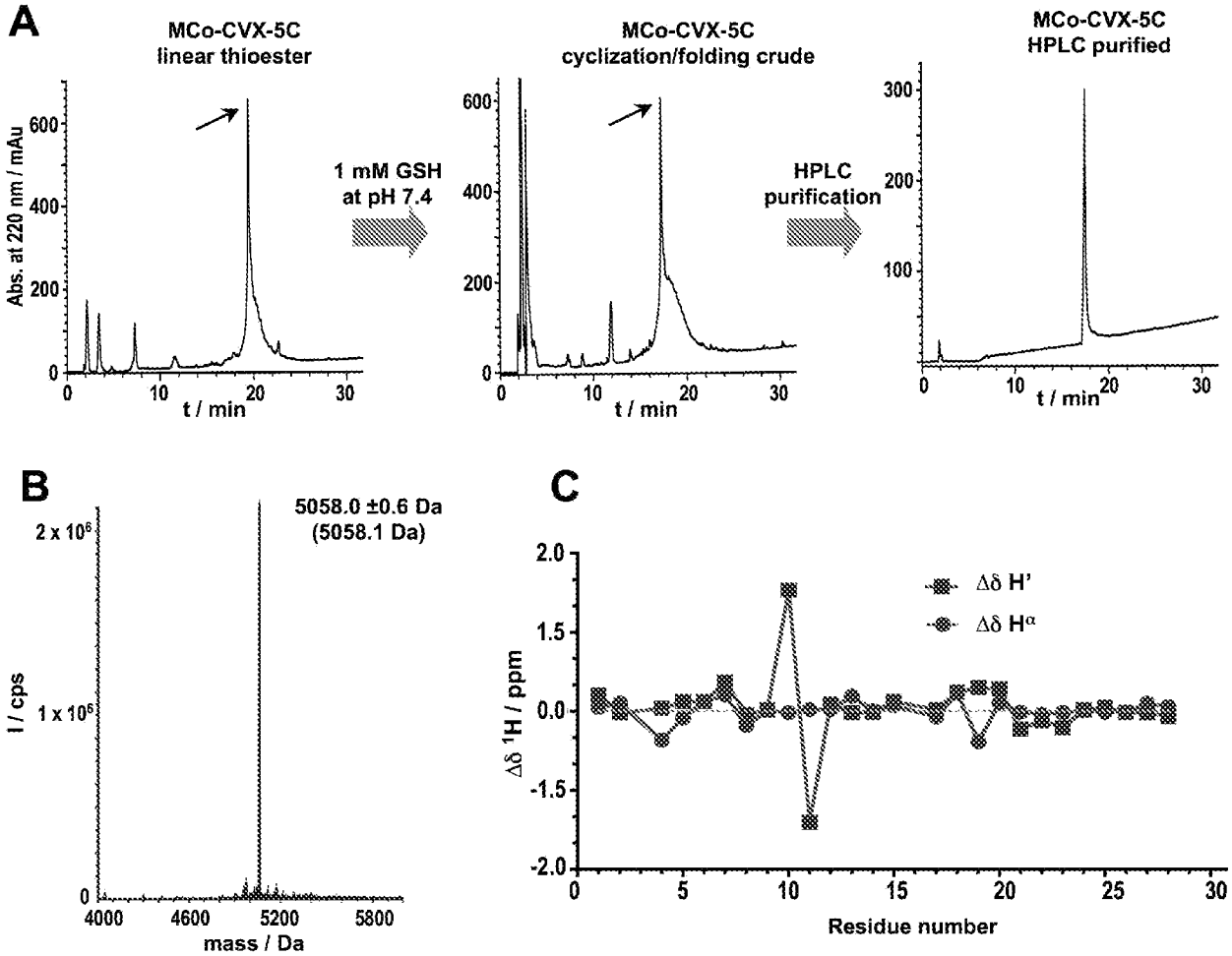

A
MCo-CVX-5C
linear thioester
Abs. at 220 nm / mAu
t / min
1 mM GSH
at pH 7.4
MCo-CVX-5C
cyclization/folding crude
t / min
HPLC
purification
MCo-CVX-5C
HPLC purified
t / min
B
5058.0 ±0.6 Da
(5058.1 Da)
I / cps
mass / Da
C
Δδ H'
Δδ Hα
Δδ ¹H / ppm
Residue number

FIG 2

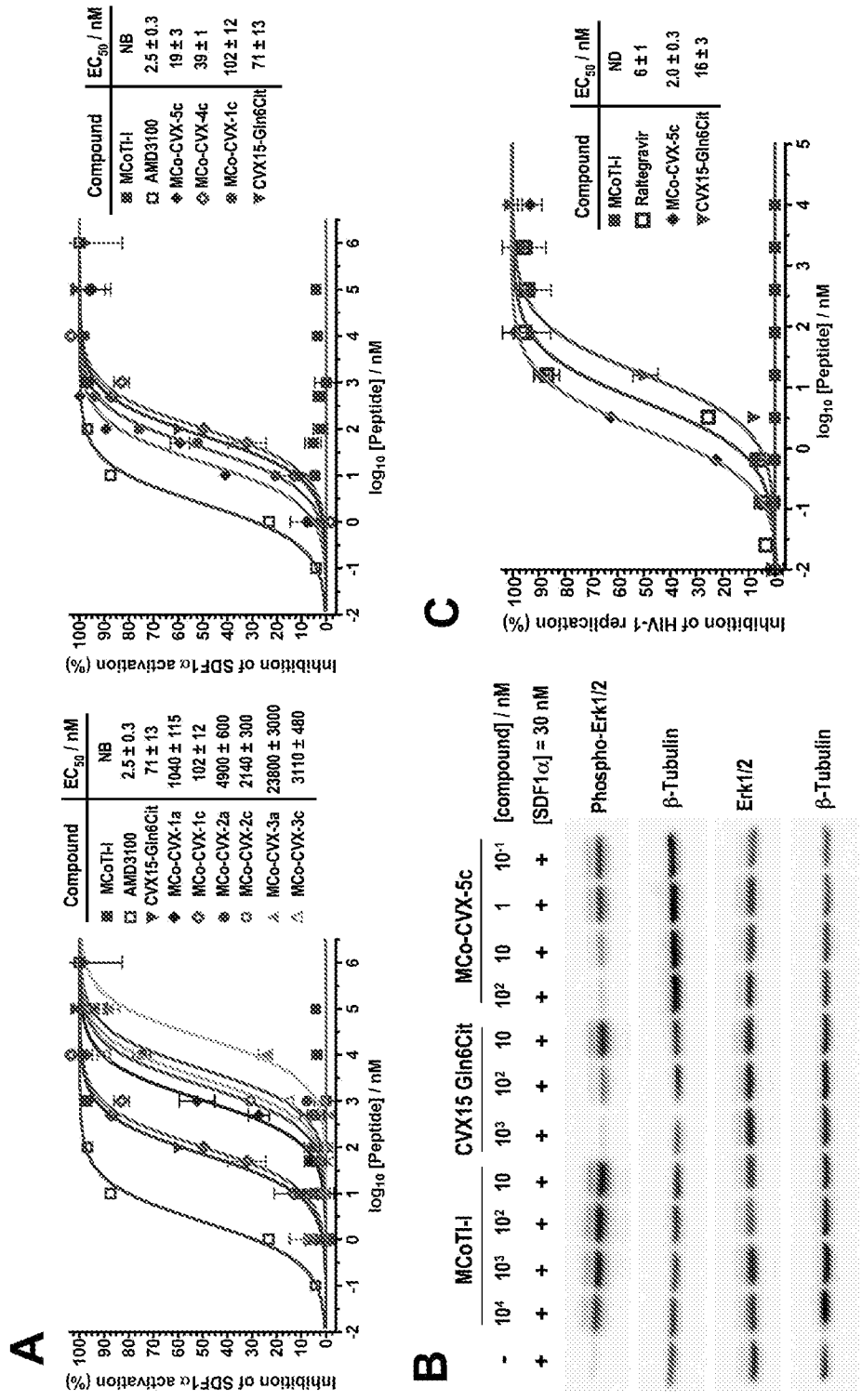

A
Inhibition of SDF1α activation (%)
log10 [Peptide] / nM
Compound
EC50 / nM
MCoTI-I
NB
AMD3100
2.5 ± 0.3
CVX15-Gln6Cit
71 ± 13
MCo-CVX-1a
1040 ± 115
MCo-CVX-1c
102 ± 12
MCo-CVX-2a
4900 ± 600
MCo-CVX-2c
2140 ± 300
MCo-CVX-3a
23800 ± 3000
MCo-CVX-3c
3110 ± 480
MCoTI-I
AMD3100
CVX15-Gln6Cit
MCo-CVX-1a
MCo-CVX-1c
MCo-CVX-2a
MCo-CVX-2c
MCo-CVX-3a
MCo-CVX-3c
B
MCoTI-I
CVX15 Gln6Cit
MCo-CVX-5c
[compound] / nM
[SDF1α] = 30 nM
Phospho-Erk1/2
β-Tubulin
Erk1/2
β-Tubulin
C
Inhibition of HIV-1 replication (%)
log10 [Peptide] / nM
Compound
EC50 / nM
MCoTI-I
ND
Raltegravir
6 ± 1
MCo-CVX-5c
2.0 ± 0.3
CVX15-Gln6Cit
16 ± 3

FIG 3

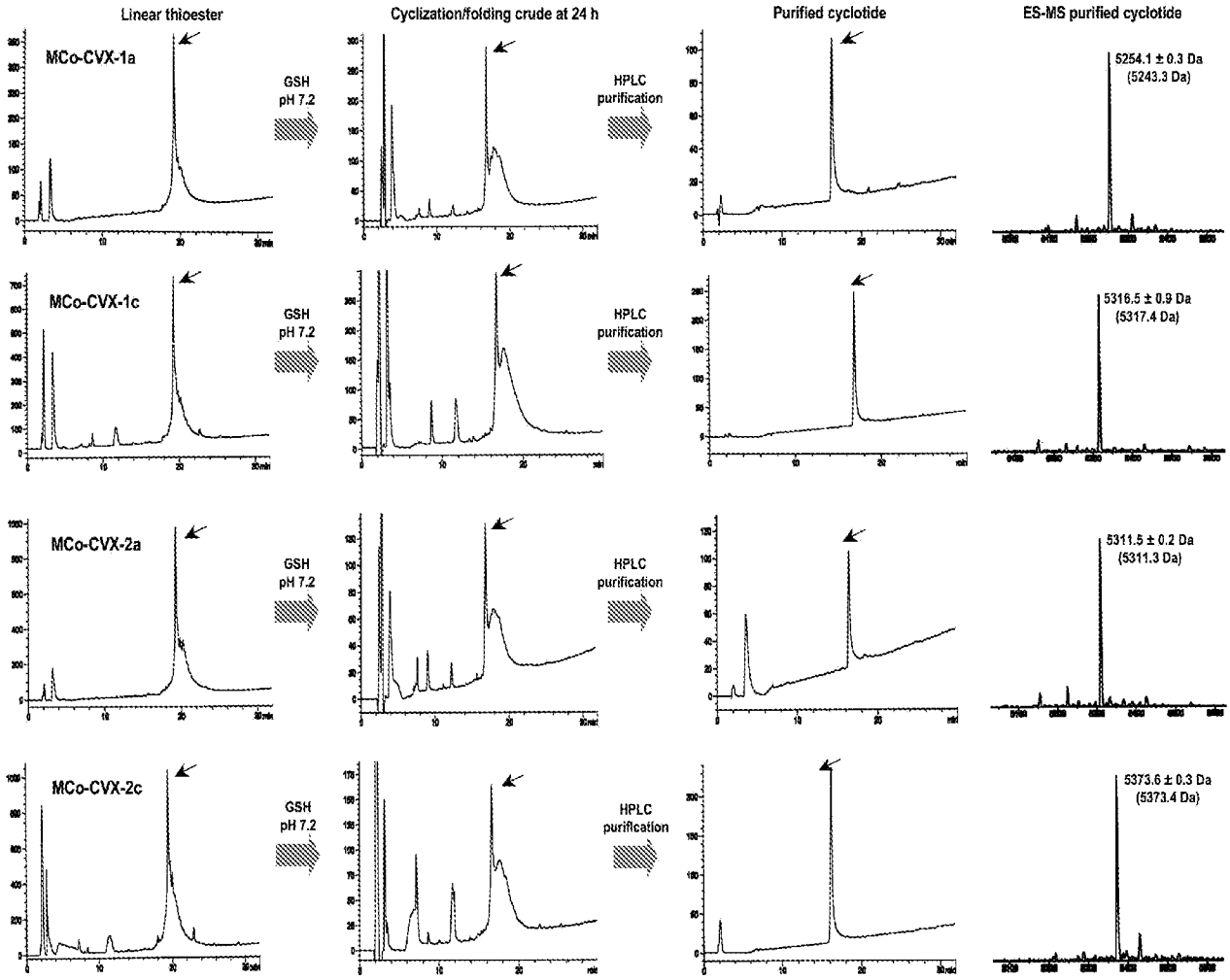

Linear thioester
Cyclization/folding crude at 24 h
Purified cyclotide
ES-MS purified cyclotide
MCo-CVX-1a
GSH pH 7.2
HPLC purification
5254.1 ± 0.3 Da (5243.3 Da)
MCo-CVX-1c
GSH pH 7.2
HPLC purification
5316.5 ± 0.9 Da (5317.4 Da)
MCo-CVX-2a
GSH pH 7.2
HPLC purification
5311.5 ± 0.2 Da (5311.3 Da)
MCo-CVX-2c
GSH pH 7.2
HPLC purification
5373.6 ± 0.3 Da (5373.4 Da)

FIG 4

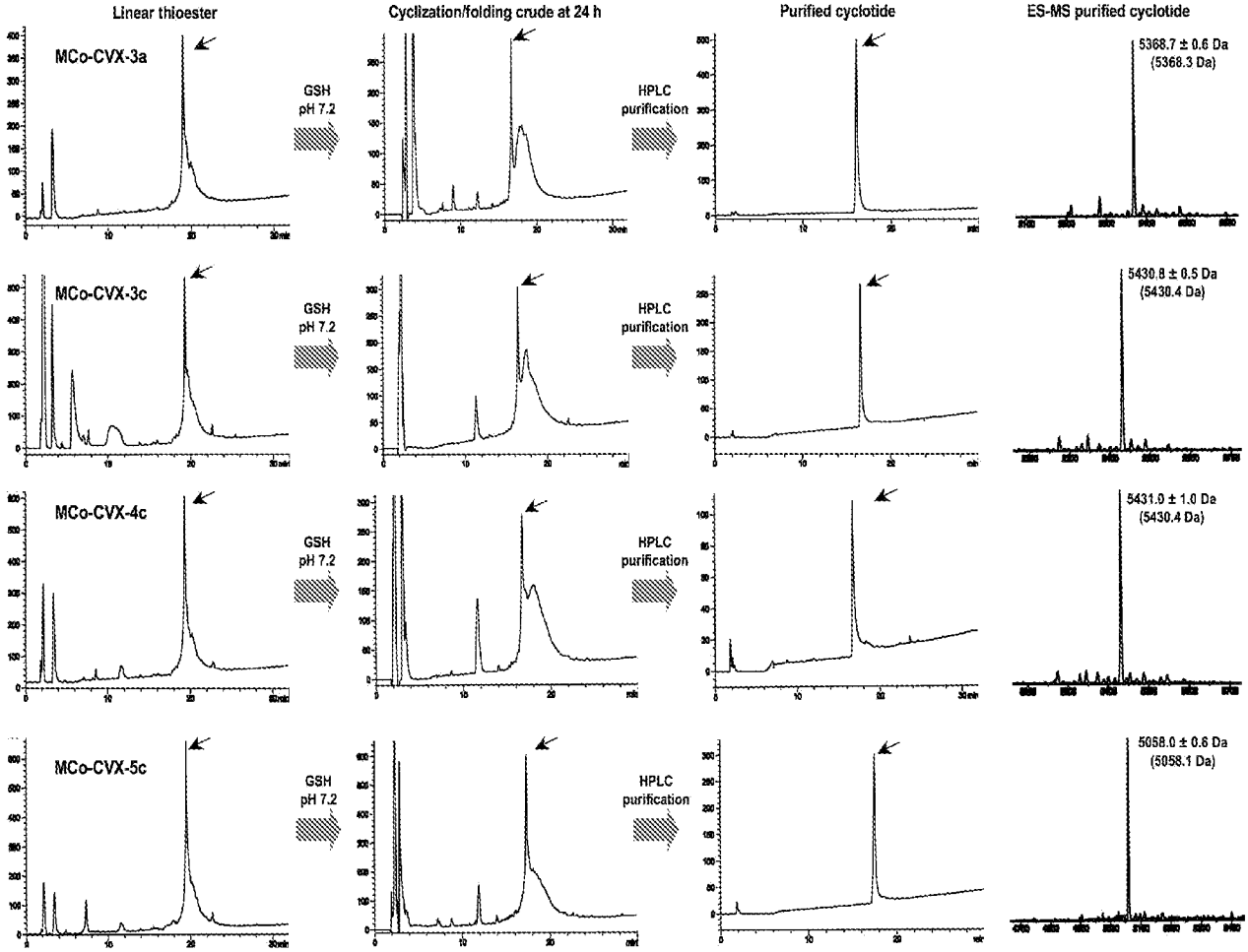


FIG 4 (continued)

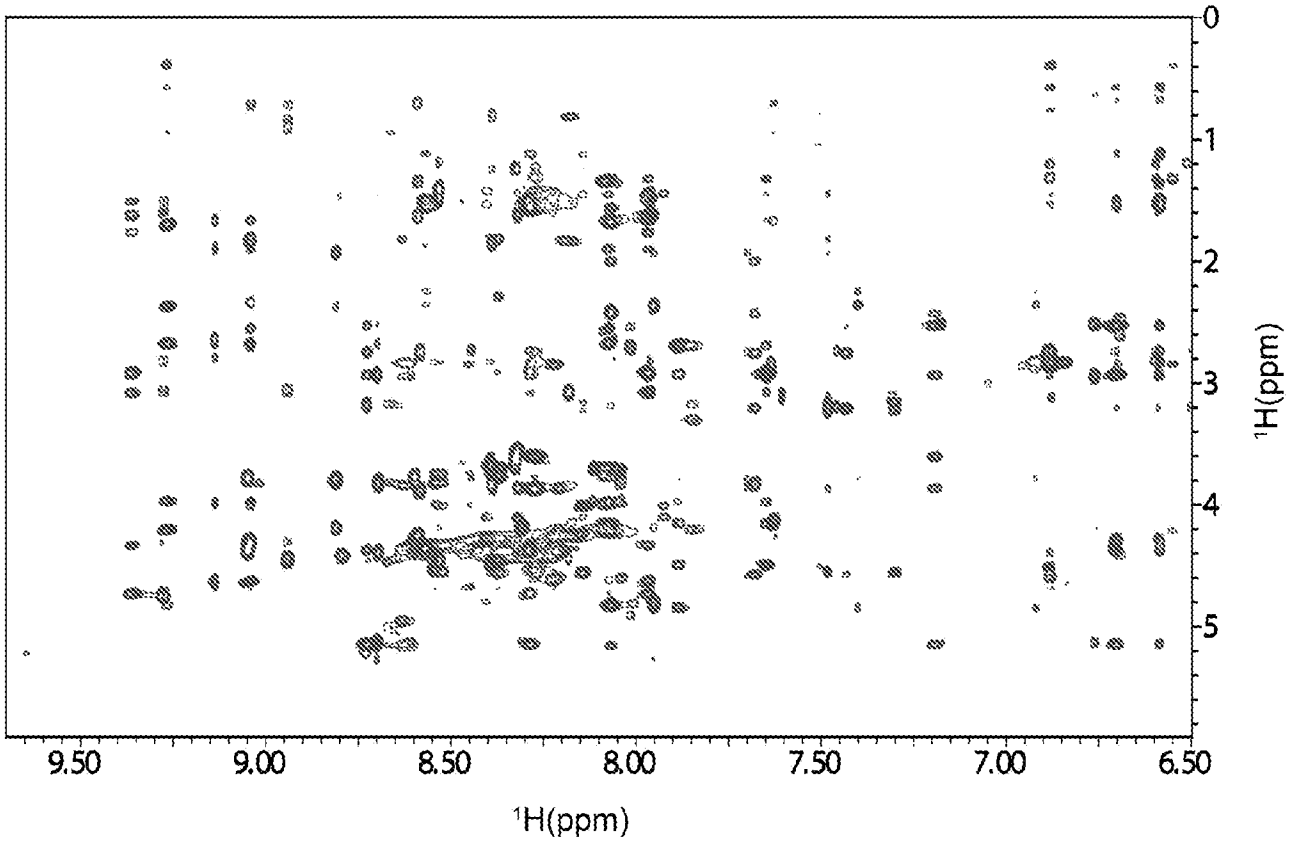


FIG 5

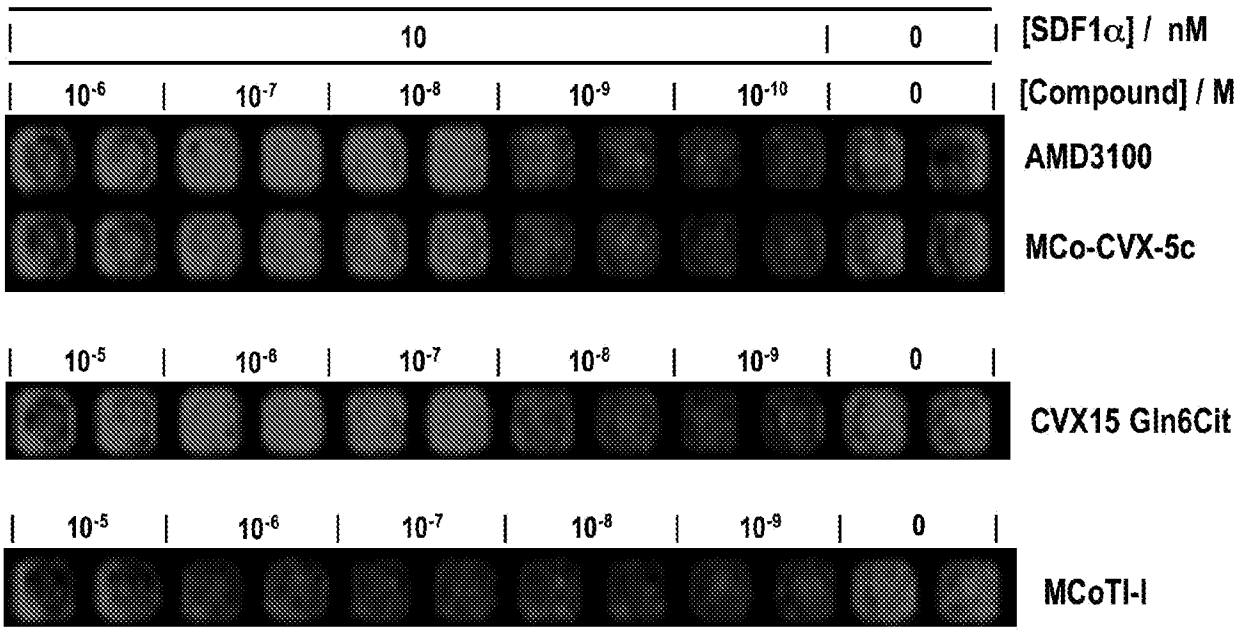

10
0
[SDF1α] / nM
10-6
10-7
10-8
10-9
10-10
0
[Compound] / M
AMD3100
MCo-CVX-5c
10-5
10-6
10-7
10-8
10-9
0
CVX15 Gln6Cit
10-5
10-6
10-7
10-8
10-9
0
MCoTI-I

FIG 6

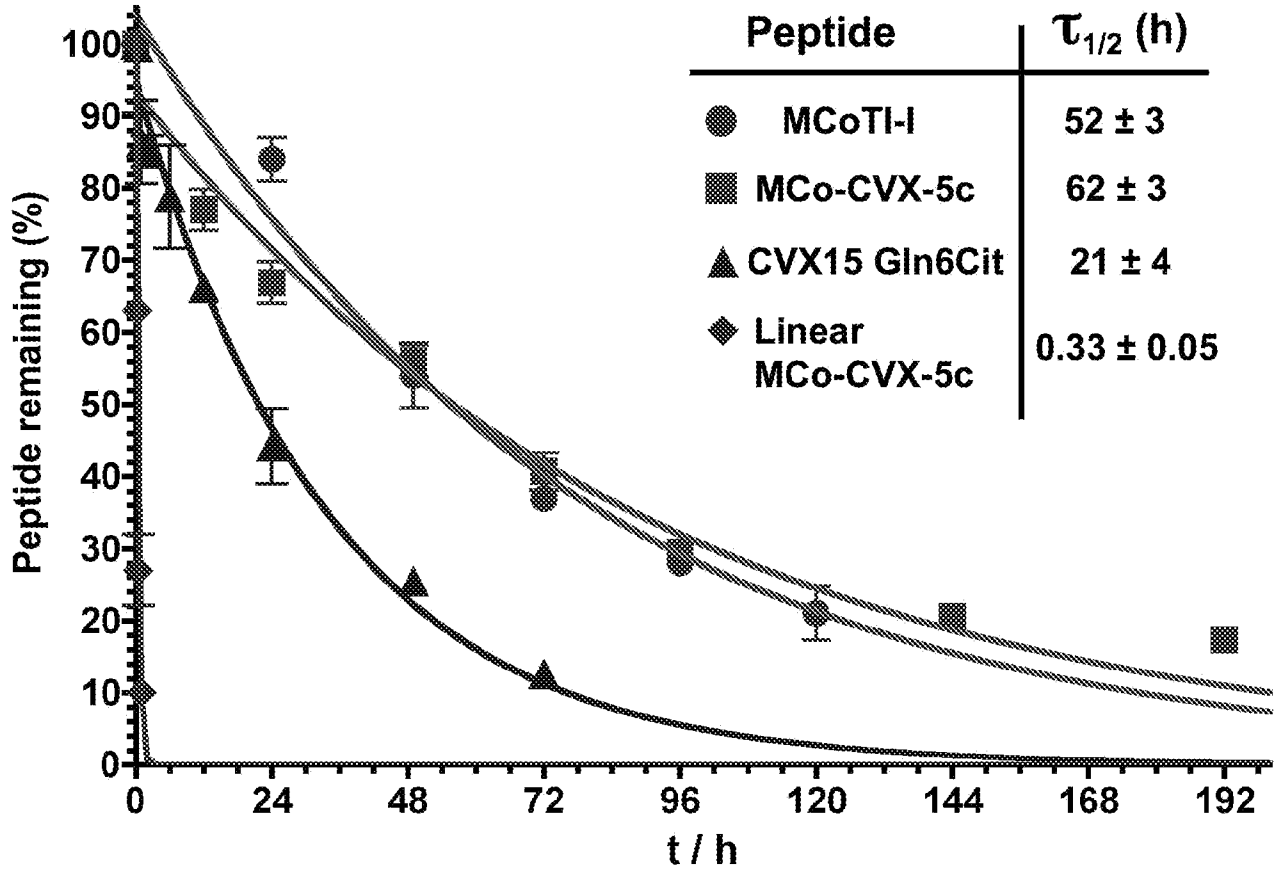

Peptide
τ1/2 (h)
MCoTI-I
52 ± 3
MCo-CVX-5c
62 ± 3
CVX15 Gln6Cit
21 ± 4
Linear MCo-CVX-5c
0.33 ± 0.05
Peptide remaining (%)
100
90
80
70
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
192
t / h

FIG 7

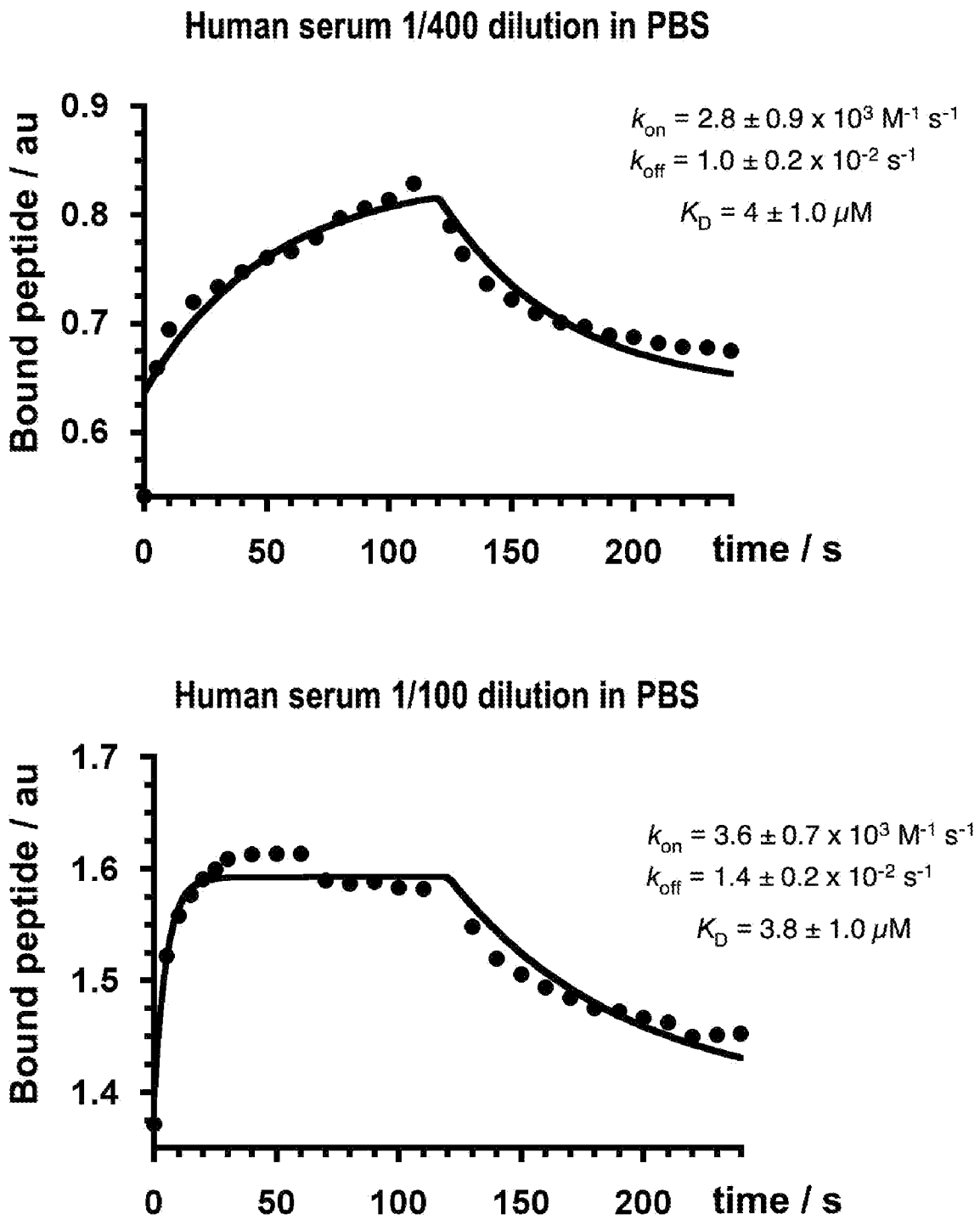

Human serum 1/400 dilution in PBS
Bound peptide / au
0.9
0.8
0.7
0.6
k_{on} = 2.8 ± 0.9 x 10^3 M^{-1} s^{-1}
k_{off} = 1.0 ± 0.2 x 10^{-2} s^{-1}
K_D = 4 ± 1.0 μM
0
50
100
150
200
time / s
Human serum 1/100 dilution in PBS
Bound peptide / au
1.7
1.6
1.5
1.4
k_{on} = 3.6 ± 0.7 x 10^3 M^{-1} s^{-1}
k_{off} = 1.4 ± 0.2 x 10^{-2} s^{-1}
K_D = 3.8 ± 1.0 μM
0
50
100
150
200
time / s

FIG 8

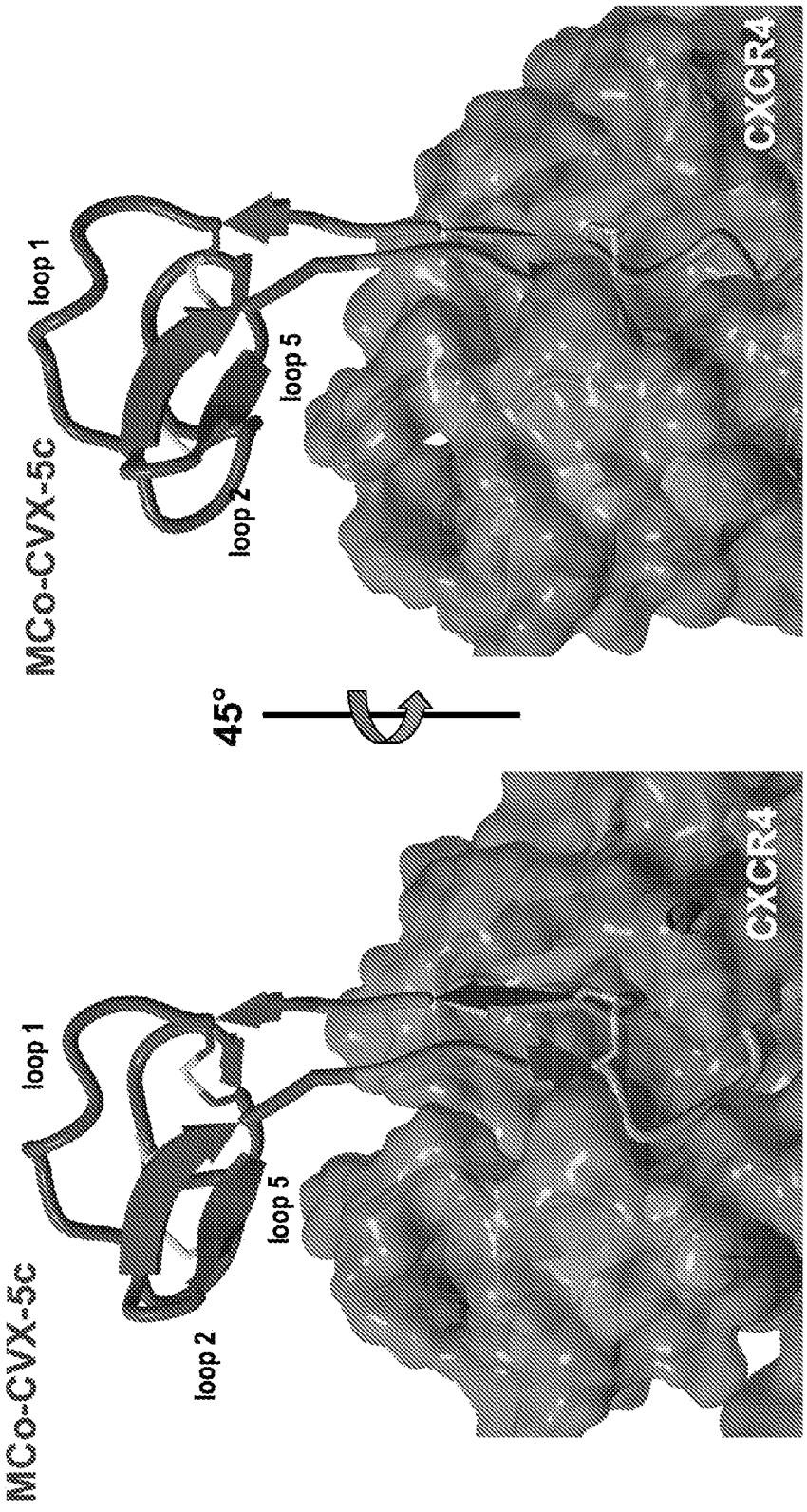

MCo-CVX-5c
loop 1
loop 2
loop 5
CXCR4
45°
MCo-CVX-5c
loop 1
loop 2
loop 5
CXCR4

FIG 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/031775

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00 (2006.01) A61K 38/16 (2006.01) A61P 31/18 (2006.01) A61P 35/00 (2006.01) C07K 14/415 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, CAplus, WPIDS, Biosis, Biotechabs: keywords: cyclotide, kalata, MCoT?, cyclic cystine knot, cyclic cysteine knot, scaffold?, engineer?, graft?, framework, CXCR?, CVX15, CD184, fusin.

PubMed: keywords: CXCR4 antagonist peptide

Registry, CAplus: subsequence search of SEQ ID NOs: 2-10

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 2013	13 June 2013

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2013/031775**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 7960340 B2 (CRAIK et al.) 14 June 2011 See claim 1; Examples 6, 7, 10-15; column 12	1-27
Y	GOULD, A. et al. 'Cyclotides, a novel ultrastable polypeptide scaffold for drug discovery', Current Pharmaceutical Design. 2011, vol. 17, pages 4294-4307 See pages 4302-4303; Figure (6), page 4302; page 4294, first paragraph	1-27
Y	CHAN, L.Y. et al. 'Engineering pro-angiogenic peptides using stable, disulfide-rich cyclic scaffolds', Blood. 2011, vol. 118, pages 6709-6717 See Abstract; Figures 1 and 4, pages 6710 and 6713; page 6709; page 6716, right column	1-27
Y	WU, B. et al. 'Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists', Science. 2010, vol. 330, pages 1066-1071 See page 1066; Figure 2 (B) and (D), page 1067	1-27
Y	DEMARCO, S.J. et al. 'Discovery of novel, highly potent and selective β-hairpin mimetic CXCR4 inhibitors with excellent anti-HIV activity and pharmacokinetic profiles', Bioorganic and Medicinal Chemistry. 2006, vol. 14, pages 8396-8404 See Scheme 1, page 8399; pages 8399-8401; Figures 1 and 2, page 8397; Abstract; page 8396	1-27
P,X	ABOYE, T.L. et al. 'Design of a novel cyclotide-based CXCR4 antagonist with anti-human immunodeficiency virus (HIV)-1 activity', Journal of Medicinal Chemistry. 2012, vol. 55, pages 10729-10734 (published 14 November 2012) See Abstract; Figure 1, page 10730; Table 1, page 10731	1-27

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2013/031775**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 7960340 B2	14 Jun 2011	AU 784222 B2	23 Feb 2006
		AU 1007101 A	23 Apr 2001
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		EP 1230263 A1	14 Aug 2002
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		US 2010298528 A1	25 Nov 2010
		US 7960340 B2	14 Jun 2011
		US 2003158096 A1	21 Aug 2003
		US 2011244564 A1	06 Oct 2011
		WO 0127147 A1	19 Apr 2001

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.