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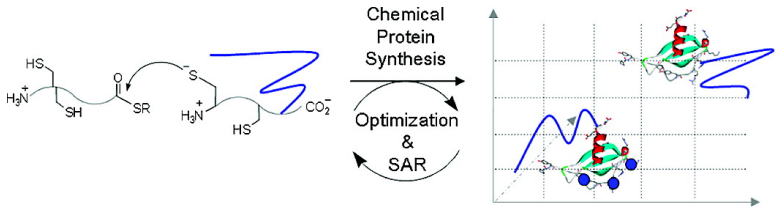

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A Chemical Approach to the Pharmaceutical Optimization of an Anti-HIV Protein

Les P. Miranda,*,† Haiyan Shao,‡ Jason Williams, Shiah-Yun Chen, Ting Kong, Rod Garcia, Yvonne Chinn, Nathalie Fraud, Bill O'Dwyer, Jay Ye, Jill Wilken, Donald E. Low, E. Neil Cagle, Maia Carnevali,# Alexander Lee, Di Song,|| Ada Kung, James A. Bradburne, Xavier Paliard,⊥ and Gerd G. Kochendoerfer§

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Abstract: Chemical protein synthesis is important for dissecting the molecular basis of protein function. Here we advance its scope by demonstrating the significant improvement of the multifaceted pharmaceutical profile of small proteins exclusively via a chemical-based approach. The focus of this work centered on CCL-5 (RANTES) derivatives with potent anti-HIV activity. The overall chemical strategy involved a combination of coded and noncoded amino acid mutagenesis, peptide backbone engineering, and site-specific polymer attachment. The ability to alter specific protein residues, as well as precise control of the position and type of polymer attachment, allows for the exploration of specific molecular designs and resulted in novel CCL-5 analogues with significant differences in their respective biochemical and pharmaceutical properties. Using this approach, the complex-interplay of variables contributing to the noncovalent self-association (aggregation) state, CCR-5 specificity, in vivo elimination half-life, and anti-HIV activity of CCL-5-based protein analogues could be empirically evaluated via total chemical synthesis. This work has led to the identification of potent (sub-nanomolar) anti-HIV proteins with significantly improved pharmaceutical profiles, and illustrates the increasing value of protein chemical synthesis in contemporary therapeutic discovery. These antiviral molecules provide a novel mechanism of action for the development of a new generation of anti-HIV therapeutics which are still desperately needed.

Introduction

Methods that permit the precise investigation, attenuation, and optimization of proteins are of high value for mechanistic, engineering, and pharmaceutical discovery studies. Historically, studies of proteins have been limited to the 20 naturally encoded amino acid residues through conventional recombinant protein expression techniques, although there are a few exceptions, such as selenocysteine incorporation.[1] However, recent advances in both chemical synthesis[2] and unnatural protein expression[3,4] techniques are enabling the site-specific incorporation of a large repertoire of unnatural amino acids into proteins. One of the general objectives of such advances is to provide methods for the modification and optimization of proteins in a manner analogous to those used to explore and improve the medicinal properties of small molecule-based drugs over many decades. Moreover, the introduction of certain unnatural amino acid residues can also be used as site-specific mechanistic probes, labels for bioassays, or chemoselective derivatization sites to avoid random biochemical modifications.

In this work we examined the utility of total chemical synthesis for the iterative design and multifaceted optimization of an anti-HIV small protein, CCL-5 (RANTES), with improved pharmaceutical properties. CCL-5 is the natural ligand of the CC-chemokine receptor 5 (CCR-5) and also signals through CCR-1 and CCR-3.[5] CCL-5 is known to self-associate, forming high molecular mass aggregates (>100 kDa) via a dynamic process.[6] Hydrophobic N-terminal modification of CCL-5 leads to derivatives that inhibit cell entry of HIV strains requiring CCR-5 to enter.[7−9] The emergence of HIV strains that are resistant to highly active antiretroviral therapy (HAART)[10]

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Table 1. Polymer-Substituted CCL-5 Derivatives with Varying Backbone Sequences and Polymer Attachment Positions[a]

	CCR-5 interface			dimer interface	CCR-1 and 3 interface		aggregation interface	aggregation interface GAG binding site			aggregation interface	C-terminal extensions		anti-HIV IC_{50}
entry	1	2	3	7	14	17	26	44	45	47	66	69	70	(nM)
1	NNY	SPro	Chg	T	Y	R	E	R	K	R	E	bPol	L	0.62
2	NNY	SPro	Chg	T	A	A	A	A	A	A	S	bPol	L	
3	NNY	SPro	Chg	N^{Me}-T	A	A	A	A	A	A	S	bPol	L	
4	NNY	SPro	Chg	T	A	A	A	A	bPol	A	*des*	*des*	*des*	>1000
5	NNY	SPro	Chg	N^{Me}-T	A	A	A	R	bPol	R	*des*	*des*	*des*	47
6	NNY	SPro	Chg	T	Y	R	E	R	bPol	R	*des*	*des*	*des*	0.76
7	NNY	SPro	Chg	T	Y	R	E	R	PEG	R	*des*	*des*	*des*	0.80

[a] Starting base CCL-5 sequence, **SPY**SSD**T**TPCCFA**Y**IA**R**PLPRAHIK**E**YFY-TSGKCSNPAVVFVT**RK**N**R**QVCANPEKKWVREYINSL**EMSX$_{aa}$X$_{aa}$**. The postulated functional properties are indicated above the respective residue positions. Residues that are substituted or deleted (*des*) are underlined. NNY, SPro, and Chg represent nonanoyl, thiaproline, and cyclohexylglycine, respectively. bPol and PEG indicate a 16 KDa branched polyamide polymer[17] and 20 kDa monomethoxyl-polyethylene glycol, respectively. The in vitro anti-HIV activity (92/US/712) of the compounds in a PBMC assay is shown in the far right column.

have increased interest in the development of HIV entry inhibitors.[11] In spite of the development and evolution of HAART, new therapeutic agents capable of preventing HIV transmission or replication are still desperately needed. In 2003, it is estimated HIV/AIDS resulted in 3 million deaths and 5 million newly infected people.[12]

PSC-RANTES, *des*-Ser^1,N^{α}-nonanoyl[(L)-thiaproline^2, (L)-cyclohexylglycine^3]-RANTES(2−68), a chemically synthesized derivative of CCL5 has been used to prevent simian−human immunodeficiency virus (SHIV) infection in vaginally challenged rhesus macaques as a topical vaginal microbiocide.[13,14] To complement these efforts, our objective was to determine if total chemical synthesis could be used to engineer and identify a CCL-5 derivative more suitable for development as a potential injectable anti-HIV therapeutic. Access to the precise protein architectural designs described here were only achievable by protein chemical synthesis, since they still remain outside the reported capabilities of unnatural protein expression methodologies.[3,4] Consequently, using an empirical protein analoging approach, enabled via total protein-polymer conjugate chemical synthesis, we have identified CCL-5 derivatives with highly potent anti-HIV activity, improved CCR-5 specificity, prolonged in vivo circulation, and well-defined homodimeric structure.

Materials and Methods

Chemical Protein Synthesis. Single letter amino acid representation code was used in accordance to the Nomenclature and Symbolism for Amino Acids and Peptides by the UPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN).[15] The amino acid peptide sequence of **1** (1−70) is BXZSSDTTPC CFAYIARPLP RAHIKEYFYT SGKC-SNPAVV FVTRKNRQVC ANPEKKWVRE YINSLEMSUL-OH, where B is N^{α}-nonanoyl, X is (L)-thiaproline, Z is (L)-cyclohexylglycine, and U is lysine(N^{ϵ}-levulinyl). The changes in amino acid sequence between analogues **1** to **7** are listed in Table 1. Analogues **1**−**6** were synthesized in the same methodological manner. Analogue **1** was synthesized from two peptides, CCL-5(1−33)-α-thioester and CCL-5(34−70)-carboxylate prepared by *tert*-butyloxycarbonyl (Boc)-based solid-phase peptide synthesis (SPPS) using 0.5 M 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*,*N*-dimethyl-formamide (DMF)/*N*,*N*-diisopropylethyl amine (DIEA) in situ neutralization chemistry.[16] Peptides were synthesized (0.2 mmol scale) on either -O-CH2-Pam (phenylacetamidomethyl) PS−DVB based resin or a thioester generating resin.[13,17,18] The following side-chain protecting groups were used: Arg(Tos), Asp(OChx), Asn(Xan), Cys(pMeBzl), Glu(OChx), His(Dnp), Lys(2-ClZ), Lys(Fmoc), Ser(Bzl), Thr(Bzl), Trp-(Formyl), and Tyr(2-BrZ). Levulinyl functionality was introduced on-resin at a Lys(Nϵ-Fmoc) residue following on-resin peptide assembly as previously described.[19] The peptide-resin product was cleaved with anhydrous hydrogen fluoride (HF) containing *p*-cresol (5% v/v, 20 mL per gram of resin) for 1 h at 0 °C. Following evaporation of HF, the crude peptides were isolated by precipitation with diethyl ether, dissolved in aqueous acetonitrile solutions, and lyophilized to give a white powder. The crude peptides were dissolved in 6 M guanidine·HCl, 0.1 M sodium acetate, pH 4 and purified by preparative reversed-phase HPLC as described below. Fractions containing target peptide segment were identified by ESI-MS, pooled, and lyophilized.

For the synthesis of analogues **1** to **6**, the 16 kDa aminooxy-derivatized branched polymer[19] (structure shown in Supporting Information) was attached to the lysine(N^{ϵ}-levulinyl) functionalized C-terminal segment by co-dissolution at a molar ratio of 1:1.2 to 1.5 in 50% aqueous acetonitrile containing 0.1% TFA at a peptide concentration of 10 mg/mL and lyophilized. The polymer-conjugated peptide was separated from unmodified polypeptide and unreacted polymer by preparative RP-HPLC with a linear water/acetonitrile gradient. Fractions containing the expected polymer-conjugated polypeptide were identified by ESI-MS, pooled, and lyophilized.

The full-length CCL-5 analogue backbone was formed by native chemical ligation[2] of the N-terminal α-thioester peptide with the C-terminal polymer-conjugated peptide segment possessing an N-terminal cysteine residue. A 50% molar excess of purified α-thioester peptide CCL-5(1−33) was added to the purified polymer-conjugated peptide conjugate (concentration 2 mM; 30 mg) dissolved in 6 M guanidine·HCl, 100 mM sodium phosphate, pH 7.5 and 0.5% v/v thiophenol. The reaction mixture was stirred overnight at room temperature, and the reaction was monitored by analytical RP-HPLC

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and ESI-MS. After the reaction was complete, β-mercaptoethanol was added to 20% by volume and left for 30 min to remove any residual His(Dnp) protecting groups and reduce disulfide bonds. Reactants and products were separated by preparative RP-HPLC with a linear gradient of 25−45% buffer B versus 0.1% aqueous TFA over 20 min at 40 °C. Fractions containing full-length CCL-5 analogue were identified by ESI-MS, pooled, and lyophilized. For folding, lyophilized linear conjugate (2.0 mg) was dissolved in buffer (1 mL) containing 2 M guanidine·HCl in 100 mM Tris, pH 8.5, containing cysteine (1 mM) and cystine (0.1 mM). It was then dialyzed overnight against 60x (v/v) folding buffer, 0.6 M guanidine·HCl,100 mM Tris, pH 8.5. The folded protein was purified by RP-HPLC using a gradient of 25−45% buffer B versus 0.1% aqueous TFA over 80 min. Fractions containing the desired folded protein were identified by ESI-MS, pooled, and lyophilized. Compound **1** calcd for $C_{1108}H_{1932}N_{210}O_{368}S_6$, 24277 Da (av); exptl observed mass (ESI-MS), 24279 Da. RP-HPLC (C4, Vydac 300 Å) purity >98%; yield, 11 mg (31%). Compound **2** calcd for $C_{1086}H_{1896}N_{200}O_{364}S_6$, 23773 Da (av); exptl observed mass (ESI-MS), 23776 Da. Purity by RP-HPLC (Vydac C4, 5 μm, 300 Å, 1%/min 0−60%B) >98%; yield, 5.1 mg (24%). Compound **3** calcd for $C_{1087}H_{1898}N_{200}O_{364}S_6$, 23787 Da (av); exptl observed mass (ESI-MS), 23790 Da. RP-HPLC (C4, Vydac 300 Å) purity, 99%; yield: 3.6 mg (22%). Compound **4** calcd for $C_{1071}H_{1870}N_{196}O_{358}S_6$, 23501 Da (av), exptl observed mass (ESI-MS), 23505 Da. RP-HPLC (C4, Vydac 300 Å) purity, >97%; yield, 0.8 mg (12%). Compound **5** calcd for $C_{1081}H_{1891}N_{203}O_{360}S_6$, 23686 Da (av); exptl observed mass (ESI-MS), 23691 Da. RP-HPLC (C4, Vydac 300 Å) purity, 96%; yield, 6.4 mg (26%). Compound **6** calcd for $C_{1091}H_{1902}N_{206}O_{363}S_6$, 23907 Da (av); exptl observed mass (ESI-MS), 23,910 Da. RP-HPLC (C4, Vydac 300 Å) purity, 97%; yield, 15.7 mg (34%).

Yield percentages are based upon the limiting reagent (C-terminal polymer-conjugated peptide segment) used in the native chemical ligation reaction used for each respective analogue and represent the final isolated yield after ligation and folding. PSC-RANTES was synthesized and characterized as published previously.[13] For the synthesis of analogue **7**, the 20 kDa monomethoxyl−polyethylene glycol aldehyde (Nektar Therapeutics, San Carlos, CA) was attached to the [*N*ϵ-aminooxylacetyl-lysinyl[45]]CCL-5(34−70)-carboxylate by co-dissolution at a molar ratio of 1:1.2 to 1.5 in 50% aqueous acetonitrile containing 0.1% TFA at a peptide concentration of 10 mg/mL and lyophilized The PEGylated-peptide was separated from unmodified polypeptide and unreacted polymer by preparative RP-HPLC with a linear 0.1% TFA water/acetonitrile gradient. Fractions containing the expected polymer-conjugated polypeptide were identified by SDS-PAGE and then pooled lyophilized.[17]

Reversed-Phase HPLC. Reversed-phase high-performance liquid chromatography was performed on an analytical (C4, 5 μm, 0.46 cm × 25 cm) or a preparative (C4, 10 μm, 2.2 cm × 25 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% ACN, 10% H2O, 0.09% TFA), typically 5−95% over 35 min at a flow rate of 1 mL/min for analytical analysis and 5−65% over 60 min at 13 mL/min for preparative separations. The purity of the crude peptides was assessed by HPLC with absorbance detection at 214 nm. Analytical and preparative HPLC fractions were characterized by ESMS and HPLC, combined and lyophilized to give white powders.

Mass Spectrometry. Mass spectra were acquired on a triple quadrupole mass spectrometer equipped with an ionspray atmospheric pressure ionization source. Samples (10 μL) were injected into a moving solvent (10 μL/min; 50:50 ACN/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100−120 μm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range 500−2200 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed *m*/*z* values.

Size-Exclusion Chromatography with On-Line Light Scattering, Absorbance, and Refractive Index Detectors (SEC-MALS). SEC experiments with on-line light scattering and refractive index detection were performed on an Agilent 1100 HPLC system coupled to a miniDawn multiangle light scattering and an Optilab refractive index detector (both Wyatt Technology) using a Superdex 200 10/30 column (Pharmacia) in the presence of 0.5 M NaCl to avoid nonspecific interactions. Molecular weights were calculated with a dn/dc value of 0.185 for proteins and 0.132 for PEG using the Astra software package (Wyatt Technology). Typically, SEC-MALS analysis was performed on the crude folding products derived from folding 2 mg of total protein amount for each analogue. Since MALS analysis is performed on eluent from the SEC column protein concentration varies across each peak. The approximate protein concentration from the SEC column for MALS analysis was 40 μg/mL.

HIV Antiviral Assay. Peripheral blood mononuclear cells (PBMCs) obtained from HIV-1 negative donors were purified over a Ficoll Hypaque gradient and activated with PHA and IL-2 for 48−72 h. PBMCs were cultured in IL-2 containing media until use. PHA-blasted PBMCs from three donors were cultured for 6 days at 106 per mL in triplicate in the presence of the primary HIV-1 clinical isolate 92/US/712 (AIDS research and reference reagent program, Bethesda, MD) and 0.5 log dilutions of CCL-5 or CCL-5 compounds **1**−**7** starting at 1 μM). Viral replication was assessed by measuring the p24 HIV-1 capsid antigen by ELISA (Beckman Coulter, Brea CA) in 6-day culture supernatants. IC_{50} and IC_{90} (concentration resulting in respectively 50 and 90% inhibition of viral replication) was determined by 4-parameter fit.

Pharmacokinetics. 1, **6**, and **7** were administered intravenously (iv) at 1 mg/kg to four male rats on day 1. Blood samples were collected from subsets of two rats at the time-points of 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, and 72 h. Blood was collected from 2 extra, untreated animals to serve as pre-dose samples (~1 mL serum per animal) without use of an anticoagulant and frozen at −70−−80 °C until shipped. The Quantikine ELISA (R&D System) for CCL-5 was used to assay plasma concentration of all analogues. A least-squares analysis of the logarithms of the concentrations was performed and the pharmacokinetic parameters extracted.

CCR-5 Calcium Flux Assay. The CCR5 transfected CHO-K1 cell line was grown in MEM supplemented with 10% FBS (heat inactivated) and 500 μg/mL of Hygromycin in a 37 °C incubator with 5% CO_2. CHO-K1 cell lines were seeded on 96-well plates at 50000 cells per well per 100 μL of media and the plates were incubated for 24 h prior to the start of the assay. The assay was performed using the Calcium 3 Assay Kit for FLEXstation (Molecular Devices, Inc.).

CCR-1/CCR-3 Assay. Analogues were tested using the AequoScreenTM assay (Euroscreen) for agonist activity on the CCR-1 and CCR-3 human recombinant receptors. MIP-1α and eotaxin served as reference standards for the CCR-1 and CCR-3 receptor activation assays, respectively. Activities were tested in duplicate at ten concentrations: 100, 33, 11, 3.7, 1.2, 0.41, 0.14, 0.046, 0.015, and 0.005 nM. AequoScreenTM cells were harvested with PBS-EDTA, washed, and resuspended in BSA-DMEM medium. Suspended cells were then incubated with coelenterazine; 50 μL of cell suspension were mixed with 50 μL of test or reference agonist in a 96-well plate. The resulting emission of light was recorded using the Hamamatsu Functional Drug Screening System 6000. Following an incubation of 15−30 min after the first injection, 100 μL of the resulting cell suspension containing the test compounds was mixed with 50 μL of the reference agonist in the 96 well test plate and the emission recorded. To standardize the emission of recorded light across plates and across different experiments, some of the wells contained 100 μM digitonin, Triton X 100 at 0.2% v/v, a saturating concentration (20 μM) of ATP, and a concentration of reference agonist at a concentration equivalent to the EC80

obtained during test validation. Agonist activity of test compounds at the given receptor is reported as a percentage of the activity of the reference compounds at their EC_{80}.

Results and Discussion

Starting from the native 68-residue CCL-5 sequence, the overall chemical synthesis strategy involved a combination of coded and noncoded amino acid mutagenesis, peptide backbone engineering, and site-specific polymer attachment. We have sought to determine if the above strategy could be used to identify polymer-conjugated CCL-5 derivatives with (1) subnanomolar (nM) in vitro antiviral activity against HIV clinical isolates, (2) a well-defined quaternary structure, such as a homodimer, through reduction of aggregation propensity, (3) increased CCR-5 specificity over CCR-1 and CCR-3 signaling, and (4) prolonged in vivo circulation. The identification of a molecule with the above profile would be a more suitable and attractive starting point for the development of an anti-HIV therapeutic. For protein therapeutics, the presence of aggregates is typically considered to be undesirable because of the concern that the aggregates may result in an immunogenic reaction.[20] The generation of antibodies against a therapeutic protein could possibly lead to loss of efficacy and neutralization of the endogenous protein with essential biological functions. Furthermore, the complex and dynamic nature of protein aggregates render thems difficult to characterize, control, and reproduce.

From earlier reports it was known that highly potent anti-HIV activity through CCR-5 could be obtained through hydrophobic N-terminal modifications such as *des*-Ser[1],N^{α}-nonanoyl-[(L)-thiaproline[2],(L)-cyclohexylglycine[3] at CCL-5 residue positions 1, 2, and 3.[13] Native CCL-5 (RANTES) is not a potent anti-HIV protein (SF-162 and E80 anti-HIV activity $>3\ \mu$M),[21] and it was the discovery of the anti-HIV activity of recombinant Met-RANTES that led to the initial notion that lipophilic groups at the N-terminus of CCL-5 can lead to potent antiviral activity against R5 (HIV) viruses.[21−23] It had also been reported that CCL-5 signaling through CCR-1 and CCR-3 can be influenced[24] in part by residues Tyr[14] and Arg[17]. Furthermore, previous reports had indicated that mutations to residues Thr[7], Glu[26], Arg[44], Lys[45], Arg[47], and Glu[66], in part, reduced the extent of CCL-5 noncovalent self-association (aggregation).[25−27]

To meet the objectives of this work several natural and unnatural residues needed to be incorporated into CCL-5 analogues baring polymer substituents at varying attachment positions (Table 1). Given the number and nature of the unnatural amino acid substitutions and the precise requirement for site-specific polymer attachment with these CCL-5 derivatives, the only available method for their preparation remained chemical synthesis. Accordingly, these analogues **1**−**7** were chemically synthesized in a convergent manner using *tert*-butoxycarbonyl (Boc)-based solid-phase peptide synthesis (SPPS)[28] and a native chemical ligation[2] strategy at the Lys[33]−Cys[34] site. This required the preparation of N-terminal peptide segments with C-terminal lysinyl-$^{\alpha}$thioester functionality and *C*-terminal peptide segments with an N-terminal cysteinyl-residue (Scheme 1). Following the preparation of the respective peptide segments, the *C*-terminal peptide segments were conjugated to the polymer group at the sites specified in Table 1. Oximation chemistry was used for the site-specific attachment of functional polymer groups to the protein backbone as previously described.[17,19,29] For analogues **1**−**6**, a lysinyl(N^{ϵ}-levulinyl) residue on the *C*-terminal peptide segment was reacted with a 16 kDa aminooxyacetyl-derivatized branched polyamide polymer in acidic aqueous acetonitrile. The branched polymer consisted of (i) a chemoselective linker, (ii) a hydrophilic succinyl-(4,7,10)-trioxatridecane-1,13-diamine spacer, (iii) a core structure with four lysinyl-based branch points, (iv) a linear polymer with twelve succinyl-(4,7,10)-trioxatridecane-1,13-diamine repeat units attached to each branch point, and (v) a negative charge-control unit at the end of each linear polymer. Additional chemical synthesis was required to prepare the branched monodisperse polymer; however, compared to conventional PEGylation approaches, the attachment of the monodisperse polymer allowed facile analysis of polymer-conjugated products by conventional electrospray mass spectrometry. The branched polymer exhibited good solubility in aqueous and organic solvents. For analogue **7**, a lysinyl (N^{ϵ}-aminooxyacetyl) residue on the *C*-terminal peptide segment was reacted with 20 kDa propionaldehyde-derivatized monomethoxy-poly(ethylene glycol) (mPEG, Nektar Therapeutics).[30] The C-terminal polymer-derivatized peptide constructs were then purified by reversed-phase (RP) HPLC and then ligated to the N-terminal peptide thioester segment in pH 7.5 6 M Gn·HCl, 100 mM sodium phosphate, containing 0.5% v/v thiophenol. Following the assembly and isolation of each full length linear polymer-conjugated CCL-5 analogue, they were subjected to pH 8.5 folding conditions and then purified by RP-HPLC. The expected molecular weight and secondary structure for each CCL-5 analogue were confirmed by electrospray mass spectrometry and circular dichroism (CD), and the estimated purities by analytical RP-HPLC were >95%. In vitro anti-HIV activities of CCL-5 analogues were examined against HIV-1 clinical isolate 92/US/712 in a peripheral blood mononuclear cell (PBMC) assay (Table 1).

Although CCL-5 elutes as a symmetrical peak by RP-HPLC, size exclusion chromatography (SEC) and sedimentation analysis[25] reveal the clear presence of a complex high-order quaternary structure. We sought to inhibit or control CCL-5 self-association (aggregation) by disrupting key protein−protein interfaces through amino acid residue substitutions and the introduction of polymer substituents. To assess the effectiveness of amino acid residue and polymer changes on the reduction self-association we performed SEC-multiangle light scattering

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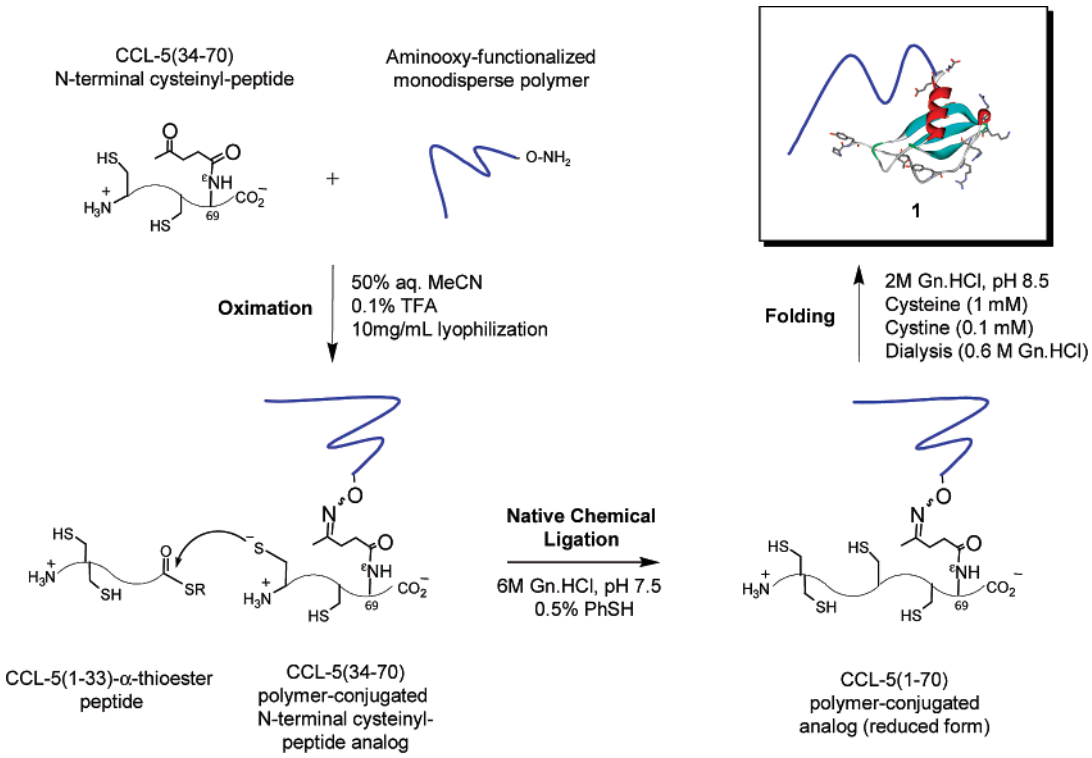
Scheme 1. General Synthetic Scheme for the Preparation of Polymer Substituted CCl-5 Analogues[a]



[a] Scheme depicts process used for the preparation of **1**. CCL-5(1−33) sequence, N^{α}-nonanoyl-[ThiaPro^{2},(L)-Chg^{3}] SSDTTPCCFAYIARPLPRAHIKEY-FYTSGK, CCL-5(34−70) sequence, CSNPAVVFVTRKNRQVCA-NPEKKWVREYINSLEMSK$^{(N\epsilon-levulinyl)}$L. Aminooxylacetyl-functionalized monodisperse polymer is a 16 KDa branched polyamide polymer.[19]

(MALS)[31] analysis on the crude folding products (Figure 1). MALS is a well-established technique compatible with the on-line determination of the absolute molecular weight of proteins and other polymers.[31] We found that conjugation of a 16 kDa polymer to residue 69 (Figure 2A) in the extended C-terminus of PSC-RANTES, **1** (cell-based anti-HIV IC_{50} 0.62 nM) did not inhibit aggregation and gave a SEC chromatographic profile similar to wild-type CCL-5 and PSC-RANTES. The self-association of **1** appeared heterogeneous and contained significant amount of components corresponding to noncovalent heptameric and octameric forms. The aggregation state of CCL-5 and **1** were not reduced by any appreciable extent by either dilution, increasing or decreasing the ionic strength of the solution, or treatment with chaotropic agents such as 6 M guanidine hydrochloride at varying pH. Given that not all chemokines form high-molecular mass aggregates, the role of self-association with respect to biological significance is somewhat unclear.[25] The solution concentrations of CCL-5 and CCL-5 polymer derivatives studied here are much higher than endogenous concentrations but nevertheless are of significant relevance to their preparation for preclinical and clinical applications. CCL-5 analogue **2** differed from analogue **1** by the alanyl substitution of residues 26, 44, 45, 47, and 66, which have been reported to reduce self-association. Surprisingly, SEC-MALS analysis of the crude folding product of **2** indicated the changes still resulted in a significant aggregate population but now also resulted in the formation of some monomeric and dimeric populations. To investigate if the dimeric population could be driven into the monomeric population, an N^{α}-methyl group was introduced at the homodimerization interface, threonyl[7], to give analogue **3**. SEC-MALS analysis of the crude folding product of **3** indicated that the dimeric population could indeed be diminished; however, the overall ratio of high-order aggregation to monomer did not improve to an appreciable extent. Although the folding and aggregation mechanism of CCL-5 is poorly understood, it appears that the mechanism of homodimerization formation is different from that of oligomerization. Moreover, the monomeric, dimeric, and high-molecular mass aggregate forms of CCL-5 analogue **3** and other analogues reported here could be isolated by size exclusion chromatography, provided they were present in the crude folding product. Upon standing for days, the isolated quaternary forms did not undergo any appreciable interconversion into other forms as determined by repeated SEC-MALS analysis (data not shown).

An alternate approach to reduce CCL-5 self-association involved the precise repositioning of the 16 kDa branched polymer from the C-terminus residue lysinyl^{69} to lysinyl^{45} (Figure 2B). Analogue **4** consisted of a polymer group at lysinyl^{45} in combination with alanine^{44,47} substitution and C-terminal truncation (residues 66−70). In this case, SEC-MALS analysis indicated the crude folding product of **4** was similar to analogue **2** and did not prevent the occurrence of aggregates. Importantly, these structural changes resulted in analogue **4** losing its in vitro anti-HIV activity (IC_{50} >1000 nM). In analogue **5** argininyl^{44} and argininyl^{47} were reintroduced with the polymer group still at position 45 in conjunction with N^{α}-methylation of threonyl.[7] Analogue **5** regained good cell-based anti-HIV activity (IC_{50} 47 nM); however, SEC-MALS analysis indicated a result similar with respect to self-association to that of analogue **3** was obtained. Surprisingly, analogue **6**

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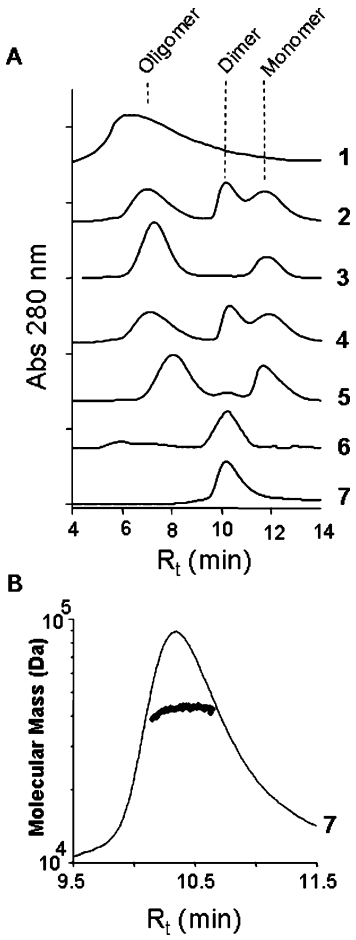

Figure 1. (A) Size exclusion chromatogram (SEC) of CCL-5-polymer conjugate after reversed phase HPLC purification. (B) Close-up zoom of SEC multilight angle scattering (MALS) chromatogram of CCL-5 analogue **7**.

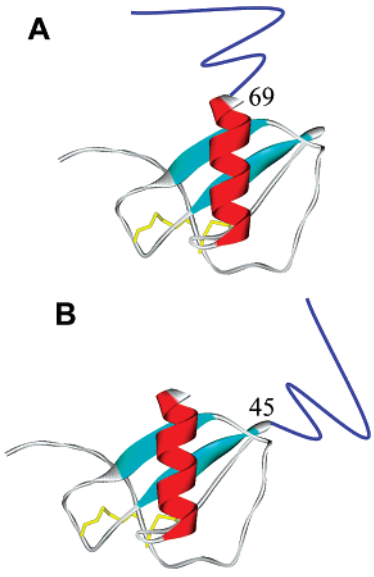

Figure 2. Model structure of CCL-5 derived from PDB file 1B3A indicating the general polymer attachment sites. The backbone secondary structure of CCL-5 is shown and the location of polymer group indicated arbitrarily as a blue line: (A) polymer position at 69; (B) polymer position at residue 45.

that further reintroduced wild-type CCL-5 residues at residues 7, 14, 17, and 26 resulted in a significant reduction in the aggregate population and the predominant formation of a homodimeric form as determined by SEC-MALS analysis. Furthermore, the introduction of these residues in analogue **6** significantly increased its cell-based anti-HIV potency (IC_{50} 0.76 nM). A very similar result was also obtained with analogue **7** which differs from analogue **6** by the incorporation of a linear 20 kDa PEG polymer instead of the branched polymer. Analogue **7** displayed potent cell-based anti-HIV activity (IC_{50} 0.80 nM). SEC-MALS analysis of analogue **7** estimated an absolute molecular weight of 42.6 kDa for the primary component (Figure 1B), which corresponds reasonably well to the structure of a homodimerized polymer−conjugate CCL-5. The contacts between monomers that are involved in the formation of homodimers has been previously reported for CCL-5 and CCL-5 derivatives, such as AOP-RANTES, by both NMR and X-ray crystallography studies, respectively.[32,33] As mentioned above, although the aggregation mechanism is unclear, the ability of **6** and **7** to form stable homodimeric structures suggests oligomerization is not governed by dimerization, and that the respective mechanisms are different. The CD spectrum for analogue **6** (Supporting Information) is indicative of a helical containing structure (characterized by minima at 208 and 222 nm) and is consistent with published CCL-5 structures.[32,33] The CCL-5 structure contains a three-stranded antiparallel β sheet flanked by a carboxy-terminal α-helix between residues lysinyl[56]−glutamyl.[66] The similar anti-HIV activities of oligomeric **1** and homodimeric analogues **6** and **7** suggest that the presence of an oligomeric form, at least in the dosing solution, is not required for anti-HIV activity.

Another objective of this work was to improve CCR-5 specificity of a polymer-modified CCL-5 derivative with respect to CCL-5. CCL-5 is an endogenous ligand to CCR-1, CCR-3, and CCR-5 and, as expected, induced in vitro receptor activation (Figure 3). CCL-5, PSC-RANTES, **1**, **6**, and **7** all possessed the ability to activate CCR-5, although **1**, **6**, and **7** displayed increased signaling at higher concentrations. Chemokine oligomerization was not important for CCR-5 signaling, and this general observation is consistent with studies on monomeric monocyte chemoattractant protein-1 (MCP-1).[34] However, we found that the incorporation of a polymer group in aggregated analogue **1**, homodimeric analogue **6**, and homodimeric analogue **7** all resulted in low CCR-1 and CCR-3 signaling compared to CCL-5. At micromolar or higher concentrations, analogue **1** appeared to have more signaling activity at CCR-3. The mechanism by which chemokines bind and activate more than one receptor is not well understood, although there is evidence that CCL-5 interacts with each of its receptors (CCR-1, CCR-3, and CCR-5) in a distinct way.[5] It appears the improved selectivity of analogues **1**, **6**, and **7** with respect to CCL-5 is a result of hydrophobic substitutions made at the N-terminus in combination with the introduction of a polymeric substituent.

We have found that in vitro anti-HIV activity of polymer-modified CCL-5 derivatives correlates closely with CCR-5 signaling; elimination of CCR-5 signaling leads to an abolish-

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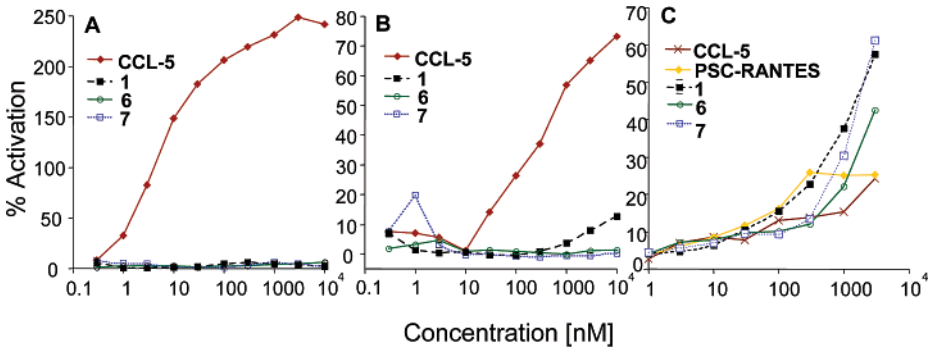

Figure 3. Comparative GPCR receptor activation of CCL-5 and analogues **1**, **6**, and **7**: (A) CCR-1, (B) CCR-3, and (C) CCR-5.

ment of anti-HIV activity. In vitro assaying against HIV-1 clinical isolate 92/US/712 with the PSC-RANTES, the most potent CCL-5 based anti-HIV protein reported to date, gave an IC_{50} of 0.5 nM, whereas the homodimeric CCL-5 compounds **6** and **7** were found to have comparable anti-HIV IC_{50} values of 0.76 and 0.80 nM, respectively. The specific attachment of the polymer substituents at lysinyl[45] in **6** and **7** apparently does not significantly disrupt CCR-5 interactions, and thus allowed for retention of potent sub-nanomolar anti-HIV activity. For comparison to T-20, a reported and clinically relevant anti-HIV fusion inhibitor,[11,35] the cell potencies of **6** and **7** against HIV-1 clinical isolate 92/US/712 are approximately 20-fold more potent than T-20 (cell-based anti-HIV IC_{50}, 13 nM). It should be noted that the mechanism of action for T-20 and the CCL-5 analogues reported here are different, and the respective mechanisms may be complementary for the treatment of resistant HIV strains. Pharmacokinetic analysis indicates the clearance in rats after bolus iv injection (1 mg/kg) of polymer-conjugated analogue **6** (0.10 L/hr/kg) and **7** (0.07 L/hr/kg) was significantly reduced in comparison to PSC-RANTES (0.76 L/hr/kg). The difference in clearance is attributed to the presence of the 16 and 20 kDa polymeric substituents on **6** and **7**, respectively, increasing their hydrodynamic radius.

Conclusions

When studying relatively large macromolecular structures and interactions, the inherent complexities of the system are difficult to fully understand and predict. As we have found in this work, relatively small changes in large macromolecular structures can lead to surprising and unexpected properties and underscore the value of precise synthesis methodologies for the preparation and study of such systems. Medicinal chemistry strategies can be successfully applied to proteins, including those bearing large polymeric substituents, via total chemical synthesis. Although protein chemical synthesis is not applicable in all cases, the benefits of being able to precisely access specific small protein structures outside of the reach of conventional approaches are evident. Here, we have reported that protein chemical synthesis has been exclusively and successfully applied to the multifaceted pharmaceutical optimization of a small anti-HIV protein, simultaneously addressing and balancing potency retention, receptor selectivity, aggregation, and pharmacokinetic issues and, in so doing, possibly increasing the therapeutic index of these novel anti-HIV protein derivatives. More specifically, two highly potent (sub-nanomolar) anti-HIV polymer-conjugated CCL-5 analogues, **6** and **7**, with significantly improved CCR-5 selectivity over CCR-1 and CCR-3, increased in vivo circulation, and well-defined homodimeric structure have been identified. In view of the ongoing problem of resistance with the treatment of HIV infection and persistance,[10] CCL-5 analogue **6** and **7** provide a novel mechanism of action for the development of a new generation of anti-HIV therapeutics which are still desperately needed. In general, this work illustrates the increasing value of protein chemical synthesis in contemporary therapeutic discovery and serves to encourage the exploration of more chemical protein architectural space to improve our understanding of protein function, design, and construction across many disciplines.

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Supporting Information Available: Complete ref 13, 19, and 25; the chemical structure of the N-terminus of PSC-RANTES and the 16 kDa branched polymer; and the circular dichroism (CD) spectra of compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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