JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
pubs.acs.org/JACS
Communication

Discovery of Potent Cyclic Sulfopeptide Chemokine Inhibitors via Reprogrammed Genetic Code mRNA Display

Jason Johansen-Leete,[▽] Toby Passioura,[▽] Simon R. Foster, Ram Prasad Bhusal, Daniel J. Ford, Minglong Liu, Seino A. K. Jongkees, Hiroaki Suga, Martin J. Stone, and Richard J. Payne*

Cite This: J. Am. Chem. Soc. 2020, 142, 9141−9146

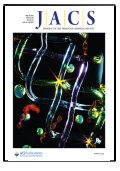
ABSTRACT: Targeting chemokine signaling is an attractive avenue for the treatment of inflammatory disorders. Tyrosine sulfation is an important post-translational modification (PTM) that enhances chemokine−receptor binding and is also utilized by a number of pathogenic organisms to improve the binding affinity of immune-suppressive chemokine binding proteins (CKBPs). Here we report the display selection of tyrosine-sulfated cyclic peptides using a reprogrammed genetic code to discover high-affinity ligands for the chemokine CCL11 (eotaxin-1). The selected cyclic sulfopeptides possess high affinity for the target chemokine (as well as one or more of the related family members CCL2, CCL7 and CCL24) and inhibit CCL11 activation of CC chemokine receptor 3 (CCR3). This work demonstrates the utility of exploiting native PTMs as binding motifs for the generation of new leads for medicinal chemistry.

Chemokines are a family of small cytokines (∼8−14 kDa) that direct the trafficking of leukocytes during inflammation by activating G protein-coupled receptors on leukocyte surfaces. Specifically, leukocytes are recruited to the site of inflammation (in a process called chemotaxis) by following a concentration gradient of secreted chemokines.[1] In view of the crucial role that chemokines play in orchestrating the inflammatory response, modulation of chemokine signaling is a promising approach for the treatment of inflammatory disorders.[2−5] A key example is the chemokine CCL11 (eotaxin-1), which functions as the primary chemoattractant for eosinophils by activating the receptor CCR3. Targeting the CCL11−CCR3 axis is of particular interest for treatment of allergic asthma and eosinophilia.[6−8] Most strategies for therapeutic intervention of the chemokine system have focused on blockade of chemokine receptors. However, targeting chemokines is an attractive avenue for modulation of inflammation, particularly in cases where one chemokine signals through multiple receptors.[9]

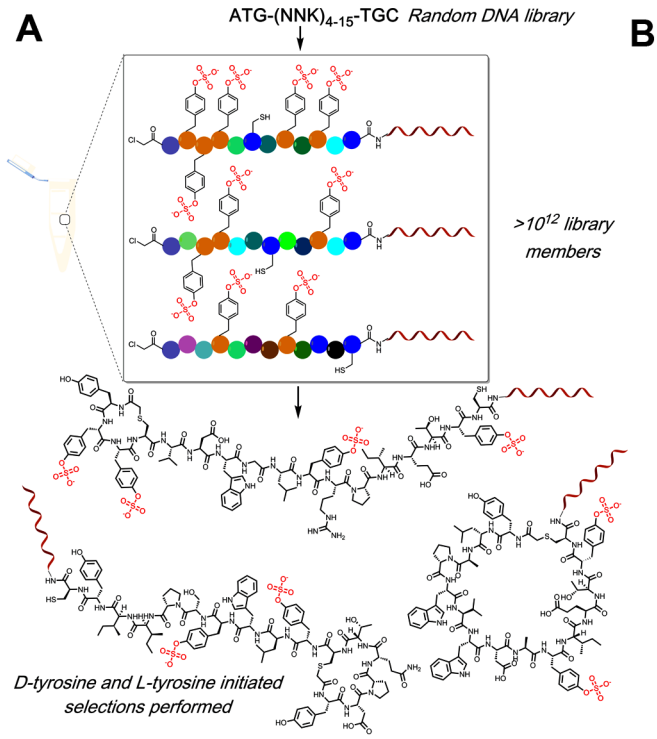
The utility of targeting chemokines to modulate inflammation is exemplified by a number of chemokine binding proteins (CKBPs) produced by pathogenic organisms to evade the host immune response. Key examples include the evasins, a family of tick salivary proteins each of which binds to several chemokines and possesses potent anti-inflammatory activity in vivo, as well as CKBPs produced by mammalian viruses.[10−13] Activation of chemokine receptors by chemokines is strongly modulated by tyrosine sulfation near the receptor N-terminus; the modification is known to significantly increase chemokine binding affinity and modulate selectivity.[14−17] Accordingly, CKBPs can also be sulfated to improve chemokine binding affinity. For example, tyrosine sulfation of the human cytomegalovirus protein UL22A resulted in a more than 300-fold increase in affinity for the chemokine CCL5 (RANTES).[18]

The relatively featureless morphology of the surface of chemokines has hindered the development of potent small-molecule inhibitors. Indeed, only compounds with micromolar affinity have been developed to date.[19,20] Given that macrocyclic peptides have been shown to be effective in disrupting protein−protein interactions and targeting proteins without defined small-molecule binding sites,[21−29] we sought to utilize this chemotype for the development of chemokine inhibitors. Specifically, we chose to use Random Nonstandard Peptide Integrated Discovery (RaPID) mRNA display technology to generate >10^{12} macrocyclic peptides that could be selected against a chemokine of choice, with a view to discovering novel leads for the treatment of inflammatory disorders (Figure 1A).[30] Importantly, the RaPID system allows genetic code reprogramming to incorporate noncanonical residues into the peptide library.[31,32] Commonly, this involves reassignment of the initiating codon to incorporate an N-chloroacetylated amino acid, thus facilitating spontaneous cyclization with the thiol side chain of a downstream cysteine residue to afford macrocyclic thioether scaffolds. Because of the importance of tyrosine sulfation for the binding and recognition of chemokines by native receptors and CKBPs, we hypothesized that incorporation of sulfotyrosine (sTyr) into the peptide library would afford privileged chemokine binders capable of inhibiting signaling. Herein we report the incorporation of the sTyr PTM via ribosomal translation together with RaPID screening to generate sulfated macrocyclic inhibitors of CCL11

Received: March 28, 2020
Published: April 24, 2020



https://dx.doi.org/10.1021/jacs.0c03152
J. Am. Chem. Soc. 2020, 142, 9141−9146



B

Peptide	Sequence
1	Y sTyr C P V N I Q W P W W V S
2	Y sTyr C P V I V W D Y P G W W S S
3	Y W L A F R W Q H sTyr A I V V Y C
4	Y L A P W V W D A sTyr I E T sTyr C
5	Y Q L R sTyr C P sTyr sTyr P I I I W T S
6	Y D P Q T C sTyr L W sTyr S P I I Y S
7	Y A H L C L F sTyr I L sTyr A P F sTyr S
8	Y sTyr C P V T I W
9	Y sTyr C P Y Q S Y Y W P Y L S Y P S
10	y sTyr Y C T D W W L sTyr R P R P L I S
11	y sTyr sTyr C V D W G L sTyr R P I E T sTyr S
12	y sTyr C sTyr sTyr A P V I L L sTyr A D sTyr S
13	y sTyr L C S W Y sTyr A P I T F Y S S
14	y sTyr C sTyr sTyr A P V
15	y H R sTyr Y C sTyr L W sTyr S P V T L Y S

Figure 1. (A) The RaPID system was used to translate a random DNA library (>10^{12} library members, 6−17 residues), affording a large peptide library whose members were selected for binding affinity against CCL11. Selections initiated with either *N*-chloroacetyl-L-tyrosine or *N*-chloroacetyl-D-tyrosine were performed because of the tendency of D-amino acids to alter peptide conformation, affording additional structural diversity in the library. No preference for sTyr position was defined in the library. (B) Fifteen peptide sequences were selected for synthesis and evaluation, nine initiated with *N*-chloroacetyl-L-tyrosine (**1**−**9**) and six initiated with *N*-chloroacetyl-D-tyrosine (**10**−**15**). NB: Peptides bearing C-terminal S residues are C-to-S mutants of the originally selected peptides; **8** and **14** are truncated variants of selected sequences.

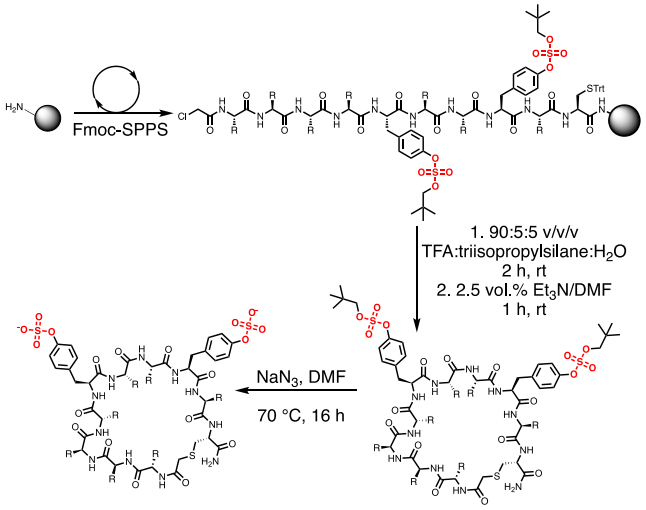
to guide future medicinal chemistry efforts targeting allergic inflammation. In a parallel set of selections (also against CCL11), we also incorporated the hydrolytically stable sulfonate mimic of sTyr, Phe(p-$CH_2SO_3^-$), as the stability of sTyr (which is known to be acid-labile[33−35]) during the selection process was uncertain.

We began with the synthesis of aminoacyl donors of sTyr and Phe(p-$CH_2SO_3^-$) derivatized as 3,5-dinitrobenzyl esters (DBEs) and cyanomethyl esters (CMEs), respectively (see the Supporting Information). These were subsequently amino-acylated onto synthetic tRNA using the corresponding flexizymes. We next assessed the translation fidelity in a flexible in vitro translation (FIT) reaction, whereby internal methionine residues were replaced with either sTyr or Phe(p-$CH_2SO_3^-$). Both amino acids were successfully incorporated and translated into a model peptide template (see the Supporting Information), and we therefore moved to performing RaPID selections under this reprogrammed genetic code. For selection, a semirandom DNA library was transcribed into mRNA, followed by covalent ligation to puromycin and translation in a genetically reprogrammed reaction to yield a cyclic peptide−mRNA fusion library in excess of 10^{12} unique molecules. Following counterselection (to remove streptavidin ligands), panning against biotinylated CCL11 immobilized on streptavidin beads was used to enrich for CCL11 affinity, and an enriched DNA library was subsequently recovered by RT-PCR. After five iterations, the final DNA library was sequenced to identify peptide ligands predicted to bind to CCL11 with high affinity (see the Supporting Information). We performed parallel selections incorporating either sTyr or Phe(p-$CH_2SO_3^-$), each initiated by either *N*-chloroacetyl-L-tyrosine or *N*-chloroacetyl-D-tyrosine, which yielded a variety of peptide sequences predicted to be high-affinity binders. We chose 15 peptides, nine L-initiated (**1**−**9**) and six D-initiated (**10**−**15**), from the selected pools for further evaluation (Figure 1B).

The 15 modified cyclic peptides were next synthesized by Fmoc-strategy solid-phase peptide synthesis (SPPS). Since the sulfonate analogue was utilized as a mimic of the native sTyr PTM for the purpose of RaPID selection, we synthesized the initial hits bearing native sTyr residues regardless of the selection from which they were obtained. Peptides were synthesized on Rink amide resin, cleaved, cyclized, and purified by HPLC (Scheme 1). The sTyr residues were incorporated using the neopentyl sulfate ester building block Fmoc-Tyr[$OSO_3CH_2C(CH_3)_3$]-OH, which enabled the sTyr residue to survive the acidic cleavage step.[36] The binding affinities of the peptides for CCL11 were assessed next using a competitive fluorescence anisotropy assay with the fluorescently labeled CCR3 N-terminal sulfopeptide Fl-R3D (see the Supporting Information).[37] Of the 15 hits identified, four peptides—two L-initiated (**4** and **6**) and two D-initiated (**11** and **12**)—exhibited exceptional affinity for CCL11, with K_d < 30 nM (see Figure 2 and Table 1 for data). We also assessed the binding affinities of unsulfated variants of peptides **4**, **6**, **11**, and **12**, which demonstrated the critical role of the sTyr modification for binding affinity to CCL11 (see the Supporting Information). Specifically, none of the unsulfated peptides showed binding to CCL11 at a concentration of 1 μM (cf. K_d = 10.4−26.6 nM for the sulfated homologues). Given that **4** and **11** were discovered through the Phe(p-$CH_2SO_3^-$) selection, we also

https://dx.doi.org/10.1021/jacs.0c03152
J. Am. Chem. Soc. 2020, 142, 9141−9146

Scheme 1. General Synthetic Scheme for the Preparation of Sulfated Cyclic Peptides via Fmoc-SPPS; The Example Shown is for a Head-to-Tail Cyclic Peptide with a C-Terminal Cys Residue (see Table 1)



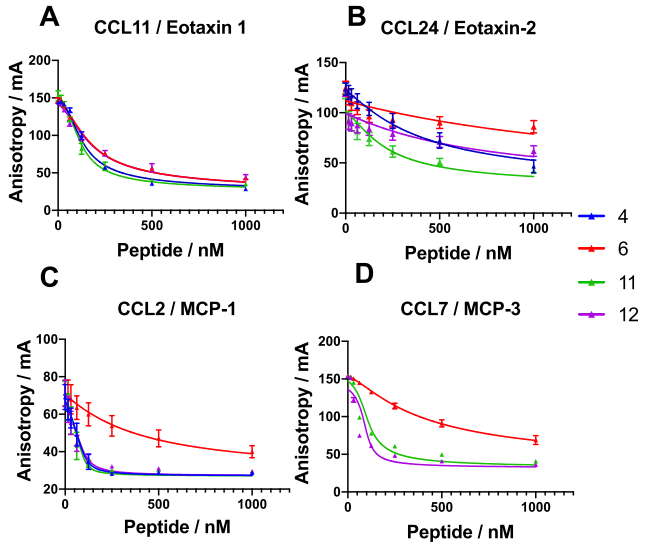


Figure 2. Fluorescence anisotropy competitive binding curves for peptides **4**, **6**, **11**, and **12** against a panel of chemokines: (A) CCL11, (B) CCL24, (C) CCL2, and (D) CCL7. Experiments used 10 nM fluorescent-receptor-derived peptide (Fl-R3D for CCL11 and Fl-R2D for CCL24, CCL2, and CCL7) and 100 nM chemokine.[37] Under these conditions, the concentrations for half-maximal inhibition do not necessary correspond to the K_d values.[39] Data are reported as mean ± SEM (n = 3, performed in duplicate). For **4**, no binding was observed at <250 nM against CCL7.

synthesized and assessed the sulfonated variants of these molecules. These were less potent binders of CCL11 (2−4-fold lower affinity) than **4** and **11** bearing sTyr, likely because of a significant difference in electronics of the sulfate moiety versus the sulfonate moiety[38] (see the Supporting Information).

Redundancy in the chemokine system provides a significant obstacle for the development of therapeutic modulators of chemokine signaling.[40] Natural CKBPs often circumvent this by binding to multiple chemokines, which signal via a shared receptor.[41] As our peptides were inspired by naturally sulfated CKBPs and receptor peptides, which can bind multiple chemokines, we next investigated whether our lead peptides could bind to other CCR3-activating chemokines, including CCL24 (eotaxin-2), CCL2 (MCP-1), and CCL7 (MCP-3) (Figure 2 and Table 1). The different sulfated cyclic peptides exhibited varied selectivity profiles for the chemokines tested. Peptide **6** was highly selective for CCL11 over CCL24 and CCL2 with modest selectivity (2-fold) over CCL7. Peptide **11** showed a broader binding profile, with moderate selectivity over CCL24 but an increase in potency against CCL2 and CCL7 compared with CCL11. Similarly, **12** was significantly less potent against CCL24 but exhibited an order of magnitude increase in binding affinity for CCL2 and CCL7 relative to CCL11. These results indicate that the RaPID platform, with the incorporation of sTyr, can yield molecules with high affinities for multiple chemokines. These selectivity profiles for the cyclic peptides were not surprising given the substantial sequence similarity among the family of chemokines and because we chose to perform selections that were not biased toward CCL11-specific binders, i.e., we did not perform counterselections with other chemokines. It should be noted that molecules that bind to several chemokines within a family (as generated here) have been shown to be more advantageous for therapeutic applications, as exemplified by the pan-chemokine binding properties of natural CKBPs (vide supra).[41] Indeed, the redundancy within the chemokine-receptor system has been recognized as a contributing factor for the failure of selective chemokine binding and receptor antagonist molecules to reach the clinic.[42]

Having identified peptides with high affinity, we next investigated their ability to inhibit CCL11 signaling via the chemokine receptor CCR3. This involved measuring the ability of the peptides to prevent CCL11-induced inhibition of cAMP production in CCR3-expressing human embryonic kidney (HEK) cells.[39,43] Peptides **4**, **6**, and **11** were all capable of inhibiting chemokine signaling, with the most potent peptide, **11**, inhibiting receptor activation with an IC_{50} of 160 nM (Figure 3 and Table 2). On the other hand, peptide **12** was ineffective at CCL11 inhibition at concentrations as high at 1 μM despite the fact that it binds to CCL11 with a K_d of 27 nM, i.e., peptide **12** blocks binding of CCL11 to Fl-R3D but does not prevent CCL11 from binding to native CCR3 on the cell surface. This result is likely due to differences in the structural and/or dynamic features of CCL11 binding to the FL-R3D-

Table 1. K_d Values for Lead Peptides against CCL11 and Other CCR3-Activating Chemokines CCL24, CCL2, and CCL7[a]

peptide	CCL11	CCL24	CCL2	CCL7
4	13.7 [7.86 ± 0.05]	120 [6.92 ± 0.08]	4.72 [8.33 ± 0.32]	ND
6	25.2 [7.60 ± 0.04]	>200	>200	59.8 [7.22 ± 0.03]
11	10.4 [7.98 ± 0.06]	53.0 [7.28 ± 0.14]	2.89 [8.54 ± 0.11]	5.04 [8.30 ± 0.11]
12	26.6 [7.58 ± 0.065]	>200	6.53 [8.19 ± 0.42]	1.96 [8.71 ± 0.24]

[a]Data are reported as K_d/nM [mean pK_d ± SEM] (n = 3, performed in duplicate). Positive control: ACA-01 evasin (K_d = 188 nM).[39]

https://dx.doi.org/10.1021/jacs.0c03152
J. Am. Chem. Soc. 2020, 142, 9141−9146

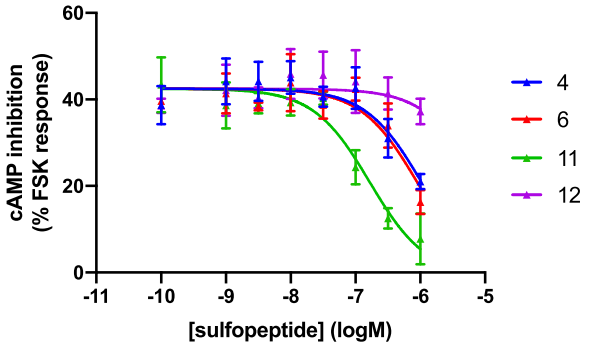


Figure 3. Concentration–response curves for inhibition of CCL11 (100 nM) signaling via receptor CCR3 in HEK cells by **4**, **6**, **11**, and **12**. Receptor activation was measured using a BRET sensor to detect forskolin (FSK)-induced production of cAMP. Data points are mean ± SEM (n = 3, performed in duplicate).

Table 2. IC_{50} Values of 4, 6, 11, and 12 for Inhibition of CCL11 Signaling via CCR3

peptide	IC_{50}/nM [pIC_{50} ± SEM][a]
4	1000 [5.98 ± 0.17]
6	900 [6.05 ± 0.17]
11	160 [6.78 ± 0.19]
12	ND

[a] n = 3, performed in duplicate. ND = negligible inhibition observed for concentrations at 1 μM. Positive control: ACA-01 evasin (IC_{50} = 1.9 μM).[39] Negative control: cyclic sulfopeptide **3** (Figure 1B), which showed no inhibition at a concentration of 500 nM.

receptor-derived peptide compared with the full-length receptor.[44,45] This result reinforces the importance of investigating receptor signaling in addition to chemokine binding for these molecules.

Finally, we assessed the plasma stabilities of peptides **4**, **6**, and **11**. Interestingly, despite the different topologies, each was resistant to proteolytic degradation in human plasma over 90 min (see the Supporting Information for data). This suggests that these molecules could also find application in vivo.

In summary, we have successfully incorporated sTyr into RaPID selections against the chemokine CCL11 via flexizyme-mediated genetic code reprogramming. While sTyr has been incorporated into proteins through amber suppression technologies,[33,46,47] to our knowledge this is the first reported incorporation of any native eukaryotic PTM into an mRNA display selection. High-affinity chemokine binders were generated that inhibited CCL11 signaling through the cognate receptor CCR3. Chemokine binders with varied selectivity profiles were discovered, including pan-CCR3 signaling chemokine binders that mimic the activity of natural CKBPs. This work highlights the utility of native PTMs to mimic natural protein binding interactions. The potent chemokine inhibitors that we have discovered in this work now serve as starting points for the development of new lead compounds targeting inflammatory disorders.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c03152.

Experimental procedures, additional data, and NMR spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Richard J. Payne − *School of Chemistry and Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Sydney, Sydney, NSW 2006, Australia;* orcid.org/0000-0002-3618-9226; Email: richard.payne@sydney.edu.au

Authors

Jason Johansen-Leete − *School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia*

Toby Passioura − *School of Chemistry, School of Life and Environmental Sciences, and Sydney Analytical, The University of Sydney, Sydney, NSW 2006, Australia; Department of Chemistry, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan;* orcid.org/0000-0002-6089-5067

Simon R. Foster − *Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia*

Ram Prasad Bhusal − *Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia*

Daniel J. Ford − *School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia*

Minglong Liu − *Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, The Netherlands*

Seino A. K. Jongkees − *Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, 3584 CG Utrecht, The Netherlands;* orcid.org/0000-0002-4796-0557

Hiroaki Suga − *Department of Chemistry, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan;* orcid.org/0000-0002-5298-9186

Martin J. Stone − *Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia;* orcid.org/0000-0002-6468-4427

Complete contact information is available at: https://pubs.acs.org/10.1021/jacs.0c03152

Author Contributions

[▽] J.J.-L. and T.P. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge funding from the National Health and Medical Research Council (Investigator Grant APP1174941, Project Grant APP1140867 to M.J.S. and R.J.P. and APP1140874 to M.J.S.) and the Horizon 2020 Research and Innovation Programme under Marie Skłodowska-Curie Grant Agreement 746631 (to S.A.K.J.).

■ REFERENCES

(1) Griffith, J. W.; Sokol, C. L.; Luster, A. D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* **2014**, *32*, 659–702.

(2) Schall, T. J.; Proudfoot, A. E. I. Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat. Rev. Immunol.* **2011**, *11*, 355–363.

(3) Viola, A.; Luster, A. D. Chemokines and their receptors: drug targets in immunity and inflammation. *Annu. Rev. Pharmacol. Toxicol.* **2008**, *48*, 171–197.

(4) Schwarz, M. K.; Wells, T. N. New therapeutics that modulate chemokine networks. *Nat. Rev. Drug Discovery* **2002**, *1*, 347−358.
(5) Proudfoot, A. E. I.; Power, C. A.; Schwarz, M. K. Anti-chemokine small molecule drugs: a promising future? *Expert Opin. Invest. Drugs* **2010**, *19*, 345−355.
(6) Fulkerson, P. C.; Rothenberg, M. E. Targeting eosinophils in allergy, inflammation and beyond. *Nat. Rev. Drug Discovery* **2013**, *12*, 117−129.
(7) Ying, S.; Meng, Q.; Zeibecoglou, K.; Robinson, D. S.; Macfarlane, A.; Humbert, M.; Kay, A. B. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (intrinsic) asthmatics. *J. Immunol.* **1999**, *163*, 6321−6329.
(8) Garcia, G.; Godot, V.; Humbert, M. New chemokine targets for asthma therapy. *Curr. Allergy Asthma Rep.* **2005**, *5*, 155−160.
(9) Déruaz, M.; Frauenschuh, A.; Alessandri, A. L.; Dias, J. M.; Coelho, F. M.; Russo, R. C.; Ferreira, B. R.; Graham, G. J.; Shaw, J. P.; Wells, T. N. C.; Teixeira, M. M.; Power, C. A.; Proudfoot, A. E. I. Ticks produce highly selective chemokine binding proteins with antiinflammatory activity. *J. Exp. Med.* **2008**, *205*, 2019−2031.
(10) Montecucco, F.; Lenglet, S.; Braunersreuther, V.; Pelli, G.; Pellieux, C.; Montessuit, C.; Lerch, R.; Deruaz, M.; Proudfoot, A. E.; Mach, F. Single administration of the CXC chemokine-binding protein Evasin-3 during ischemia prevents myocardial reperfusion injury in mice. *Arterioscler., Thromb., Vasc. Biol.* **2010**, *30*, 1371−1377.
(11) Montecucco, F.; Mach, F.; Lenglet, S.; Vonlaufen, A.; Gomes Quinderé, A. L.; Pelli, G.; Burger, F.; Galan, K.; Dallegri, F.; Carbone, F.; Proudfoot, A. E.; Vuilleumier, N.; Frossard, J.-L. Treatment with Evasin-3 abrogates neutrophil-mediated inflammation in mouse acute pancreatitis. *Eur. J. Clin. Invest.* **2014**, *44*, 940−950.
(12) Lucas, A.; McFadden, G. Secreted immunomodulatory viral proteins as novel biotherapeutics. *J. Immunol.* **2004**, *173*, 4765−4774.
(13) Alcami, A. Viral mimicry of cytokines, chemokines and their receptors. *Nat. Rev. Immunol.* **2003**, *3*, 36−50.
(14) Ludeman, J. P.; Stone, M. J. The structural role of receptor tyrosine sulfation in chemokine recognition. *Br. J. Pharmacol.* **2014**, *171*, 1167−1179.
(15) Stone, M. J.; Payne, R. J. Homogeneous sulfopeptides and sulfoproteins: synthetic approaches and applications to characterize the effects of tyrosine sulfation on biochemical function. *Acc. Chem. Res.* **2015**, *48*, 2251−61.
(16) Colvin, R. A.; Campanella, G. S.; Manice, L. A.; Luster, A. D. CXCR3 requires tyrosine sulfation for ligand binding and a second extracellular loop arginine residue for ligand-induced chemotaxis. *Mol. Cell. Biol.* **2006**, *26*, 5838−5849.
(17) Choe, H.; Moore, M. J.; Owens, C. M.; Wright, P. L.; Vasilieva, N.; Li, W.; Singh, A. P.; Shakri, R.; Chitnis, C. E.; Farzan, M. Sulphated tyrosines mediate association of chemokines and Plasmodium vivax Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). *Mol. Microbiol.* **2005**, *55*, 1413−1422.
(18) Wang, X.; Sanchez, J.; Stone, M. J.; Payne, R. J. Sulfation of the Human Cytomegalovirus Protein UL22A Enhances Binding to the Chemokine RANTES. *Angew. Chem., Int. Ed.* **2017**, *56*, 8490−8494.
(19) Veldkamp, C. T.; Ziarek, J. J.; Peterson, F. C.; Chen, Y.; Volkman, B. F. Targeting SDF-1/CXCL12 with a ligand that prevents activation of CXCR4 through structure-based drug design. *J. Am. Chem. Soc.* **2010**, *132*, 7242−7243.
(20) Ziarek, J. J.; Liu, Y.; Smith, E.; Zhang, G.; Peterson, F. C.; Chen, J.; Yu, Y.; Chen, Y.; Volkman, B. F.; Li, R. Fragment-based optimization of small molecule CXCL12 inhibitors for antagonizing the CXCL12/CXCR4 interaction. *Curr. Top. Med. Chem.* **2012**, *12*, 2727−2740.
(21) Driggers, E. M.; Hale, S. P.; Lee, J.; Terrett, N. K. The exploration of macrocycles for drug discovery-an underexploited structural class. *Nat. Rev. Drug Discovery* **2008**, *7*, 608−624.
(22) Yudin, A. K. Macrocycles: lessons from the distant past, recent developments, and future directions. *Chem. Sci.* **2015**, *6*, 30−49.
(23) Miranda, E.; Nordgren, I. K.; Male, A. L.; Lawrence, C. E.; Hoakwie, F.; Cuda, F.; Court, W.; Fox, K. R.; Townsend, P. A.; Packham, G. K.; Eccles, S. A.; Tavassoli, A. A cyclic peptide inhibitor of HIF-1 heterodimerization that inhibits hypoxia signaling in cancer cells. *J. Am. Chem. Soc.* **2013**, *135*, 10418−10425.
(24) Angelini, A.; Cendron, L.; Chen, S.; Touati, J.; Winter, G.; Zanotti, G.; Heinis, C. Bicyclic peptide inhibitor reveals large contact interface with a protease target. *ACS Chem. Biol.* **2012**, *7*, 817−821.
(25) Heinis, C. Drug discovery: tools and rules for macrocycles. *Nat. Chem. Biol.* **2014**, *10*, 696−698.
(26) Qian, Z.; Dougherty, P. G.; Pei, D. Targeting intracellular protein-protein interactions with cell-permeable cyclic peptides. *Curr. Opin. Chem. Biol.* **2017**, *38*, 80−86.
(27) Gao, M.; Cheng, K.; Yin, H. Targeting protein-protein interfaces using macrocyclic peptides. *Biopolymers* **2015**, *104*, 310−316.
(28) Nawatha, M.; Rogers, J. M.; Bonn, S. M.; Livneh, I.; Lemma, B.; Mali, S. M.; Vamisetti, G. B.; Sun, H.; Bercovich, B.; Huang, Y.; Ciechanover, A.; Fushman, D.; Suga, H.; Brik, A. De novo macrocyclic peptides that specifically modulate Lys48-linked ubiquitin chains. *Nat. Chem.* **2019**, *11*, 644−652.
(29) Yamagishi, Y.; Shoji, I.; Miyagawa, S.; Kawakami, T.; Katoh, T.; Goto, Y.; Suga, H. Natural product-like macrocyclic N-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed de novo library. *Chem. Biol.* **2011**, *18*, 1562−1570.
(30) Passioura, T.; Suga, H. A RaPID way to discover nonstandard macrocyclic peptide modulators of drug targets. *Chem. Commun.* **2017**, *53*, 1931−1940.
(31) Passioura, T.; Suga, H. Flexizyme-mediated genetic reprogramming as a tool for noncanonical peptide synthesis and drug discovery. *Chem. - Eur. J.* **2013**, *19*, 6530−6536.
(32) Morimoto, J.; Hayashi, Y.; Suga, H. Discovery of macrocyclic peptides armed with a mechanism-based warhead: isoform-selective inhibition of human deacetylase SIRT2. *Angew. Chem., Int. Ed.* **2012**, *51*, 3423−3427.
(33) Watson, E. E.; Liu, X.; Thompson, R. E.; Ripoll-Rozada, J.; Wu, M.; Alwis, I.; Gori, A.; Loh, C.-T.; Parker, B. L.; Otting, G.; Jackson, S.; Pereira, P. J. B.; Payne, R. J. Mosquito-derived anophelin sulfoproteins are potent antithrombotics. *ACS Cent. Sci.* **2018**, *4*, 468−476.
(34) Thompson, R. E.; Liu, X.; Ripoll-Rozada, J.; Alonso-Garcia, N.; Parker, B. L.; Pereira, P. J. B.; Payne, R. J. Tyrosine sulfation modulates activity of tick-derived thrombin inhibitors. *Nat. Chem.* **2017**, *9*, 909−917.
(35) Watson, E. E.; Ripoll-Rozada, J.; Lee, A. C.; Wu, M. C. L.; Franck, C.; Pasch, T.; Premdjee, B.; Sayers, J.; Pinto, M. F.; Martins, P. M.; Jackson, S. P.; Pereira, P. J. B.; Payne, R. J. Rapid assembly and profiling of an anticoagulant sulfoprotein library. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 13873−13878.
(36) Simpson, L. S.; Widlanski, T. S. A comprehensive approach to the synthesis of sulfate esters. *J. Am. Chem. Soc.* **2006**, *128*, 1605−1610.
(37) Ludeman, J. P.; Nazari-Robati, M.; Wilkinson, B. L.; Huang, C.; Payne, R. J.; Stone, M. J. Phosphate modulates receptor sulfotyrosine recognition by the chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2). *Org. Biomol. Chem.* **2015**, *13*, 2162−2169.
(38) Lam, S. N.; Acharya, P.; Wyatt, R.; Kwong, P. D.; Bewley, C. A. Tyrosine-sulfate isosteres of CCR5 N-terminus as tools for studying HIV-1 entry. *Bioorg. Med. Chem.* **2008**, *16*, 10113−10120.
(39) Hayward, J.; Sanchez, J.; Perry, A.; Huang, C.; Rodriguez Valle, M.; Canals, M.; Payne, R. J.; Stone, M. J. Ticks from diverse genera encode chemokine-inhibitory evasin proteins. *J. Biol. Chem.* **2017**, *292*, 15670−15680.
(40) Dyer, D. P.; Medina-Ruiz, L.; Bartolini, R.; Schuette, F.; Hughes, C. E.; Pallas, K.; Vidler, F.; Macleod, M. K. L.; Kelly, C. J.; Lee, K. M.; Hansell, C. A. H.; Graham, G. J. Chemokine receptor redundancy and specificity are context dependent. *Immunity* **2019**, *50*, 378−389.

https://dx.doi.org/10.1021/jacs.0c03152
J. Am. Chem. Soc. 2020, 142, 9141−9146

(41) Bonvin, P.; Power, C. A.; Proudfoot, A. E. I. Evasins: Therapeutic potential of a new family of chemokine-binding proteins from ticks. *Front. Immunol.* **2016**, *7*, 208.
(42) Horuk, R. Chemokine receptor antagonists: overcoming developmental hurdles. *Nat. Rev. Drug Discovery* **2009**, *8*, 23−33.
(43) Huma, Z. E.; Sanchez, J.; Lim, H. D.; Bridgford, J. L.; Huang, C.; Parker, B. J.; Pazhamalil, J. G.; Porebski, B. T.; Pfleger, K. D.; Lane, J. R.; Canals, M.; Stone, M. J. Key determinants of selective binding and activation by the monocyte chemoattractant proteins at the chemokine receptor CCR2. *Sci. Signaling* **2017**, *10*, No. eaai8529.
(44) Millard, C. J.; Ludeman, J. P.; Canals, M.; Bridgford, J. L.; Hinds, M. G.; Clayton, D. J.; Christopoulos, A.; Payne, R. J.; Stone, M. J. Structural basis of receptor sulfotyrosine recognition by a CC chemokine: the N-terminal region of CCR3 bound to CCL11/eotaxin-1. *Structure* **2014**, *22*, 1571−1581.
(45) Bhusal, R. P.; Foster, S. R.; Stone, M. J. Structural basis of chemokine and receptor interactions: Key regulators of leukocyte recruitment in inflammatory responses. *Protein Sci.* **2020**, *29*, 420−432.
(46) Liu, C. C.; Brustad, E.; Liu, W.; Schultz, P. G. Crystal structure of a biosynthetic sulfo-hirudin complexed to thrombin. *J. Am. Chem. Soc.* **2007**, *129*, 10648−10649.
(47) Liu, C. C.; Schultz, P. G. Recombinant expression of selectively sulfated proteins in Escherichia coli. *Nat. Biotechnol.* **2006**, *24*, 1436.

https://dx.doi.org/10.1021/jacs.0c03152
J. Am. Chem. Soc. 2020, 142, 9141−9146