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Direct Interaction between an Allosteric Agonist Pepducin and the Chemokine Receptor CXCR4

Jay M. Janz,[*,†] Yong Ren,[†] Richard Looby,[†] Manija A. Kazmi,[‡] Pallavi Sachdev,[‡,∥] Amy Grunbeck,[†] Lynn Haggis,[†] Daniel Chinnapen,[†] Amy Ying Lin,[‡] Christoph Seibert,[‡] Thomas McMurry,[†] Kenneth E. Carlson,[†] Tom W. Muir,[§,⊥] Stephen Hunt III,[†] and Thomas P. Sakmar[*,‡]

[†]Anchor Therapeutics, Cambridge, Massachusetts 02142, United States

[‡]Laboratory of Molecular Biology and Biochemistry and [§]Laboratory of Synthetic Protein Chemistry, The Rockefeller University, New York, New York 10065, United States

Supporting Information

ABSTRACT: Cell surface heptahelical G protein-coupled receptors (GPCRs) mediate critical cellular signaling pathways and are important pharmaceutical drug targets.[1] In addition to traditional small-molecule approaches, lipopeptide-based GPCR-derived pepducins have emerged as a new class of pharmaceutical agents.[2,3] To better understand how pepducins interact with targeted receptors, we developed a cell-based photo-cross-linking approach to study the interaction between the pepducin agonist ATI-2341 and its target receptor, chemokine C-X-C-type receptor 4 (CXCR4). A pepducin analogue, ATI-2766, formed a specific UV-light-dependent cross-link to CXCR4 and to mutants with truncations of the N-terminus, the known chemokine docking site. These results demonstrate that CXCR4 is the direct binding target of ATI-2341 and suggest a new mechanism for allosteric modulation of GPCR activity. Adaptation and application of our findings should prove useful in further understanding pepducin modulation of GPCRs as well as enable new experimental approaches to better understand GPCR signal transduction.

The C-X-C chemokine receptor 4 (CXCR4) mediates directed cell migration and is involved in diverse processes including development, inflammatory disease, HIV infection, and cancer metastasis.[4] CXCR4 and its agonist ligand, CXCL12, regulate the retention of polymorphonuclear and hematopoietic stem cell (HSC) and progenitor cells in the bone marrow niche.[5] The small-molecule CXCR4 antagonist AMD3100 is effective in interfering with this retention process and is currently in use as a therapeutic agent to mobilize HSCs prior to autologous bone marrow transplantation.[6] We recently reported the discovery and characterization of a new class of CXCR4 agonists that also mobilize bone marrow hematopoietic cells.[7] These novel CXCR4-specific agonists are members of a class of pharmacological agents called pepducins.[2] A pepducin is a lipid-linked peptide with an amino acid sequence derived from the intracellular loop region of a target G protein-coupled receptor (GPCR). Pepducins are typically modified at the N-terminus with palmitic acid, but the lipid moiety and amino acid sequence are subject to optimization to produce the desired pharmacology and pharmaceutical properties.[2] Pepducins are thought to act as allosteric modulators of GPCR signaling by binding to the intracellular surface of target receptors.[2,3] While there are numerous reports of biologically active pepducins targeting a variety of receptors,[2,3,8] the precise pharmacological basis of their activity is not known, and biochemical and mechanistic data are limited.

To gain mechanistic insight into the functional actions of pepducins, we set out to demonstrate the direct interaction between a pepducin and its target GPCR by a photochemical cross-linking approach. To enable these studies, we designed and characterized a series of pepducin analogues based on the previously described CXCR4 pepducin agonist ATI-2341[7] (Figure 1a). First, ATI-2755 was generated to serve as a scaffold for additional functional groups by replacing Met residues with Gly or Ala residues to limit oxidation. Subsequent structure−activity studies suggested that the Leu at position 7 of ATI-2755 tolerated substitution without loss of function and was therefore replaced with L-photo-leucine (photo-Leu). Photo-Leu resembles Leu but contains a photoactivatable diazirine ring that generates a reactive carbene intermediate upon UV photolysis and loss of nitrogen.[9] The carbene reacts to form a stable covalent cross-link with a neighboring atom, usually by insertion at a C−H bond, in a manner that is highly location-specific as a result of the short half-life of the activated carbene and the lack of any spacer moiety. We next inserted a 5(6)-carboxytetramethylrhodamine (TAMRA) fluorophore flanking the lipid linkage and immediately preceding the peptide portion of the pepducin to allow for visualization in cell-imaging and in-gel fluorescence studies. The TAMRA fluorophore was selected because of its strong emission properties and its lack of absorbance in the UV region needed to activate the photo-Leu probe [Figure S1 in the Supporting Information (SI)]. To facilitate efficient TAMRA coupling and to balance lipophilicity, 2-(undecycloxy)ethane was used in place of the C16 palmitate, a substitution that had minimal impact on function. The resulting pepducin analogue containing both the photo-Leu and the TAMRA label was termed ATI-2766.

Similar to the parent compound ATI-2341, the pepducins ATI-2755 and -2766 induced dose-dependent calcium mobilization in U87 cells stably expressing CXCR4 (Figure 1b) and had no activity on naive cells or cells stably transfected with the related chemokine receptor CCR5 (Figure 1c). Fluorescence

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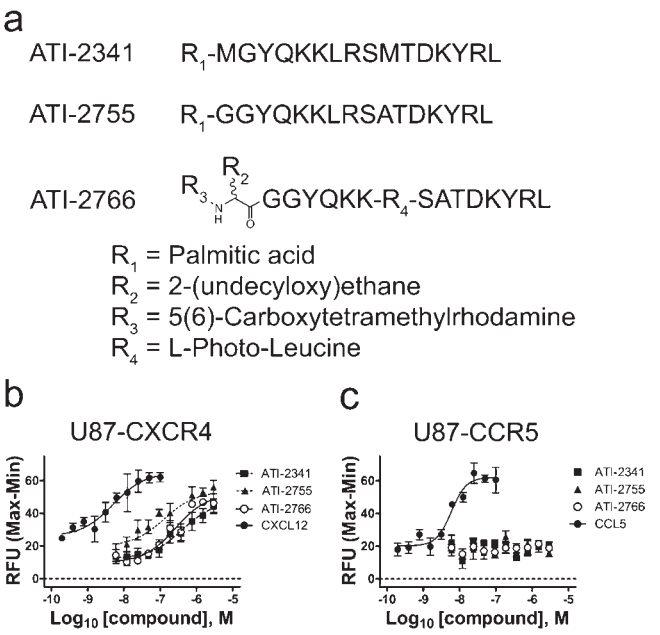

Figure 1. Characterization of CXCR4 photoaffinity-labeling pepducins. (a) Chemical structures of parental and modified CXCR4 i1 loop pepducins. Modified functional groups (R1−R4) are indicated. (b) CXCL12 and ATI-2341, -2755, and -2766 dose-dependently induce a calcium response in U87 CD4+ cells stably transfected with human CXCR4. (c) U87 CD4+ cells stably transfected with human CCR5 exhibit a dose-dependent calcium response to the CCR5 cognate ligand CCL5 but do not respond to CXCR4-derived pepducins. All experiments shown are representative examples of at least $n = 3$ sample sets.

microscopy demonstrated that ATI-2766 induced internalization of CXCR4 and colocalized with the internalized receptor, consistent with the agonist-like properties reported for the parental ATI-2341 compound (Figure S2). Collectively, these data demonstrate that despite the modifications introduced, ATI-2766 largely retained the pharmacological profile of the parent compound ATI-2341.

We next incorporated a C-terminal C9 1D4 monoclonal antibody (mAb) epitope tag into CXCR4 to facilitate immunopurification of the receptor from cell lysates.[10] We confirmed that the modification did not impair the agonist activity of the CXCR4 pepducins in calcium mobilization assays (Figure S3) and subsequently used the epitope-tagged receptor to carry out in-cell photo-cross-linking experiments. HEK-293T cells transfected with CXCR4-1D4 were incubated with ATI-2766 and then exposed to UV light. Cells were harvested and solubilized in a detergent buffer, and the 1D4 mAb was used to immunoprecipitate CXCR4 (or control receptor). The immunoprecipitate was resolved by SDS-PAGE, and covalently cross-linked ATI-2766 was detected using in-gel fluorescence imaging tuned for TAMRA detection (λ_{ex} = 532 nm, λ_{em} = 580 nm). A prominent band migrating at approximately the expected molecular mass of CXCR4 (∼45 kDa) was visualized in lanes containing immunoprecipitates from cells expressing CXCR4 treated with ATI-2766 and exposed to UV light (Figure 2a). The band was still apparent in samples treated with 10-fold less pepducin, and Western immunoblot analysis confirmed the presence of CXCR4. Taken together, these results show the existence of a UV-light-dependent covalent complex between ATI-2766 and CXCR4. Importantly, no significant cross-linked material was observed in

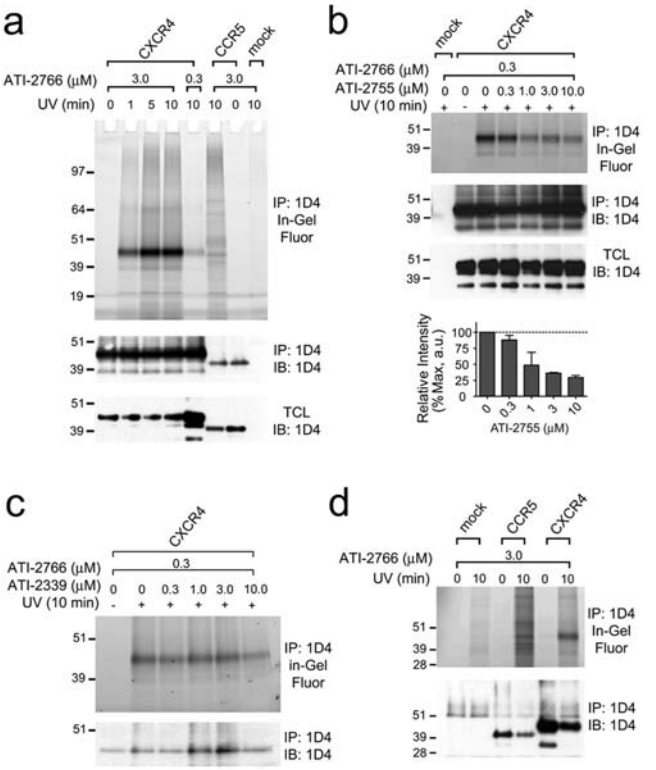

Figure 2. Photochemical cross-linking of ATI-2766 to CXCR4. (a) In-gel fluorescence and Western blot analysis of ATI-2766 cross-linked to HEK-293T cells transfected with empty vector (mock), CXCR4-1D4, or CCR5-1D4 in intact cells. Following the indicated whole-cell treatment, cell lysates were immunopurified (IP) with sepharose beads conjugated to the 1D4 antibody, resolved by SDS-PAGE, and imaged for TAMRA fluorescence (top panel). The transferred gel was immunoblotted (IB) with the monoclonal 1D4 antibody to confirm the presence of the 1D4-tagged receptors (middle panel). The total cell lysate (TCL) loading control for the immunopurification is also shown (bottom panel). The UV-dependent TAMRA signal at the molecular mass of CXCR4 (∼45 kDa) indicates that ATI-2766 is cross-linked to CXCR4. In addition, no specific signal is present in CCR5-1D4 or mock transfected cells. (b) The parental compound of ATI-2766 lacking the TAMRA and photo-Leu modifications, ATI-2755, directly competes with ATI-2766 for binding to CXCR4. Cross-linking experiments performed with increasing concentrations of ATI-2755 exhibit a dose-dependent decrease in ATI-2766 cross-linking that indicates the specificity of the binding interaction. Competition was quantified by densitometry and normalized to expressed receptor levels. (c) ATI-2339 is a truncated version of ATI-2341 that lacks the last three amino acids of the pepducin and is functionally silent in signaling assays. This molecule does not compete with ATI-2766 for cross-linking to CXCR4 (except at the highest concentration), as the intensity of the normalized TAMRA fluorescence remained consistent as ATI-2339 was titrated in. (d) In-gel fluorescence and Western blot analysis of ATI-2766 cross-linking in membrane preparations from cells transfected with CXCR4-1D4 or CCR5-1D4. ATI-2766 specifically cross-links to CXCR4 in a receptor- and UV-light-dependent manner when incubated with membranes from cells transfected with 1D4-tagged CXCR4 (top panel). The gel was subsequently transferred and blotted with the monoclonal 1D4 antibody to confirm the presence of the 1D4-tagged receptors (bottom panel). All experiments shown are representative examples of at least $n = 3$ sample sets.

samples that had not been treated with UV light or in samples that contained untransfected cells or cells expressing CCR5, a

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chemokine receptor with high homology to CXCR4. The cross-linking reaction was robust, since a strong fluorescent band was observed in SDS-PAGE gels of total cell lysates expressing CXCR4 that were run prior to immunoprecipitation (Figure S4a). While it is difficult to ascertain the precise stoichiometry of the reaction, we speculate that it is near 1:1 (CXCR4:ATI-2766) since the cross-linking did not result in a large-scale mobility shift on the gel, as might be expected if numerous ATI-2766 molecules were bound to CXCR4. In addition, the formation of the cross-linked product was not dependent on the type or location of the CXCR4 affinity tag, since similar results were obtained using an alternative affinity tag (HA) located on the N-terminus (Figure S4b).

The specificity of the cross-linking reaction was evaluated in competition experiments in which ATI-2766 cross-linking was carried out in the presence of increasing concentrations of unmodified pepducin (ATI-2755). We found that ATI-2755 competes with ATI-2766 for cross-linking to CXCR4 in a dose-dependent manner, providing further evidence that the binding interaction is specific (Figure 2b). While it is difficult to ascribe binding affinities to results obtained from cross-linking studies, a qualitative assessment indicates that the competition occurs in a concentration range consistent with the demonstrated functional activity of these compounds. The fact that the observed competition was not complete is not unexpected in view of the lipidated nature of the pepducin analogues, which leads to membrane accumulation. A pepducin lacking three C-terminal amino acid residues failed to compete for cross-linking with ATI-2766 except at the highest concentration, consistent with the limited functional activity[7] of this pepducin analogue (Figure 2c). In addition, pharmacologically active pepducins targeting other GPCRs did not compete with ATI-2766 for cross-linking to CXCR4 (data not shown). Competition experiments were also conducted using the CXCR4 inverse agonist T-140. CXCR4-derived pepducins, including ATI-2766, were not able to compete with a functional variant of T-140 bearing a photoreactive group[11] for cross-linking to CXCR4 (Figure S5). This result suggests that ATI-2766 and T-140 do not share an overlapping binding site on CXCR4. Furthermore, in contrast to numerous CXCR4-targeted small molecules, we found that the parent molecule, ATI-2341, has little to no effect on CXCL12 binding in competition binding experiments (Figure S6).

We next set out to determine whether the cross-linking reaction requires an intact viable cell or if similar results could be obtained in cell membrane preparations. HEK-293T cells expressing CXCR4-1D4 were used to generate cell membrane preparations (see Supplemental Methods in the SI), which were incubated with ATI-2766, exposed to UV light, and processed as described above. Consistent with the findings from live intact cells, a prominent fluorescent band was visible only in UV-treated membranes prepared from cells expressing CXCR4 and not in membranes prepared from cells expressing CCR5 (Figure 2d). The difference in background signal intensity for the mock and CCR5 lanes may be attributable to an increase in the amount of protein loaded in the CCR5 lane, resulting from immunoprecipitation with the 1D4 mAb, relative to the mock lane, where no epitope-tagged material was present.

The binding of CXCL12 to CXCR4 has been modeled as a two-step/two-site process.[12] In this model, CXCL12 interacts first with the CXCR4 N-terminus and subsequently with the ligand-binding cavity on the extracellular face and transmembrane

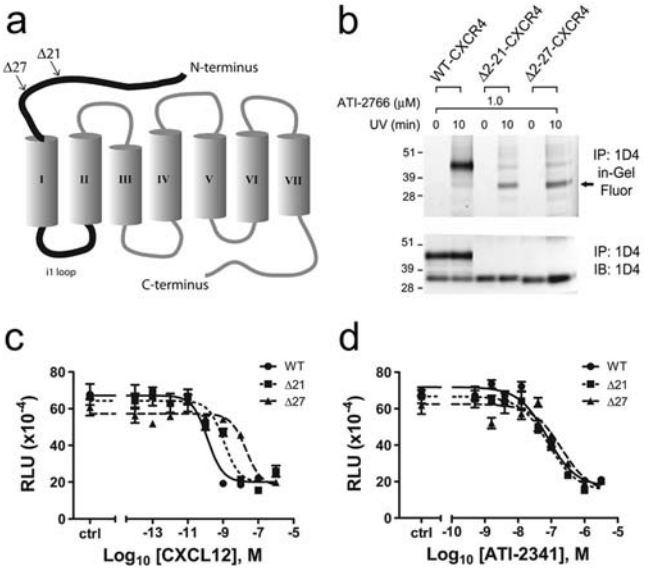

Figure 3. Allosteric interaction of ATI-2766 with CXCR4. (a) Schematic diagram of the CXCR4 receptor indicating the i1 loop region from which the active agonist pepducins are derived and highlighting the N-terminal truncation mutants Δ2−21 and Δ2−27. (b) ATI-2766 cross-links to the CXCR4 Δ2−21 and Δ2−27 truncation mutants in a UV-dependent manner (arrow). The truncation results in altered gel mobility relative to WT-CXCR4, as demonstrated in both the in-gel fluorescence image and the Western blot. (c) CRE luciferase reporter assays for CXCL12 HEK-293T cells expressing WT-CXCR4 or the Δ2−21 or Δ2−27 truncation mutations. Truncation of the N-terminal region of CXCR4 has a significant impact on the potency of the endogenous ligand CXCL12 (150-fold for the Δ2−27 mutation). (d) CRE luciferase reporter assays for ATI-2341 on HEK-293T cells expressing WT-CXCR4 or the Δ2−21 or Δ2−27 truncation mutations. In contrast to the potency shift observed for CXCL12, ATI-2341 signaling was minimally affected in HEK-293T cells expressing WT-CXCR4 or the N-terminal truncation mutations (2-fold shift for the Δ27 mutation). All results are representative examples of at least three independent experiments.

domain, leading to receptor activation and signaling. Previous studies suggested that the N-terminus of CXCR4 is critical to both CXCL12 binding and receptor activation, as mutations and truncations in this region impair ligand binding and impair or abolish signaling.[13] We generated two N-terminal truncation mutations: Δ2−21, which effectively removes all of the residues modified by Tyr sulfation, sites known to be important for binding;[14] and Δ2−27, which truncates the receptor to a Cys residue involved in a conserved disulfide bond. We then conducted functional cyclic adenosine monophosphate (cAMP) accumulation assays and photochemical cross-linking experiments (Figure 3).

We found that ATI-2766 cross-links to these truncated CXCR4 variants in a UV-light-dependent manner similar to results observed for wild-type (WT) CXCR4 (Figure 3b). As expected, these mutants exhibited greatly reduced potency to CXCL12 (150-fold for Δ2−27 relative to WT) in a cAMP response element (CRE) luciferase reporter assay (Figure 3c). In contrast, these truncation mutations had little to no effect on the efficacy or potency of pepducin-mediated signaling (2-fold for Δ2−27 relative to WT) (Figure 3d). Collectively, these results indicate that CXCR4-directed agonist pepducins use a binding mechanism distinct from that used by CXCL12 and furthermore show that they modulate receptor activity allosterically.

Recent studies have shown that pepducins have significant potential as therapeutics.[3,15] In unpublished studies focused on the CXCR4 receptor, we have elucidated a robust structure—activity relationship for agonist activity demonstrating both an improvement in potency and an enhancement of pharmaceutical properties without compromising selectivity. Specifically, we have shown that alteration of selected residues in the peptide core (while retaining the critical N-terminal KYRL motif) has yielded full agonists with 8−10-fold improved potency relative to ATI-2341 along with greatly improved human plasma stability. In addition, excellent bioavailability following subcutaneous injection (88%) has been demonstrated along with dose-responsive in vivo efficacy in the mouse. Taken together, these data illustrate the promising evolution of pepducins from research tools toward an established class of pharmaceuticals.

In conclusion, using a chemically directed cell-based photocross-linking strategy, we have demonstrated a unique and specific direct physical interaction between a pepducin and its targeted receptor, CXCR4. The approach and new molecular tools we have described should prove useful in further elucidating the mechanism of action for this important new class of therapeutics as well as increasing our understanding of the complex processes involved in GPCR-mediated signal transduction.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis methods and experimental details; steady-state fluorescence spectra; colocalization images; calcium mobilization assays; photochemical cross-linking characterization and competition experiments; and ligand binding assays. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

jjanz@anchortx.com; Sakmar@rockefeller.edu

Present Addresses

[‖]Eisai, Inc., Woodcliff Lake, NJ 07677.

[⊥]Department of Chemistry, Princeton University, Princeton, NJ 08544.

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