Insights into the Mechanism of Inhibition of CXCR4: Identification of Piperidinylethanamine Analogs as Anti-HIV-1 Inhibitors

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Running Title: Piperidinylethanamine Analogs as anti-HIV-1 Inhibitors

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ABSTRACT

The cellular entry of HIV-1 to CD4+ T-cells requires ordered interactions of HIV-1 envelope glycoprotein with CXCR4 receptors. However, such interactions, which should be critical for rational structure-based discovery of new CXCR4 inhibitors, remain poorly understood. Herein, we first determined the effects of amino acid substitutions in CXCR4 on HIV-1NL4-3 glycoprotein-elicited fusion events using site-directed mutagenesis-based fusion assays and identified eleven potentially key amino acid substitutions, including D97A and E288A, which caused a >30% reduction of the fusion. We subsequently carried out computational search of a screening library containing ~604,000 compounds in order to identify potential CXCR4 inhibitors. The computational search used the shape of IT1t, a known CXCR4 inhibitor, as a reference and employed various algorithms including shape similarity, isomer generation, and docking against a CXCR4 crystal structure. Sixteen small-molecules were identified for biological assays based on their high shape similarity to IT1t and their putative binding modes formed hydrogen bond interactions with amino acids identified above. Three compounds, having a piperidinylethanamine core, showed activity and were resynthesized. One molecule, designated CX6, was proven to significantly inhibit X4-HIV-1NL4-3 glycoprotein-elicited fusion (IC\textsubscript{50}: 1.9 µM), inhibit SDF-1α-elicited Ca\textsuperscript{2+} flux (IC\textsubscript{50}: 92 nM), and exert anti-HIV-1 activity (IC\textsubscript{50}: 1.5 µM). Structural modeling demonstrated that CX6 bound to CXCR4 through hydrogen-bond interactions with Asp97 and Glu288. Our study suggests that targeting CXCR4 residues important for HIV-1-envelope glycoprotein-elicited fusion should be a useful and practically feasible approach in identifying novel CXCR4 inhibitors.
inhibitors and sheds important insights into the mechanism by which small-molecule CXCR4 inhibitors exert their anti-HIV-1 activity.
INTRODUCTION

Over the last thirty years, the human immunodeficiency virus (HIV-1) has become responsible for more than thirty million deaths worldwide, and approximately 35 million people are estimated to be currently infected by the virus (United Nations AIDS Global report, 2013). Major innovations and advancements have led to the current availability of many anti-HIV-1 inhibitors; however continued discovery and development of novel inhibitors against existing and newly discovered targets are imperative to overcome a number of inherent problems in the current antiretroviral therapy (ART) including toxicities and HIV-1’s acquisition of drug resistance (1).

C-X-C chemokine receptor type 4 (CXCR4) and C-C-chemokine receptor type 5 (CCR5) are essential co-receptors for the entry of HIV-1 into the host cell. Both CXCR4 and CCR5 are G-protein coupled receptors (GPCR) with seven transmembrane helical structures. Maraviroc is the one and only small molecule FDA-approved therapeutic targeting CCR5. Compared to CCR5 inhibitors, a lesser number of CXCR4 inhibitors have been reported as potential therapeutics for treating HIV-1 infection. In fact, no CXCR4 inhibitor, as an anti-HIV-1 agent, has been approved for clinical use as of today and there is an urgent need for novel small molecule inhibitors targeting CXCR4. Such a molecule, by itself, and in particular, in combination with a CCR5 antagonist, should greatly improve the treatment options available to patients predominantly infected with X4- and dual tropic HIV-1 strains.

Initial reports identified several peptides, such as T140 and macrocycles such as AMD3100 that targeted CXCR4 (2-4). To improve the oral bioavailability, attempts to replace or decrease the size of the macrocycles while retaining anti-HIV-1 potency were
made. One such effort led to the discovery of AMD070, a molecule with benzoimidazol and tetrahydroquinoline groups (5, 6). AMD070 is orally bioavailable, and has good safety and pharmacokinetic profiles (7, 8). Jenkinson et al. reported on the anti-HIV-1 and pharmacological profiles of GSK812397, a molecule with some structure similarity with AMD070 (9). Thoma et al. identified several isothiourea derivatives that bind to CXCR4 and inhibit HIV-1 infection (10). The crystal structures of CXCR4 in complex with a small molecule (IT1t), and with a 16-residue cyclic peptide (CVX15) were determined (11). The structures demonstrated important features of CXCR4, but further understanding of the mechanism of antiviral activity exerted by small molecule inhibitors is required for a rational structure-based design of new CXCR4 inhibitors. Moreover, only a limited number of studies have utilized the recent crystal structures of various GPCRs in the discovery of novel chemotypes or in the optimization of existing candidates. Part of the reason might be because inhibitors may bind to the binding sites of GPCRs in an orthosteric or allosteric fashion. The orthosteric inhibitors directly bind to the active site and competitively inhibit the natural substrate or ligand, while the allosteric modulators show their effect distal from their binding location (12). Thus, the functional significance of each binding site residue, and residues that need to be selectively targeted, based on a mechanism of action, needs to be elucidated for designing and discovering new inhibitors.

The interaction of the HIV-1 envelope glycoprotein gp120 with CXCR4 enables the virus to gain entry to cells. We wanted to better understand the structural and functional importance of CXCR4 residues implicated in gp120 protein-elicited fusion and determine if preferential interaction of an inhibitor with such residues may give rise to
inhibition of the fusion event and anti-HIV-1 activity. In the current study, we first introduced a variety of amino acid substitutions in CXCR4 to determine residues that are important for the interaction of CXCR4 with the gp120 envelope protein. We then hypothesized that molecules that formed critical polar interactions with such potentially key residues were likely to interfere with the binding and interactions of CXCR4 with gp120, exerting antiviral activity. To identify such molecules, we utilized the crystal structure of CXCR4 in complex with IT1t (11) as follows. First, we carried out a computational search that identified molecules that had a high shape similarity with IT1t. The putative interactions of such molecules with CXCR4 were examined by docking simulations against the crystal structure of CXCR4. Sixteen molecules that had hydrogen bond interactions with at least two CXCR4 amino acid residues determined to be important for gp120-elicited fusion were selected for assays. Three piperidinylethanamine (PEA) derivatives were identified to have binding to CXCR4 in Ca\textsuperscript{2+} flux assays in Molt4 cells and showed anti-HIV-1 activity against X4-HIV-1\textsubscript{NL4-3}. The PEA derivatives also inhibited the fusion of HIV-1\textsubscript{NL4-3} envelope gp120 with CXCR4. We subsequently synthesized several PEA derivatives and assays confirmed their activity and specificity as anti-HIV-1 inhibitors targeting CXCR4. Our study sheds important insight on the mechanism of inhibition and suggests that interactions of molecules with CXCR4 residues that are important for gp120-elicited fusion might be a worthwhile strategy for discovering new inhibitors of CXCR4. The present study should provide a useful platform for structure-based discovery of inhibitors of CXCR4. Similar strategies, based on residue-specific interactions responsible for a mechanism may be used for the discovery of novel chemotypes for other GPCRs.
MATERIALS AND METHODS

Cells and Viruses. MT-4 cells were grown in an RPMI-1640 culture medium supplemented with 10% fetal calf serum (FCS; GEMINI Bio-Products). The multinuclear activation of the galactosidase indicator (MAGI) cell line (13) and the U-373 MAGI cell line (14) were provided by NIH AIDS Research and Reference Reagent Program and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 200 µg/ml G418, and 100 µg/ml hygromycin B. The 293T cells were cultured in DMEM with 10% FCS. Peripheral blood mononuclear cells (PBM) were isolated from buffy coats of HIV-1 seronegative individuals and activated with 10 µg/ml phytohemagglutinin (PHA) prior to use as previously described (15). Two HIV-1 strains, HIV-1_NL4-3 (16) and HIV-1 Ba-L (17), were employed for drug susceptibility assays.

HIV-1-gp120-Elicited Cell-Cell Fusion Assay. The HIV-1-gp120-elicited cell-cell fusion assays using a panel of CXCR4 mutants were conducted as previously reported (18) with minor modifications. A CXCR4 expression vector, pcDNA3.1-CXCR4 (UMR cDNA Resource Center, Rolla, MO) was employed and a variety of plasmids carrying a mutant CXCR4-encoding gene (pcDNA3.1-CXCR4<sub>WT</sub>) were subsequently generated by employing the site-directed mutagenesis technique. An HIV-1-envelope expression vector, pCXN-NL4-3<sub>env</sub> was generated by replacing JRFL envelope gene of pCXN-JR<sub>env</sub> (19) with NL4-3 envelope gene.

The envelope expression vector (pCXN-NL4-3<sub>env</sub>) and tat expression vector (18) (0.5 µg each) were co-transfected into 293T cells (2 x 10<sup>5</sup>, 3 ml in 6-well microculture plates) using Lipofectamine 2000 (Invitrogen), while the CXCR4<sub>WT</sub> or mutant CXCR4 expression vector and pLTR-LucE (0.5 µg each) were co-transfected into U373-MAGI.
cells ($2 \times 10^5$, 3 ml in 6-well microculture plates) since they do not endogenously express CXCR4. On the next day, both the co-transfected cells were harvested and mixed in a well of 96-well plates ($2 \times 10^4$ cells each). The co-transfected cells were incubated further for 6 hrs and the luciferase activity in each well was detected using Bright-Glo Luciferase Assay System (Promega) and its luminescence level was measured using Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). Non-specific luciferase activity was determined in the well containing control Tat', env' 293T cells and Luc', CXCR4' U373-MAGI cells and the value of the non-specific luminescence level was subtracted from each experimental luminescence level. The inhibition of cell-cell fusion by the test compounds was also determined under the same condition but at various drug concentrations and their half maximal inhibitory concentrations (IC$_{50}$) were determined.

Computational Screening. We searched the screening libraries from ChemBridge™ (http://www.chembridge.com) to identify potential small molecule inhibitors of CXCR4. In brief, the computational search identified potential molecules by the following two simulations: i) initial identification of molecules that had a high shape similarity to IT1t (11), a known CXCR4 inhibitor, and ii) subsequent determination of the putative binding mode and interaction of these molecules to potentially key CXCR4 amino acid residues. A flow diagram of the screening protocol is shown in Fig. 1. Initially, known aggregators, molecules with more than 20 rotatable bonds or with a molecular weight below 350 Dalton and above 750 Dalton were eliminated from consideration. In order to determine the molecules that had a high shape similarity to IT1t, the possible stereoisomers and three dimensional conformations were generated using Omega (version 2.3.2, OpenEye Scientific Software, Inc. Santa Fe, New Mexico), and the shape overlay program ROCS
(version 3.0.0, OpenEye Scientific Software) was used (20, 21). The crystal structure conformation of IT1t (11) was used as the query template against which molecules from the chemical database were aligned. Molecules, whose shape similarity Tanimoto coefficient was at least 0.7 with IT1t, were retained for molecular docking. In order to eliminate lack of compatibility between software suites from different sources, the possible ionization states at pH 7±2, tautomers, stereoisomers, and ring conformations of these molecules were again generated (LigPrep version 2.4, Schrödinger, LLC). These structures were docked into CXCR4 using Glide (version 5.6, Schrödinger, LLC) (22, 23). The crystal water molecules were removed, the docking grid around the binding site of IT1t to CXCR4 was generated, and molecules were docked. Molecules that did not form at least two hydrogen bond interactions with two of the following residues determined to be important for the fusion of gp120-envelope-protein to CXCR4 were eliminated (Asp97, Tyr116, Phe174, Ala175, Asp182, Asp187, Arg188, Tyr190, Asp262, Glu288 and Phe292). Selection of the candidate molecules was made by analyzing the putative binding mode and interaction with CXCR4 residues, without explicit consideration of the glide docking scores.

**Determination of Inhibition of Chemokine Binding to CXCR4.** In order to determine if the selected compounds bind to CXCR4, calcium flux binding inhibition assay was conducted using Fluo-4 DirectTM calcium reagent (Invitrogen) according to the manufacturer’s protocol. In brief, Molt4 cells (5 x 10^5 cells) were exposed to Fluo-4 DirectTM calcium reagent for 60 min at 37°C in RPMI containing 5% FCS. A test compound was added at various concentrations, incubated for 30 min, and the cells were exposed to stromal cell-derived factor 1 (SDF-1α) at a concentration of 1 nM. Relative
increases of the cytosolic Ca\(^{2+}\) level after the SDF-1\(\alpha\) exposure were determined with fluorescence-activated cell sorting (FACS) Calibur (BD Biosciences) and IC\(_{50}\)s of cytosolic Ca\(^{2+}\) mobilization (Ca\(^{2+}\) flux) by test compounds were determined by comparison with the Ca\(^{2+}\) flux level in drug-free control samples.

**Drug Susceptibility and Cytotoxicity Assay.** The susceptibility of HIV-1 strains against various drugs was determined as previously described (15, 24) with minor modifications. In the methylthiazol tetrazolium (MTT) assay, MT-4 cells (5x10\(^4\)/ml) were exposed to 100 TCID\(_{50}\) (50% tissue culture infective dose) of HIV-1 in the presence of various concentrations of drugs in 96-well microculture plates and incubated at 37°C for 5 days. After culture, 10 \(\mu\)l of cell staining solution (Cell Counting Kit-8, Dojindo Molecular Technologies, Inc. Japan) was added to each well in the plate, followed by incubation at 37°C for 1-2 hrs and the optical density was measured in a microplate reader (Model 3550, Biorad). All assays were performed in duplicate, and data shown represent mean values (± 1-standard deviation) derived from results of at least two independent experiments.

PHA-PBMC (1x10\(^6\)/ml) were exposed to 50 TCID\(_{50}\) of HIV-1\(_{NL4-3}\) in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates. All assays were performed in triplicate. The amounts of p24 antigen produced by the cells were determined on day 7 in culture using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (PerkinElmer). Drug concentrations which resulted in 50% inhibition (IC\(_{50}\)) of p24 antigen production were determined by comparison with the p24 production level in drug-free control cell cultures. Antiviral assays using MAGI cells (MAGI assay) were also conducted as
previously reported (15).

Cytotoxicity of a compound in MT-4 cells was also determined using MTT assay.

Cells were plated in a 96-well plate, exposed to various concentrations of a compound and were cultured in the same condition as anti-HIV-1 assay. The number of viable cells in each well was determined using Cell Counting Kit-8.

**Synthesis of Piperidinylethanamine (PEA) derivatives.** Synthesis of a PEA derivative (inhibitor CX6) is shown in Fig. 2. Reductive amination of ethylpiperidine-3-carboxylate with cyclopentanone provided amine 1 in 81% yield. Dibal-H reduction at -78°C afforded aldehyde 2 in 75% yield. Amine 4 was prepared by alkylation of piperdine with commercially available bromide 3. Reductive amination of aldehyde 2 with amine 4 in the presence of Ti(O-iPr)₄ furnished secondary amine 5. Amine 5 was then subjected to another reductive amination with 3-hydroxybenzaldehyde to provide compound 6 (CX6) in 78% yield, and 97% purity as determined by HPLC. The detailed experimental procedure and characterization is given in the supplemental material. The characterization of other synthesized PEA derivatives (CX6, CX21, CX22, CX23, CX24, CX25 and CX27) is given in the supplemental material.
RESULTS

Effects of Amino Acid Substitutions in CXCR4 on HIV-1-envelope-induced Cell Fusion. The HIV-1 envelope glycoprotein gp120 interacts with CXCR4 for entry to CD4+ T-cells. The gp120-CXCR4 interaction is described by a two-site model in which the N-terminus of CXCR4 interacts with the base of the hypervariable region 3 (V3) loop of gp120 (site-1) and CXCR4 residues in transmembrane (TM) helices and the second-extracellular loop (ECL2) interact with the tip of V3 (site-2) (11). The two-site model suggests that certain transmembrane and extracellular residues are important for gp120 binding, and we hypothesized that small molecules that bind to such residues in the orthosteric binding site would likely inhibit the CXCR4-gp120 interactions. To determine these residues, fifty-six amino acid residues in the extracellular and transmembrane regions of CXCR4 were selected for introducing amino acid substitution(s) and the HIV-1-gp120-elicited cell fusion levels were determined and compared to that of wild type CXCR4. The intracellular residues are important for signal transduction but do not seem to directly affect anti-HIV-1 inhibitor binding or gp120 fusion; hence they were not considered. The significance of some of the residues selected for substitution are as follows: Asp133, Arg134, and Tyr135 in TM-3 are the conserved Asp-Arg-Tyr motif in various GPCRs and are reportedly important in triggering ligand-induced conformational changes that lead to receptor activation (25). Asp171 (TM4), Gly207 (TM5), Asp262 (TM6), Asp187 (ECL2), Arg188 (ECL2), Tyr190 (ECL2), His281 (ECL3) are important in the binding of T140, AMD3100, or AMD3465 to CXCR4 (26-28). The second extracellular loop (ECL2) is known to be important for the structure and function of CXCR4, CCR5 and other GPCRs (29-31); therefore, several residues in ECL2 were
selected. Cys109 located in the extracellular region of TM3 forms a disulfide bond with Cys186 in ECL2. This disulfide linkage is conserved for class-A GPCRs and is known to be important for their structures and functions (11, 31). The effect on HIV-1 glycoprotein-elicited fusion by substitution of some of these residues was reported before (32); however the coverage was partial and we carried out a comprehensive analysis of the effect of CXCR4 amino acid residues on viral induced fusion.

Fig. 3A shows the changes in the magnitude of cell-cell fusion levels with wild type or mutant CXCR4. Eleven amino acid(s) substitutions (D97A, Y116A, F174A, A175F, D182A, D187A, R188A, Y190A, D262A, E288A, and F292A) resulted in a substantial reduction in the HIV-1-envelope protein-elicited fusion level by more than 30%. Amino acid substitutions C109A, D133A, C186A, and D193A drastically reduced CXCR4-gp120 fusion; however they also had a critical decrease in CXCR4 expression levels on the cell surface as determined by FACS analysis. Thus, we could not conclude if these reductions were because of their functional importance or because of the decrease of expression levels. Substitutions of Asn176 (ECL2) had no effects on the fusion level, in agreement with the data of Brelot et al. (32). It is noteworthy that there are a number of negatively charged acidic residues (D97A, D182A, D187A, D262A, and E288A) whose substitution significantly decreased gp120 fusion (Fig. 3A). These acidic residues may interact with the basic residues of gp120 to affect co-receptor selectivity. Negatively charged acidic residues have also been shown to be important for the binding of SDF-1 and for CXCR4 co-receptor function in HIV-1 entry (32).

We analyzed the crystal structures of CXCR4 (11) to understand the interactions and orientation of the residues whose substitution adversely impacted the interactions of
CXCR4 with the HIV-1-envelope protein (Fig. 3B). Six amino acid substitutions (F174A, A175F, D182A, D187A, R188A, and Y190A) that impacted the fusion event were identified in or near ECL2 (Fig. 3B), strongly suggesting that this region as a whole probably affects fusion more than any other loop or transmembrane region of CXCR4. Asp187 and Arg188 have a hydrogen bond interaction with each other through their side chains and form part of the binding cavity. However, Phe174, Ala175 and Tyr190 are not part of the ligand binding pocket of IT1t. Asp262, located in TM6, is not close enough to the ligand binding pocket of IT1t, but forms hydrogen bond interactions with the much larger sixteen residue peptide CVX15 (11). Site-directed mutagenesis studies have suggested that Asp262 is important for the binding of AMD3100 (26). Glu288 (TM7) has a hydrogen bond with the side chain of Tyr116 (TM3) and an intra-helix hydrogen bond with Phe292 (TM7), and these three residues form part of the binding pocket within the transmembrane domain. The side chain of Asp97 forms part of the binding pocket for small molecules whereas the carboxylate side chain of Asp182 is oriented towards the extracellular region and away from the binding pocket located inside the transmembrane domain. Substitution of residues in TM5 or ECL3 did not affect the fusion event in the assay (Fig. 3A), indicating that these regions are not involved in the interaction of CXCR4 with the HIV-1-envelope protein.

**Computational Examination of Molecules for the Identification of CXCR4 Inhibitors.** We searched a general screening library from ChemBridge™, which has more than six hundred thousand molecules, to select molecules for assays as potential inhibitors of CXCR4. Known aggregators and molecules that did not have a molecular weight between 350 and 750 Daltons were eliminated (Fig. 1), and the possible
stereoisomers and conformations of the rest were determined with OMEGA (version 2.3.2, OpenEye Scientific Software, Inc. Santa Fe, New Mexico) (20). Previous studies have shown that determination of molecular similarity plays a critical role in analyzing large compound databases in chemical and pharmaceutical research (33). Therefore, using the software tool ROCS (21), we determined the shape similarity of the molecules, from the ChemBridge library to IT1t, a molecule that binds with high affinity to CXCR4 and demonstrates anti-HIV-1 activity (10, 11). The highest shape similarity Tanimoto coefficient to IT1t from the database was 0.85. Seven-hundred-fifty-three (753) unique molecules with one-thousand-five (1005) configurations/conformations had a shape Tanimoto coefficient of at least 0.70. The binding mode and interactions of these molecules with CXCR4 were determined by molecular docking using Glide (version 5.6, Schrödinger, LLC) (22, 23). To avoid any issues that may arise through using conformations generated by Omega with Glide docking, Ligprep was used to generate molecular configurations and conformations for docking, and it generated fourteen-thousand-two-hundred-twenty-six (14,226) of them. These configurations/conformations were docked to the crystal structure of CXCR4 to determine their possible binding modes. Since it had been determined that CXCR4 transmembrane residues Asp97, Tyr116, Asp262, Glu288, Phe292 and ECL2 residues Phe174, Ala175, Asp182, Asp187, Arg188 and Tyr190 appeared to be important for the cell fusion event (Fig. 3A), we hypothesized that molecules that bound around the active site determined in the crystal structure, and formed hydrogen bond interactions with at least two of these residues are likely to competitively inhibit the interactions of CXCR4 with gp120. Our candidate molecule selection was based on shape similarity to a known inhibitor (IT1t) and putative binding
mode and interactions with residues that were determined to be important for the HIV-1-gp120-elicited cell fusion event with CXCR4. Of note, we did not use any energy-based or empirical scoring functions for estimating the relative affinity for the selection of compounds. Based on the hypothesis described above, we selected sixteen compounds (named CX1 to CX16) for biological assays from ChemBridge™ general screening library. Three compounds were piperidinylethanamine derivatives, and four compounds were tetrahydro-β-carboline derivatives. The other compounds had quinoxaline, indole, indane, imidazole, imidazopyridine, imidazothiazole, piperazine, morpholine, and diazepane moieties in them. In computational examination of screening libraries, in some instances completely different cores are selected for assays, but we chose multiple piperidinylethanamine and tetrahydro-β-carboline derivatives as the binding modes were thought to be most substantive. While there are certain advantages to selecting only one compound from one core, we decided not to take that approach because we did not want to eliminate an active core if the only compound we chose happened to be inactive.

**Inhibition of Chemokine Binding to CXCR4 by Piperidinylethamine derivatives.** We asked whether the sixteen selected compounds bound to CXCR4 by blocking the intracellular Ca^{2+} mobilization induced by SDF-1α, whose primary receptor is CXCR4. One of the compounds, described as CX6 (Fig. 4) blocked the SDF-1α-induced Ca^{2+} mobilization in Molt4 cells with IC_{50} value of 92 nM (Table 1, Fig. S1, supplemental material). In the same assay, AMD3100 showed inhibition of SDF-1α-induced Ca^{2+} mobilization with an IC_{50} value of around 10 nM (Fig. S1, supplemental material). Two other compounds, CX11 and CX13, both piperidinylethananine (PEA) derivatives like CX6 (Fig. 4), blocked SDF-1α-induced Ca^{2+} mobilization in Molt4 cells.
with IC$_{50}$ values of 161 and 149 nM, respectively (Table 1). None of the other compounds (Fig. S2, supplemental material) bound to CXCR4. Of note, all three PEA derivatives (CX6, CX11 and CX13) failed to block the Ca$^{2+}$ mobilization induced with RANTES, whose receptor is CCR5, indicating that these compounds did not bind to CCR5. When we asked if these three compounds had agonistic effects to induce Ca$^{2+}$ mobilization in CXCR4$^+$ cells, none induced Ca$^{2+}$ mobilization, suggesting that CX6, CX11, and CX13 were antagonists of CXCR4 (Fig. S3, supplemental material). In summary, the above data strongly suggest that the PEA derivatives bound to CXCR4 with specificity and were antagonists of CXCR4. Subsequently, we newly synthesized CX6 (Fig. 2 and supplemental material). Other PEA derivatives in high purity were also synthesized and their characterization is given in the supplemental material.

**Inhibition by Piperidinylethanolamine (PEA) derivatives of the Fusion Elicited by HIV-1$_{NL4-3}$ Envelope Protein Interactions with CXCR4.** The fusion event elicited by the interactions of HIV viral envelope glycoprotein with CXCR4 enables the virus to gain entry to cells, eventually leading to viral replication. The sixteen compounds were selected through docking simulations suggesting that they bound to a potential orthosteric binding site of CXCR4. These compounds formed hydrogen bonds to at least two amino acid residues most likely to be important for the fusion event, and thereby have the potential to competitively inhibit the fusion event. We thus determined if the compounds were actually able to inhibit the interactions of the HIV-1$_{NL4-3}$ envelope protein with CXCR4, and to block the fusion event in the HIV-1-gp120-elicited cell-cell fusion assays with the wild-type CXCR4. Both CX6 and CX11 blocked the fusion with IC$_{50}$ values of 1.9 µM and 7.9 µM (Table 1). Moreover, none of the compounds including CX6 and
CX11 inhibited the fusion event as examined with the HIV-1-gp120-elicited cell-cell fusion assays using the cells expressing the wild-type CCR5-derived from HIV-1BaL (R5-HIV-1) as previously described (18, 29). This suggested that CX6 and CX11 inhibited the fusion event associated with CXCR4 but not with CCR5.

**Interactions of Piperidinylethanamine derivatives with CXCR4.** The three-dimensional shape overlay of CX6 (shown in gray sticks) with IT1t (shown in green sticks), as determined by ROCS (version 3.0.0, OpenEye Scientific Software), is shown in Fig. 5-A. The molecules have an excellent shape Tanimoto overlap coefficient of 0.73. The imidazothiazole ring of IT1t overlays with the cyclopentylpiperidinyl group of CX6. The interactions of the identified hit compound (CX6) with CXCR4 were deduced by molecular docking. The interactions of CX6 with CXCR4 are illustrated in Fig. 5 B-E. The following amino acid residues of CXCR4 were seen to form the active site (<4Å from the inhibitor) for the binding of CX6: Glu-32 of the N-terminus of CXCR4; Phe-36, Asn-37, Leu-41, and Tyr-45 of TM1; Trp94, Asp97, and Ala98 of TM2; Trp-102 of ECL1; Val-112, His-113, and Tyr-116 of TM3; Cys-186 and Arg-188 of ECL2; and His-281, Ile-284, Ser-285, and Glu-288 of TM-7. Thus, the molecule CX6 bound in the active site predominantly formed by residues from transmembranes 1, 2, 3, 7, and ECL2. As expected, no amino acid residues of TM4, TM5, and TM6 had interactions with CX6. The nitrogens of both piperidine groups were determined to be protonated (LigPrep version 2.4, Schrödinger, LLC). For CX6, the protonated nitrogen of cyclopentylpiperidinyl formed hydrogen bond interactions with Glu-288 and the protonated nitrogen of piperidinylethanamine formed hydrogen bond interactions with Asp97. The phenol group of CX6 interacted with Glu-32 located in the N-terminus of
CXCR4. Asp97 has been shown to be important for the binding of AMD070 as well as for CXCR4-gp120-elicited fusion. Glu288 is important for the fusion event as it is shown that substitution of Glu288 with alanine results in loss of the CXCR4-gp120-elicited fusion. Sequence alignment indicated that the residue corresponding to Glu288 of CXCR4 is Glu283 of CCR5. Therefore, E283 for CCR5 should be important for the binding of CCR5 antagonist aplaviroc and its analogs, in line with previously published results (18, 29). Indeed, the substitution of E283 of CCR5 resulted in loss of the CCR5-gp120-elicited fusion event, as previously described (18, 29).

Both E283 of CCR5 and E288 of CXCR4 represent the 6th residue of TM7 in both chemokine receptors. Glutamic acid at position 6 in TM7 is a highly conserved amino acid residue in chemokine receptors and has been demonstrated to be important for the binding of non-peptidic ligands to chemokine receptors CCR1 and CCR2 (34). Our current study suggests that Glu288 is an important residue to target for rational structure based design and discovery of CXCR4 inhibitors.

**Binding of PEA derivatives to CXCR4 and Antiviral Activity against X4-HIV-1.** Finally, we determined the anti-HIV-1 activity and cytotoxicity of CX6 with the MTT assay using X4-HIV-1NL4-3 and MT4 as target cells. CX6 suppressed the infectivity and replication of HIV-1NL4-3 in a good dose-response fashion (Fig. 6). As shown in Table 1, CX6 exerted substantial activity against HIV-1NL4-3 and HIV-1LAI, both of which are X4-HIV-1 strains, with IC50 values of 1.5 and 3.0 µM, respectively. The cytotoxicity CC50 of CX6 was 58 µM with a therapeutic index of 39. CX6 blocked the fusion event as examined in the fusion inhibition assay using HIV-1NL4-3-derived envelope protein. In a Ca2+-flux inhibition assay, in which SDF-1α-elicited Ca2+-flux levels are determined in...
the presence or absence of a potential small molecule inhibitor, CX6 was found to block the flux at an IC$_{50}$ value of 92 nM (Table 1). However, as expected, CX6 failed to block the infectivity and replication of an R5-HIV-1 (HIV-1$_{BaL}$) (IC$_{50}$: $>$10 µM) as examined in an anti-HIV assay using HIV-1$_{BaL}$ and MAGI cells. CX11 and CX13 similarly exerted activity against X4-HIV-1$_{NL4-3}$ and X4-HIV-1$_{LAI}$, but failed to block the infectivity and replication of R5-HIV-1$_{BaL}$ (Table 1). Of note, CX13 was least active among the three PEA derivatives initially examined and only marginally suppressed the fusion event (25 to 27% reduction at 10 µM) as examined using the HIV-1$_{NL4-3}$-derived envelope protein.

The cyclopentylpiperidinyl methyl piperidinyl ethylamine (CpPMPEA) is the pharmacophore of these PEA derivatives (Fig. 4). Substitution of the cyclopentane piperidinyl group with an m-methoxybenzene moiety abolished activity, confirming that CpPMPEA is critical for the observed activities.

After establishing the activity and specificity of the PEA derivatives as described before, we further examined the activity of other PEA derivatives with the CpPMPEA pharmacophore (Fig. 4). CX20 and CX26 similarly exerted substantial activity against X4-HIV-1$_{NL4-3}$ and X4-HIV-1$_{LAI}$, but failed to block the infectivity and replication of R5-HIV-1$_{BaL}$ (Fig. 4, Table 1). Of note, CX20 exerted the most potent activity against X4-HIV-1$_{NL4-3}$ and X4-HIV-1$_{LAI}$, had a therapeutic index of 67, and most potently inhibited the CXCR4-associated fusion and the SDF-1-induced Ca$_{2+}$-flux among the eight PEA derivatives examined. In this regard, a docking analysis demonstrated that the larger benzimidazol group of CX20 forms a better hydrophobic contact with CXCR4 than CX6 containing a phenol moiety, thus probably giving rise to its more potent anti-HIV-1 activity and greater binding affinity. It is also of note that CX26 and CX6 are isomers.
and differ in the position of the alcohol group in phenol (CX26 has an o-phenol, while CX6 an m-phenol). CX26 had comparable anti-HIV-1 activity with an IC₅₀ value of 2.6 µM as compared to CX6, suggesting that the phenol moiety should have good interactions with CXCR4 receptors. We synthesized six other PEA derivatives (CX21, CX22, CX23, CX24, CX25 and CX27) and determined their anti-HIV-1 activities. As assessed in the MTT assay, the compounds had activity against X4-HIV-1NL4-3 with IC₅₀ values between 0.6 to 2.9 µM (Fig. 4), further supporting that the CpPMPEA pharmacophore plays significant roles in the interactions and activity of these derivatives with CXCR4.

**DISCUSSION**

As of this writing, maraviroc, active against R5-HIV-1, is the one and only CCR5 inhibitor in clinical use. Many infected patients carry both R5-HIV-1 as well as X4-HIV-1 strains and no small molecule inhibitor specifically targeting CXCR4 is in clinical use. Moreover, X4-HIV-1 may become predominant when HIV-1 disease progresses. Thus, availability of a CXCR4 inhibitor would greatly increase the treatment options for patients infected with X4- and dual-tropic X4/R5-HIV-1 (1). *In vitro* studies suggest that CCR5 and CXCR4 inhibitors together provide a greater synergistic effect (35). Therefore, the use of CCR5 and CXCR4 inhibitors together should have a significant potential to provide greater benefits to patients, in particular, over those receiving maraviroc alone.

CCR5 has been actively pursued as a promising target as some patients with a natural deletion of the CCR5 gene are apparently physiologically normal. CXCR4 belongs to the family of chemoattractant cytokines and is reportedly functionally
important for angiogenesis, angiostasis, embryogenesis, cancer and inflammatory
diseases (36). These putative functions indicate challenges involved in developing a safe
CXCR4 antagonist(s) for clinical use. In fact, clinical trials of AMD3100 were
discontinued for HIV-1 due to adverse toxicity, though it was later approved as a stem
cell mobilizer. An ideal solution to CXCR4 inhibitor-induced toxicity would be to create
a molecule that when bound to CXCR4 would prevent HIV-1 fusion, without interfering
with normal CXCR4 functions. Besides discovering a molecule with a high selectivity
index, an optimum dosage might need to be determined. It is quite possible that having a
specific dose of a CXCR4 inhibitor could lead to effective HIV intervention, while
minimizing toxicity. If these challenges are overcome, CXCR4 inhibitors are likely to
play a significant role in ART regimens.

Besides inhibitors targeting CCR5 and CXCR4, inhibitors such as AR177
(Zintevir) that target gp120, and demonstrate anti-HIV activity have been reported (37).
Virtually all inhibitors of CXCR4-gp120 interactions previously reported in the literature
were discovered and optimized before the crystal structure became available. The crystal
structure reported by Wu et al. (11), showing an orthosteric binding site, opened up the
possibility of discovering novel inhibitor chemotypes and rational optimization of
inhibitors. We did a comprehensive examination of the effects of amino acid substitutions
in the transmembrane and extracellular domains of CXCR4 on HIV-1-gp120-elicited cell
fusion and identified eleven residues important for preserving the interaction of CXCR4
with the gp120 protein. We hypothesized that molecules that formed hydrogen bond
interactions with at least two of these residues might competitively inhibit the interaction
of CXCR4 and gp120 and exert antiviral activity. A general screening library was
searched to identify molecules with a high three-dimensional shape similarity to a known CXCR4 inhibitor and formed hydrogen bond interactions with at least two of these eleven residues. Using a computational search that took account of the residue-by-residue interaction analysis of CXCR4, we identified piperidinylethanamine derivatives as a novel antagonist family of CXCR4. The most promising PEA derivative (CX20) inhibited SDF-1α-induced Ca\(^{2+}\) influx with CXCR4 in Molt4 cells with an IC\(_{50}\) of 76 nM, and exerted anti-HIV-1 activity with an IC\(_{50}\) value of 600 nM. AMD3100 and AMD11070 inhibited SDF-1α-induced Ca\(^{2+}\) influx with CXCR4 with IC\(_{50}\) values of 12 nM and 32 nM respectively (Table 1). The PEA derivatives did not have any activity against R5-HIV-1_BaL. In summary, our Ca\(^{2+}\) flux, fusion, antiviral and cytotoxicity assays demonstrate that PEA derivatives exert their activity through strong and specific interaction with CXCR4 and not with CCR5. The current study provides a platform for structure-based discovery of CXCR4 inhibitors. The study also sheds important insight into the mechanism of antiviral activity of inhibitors targeting CXCR4 and our approach may be useful in exploring antagonists against other novel targets by understanding the mechanism of action.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG 1 Flow-diagram of computational screening protocol. Starting from over six hundred thousand compounds from a general screening library, molecules that were too flexible or did not meet our desired molecular weight range were eliminated. The three dimensional shape similarity of molecules with IT1t, a known inhibitor was determined and molecules with a Tanimoto shape similarity of at least 0.7 were retained. The interactions of these molecules with CXCR4 were determined by molecular docking. Sixteen molecules were selected based on their ability to form hydrogen bonds to at least two critical residues as determined by CXCR4-mutant-HIV-1-envelope protein fusion assays, and by their binding mode and location in the active site. Ca\textsuperscript{2+} binding, cell-cell fusion, and antiviral assays identified three compounds, with a PEA core that bound to CXCR4 and was active against X4- HIV-1.

FIG 2 Synthesis of inhibitor 6 (CX6).

FIG 3A Effect of CXCR4 amino acid substitution on HIV-1-envelope-elicited cell fusion. Tat\textsuperscript{+}, X4-envelope\textsuperscript{+}-293T cells were co-cultured with U373-MAGI cells expressing LTR-luciferase and CXCR4 (wild-type (WT) or a mutant) in a ninety-six well plate for six hours. The luciferase activity (Y-axis) that determined cell-cell fusion activity was measured and the value in wells co-cultured with CXCR4\textsubscript{WT}\textsuperscript{+}-U373-MAGI is shown as 100\% (black bar). For CXCR4\textsubscript{mutant}\textsuperscript{+}-U373-MAGI, activity less than 70\% compared to that of the wild-type control is shown by magenta bars, whereas activity more than 70\% are shown with a blue bar. The white bars indicate substitutions that had both low cell expression as well as low luciferase activity. The data shown represent mean values (± 1standard deviation) derived from results of at least two independent experiments.
FIG 3B Location of CXCR4 amino acids whose substitution decreased HIV-1-envelope-elicited cell fusion. A ribbon and van der Waals surface diagram of CXCR4 with the location of residues whose substitution resulted in more than a 30% decrease (magenta colored bars in Fig. 3A) of HIV-1-envelope-elicited cell fusion is shown. The red spheres indicate the location of the alpha carbons of these residues. The figure was generated using Maestro version 9.3 from Schrödinger, LLC.

FIG 4 Structure of CXCR4 inhibitors. The structure of IT1t (10), a known inhibitor, and CX6 is shown. The newly identified inhibitors have a cyclopentylpiperidinyl methyl piperidinyl ethylamine (CpPMPEA) pharmacophore. The R-groups and anti-HIV-1 activity of the other derivatives are shown.

FIG 5 (A) ROCS shape overlap and similarity of the query (IT1t) and an identified hit molecule (CX6). The figure shows a representation of the three dimensional shape (volume) overlap and similarity between IT1t (green carbons) and the identified lead molecule CX6 (grey carbons). The surface of IT1t is shown in grey and that of the identified inhibitor CX6 is shown in orange. The molecules are structurally different but have a Tanimoto shape similarity of 0.73. (B-C) A model of the interaction of CX6 with CXCR4 is shown. The model suggests that CX6 forms polar interactions with Asp97 and Glu288 – residues important for the fusion elicited by HIV-envelope protein interactions with CXCR4. A side view and view from top of the molecular surface of CX6 bound in the active site of CXCR4 is shown in D and E, respectively. The figure was generated using Maestro version 9.3 from Schrödinger, LLC.

FIG 6 Antiviral activity of PEA derivatives against HIV-1NL4-3 (X4-HIV-1). The dose-response curves determining the antiviral activities by MTT assay using MT4 cells and
HIV-1<sub>NL4-3</sub> are shown. All assays were conducted in duplicate, and the data shown represent mean values (± 1 standard deviation) derived from results of at least two independent experiments.
- Eliminate aggregators / compounds with more than 20 rotatable bonds
- Retain compounds with MW between 350 and 750 Daltons
- Generate stereoisomers / conformations using OMEGA

- Determine shape similarity to IT1t using ROCS
- Retain molecules with ROCS Tanimoto shape similarity of at least 0.7 to IT1t
- Generate stereoisomers / conformations using Ligprep

- Dock against CXCR4 crystal structure
- Selection by
  - Polar interaction to at least two critical residues
  - Binding mode inside the cavity
  - Visual inspection

- Biological assays (Ca^{2+}-flux binding, fusion inhibition, cell-based antiviral assays etc.)
FIG 2

Chemical reaction diagram:

1. $\text{NaBH}_3(\text{OAc})_3, \text{AcOH}, \text{CH}_2\text{Cl}_2$, 81%

2. $\text{DIBAL-H}, \text{CH}_2\text{Cl}_2$, 75%

3. $\text{Br}_3$, 1. piperidine, $\text{K}_2\text{CO}_3$, DMF, 75%

4. $2. 3\text{N HCl}$, 69%

5. $\text{EtOH}, \text{NaBH}_4$, $\text{Ti(PhO)}_4$, 84%

6. $\text{NaBH}_3(\text{OAc})_3, \text{AcOH}, \text{CH}_2\text{Cl}_2$, 78%
FIG 4

CX11
IC_{50} 2.6 µM

CX13
IC_{50} 2.6 µM

CX20
IC_{50} 0.6 µM

CX21
IC_{50} 0.6 µM

CX22
IC_{50} 1.8 µM

CX23
IC_{50} 1.4 µM

CX24
IC_{50} 0.9 µM

CX25
IC_{50} 1.9 µM

CX26
IC_{50} 2.6 µM

CX27
IC_{50} 2.9 µM

CpPMPEA

IT1t
FIG 5

A. CX6

B. IT1t

C. CX6

D. CX6

E. CX6
Table 1. Anti-HIV activities, SDF-1α binding inhibition (IC₅₀), and cytotoxicity (CC₅₀) of selected CXCR4 inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTT HIV-1NL4-3(X4)</th>
<th>IC₅₀ (µM)</th>
<th>P24 HIV-1NL4-3(X4)</th>
<th>IC₅₀ (µM)</th>
<th>MAGI HIV-1NL4-3(R5)</th>
<th>IC₅₀ (µM)</th>
<th>MAGI HIV-1NL4-3env (X4)</th>
<th>IC₅₀ (µM)</th>
<th>Fusion Ca²⁺ Flux</th>
<th>Molt4 cell</th>
<th>Binding IC₅₀ (nM)</th>
<th>MTT</th>
<th>CC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX6</td>
<td>1.5 ± 0.4</td>
<td>3.0 ± 0.8</td>
<td>3.0 ± 1.5</td>
<td>&gt;10</td>
<td>1.9 ± 0.4</td>
<td>92 ± 20</td>
<td></td>
<td>58 ± 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX11</td>
<td>2.6 ± 0.3</td>
<td>4.6 ± 2.0</td>
<td>5.2 ± 3.0</td>
<td>&gt;10</td>
<td>7.9 ± 1.1</td>
<td>161 ± 6</td>
<td></td>
<td>96 ± 22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX13</td>
<td>2.6 ± 1.0</td>
<td>6.7 ± 2.2</td>
<td>7.8 ± 1.3</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>149 ± 19</td>
<td></td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX20</td>
<td>0.6 ± 0.5</td>
<td>1.0 ± 0.5</td>
<td>1.2 ± 0.5</td>
<td>&gt;10</td>
<td>1.5 ± 0.2</td>
<td>76 ± 10</td>
<td></td>
<td>40 ± 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX26</td>
<td>2.6 ± 1.3</td>
<td>2.6 ± 1.1</td>
<td>7.3 ± 1.1</td>
<td>&gt;10</td>
<td>2.9 ± 0.5</td>
<td>159 ± 20</td>
<td></td>
<td>36 ± 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMD3100</td>
<td>0.02 ± 0.01</td>
<td>n.d.</td>
<td>0.011 ± 0.008</td>
<td>&gt;10</td>
<td>n.d.</td>
<td>12 ± 1</td>
<td></td>
<td>&gt;10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMD11070</td>
<td>0.026 ± 0.01</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>32 ± 4</td>
<td></td>
<td>&gt;10</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

CX6, CX11 and CX13 were among the sixteen compounds initially selected from the general screening library. CX20 and CX26, containing the CpPMPEA pharmacophore were subsequently analyzed. Anti-HIV activity and binding data for known CXCR4 inhibitors AMD3100 and AMD11070 are included for comparison. All assays were conducted in duplicate, and data shown represent mean values (± 1 standard deviation) derived from results of at least two independent experiments.

n.d.: not determined