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Interaction of small molecule inhibitors of HIV-1 entry with CCR5

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Abstract

The CC-chemokine receptor 5 (CCR5) is the major coreceptor for macrophage-tropic (R5) HIV-1 strains. Several small molecule inhibitors of CCR5 that block chemokine binding and HIV-1 entry are being evaluated as drug candidates. Here we define how CCR5 antagonists TAK-779, AD101 (SCH-350581) and SCH-C (SCH-351125), which inhibit HIV-1 entry, interact with CCR5. Using a mutagenesis approach in combination with a viral entry assay to provide a direct functional read out, we tested predictions based on a homology model of CCR5 and analyzed the functions of more than 30 amino acid residues. We find that a key set of aromatic and aliphatic residues serves as a hydrophobic core for the ligand binding pocket, while E283 is critical for high affinity interaction, most likely by acting as the counterion for a positively charged nitrogen atom common to all three inhibitors. These results provide a structural basis for understanding how specific antagonists interact with CCR5, and may be useful for the rational design of new, improved CCR5 ligands.
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Introduction

HIV-1 cellular entry inhibitors are a promising new class of potential anti-HIV-1/AIDS drugs (Michael and Moore, 1999; Moore and Stevenson, 2000; LaBranche et al., 2001; De Clercq, 2002). By interacting with the viral envelope glycoproteins (gp120 or gp41), with CD4 or with the coreceptors, these inhibitors block different steps in the complex sequence of events leading to virus–cell fusion (Michael and Moore, 1999; Moore and Stevenson, 2000; LaBranche et al., 2001; De Clercq, 2002). The HIV-1 coreceptors are particularly attractive targets for entry inhibitors, because they belong to the protein superfamily of G protein-coupled receptors (GPCRs) (Schwarz and Wells, 2002; Kazmierski et al., 2003; Horuk, 2003; Seibert and Sakmar, 2004).

Although several chemokine receptors are able to mediate HIV-1 entry in vitro, only CCR5 and CXCR4 are likely to be of major importance in vivo and hence relevant targets for pharmaceutical intervention (Zhang and Moore, 1999; Zhang et al., 2000). CCR5 in particular is the principal coreceptor for the HIV-1 strains that are most commonly transmitted between individuals and which predominate during the early years of infection (Berger et al., 1999; Douek et al., 2003). The clinical relevance of the predominant use of CCR5 by

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HIV-1 is demonstrated by the impact of a naturally occurring CCR5 mutation, CCR5-Δ32, that generates a non-functional coreceptor (Berger et al., 1999; Liu et al., 1996; Samson et al., 1996; Dean et al., 1996; Wu et al., 1997; O'Brien and Moore, 2000). Individuals who are homozygous for this mutation are strongly protected against HIV-1 infection, while infected, heterozygous individuals progress less rapidly to disease and death. Furthermore, no obvious adverse effects on health have been observed in individuals lacking functional CCR5.

Several different types of inhibitors for CCR5-mediated HIV-1 entry have now been identified and are in pre-clinical or clinical development as drug candidates. These include CC-chemokine derivatives, anti-CCR5 monoclonal antibodies and small molecule receptor antagonists (Michael and Moore, 1999; Moore and Stevenson, 2000; LaBranche et al., 2001; De Clercq, 2002; Schwarz and Wells, 2002; O'Hara and Olson, 2002; Kazmierski et al., 2003; Horuk, 2003). High-throughput screening of compound libraries followed by medicinal chemistry-based optimization of lead structures allowed the discovery of several potent, small molecule antagonists of CCR5-mediated chemokine signaling and HIV-1 entry (Schwarz and Wells, 2002; Kazmierski et al., 2003; Horuk, 2003; Seibert and Sakmar, 2004). TAK-779, a quaternary ammonium anilide (Fig. 1), was the first small molecule CCR5 antagonist reported (Baba et al., 1999). Other small molecule CCR5 antagonists with improved potency and/or pharmacological properties have since been described (Schwarz and Wells, 2002; Kazmierski et al., 2003; Horuk, 2003; Seibert and Sakmar, 2004). For example, SCH-C (SCH-351125) is an oximino-piperidino-piperidine amide (Fig. 1) (Palani et al., 2001, 2002; Strizki et al., 2001) that has antiviral activity in HIV-1-infected humans (Reynes et al., 2002). AD101 (SCH-350581) (Fig. 1), a piperidino-piperazine based compound, is about 10-fold more potent than SCH-C but has poor pharmacological properties (Tagat et al., 2001a, 2001b). SCH-D (SCH-417690), which is now in clinical development but was not available when this study was conducted, is structurally related to AD101 and has both improved potency and better pharmacological properties compared to SCH-C (Tagat et al., 2004; Schurmann et al., 2004).

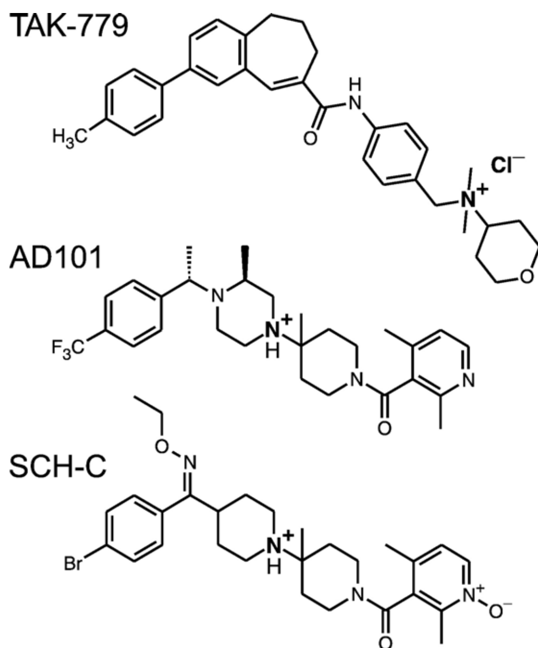

Fig. 1. Chemical structures of TAK-779, AD101 and SCH-C. TAK-779, a quaternary ammonium ion, is shown with a chloride counterion. AD101 and SCH-C are shown in the protonated state that predominates at physiological pH. In each structure, the positively charged nitrogen atom is highlighted in bold. On the basis of pK_a prediction methods (Perrin et al., 1981), we estimated that the second nitrogen atom in the AD101 piperazine ring is not protonated at physiological pH.

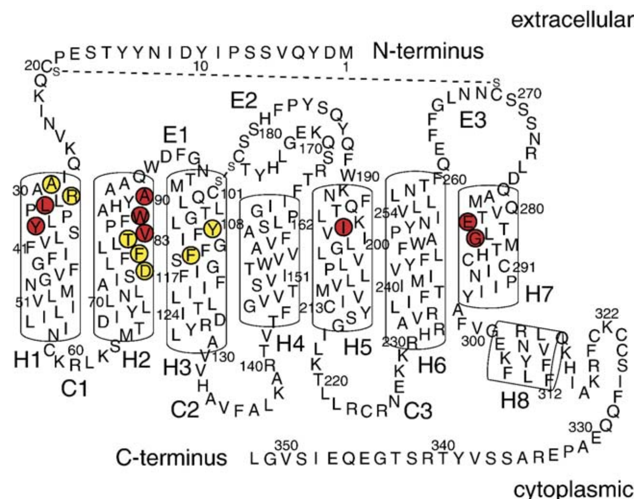

Fig. 2. CCR5 residues involved in the interaction with TAK-779, AD101 or SCH-C. The seven transmembrane helices (H1–H7) and helix 8 (H8) are symbolized as cylinders. The assignment of the helical segments is based on the transmembrane topology of rhodopsin (Palczewski et al., 2000). Extracellular and cytoplasmic loops are labeled E1, E2, E3 and C1, C2, C3, respectively. The amino acid sequence is represented in single-letter code (excluding the C-terminal hemagglutinin affinity tag). Residues highlighted in red have been shown to be required for efficient interaction with TAK-779, AD101 and SCH-C; residues labeled in yellow have been shown to be required for a subset of these inhibitors only (A29, R31 for TAK-779; F79 for AD101; D76, F113 for AD101 and SCH-C; Y108 for TAK-779 and AD101).

TAK-779, SCH-C and AD101 all inhibit HIV-1 entry into target cells by blocking the interaction between the gp120/CD4 complex and CCR5 (Dragic et al., 2000; Tsamis et al., 2003). The underlying molecular mechanism of this activity is not known. Available evidence from mutagenesis and immunological studies suggests that the gp120 binding site on CCR5 is located within its N-terminus (Nt) and the second extracellular loop (E2) (Rucker et al., 1996; Ross et al., 1998; Blanpain et al., 1999; Howard et al., 1999; Dragic, 2001; Dragic et al., 1998; Rabut et al., 1998; Farzan et al., 1998; Doranz et al., 1997). The TAK-779, AD101 and SCH-C molecules, however, are believed to bind predominantly within the transmembrane domain of CCR5 (Dragic et al., 2000; Tsamis et al., 2003). Several CCR5 residues required for these compounds to inhibit HIV-1 entry have been identified using alanine scanning mutagenesis (Fig. 2) (Dragic et al., 2000; Tsamis et al., 2003). However, a detailed side-by-side comparison of several

inhibitors with the aim of identifying their mechanism of action has not been reported.

We hypothesized that common structural elements among the inhibitors interact with a common ligand binding pocket in the transmembrane domain of CCR5, while other compound-specific elements provide additional receptor contacts (Tsamis et al., 2003). To test this hypothesis, we constructed a three-dimensional model of the transmembrane domain of CCR5 based on the rhodopsin crystal structure (Palczewski et al., 2000). We used this model to analyze data from previous mapping studies (Dragic et al., 2000; Tsamis et al., 2003), and to design a series of receptor mutants, which were tested for inhibitor sensitivity in an HIV-1 entry assay. The results confirm the fidelity of the model of CCR5 topology and support the hypothesis of a common inhibitor-binding site. Furthermore, the specific contributions of key residues involved in a direct interaction between CCR5 and each of the three small molecule inhibitors were defined, and largely account for differences in their potency and specificity. These results provide a structural basis for understanding how small molecules antagonize HIV-1 entry via CCR5, and may be useful for the rational design of more effective inhibitors.

Results

The role of CCR5 extracellular domain residues

The TAK-779, AD101 and SCH-C structures contain hydrophobic aromatic groups as well as more hydrophilic regions that include a positively charged nitrogen atom (Fig. 1). While TAK-779, a quaternary ammonium ion, has a permanent positive charge, AD101 and SCH-C respectively possess basic piperazine or piperidine nitrogens that are protonated at physiological pH (Perrin et al., 1981). Our initial hypothesis was that the hydrophobic parts of TAK-779, AD101 and SCH-C might bind to the transmembrane domain of CCR5 while the more hydrophilic regions, including the positively charged nitrogen, would interact with the extracellular domain. However, this hypothesis was not supported by the results of an extensive alanine scanning mutagenesis screen of the entire extracellular domain of CCR5 (Dragic et al., 2000; Tsamis et al., 2003). We designed and tested additional mutants to determine whether the contribution of residues in the N-terminus (Nt) or the extracellular loops (E1, E2 and E3) might have escaped detection by the alanine mutagenesis approach (see legend to Table 1).

We first investigated whether any of the acidic residues D2 (Nt), D11 (Nt), E18 (Nt), D95 (E1), E172 (E2), E262 (E3), D276 (E3) or the tyrosine residues Y3 (Nt), Y10 (Nt), Y14 (Nt) and Y15 (Nt) that are potentially sulfated (Farzan et al., 1999; Seibert et al., 2002) (see Fig. 2), could be involved in the interaction with the positively charged nitrogen that is common to TAK-779, AD101 and SCH-C (Fig. 1). We changed each of these residues to lysine. Also, we tested other non-alanine mutants that involve a significant change in either the size or charge of the side chain (see legend to Table 1). When assayed for inhibition of HIV-1 entry, none of these mutants differed significantly from wild-type CCR5 for any of the three inhibitors (see legend to Table 1). In accordance with our previous studies (Dragic et al., 2000; Tsamis et al., 2003), we find no evidence that TAK-779, AD101 or SCH-C interacts with extracellular regions of CCR5. In particular, there are no ionic interactions between acidic residues in the CCR5 extracellular domain and the positively charged nitrogen atoms of the antagonists.

Table 1
Effects of substitutions of CCR5 residues on inhibition of HIV-1 entry

Mutant[a]	% HIV-1 JR-FL entry[b]		
	TAK-779	AD101	SCH-C
WT-CCR5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A29L	73.5 ± 10.1	0.6 ± 1.9	0.0 ± 0.0
L33A	40.0[c]	34.2 ± 2.2[d]	19.9 ± 2.7[d]
L33V	30.2 ± 6.8	6.3 ± 0.9	15.1 ± 0.6
L33I	25.3 ± 5.3	5.3 ± 2.9	6.0 ± 5.3
L33N	68.5 ± 8.4	24.0 ± 3.3	41.8 ± 3.3
L33T	59.0 ± 5.4	43.0 ± 6.8	63.5 ± 3.5
L33M	27.3 ± 3.2	21.3 ± 5.7	40.3 ± 5.9
Y37A	74.0[c]	71.5 ± 2.2[d]	32.1 ± 5.5[d]
Y37F	43.5 ± 9.8	0.0 ± 0.0	0.8 ± 0.8
Y37W	60.3 ± 1.4	3.7 ± 2.2	0.0 ± 0.0
Y37W/W86Y	77.6 ± 13.0	48.8 ± 3.2	57.3 ± 6.3
D76A	1.0[c]	44.7 ± 5.1[d]	20.6 ± 3.3[d]
D76E	4.8 ± 2.8	8.3 ± 5.3	22.0 ± 2.7
D76K	4.6 ± 3.5	34.6 ± 8.5	68.2 ± 19.6
V83A	18.8 ± 3.6	18.6 ± 2.5	21.1 ± 0.1
W86A	79.0[c]	93.8 ± 12.4[d]	62.0 ± 0.8[d]
W86F	56.3 ± 4.2	39.8 ± 8.9	51.7 ± 5.4
W86Y	60.3 ± 5.3	42.2 ± 4.0	74.5 ± 4.2
A90L	36.1 ± 6.0	43.1 ± 4.3	61.0 ± 7.1
L104E/E283L	71.4 ± 4.9	97.2 ± 4.8	77.4 ± 11.7
Y108A	100.0[c]	42.6 ± 1.0[d]	9.6 ± 0.2[d]
Y108F	78.5 ± 2.1	24.2 ± 2.6	8.5 ± 1.4
Y108Q	33.3 ± 6.3	39.6 ± 2.4	11.3 ± 0.6
E283A	27.0[c]	118.7 ± 18.4[d]	94.6 ± 13.3[d]
E283Q	28.1 ± 4.3	61.7 ± 4.9	122.6 ± 22.8
E283L	84.7 ± 1.3	73.1 ± 3.6	90.4 ± 11.1
E283D	39.8 ± 4.4	98.5 ± 11.0	73.7 ± 5.3
E283K	81.0 ± 10.5	106.7 ± 13.2	87.5 ± 11.2
G286A	48.1 ± 2.8	2.7 ± 0.2	3.4 ± 1.6
G286V	61.6 ± 9.4	65.9 ± 9.4	66.9 ± 12.3

[a] The following mutations in CCR5 had no significant effect on HIV-1 entry in the presence of any of the three inhibitors: D2K, Y3K, Y10K, D11K, Y14K, Y15F, Y15D, Y15K, E18K, N24Q, A30L, V40A, V40I, D76N, V83I, V83L, V83T, A91L, A92L, A92V, D95N, D95K, L104E, E172K, N192K, E262K, N268D, S270L, S271L, D276N, D276L, D276K, A278L.

[b] Relative HIV-1 entry in the presence of inhibitors normalized to entry in the absence of inhibitors. All values are means ± standard error of at least three independent experiments. A value of 100% means that a mutant is insensitive to the inhibitor, a value of 0% means that a mutant is as sensitive to the inhibitor as wild-type CCR5.

[c] Data from Dragic et al. (2000).

[d] Data from Tsamis et al. (2003).

Structural model of the CCR5 transmembrane domain

Next, we focused on defining the specific function of transmembrane domain residues in the interaction of CCR5 with TAK-779, AD101 and SCH-C. We previously used a low resolution rhodopsin model (Shieh et al., 1997), derived from

electron diffraction and NMR studies, as a template to construct a model of the CCR5 transmembrane domain that was useful for mapping the TAK-779 binding site (Dragic et al., 2000). Here, we prepared an improved model of CCR5 based on the crystal structure of rhodopsin, which was solved at 2.8 Å resolution (Palczewski et al., 2000). This CCR5 model includes the seven α-helical transmembrane segments H1 (K26-C58), H2 (S63-W94), H3 (F96-V131), H4 (T141-I165), H5 (W190-I217), H6 (K228-Q261) and H7 (L275-G301). Not included are the N- and C-terminus (Nt, Ct), the extracellular and cytoplasmic loop regions (E1–E3, C1–C3) and the putative helix 8 (H8). The new model redefines the assignments of the transmembrane helices. Furthermore, the relative orientations of the helices and the positions of the amino acid side chains are also significantly different compared to the previous model. The new model proved to be generally more reliable in predicting and explaining the results from our mutagenesis studies than the previous model (see below). The model is depicted in Fig. 3, which also presents information on the inhibitor-binding site that was derived from the experiments described below. The model coordinates are available upon request.

The CCR5 binding pocket for small molecule inhibitors

To localize the small molecule binding site on CCR5, we first reanalyzed data from our previous mapping studies (Dragic et al., 2000; Tsamis et al., 2003) (Fig. 2) using the refined CCR5 model (Fig. 3). For completeness, we included information on residues A29, V83, A90 and G286 in the analysis although these residues were only identified later in this study. While amino acid substitutions of residues L33, Y37, V83, W86, A90, I198, E283 and G286 reduced the inhibitory effect of all three compounds in an HIV-1 entry assay, certain changes at other residues had a more specific effect (Fig. 2). For example, substitutions of D76 and F113 adversely influenced the inhibitory activities of AD101 and SCH-C, but not of TAK-779. Likewise, changing residue Y108 affected the activity of TAK-779 and AD101, but not of SCH-C. Only TAK-779 activity was sensitive to alterations at residues A29, R31 and T82, and only AD101 was affected by substitutions of F79.

The majority of the residues important for the interactions of CCR5 with TAK-779, AD101 and/or SCH-C are clustered

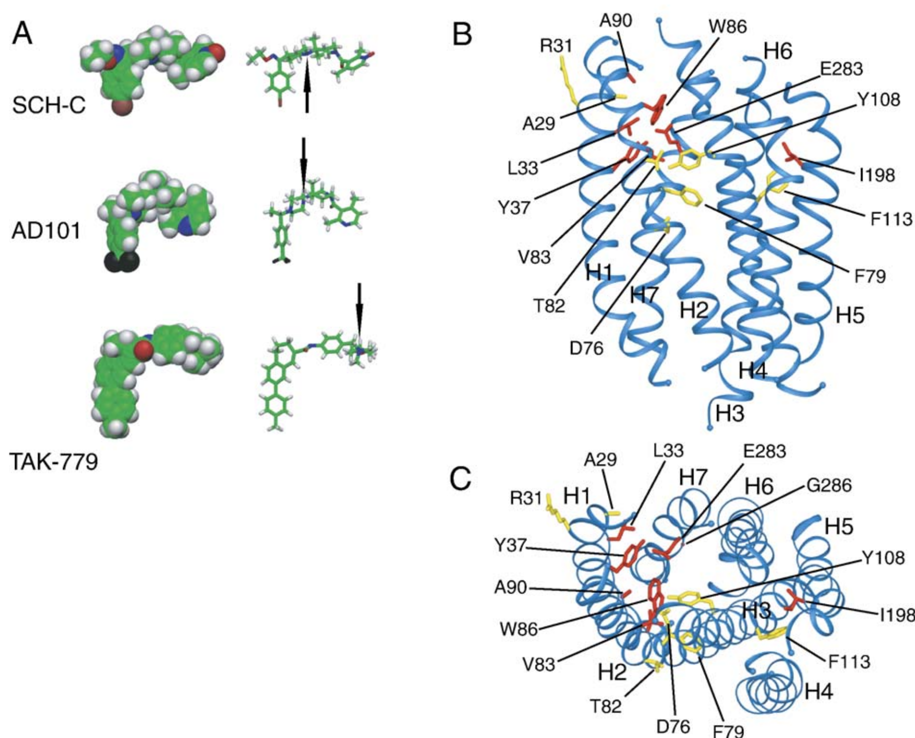

Fig. 3. Model of the transmembrane domain of CCR5 with energy-minimized structures of TAK-779, AD101 and SCH-C. (A) Energy-minimized structures of TAK-779, AD101 and SCH-C in space-filling and stick representation. Atoms are color-coded: carbon, green; oxygen, red; nitrogen, blue; hydrogen, grey; fluorine, black; bromine, brown. Note the similarities between the three structures: The molecules adopt a broadly similar L-shape, the left segment consists of hydrophobic aromatic groups (facing downwards), the right part contains a positively charged nitrogen atom (arrow mark). (B) Structural model of the transmembrane domain of CCR5 viewed from within the plane of the membrane. The extracellular surface is oriented towards the top of the figure, the cytoplasmic surface towards the bottom. The seven α-helical transmembrane segments (H1–H7, Cα-traces only) are depicted as blue-colored ribbons. Amino acid residues required for efficient interaction of CCR5 with TAK-779, AD101 or SCH-C are shown in stick representation. The color coding scheme is the same as in Fig. 2: substitution by alanine (or leucine) of red-colored residues (L33, Y37, W86, A90, I198, E283, G286) affected TAK-779, AD101 and SCH-C activity, replacement of yellow-colored residues affected a subset of these inhibitors only (A29, R31: TAK-779; F79: AD101; D76, F113: AD101 and SCH-C; Y108: TAK-779 and AD101). (C) View of the CCR5 model from the extracellular side of the membrane after rotating the model by approximately 90° out of the paper plane from the orientation in panel B. Labeling and color coding are the same as in panel B. The models in panels A, B and C are shown at the same scale.

in transmembrane helices H1, H2, H3 and H7. They span the distance from the extracellular border to about one-third of the way into the transmembrane domain and surround a cavity that is believed to be the small molecule binding pocket (Figs. 3B, C). According to the CCR5 model, residues V83, W86, A90, Y108 and E283 point toward the center of the helical bundle (Fig. 3C). These residues are, therefore, particularly good candidates for a direct interaction with the small molecule inhibitors. Residues A29, L33 and Y37 are also located inside the helical bundle but tend to be oriented towards the cleft between transmembrane helices H1 and H7 (Fig. 3C). Hence, this cleft might also participate in inhibitor binding. Residue G286 on the other hand is roughly oriented towards H3 and H6.

To gain insight into how TAK-779, AD101 and SCH-C might fit into this putative binding pocket, we calculated their energy-minimized structures. All three inhibitors most likely adopt a broadly similar, bent conformation (Fig. 3A). In the case of TAK-779, the calculated structure was confirmed to be accurate by using NMR spectroscopy (data not shown). Juxtaposing the models for CCR5 (Figs. 3B, C) and the small molecules (Fig. 3A) suggests that several of the residues that were previously identified by alanine-scanning mutagenesis might not interact directly with the inhibitors. In particular, the side chains of residues R31, D76, F79, T82, F113 and I198 are either located outside the helical bundle or are likely to be too distant from the putative binding pocket. Due to the inherent limitations of the CCR5 homology model and because the specific receptor and inhibitor conformations in the complex are currently not known, we do not present any computational docking studies.

The role of E283 and D76

Our observation that none of the negatively charged residues in the extracellular domain of CCR5 is involved in the interaction with the cationic small molecule inhibitors (Table 1) focused our attention on acidic residues in the transmembrane domain of the receptor. Alanine scanning mutagenesis of the transmembrane domain identified two acidic residues, E283 and D76 (Dragic et al., 2000; Tsamis et al., 2003). Of these residues, only E283 in H7 is critical for CCR5 to interact efficiently with TAK-779, AD101 and SCH-C. Substitution of D76 in H2, on the other hand, affects the activity of AD101 and to a lesser extent SCH-C, but not TAK-779. According to the CCR5 model, the carboxyl group of residue E283 is located in the putative binding pocket and is hence well positioned to serve as the counterion for the charged nitrogen atom on each inhibitor (Fig. 4). Importantly, E283 is not surface accessible and does not have an apparent positive counterion in the CCR5 model. E283 is predicted to be protonated and hydrogen bonded to Y37. The absence of a positive counterion in the vicinity of E283 in CCR5 should facilitate an ionic interaction with the positively charged inhibitors. A direct interaction between D76 and the small molecules is less likely to occur, however, because this residue is located in the center of the transmembrane domain (Fig. 4). Moreover, D76 is located in a polar pocket and

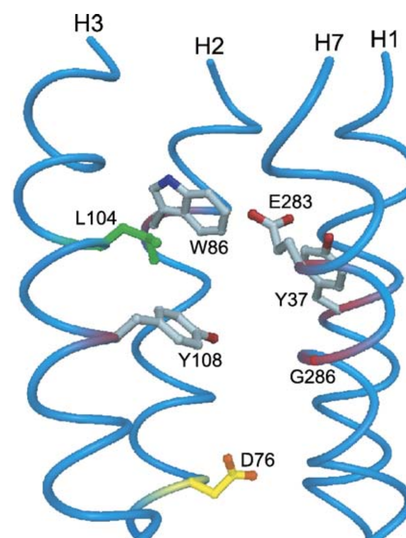

Fig. 4. Orientation of key acidic and aromatic residues in the small molecule binding site. Close-up view on the putative binding site from within the plane of the membrane showing the Cα-traces of transmembrane helices H1, H2, H3 and H7 (blue-colored tubes). The extracellular surface is oriented towards the top of the figure, the cytoplasmic surface towards the bottom. Key residues E283, Y37, W86 and Y108 are colored in grey, G286 is shown in red, D76 is colored in yellow and L104 is depicted in green. Side chain oxygen atoms are labeled in red, and side chain nitrogen atoms are labeled in blue.

part of a hydrogen-bonding network that includes N48, H289 and C290.

To clarify the role of E283 and D76 in small molecule inhibition of HIV-1 entry, we introduced non-alanine substitutions at both positions (Table 1). Replacing E283 with glutamine reduced the ability of each of the three compounds to inhibit HIV-1 entry, the effect being more pronounced for AD101 and SCH-C than for TAK-779 (Table 1). For AD101 and SCH-C, the effect of the E283L substitution was not significantly different from that seen with E283A and E283Q (Table 1). However, the E283L substitution was more effective than either E283A or E283Q at impairing the inhibitory activity of TAK-779 (Table 1). This difference between AD101/SCH-C and TAK-779 might be caused by steric interference between TAK-779 and a leucine side chain at residue 283.

These results indicate that a negative charge at position 283 is required for efficient interaction of CCR5 with the small molecule inhibitors, most likely by acting as a counterion for the positively charged nitrogen atom. Substituting E283 with a positively charged lysine residue not surprisingly caused a strong reduction in inhibitory activity for TAK-779, AD101 and SCH-C (Table 1). However, the conservative substitution of E283 with an aspartate residue (E283D) created a coreceptor with properties similar to those of the E283A and E283Q mutants (Table 1). This result suggests that the shorter alkyl chain of aspartate compared to glutamate prevents the aspartate carboxyl group from protruding far enough into the binding pocket to interact efficiently with the inhibitors, or otherwise positions the carboxyl group inappropriately. To probe further the role of E283, we swapped the position of the glutamate side chain of residue E283, by creating the L104E/E283L double mutant. According to the CCR5 model, the side chain of residue

L104 in H3 is oriented towards the carboxyl group of residue E283 (Fig. 4). The L104E substitution did not itself affect HIV-1 entry inhibition by TAK-779, AD101 or SCH-C, so an additional acidic residue can be tolerated within the binding pocket. However, the L104E/E283L double mutant behaved similarly to the E283L mutant, and differently from the L104E mutant, in that it was only weakly sensitive to the three inhibitors (Table 1).

In contrast to what was observed for E283, replacement of D76 with asparagine resulted in a coreceptor with wild-type CCR5 characteristics (Table 1). This clearly shows that a negative charge on the side chain of D76 is not required for the interaction with the antagonists. However, replacement of D76 with glutamate led to a loss in sensitivity towards SCH-C only, while its replacement with lysine reduced sensitivity to AD101 and SCH-C but not TAK-779 (Table 1). These results suggest that a hydrogen acceptor/donor group at residue 76 is required for the efficient interaction with AD101 and SCH-C but not TAK-779. We cannot rule out completely that D76 might interact directly with functional groups in AD101/SCH-C. However, because of the unfavorable location of D76 for such an interaction (Fig. 4), it is more likely that a hydrogen bond between D76 and another CCR5 residue is responsible for the observed effects. As noted above, D76 in H1 is predicted to interact with both N48 in H2 and H289/C290 in H7. Disrupting this hydrogen-bonding network in the D76A and D76K mutants would likely change the position or orientation of H1, H2 or H7.

Interaction of inhibitors with key CCR5 aromatic residues

The CCR5 model predicts that the aromatic rings of Y37 and W86 constitute a large area of the small molecule binding interface. Furthermore, they are located in close proximity to the carboxyl group of E283 (Fig. 4). Another aromatic residue, Y108, is located directly below these residues with its hydroxyl group pointing towards the center of the helical bundle (Fig. 4). Substituting Y108 with alanine significantly reduced the inhibitory activity of TAK-779 and AD101, but not of SCH-C (Dragic et al., 2000; Tsamis et al., 2003) (Table 1), which is surprising because AD101 is structurally related to SCH-C but not TAK-779 (Fig. 1).

To define the specific, individual contributions of residues Y37, W86 and Y108 to the interaction of the inhibitors with CCR5, we first addressed whether it was the hydroxyl group or the aromatic moiety of tyrosine residue Y37 that was the more important. The Y37F mutant remained sensitive to AD101 and SCH-C (Table 1), so the aromatic ring but not the hydroxyl group of Y37 must be involved in interacting with these inhibitors. To further define the side-chain requirements for Y37, we made a tryptophan substitution. The Y37W mutant remained fully sensitive to both AD101 and SCH-C (Table 1). However, TAK-779 sensitivity was severely reduced by the loss of the phenolic hydroxyl group in both the Y37F and Y37W mutants (Table 1), suggesting that the hydroxyl group might be involved in a specific interaction with the TAK-779 molecule. Alternatively, the hydroxyl group could participate in an intramolecular interaction within CCR5 that might be required for binding TAK-779, but not AD101 or SCH-C.

Next, we replaced W86 with phenylalanine or tyrosine. The CCR5 model predicts that the smaller aromatic side-chain of these residues will not reach very deeply into the inhibitor-binding pocket, but will instead point towards H1 (Fig. 4). The HIV-1 entry inhibition experiments support this prediction (Table 1). Thus, the W86F and W86Y mutant coreceptors are relatively insensitive to the three compounds, indicating that phenylalanine and tyrosine cannot fully replace W86 in the binding pocket. Moreover, HIV-1 entry data for the Y37W/W86Y double mutant indicate that the mere presence of an indole ring system is not sufficient; its precise location within the binding pocket is also crucial. The inhibitor sensitivity of the Y37W/W86Y mutant coreceptor resembles that of the W86Y mutant (Table 1), so swapping the positions of these two key aromatic residues does not permit CCR5 to interact properly with any of the inhibitors.

We then analyzed the side-chain requirements for residue Y108. The inhibitor-selective effect of the tyrosine to phenylalanine substitution, Y108F, implies that the phenolic hydroxyl group is important for CCR5 to interact with TAK-779, less critical for AD101 and not required at all for SCH-C (Table 1). However, when a hydrogen-bond acceptor/donor group was retained on a non-aromatic residue at position 108, by replacing the hydroxyl group with an amide to make the Y108Q mutant, CCR5 remained fully sensitive to SCH-C but not to either TAK-779 or AD101 (Table 1). Thus, the aromatic moiety of Y108 might also be involved in the interaction of CCR5 with TAK-779 and AD101. This could be a direct effect or, more likely, an indirect one, for example by affecting the precise positioning of the hydroxyl group.

The role of aliphatic amino acid residues

We next addressed the role that certain aliphatic amino acid residues play in the interaction of CCR5 with TAK-779, AD101 and SCH-C. According to the CCR5 model, several small aliphatic side chains are located at the extracellular border of transmembrane helices H1, H2, H3 and H7 (Fig. 5)—a region predicted to be the entrance of the binding pocket. A29 and A30 in H1 are oriented not towards the binding pocket but towards neighboring helices, respectively H7 and H2 (Fig. 5). Replacing A30 with leucine had no effect on the activity of the three inhibitors, whereas substituting A29 with leucine strongly impaired the activity of TAK-779 but did not affect AD101 and SCH-C (Table 1). Hence, the predicted orientations of A29 and A30 are likely to be accurate. The strong impairment of only TAK-779 activity caused by the A29L substitution suggests that this inhibitor binds differently from the other two. TAK-779 is larger than AD101 and SCH-C, so its binding may also depend on amino acid side chains located in the cleft between H1 and H7.

The extracellular border of H2 contains a cluster of three consecutive alanine residues (Fig. 5). Of these, only A90 is oriented towards the putative binding pocket, one helical turn above W86, a residue critical for HIV-1 entry inhibition by all

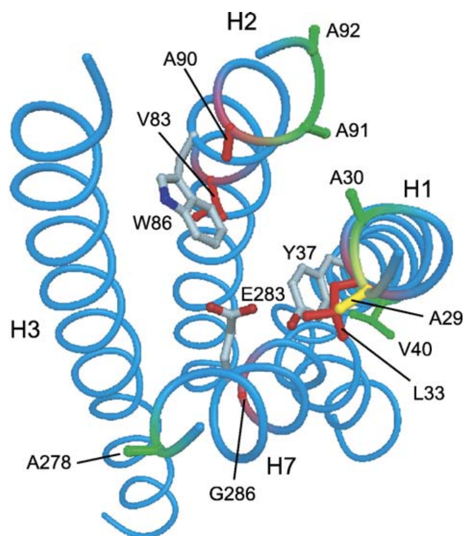

Fig. 5. Orientation of aliphatic side chains relative to the small molecule binding site. View on the putative binding site from the extracellular side of the membrane showing the Cα-traces of transmembrane helices H1, H2, H3 and H7 (blue-colored tubes). Key residues E283, Y37 and W86 are colored in grey with side chain oxygen atoms labeled in red and side chain nitrogen atoms labeled in blue. Changing the side chain size of aliphatic residues colored in red (L33, V83, A90, G286) affected the activity of TAK-779, AD101 and SCH-C. In contrast, substitutions of residues colored in green (A30, V40, A91, A92, A278) had no effect on any of the three inhibitors and substitution of A29 (labeled in yellow) by leucine only reduced the effect of HIV-1 entry inhibition by TAK-779.

three compounds. Residue A91, however, is oriented towards H1 and residue A92 faces outwards, away from the helical bundle (Fig. 5). The CCR5 model suggests that replacing A90, but not A91 or A92, with a large and bulky residue like leucine should block the interaction with all three small molecule inhibitors. Again, the experimental results confirm this prediction (Table 1). As a negative control, we also included A278 in the leucine substitution series. This residue is located on top of H7 and predicted to point outwards, away from the binding pocket (Fig. 5). As expected, we saw no effect of the A278L substitution on HIV-1 entry inhibition by any of the compounds (Table 1).

Having defined the extracellular border of the putative binding pocket, we further explored the role of aliphatic residues that reach deeper into the CCR5 transmembrane domain. In particular, we were interested in L33 because this residue, located about one helical turn above the aromatic key residue Y37 in H1, is predicted to border the small molecule binding pocket at the H1–H7 cleft (Fig. 5). In previous studies (Dragic et al., 2000; Tsamis et al., 2003), it was shown that substitution of L33 with a much smaller alanine residue resulted in reduced sensitivity towards TAK-779, AD101 and SCH-C (Table 1). To further explore the function of L33, we first analyzed the effect that a polar side chain would have at this position by replacing L33 with threonine or asparagine. The results for the L33T and L33N mutants indicate that a polar side chain interferes with the CCR5 interactions of TAK-779, AD101 and SCH-C (Table 1). Next, we tested the effect of conservative amino acid changes. Replacing L33 with either isoleucine or valine had a substantial effect only for TAK-779 (Table 1). However, introducing a methionine at this position reduced the inhibitory activities of all three compounds (Table 1).

Another aliphatic residue, V40, is located about one helical turn below Y37 and so is not directly associated with the surface of the binding pocket (Fig. 5). Furthermore, relative to Y37 and L33, the side chain of V40 is rotated away from the transmembrane helical bundle. In agreement with the predictions of the CCR5 model, none of the substitutions for V40 resulted in a loss of sensitivity for TAK-779, AD101 or SCH-C (Table 1).

Similar to V40, residue V83 is also located about one helical turn below the second key, aromatic residue W86 in H2 (Fig. 5). In contrast to V40, however, V83 is oriented towards the transmembrane helical bundle. Substituting V83 with alanine resulted in a moderate reduction in sensitivity towards TAK-779, AD101 and SCH-C (Table 1). However, more subtle variations in the size or polarity of the side chain at residue 83 are well tolerated: replacing V83 with leucine, isoleucine, or threonine did not result in a significantly altered response to any of the three inhibitors (Table 1). In agreement with the CCR5 model, V83 most likely is located at the periphery of the binding site; its interaction with the small molecule inhibitors might involve only one of its two side chain methyl groups.

Finally, we focused our attention on residue G286, which is located below the key acidic residue, and putative counterion, E283 in H7. According to the CCR5 model, G286 is inside the transmembrane helical bundle but predicted to be oriented towards H3 and H6 rather than towards the center of the putative binding pocket (Fig. 5). Introduction of an alanine residue at position 286 is sufficient to inhibit the interaction with TAK-779 (Table 1), but a considerably larger valine side chain is required to also affect AD101 and SCH-C (Table 1). A possible explanation for these observations would be that a large residue at position 286 protrudes into the binding site and interferes directly with small molecule binding. Alternatively, it is possible that increasing the size of residue 286 perturbs the small molecule binding site more indirectly or interferes with the mechanism by which the binding of a small molecule inhibits HIV-1 entry. For example, glycine residues have been shown to be involved in helix–helix interactions in membrane proteins (Javadpour et al., 1999; Eilers et al., 2002). Hence, it is likely that substitution of G286 might affect the interaction of H7 with H3 and/or H6.

Discussion

We carried out a targeted mutagenesis screen to define the amino acid residues in CCR5 that form a ligand binding pocket for a series of small-molecule inhibitors of cellular HIV-1 entry. Our results indicate that side chains from residues E283, W86, Y37, Y108, L33, V83, A90, A29 and G286 line a cavity formed by transmembrane helices H1–H3 and H7 that is the small molecule binding site. Furthermore, the results suggest that E283 serves as the counterion for the positively charged nitrogen atom common to TAK-779, AD101 and SCH-C (Fig. 1). Another key element of the putative binding pocket is a

cluster of aromatic residues. In particular, W86 is indispensable for the interaction of CCR5 with TAK-779, AD101 and SCH-C. A likely scenario would be for the large indole ring of W86 to interact with hydrophobic regions in the antagonists. However, due to the quadrupole moment of the indole ring, interactions of W86 with polar groups on the compounds are also possible (Burley and Petsko, 1988).

Y37 is also involved in the interaction of CCR5 with all three of the compounds tested. In contrast to W86, however, the specific function of Y37 seems to be different for AD101 and SCH-C versus TAK-779. In the interaction with AD101 and SCH-C, Y37 can be replaced by any aromatic residue, which shows that only the aromatic moiety but not the phenolic hydroxyl group is required for this interaction. We propose that Y37 participates in a type of interaction with AD101 and SCH-C similar to that of W86. The interaction with TAK-779, however, specifically requires a tyrosine at residue 37. Hence, the phenolic hydroxyl group of Y37 may be engaged in a specific hydrogen bond, the formation of which is required for the inhibitory activity of TAK-779. It is not clear whether this hydrogen bond involves TAK-779 itself, or rather another CCR5 residue. For example, the CCR5 model predicts a potential hydrogen bond between the hydroxyl group of Y37 and the carboxyl group of E283. It is also not clear what role is played by the aromatic moiety of Y37 in the interaction with TAK-779. The same conclusions seem to apply to the involvement of Y108 in the interactions with TAK-779 and AD101, which both require the phenolic hydroxyl group. However, in contrast to Y37, neither the aromatic moiety nor the hydroxyl group of Y108 is important for the interaction with SCH-C.

Aliphatic residues of CCR5 appear to participate in interactions with non-polar groups in the antagonists, which could be either aromatic or aliphatic. The interaction with TAK-779, AD101 and SCH-C was disturbed by modifications in the polarity and/or size of the side chain at position 33. Of note is that TAK-779 was more sensitive than AD101 and SCH-C toward modest changes in the side chain structure, whereas all three compounds were similarly affected by more drastic changes. This suggests that the larger TAK-779 molecule might be more constrained than AD101 and SCH-C within the binding pocket. For V83, on the other hand, we could find no significant reduction in inhibitor sensitivity upon substitution with either polar or aliphatic side chains of similar size. However, a moderate reduction in the inhibitory activity of TAK-779, AD101 and SCH-C was seen when V83 was replaced with alanine. These observations suggest that V83 might be located at the periphery of the binding pocket where only a small section of its side chain would interact with the antagonist molecules—an interpretation supported by the CCR5 model.

Residues A90 and G286, which we believe define the entrance and the bottom of the putative binding pocket, respectively, are not likely to contribute much binding energy because of their small side chains. However, these residues might be important in shaping the binding pocket and providing sufficient space for the antagonist molecules to fit into. Indeed, substitution of A90 and G286 with large aliphatic residues blocked the interaction of CCR5 with TAK-779, AD101 and SCH-C. Interestingly, substituting A29, which is also at the opening of the binding pocket but protruding into the H1–H7 cleft, affected only TAK-779 activity. Hence, TAK-779 might extend into this cleft, a hypothesis that would also explain the sensitivity of TAK-779 to small changes at L33.

The CCR5 model predicts that several other residues affecting inhibitor activity are spatially separated from the proposed binding pocket. For example, the side chains of residues R31 and F113 face away from the binding pocket, and residue I198 is at the opposite end of the helical bundle, far from the putative binding pocket (Fig. 3). Changing the identities of these residues could affect the conformation of the small molecule binding site indirectly. Alternatively, it is possible that the inhibitory effect of the small molecules requires conformational changes within CCR5 and that such changes might not occur efficiently in some of the distal site CCR5 mutants. Substitutions at residues D76, F79 and T82, which are located near the binding pocket (Fig. 3), could also work in one of these indirect ways. However, it is also possible that these residues might interact directly with peripheral regions of the inhibitors. It is notable that D76, a residue highly conserved among Family A GPCRs, is believed to be involved in agonist-induced conformational changes that lead to receptor activation (Strader et al., 1994, 1995). For example, it has been shown in a previous study that the D76N variant has wild-type CCR5 characteristics with regard to coreceptor activity and chemokine binding (Farzan et al., 1997). However, chemokine binding does not induce receptor activation in the D76N variant (Farzan et al., 1997).

While we believe that the CCR5 residues we have identified so far are the major interaction sites for TAK-779, AD101 and SCH-C, there may be other, additional residues that might constitute minor interaction sites. Such weak interactions could have escaped detection due to the sensitivity limit of the HIV-1 entry assay or because of the limitations of the alanine mutagenesis approach itself. Furthermore, it is possible that hydrogen bond interactions between the peptide main chain and the small molecule inhibitors exist. Such interactions would remain undetected by a conventional mutagenesis approach that necessarily focuses on the peptide side chains. Therefore, while we have no indication for any interactions between extracellular domain residues and TAK-779, AD101 or SCH-C, we cannot completely rule out the possibility that some might exist.

Although the structural differences between TAK-779 and AD101/SCH-C are substantial (Fig. 1), these inhibitors do interact with a common set of CCR5 residues. Most likely, this is accomplished by a set of common structural elements. The importance of the positively charged nitrogen atoms and hydrophobic aromatic groups, in particular, for inhibitor effectiveness has been demonstrated by SAR studies (Shiraishi et al., 2000; Palani et al., 2001, 2002, 2003a, 2003b; Tagat et al., 2001a, 2001b). Furthermore, computational chemistry methods predict that TAK-779, AD101 and SCH-C adopt a similar, bent conformation (Fig. 3A), a structure that has been confirmed for TAK-779 by NMR spectroscopy (data not shown). So far, it is

not known whether or how the inhibitors' conformations change upon binding to CCR5. However, it has been shown that four rotational isomers of SCH-C exist under physiological conditions (Palani et al., 2001, 2003a, 2003b). These rotamers are caused by hindered rotation at both the amide bond and the bond linking the amide carbonyl to the unsymmetrical nicotinamide-*N*-oxide (Fig. 1). Evaluation of the inhibitory activities of the individual rotamers using both RANTES binding and HIV-1 entry assays revealed that one was at least 10-fold more potent than the other three, indicating a clear preference for interaction of CCR5 with this specific rotamer (Palani et al., 2003a, 2003b).

Recently, a model for TAK-779 binding to CCR5 was proposed based on computer docking simulations (Paterlini, 2002). This docking model agrees with some of our experimental findings. In particular, it predicts an interaction between E283 and the positively charged quaternary ammonium group in TAK-779 (Fig. 1). Furthermore, extracellular domain residues of CCR5 are not involved in the interaction with TAK-779, according to the docking model. However, the model places only the *N*,*N*-dimethyl-*N*-benzyl-tetrahydro-2*H*-pyran-4-aminium moiety of TAK-779 (Fig. 1, right half of TAK-779) in the pocket formed by H1, H2, H3 and H7 (Fig. 3). The larger 4-methylphenyl-6,7-dihydro-5*H*-benzocycloheptenyl moiety (Fig. 1, left half of TAK-779) on the other hand is predicted to bind outside this pocket and to interact with residues in H5 and H6. This latter prediction is not supported by our experimental results. Thus, alanine mutagenesis identified only a single residue, I198, in this region (Fig. 3), and mutation of I198 affected the inhibitory activity of TAK-779 only moderately (Dragic et al., 2000; Billick et al., 2004).

Several classes of small molecule CCR5 inhibitors have now been described (Horuk, 2003; Schwarz and Wells, 2002; Kazmierski et al., 2003; Maeda et al., 2004b; Seibert and Sakmar, 2004). Comparison of the chemical structures and available information from SAR studies does reveal common structural elements critical for the antagonistic and antiviral activity of these compounds. In particular, a positively charged or basic nitrogen atom and hydrophobic aromatic groups are key pharmacophore elements in the majority of the CCR5 inhibitors. Hence, these might bind to CCR5 in a fashion similar to TAK-779, AD101 and SCH-C. For example, it was shown in a recent study that alanine-substitution of E283, W86 and Y108 reduced the affinity of two classes of CCR5 antagonists, 2-aryl-4(piperidin-1-yl) butanamines and 1,2,4-trisubstituted pyrrolidines (Castonguay et al., 2003). In contrast to TAK-779, AD101 and SCH-C, however, substitution of Y37 with alanine did not influence binding of these compounds to CCR5 (Castonguay et al., 2003). The same study also showed that another aromatic residue, Y251, is involved in the binding of the 2-aryl-4 (piperidin-1-yl)butanamines and 1,2,4-trisubstituted pyrrolidines (Castonguay et al., 2003). However, we could not evaluate a possible involvement of residue Y251 in interaction with TAK-779, AD101 and SCH-C in our studies, because the coreceptor activity of the Y251A CCR5 variant was too low (Dragic et al., 2000; Tsamis et al., 2003).

Since chemokine receptors are structurally conserved, a similar small molecule binding pocket may exist in other members of this GPCR subfamily. Supporting evidence is provided by the high degree of inter-receptor conservation among residues that are crucial for interaction of TAK-779, AD101 and SCH-C with CCR5. While most of these residues are conserved within the chemokine receptor subfamily, they are not generally conserved throughout GPCR family A (Paterlini, 2002). For example, Y37 is conserved in all chemokine receptors; W86 is substituted only in CCR8 and CXCR5; E283 is substituted only in CCR7, CCR9, CXCR3 and XCR1. Small molecule antagonists have been described for CC-chemokine receptors CCR1 (Hesselgesser et al., 1998; Liang et al., 2000; Naya et al., 2001b), CCR2b (Mirzadegan et al., 2000; Forbes et al., 2000) and CCR3 (Naya et al., 2001a; Ting et al., 2005) (see Schwarz and Wells, 2002; Carter, 2002; Horuk, 2003 for review). As with the CCR5 inhibitors, a positively charged or basic nitrogen atom as well as aromatic groups are key pharmacophore elements within this group of antagonists. In particular, it has been shown that the interaction of spiropiperidine-based antagonists with CCR2b critically depends on the basic nature of a specific nitrogen atom and that it requires an acidic residue in H7 of the receptor (Mirzadegan et al., 2000). This residue, E291, is equivalent to E283 in CCR5.

Certain antagonists of CXCR1, CXCR2, CCR2b and even CCR5 do, however, lack a positively charged or basic nitrogen atom (Schwarz and Wells, 2002; Carter, 2002). Such molecules might bind to their respective receptors in a manner different from TAK-779, AD101 and SCH-C. Yet in the case of CCR2b, a class of acidic 2-carboxy-pyrrole antagonists was less potent than the spiropiperidine compounds, and medicinal chemistry optimization did not yield an antagonist useful for further development (Mirzadegan et al., 2000). This again emphasizes the importance of a positively charged or basic nitrogen atom for small molecules to have potent inhibitory activity against these various receptors.

The CXCR4 antagonist AMD3100, a symmetrical bicyclam, contains eight basic nitrogen atoms and is believed to have a net charge of +4 at physiological pH (Hatse et al., 2001). Several acidic residues in H4, H6 and H7 of CXCR4 have been shown to be involved in the interaction of AMD3100 with the receptor (Labrosse et al., 1998; Gerlach et al., 2001; Hatse et al., 2001; Trent et al., 2003; Rosenkilde et al., 2003), including E288, which is the homologue of E283 in CCR5 (Trent et al., 2003; Rosenkilde et al., 2003). AMD3100 is believed to bind to a pocket formed by H4, H5, H6 and H7, which is different from the small molecule binding pocket in CCR5 (Gerlach et al., 2001; Hatse et al., 2001; Trent et al., 2003; Rosenkilde et al., 2003). AMD3100 presumably represents a category of inhibitors that is unique to CXCR4. The *N*-pyridinmethyl cyclam analog AMD3451 on the other hand has been shown to be a dual antagonist for CCR5 and CXCR4 (Princen et al., 2004).

The high degree of inter-receptor conservation among residues that are crucial for interaction of CCR5 with small molecule inhibitors raises the question of how the receptor specificity of these compounds is achieved. TAK-779, for

example, is an antagonist for CCR5 and CCR2b but has no effect on other chemokine receptors (Baba et al., 1999). AD101 and SCH-C on the other hand are highly specific for CCR5, although there is some very low residual potency for muscarinic receptors (Tagat et al., 2001b; Palani et al., 2001; Strizki et al., 2001). Our results suggest that common structural elements within the small molecules interact with the conserved residues in the chemokine receptors. Selectivity could then be provided by additional interactions involving more specific small molecule elements and variable chemokine receptor residues. Although sequence diversity among chemokine receptors is most pronounced within the extracellular domain, we could find no evidence for any involvement of this region in small molecule interactions. Thus, it is most likely that relatively subtle variations in transmembrane domain residues account for receptor specificity. Less-conserved residues could directly interact with inhibitors, increasing affinity by providing additional contacts or decreasing it due to steric hindrance. Alternatively, variable residues could indirectly influence the conformation of the binding pocket and hence the precise positions of other functionally important amino acid side chains.

AD101 and SCH-C were developed from early lead compounds that are potent antagonists for the M2 muscarinic receptor (Tagat et al., 2001a, 2001b; Palani et al., 2001, 2002). The M2 receptor does not possess an acidic residue in H7 that is equivalent to E283 in CCR5. However, an acidic residue in H3 serves as the counterion for positively charged nitrogen atoms in agonists and antagonists of muscarinic and other biogenic amine receptors (Fraser et al., 1989; Strader et al., 1995). Our results show that the precise location of the E283 carboxyl group in CCR5 is critical for inhibitor activity (Fig. 4). In particular, swapping the glutamate residue from H7 to H3 on the opposite side of the binding pocket is not tolerated. Hence, we believe that, in the optimization process creating AD101 and SCH-C, structural modifications were introduced into the inhibitors that favor the use of a counterion in H7 over a counterion in H3. For example, it has been shown that the specificity of AD101-related compounds is controlled in part by the stereochemistry of the piperazine 2-methyl substituent, which is in the vicinity of the protonated nitrogen atom (Fig. 1): the 2(*S*)-configuration favors an interaction with CCR5 while the 2(*R*)-configuration results in M2 antagonists (Tagat et al., 2001a, 2001b). Thus, it seems possible that the piperazine 2-methyl group influences receptor specificity by directing the protonated nitrogen atom towards either H3 or H7.

TAK-779, AD101 and SCH-C most likely prevent the cellular entry of HIV-1 by inhibiting the interaction of gp120 with CCR5 subsequent to CD4 binding (Dragic et al., 2000; Tsamis et al., 2003). The precise nature of the gp120-CCR5 interaction is not clear; however, available evidence indicates that the N-terminus of CCR5 interacts with residues in the β19 strand and near the base of V3, while the V3 crown may interact with ECL2 residues (Cormier and Dragic, 2002; Hartley et al., 2005). How inhibition occurs is not well understood on a molecular level. One possibility is that small molecule binding to the transmembrane domain of CCR5 selectively stabilizes or induces a receptor conformation that is not recognized by gp120 (Dragic et al., 2000; Tsamis et al., 2003; Kazmierski et al., 2003; Kenakin, 2004). Alternatively, the small molecules might compete directly with gp120 due to a partial overlap between their respective binding sites. Although we cannot completely rule out the latter alternative, we believe that the experimental evidence favors an allosteric mechanism. In particular, we could find no evidence for any interaction of TAK-779, AD101 or SCH-C with the extracellular domain of CCR5 (Dragic et al., 2000; Tsamis et al., 2003); gp120 on the other hand has been shown to interact predominantly with residues in this receptor region (Rucker et al., 1996; Ross et al., 1998; Blanpain et al., 1999; Howard et al., 1999; Dragic, 2001; Dragic et al., 1998; Rabut et al., 1998; Farzan et al., 1998; Doranz et al., 1997). Furthermore, it was shown that AD101 and SCH-C both inhibit the binding of several monoclonal antibodies that recognize conformational epitopes in E2 of CCR5 (Tsamis et al., 2003). This observation is most simply explained by the binding of AD101 and SCH-C causing conformational changes in CCR5 that alter the conformation of the antibody epitopes. These changes would also affect regions of the receptor known to interact with gp120 (Tsamis et al., 2003).

TAK-779, AD101 and SCH-C not only inhibit HIV-1 entry, they also act as classical GPCR antagonists by blocking chemokine binding to CCR5 (Baba et al., 1999; Palani et al., 2001, 2002; Tagat et al., 2001a; Strizki et al., 2001). Although the primary chemokine binding site in the extracellular domain of CCR5 seems to have no overlap with the small molecule binding site, there is evidence for a secondary chemokine interaction site in the transmembrane domain of CCR5 (Blanpain et al., 2003). This site is likely to overlap with the small molecule binding site, allowing for a direct competition between the small molecules and the chemokines. However, it has been shown that chemokine interaction with this secondary site is required only for receptor activation, and not for chemokine binding (Blanpain et al., 2003). We therefore believe that an allosteric mechanism similar to the one proposed above for the inhibition of the gp120 interaction agrees better with the experimental evidence than does a direct competition mechanism. The allosteric receptor inhibition model is supported by the results from chemokine binding competition studies with a panel of five CCR5 antagonists including TAK-779, SCH-C, SCH-D, UK-427,857 and GSK-873140 (formerly AK602) (Watson et al., 2005). These results indicate that all five antagonists bind to a common allosteric site on CCR5 and that they interfere with chemokine receptor interaction in a non-competitive fashion.

While TAK-779, AD101 and SCH-C completely block both HIV-1 entry and chemokine binding, certain other small molecules with differential effects have been described. For example, GSK-873140 has been shown to completely block HIV-1 entry; however, this compound is only a partial antagonist of chemokine binding (Maeda et al., 2004a; Watson et al., 2005). Furthermore, differential effects of certain CCR5 inhibitors are also implied by differences in their ability to block

the binding of various anti-CCR5 antibodies (Maeda et al., 2004a; Dragic et al., 2000; Tsamis et al., 2003). Within the framework of the allosteric receptor inhibition model (Kenakin, 2004, 2005), these differential effects can be explained by the conformational flexibility of GPCRs. While relatively subtle differences in the overall shape of the inhibitors or the presence of certain peripheral groups may not have a strong effect on the receptor affinity, such specific features might be crucial for inducing slightly different receptor conformations leading to different allosteric effects. For example, extensive SAR studies performed on 2-aryl-4(piperidine-1-yl)butanamines and 1,2,4-trisubstituted pyrrolidines revealed structural features that are selectively required for inhibition of HIV-1 entry but not for chemokine antagonism (see Seibert and Sakmar, 2004 for review).

Significance

We have identified CCR5 amino acid residues that are critical for three separate small molecule antagonists, TAK-779, AD101 or SCH-C, to interact efficiently with CCR5 to inhibit HIV-1 entry. The antagonists, which were the result of targeted drug discovery programs, bind to similar but not completely identical sites on CCR5. We found no evidence for any interaction of TAK-779, AD101 or SCH-C with extracellular domain residues. A homology model of CCR5 predicts that the majority of the residues identified to be important line a cavity formed by transmembrane helices H1, H2, H3 and H7. Hence, we conclude that this cavity is the small molecule binding pocket. Within this putative binding pocket, residues E283, W86, Y37, Y108, L33, V83, A90, A29 and G286 are well positioned to interact with a bound antagonist. This is the first study to report a side-by-side comparison of multiple drug candidates using a functional assay of HIV-1 entry with the aim of defining the ligand binding site on the CCR5 coreceptor. Our results provide a structural basis for understanding how the binding of small molecules leads to inhibition of the HIV-1 coreceptor and chemokine receptor functions of CCR5. Understanding how small molecule inhibitors interact with CCR5 might facilitate the rational design of improved CCR5 antagonists with activity against HIV-1 entry, and also against inflammatory and autoimmune diseases in which CCR5 is also implicated (Yang et al., 2002). In addition, this work provides a framework for understanding how rare, single amino acid polymorphisms of CCR5 might influence the antiviral responses of HIV-1-infected individuals to therapy with compounds like SCH-C and its derivatives. The eventual emergence of HIV-1 resistance to CCR5 antagonist therapies might also be better understood by knowledge of how the drugs interact with CCR5.

Materials and methods

Compounds

TAK-779 (*N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl] tetrahydro-2*H*-pyran-4-aminium chloride; M_r = 531.13) was obtained from the AIDS Research and Reference Reagent Program, NIAID, NIH, contributed by DAIDS (Baba et al., 1999; Dragic et al., 2000). AD101 (SCH-350581) (1-[(2,4-Dimethyl-3-pyridinyl)carbonyl]-4-methyl-4-[3(*S*)-methyl-4-[1(*S*)-[4-(trifluoro-methyl)phenyl]ethyl]-1-piperazinyl]-piperidine; M_r = 502.62) and SCH-C (SCH-351125) (4-[(*Z*)-(4-Bromophenyl)(ethoxyimino)methyl]-1′-[(2,4-dimethyl-3-pyridinyl)carbonyl]-4′-methyl-1,4′-bipiperidine *N*-oxide; M_r = 557.53) were synthesized as described (Palani et al., 2001, 2002; Tagat et al., 2001b). The chemical structures of these compounds are depicted in Fig. 1.

Energy-minimized structures of CCR5 inhibitors

Energy-minimized structures of TAK-779 (including chloride counterion), AD101 and SCH-C were calculated using the PM3 semi-empirical method of the *HyperChem* software (Hypercube Inc., Gainesville, FL).

Structural model of the transmembrane domain of CCR5

The crystal structure of bovine rhodopsin (PDB ID 1F88) (Palczewski et al., 2000) was used as a template to model the transmembrane domain of CCR5. The amino acid sequence of CCR5 was aligned with that of rhodopsin. The CCR5 amino acid side chains were extended from the helical backbone of the rhodopsin structure and energy-minimized with the program X-PLOR (Brunger, 1992), using Powell minimization for 10,000 cycles. Hydrogen-bonding restraints were applied between the backbone amide and carbonyl groups. This allowed the transmembrane helices (H1–H7) to maintain α-helical structure, but provided flexibility for kinks to be introduced at transmembrane prolines unique to CCR5. Hydrogen-bonding restraints were also applied between the following pairs of residues: N48-D76, N71-W153, Y214-R235, D125-R126 and N293-Y297. These residues generally are conserved in GPCRs and are thought to form key intramolecular interactions. The model predicts a large number of hydrogen-bonding interactions including Y37-E283 and D76-H289. The following residues are included in the CCR5 structural model: H1 (K26-C58), H2 (S63-W94), H3 (F96-V131), H4 (T141-I165), H5 (W190-I217), H6 (K228-Q261) and H7 (L275-G301). Molecular graphics were prepared using the program DINO (Visualizing Structural Biology (2002), http://www.dino3d.org).

Site-directed mutagenesis of CCR5

A pcDNA3.1 (Invitrogen, Carlsbad, CA)-based expression plasmid was used as the template for site-directed mutagenesis of CCR5. This plasmid contains the coding sequence for a CCR5 construct with a C-terminal hemagglutinin affinity tag (Dragic et al., 1998). Site-directed mutagenesis was performed using the QuickChange method (Stratagene, La Jolla, CA) and mutations were verified by DNA sequencing

of the entire CCR5-coding region (The Rockefeller University Protein/DNA Technology Center, New York).

HIV-1 entry assay

Human astroglioma cells stably expressing CD4 (U87-CD4 cells) were transfected with expression plasmids encoding wild-type or mutant CCR5 using either Lipofectin or Lipofect-AMINE 2000 (Invitrogen, Carlsbad, CA) (Dragic et al., 1998). Entry of HIV-1 reporter virus (NL*env*$^{-}$*luc*$^{+}$ virus) pseudotyped with envelope glycoproteins (Env) from the HIV-1 JR-FL isolate and bearing the firefly luciferase gene into the transfected U87-CD4 cells was determined by quantifying luciferase expression, as previously described (Dragic et al., 1998). The luciferase activity was directly proportional to viral entry, as confirmed by serial dilution of the virus in the absence of inhibitors (data not shown).

Effect of inhibitors on HIV-1 entry

For each CCR5 mutant HIV-1 entry, experiments were performed in the presence of 200 nM TAK-779, 100 nM AD101 or 100 nM SCH-C, and in the absence of any inhibitor. To evaluate the sensitivity of the CCR5 mutants to these inhibitors, relative entry levels were calculated as described elsewhere (Dragic et al., 2000): a value of 100% viral entry in the presence of an inhibitor means that a mutant is completely insensitive to the inhibitor. Conversely, a value of 0% viral entry in the presence of an inhibitor means that a mutant is as sensitive to the inhibitor as wild-type CCR5. Mean entry levels and standard errors were calculated from the results of at least three independent experiments with quadruplicate samples. Based on statistical parameters of the entry assay, it was estimated that mutants yielding ≥14% viral entry in the presence of an inhibitor have a significantly reduced sensitivity for the respective inhibitor (Dragic et al., 2000). CCR5 substitutions that affect HIV-1 entry also affect the sensitivity of the entry assay in the presence of an inhibitor. Thus, a few mutants, apparently more sensitive to inhibition than wild-type CCR5, yielded entry levels <0%. These effects were small and may have no functional implications. For clarity, values <0% are represented as = 0%. In the absence of inhibitors, the different CCR5 mutants supported 7–130% of the entry level for wild-type CCR5.

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