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Review

Recent Progress in Discovery of Small-Molecule CCR5 Chemokine Receptor Ligands as HIV-1 Inhibitors

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Abstract—This review addresses key pharmacology and virology issues relevant in discovery and development of CCR5 antagonists as anti-HIV drugs, such as target validation, receptor internalization, allosterism, viral resistance and tropism. Recent progress in the discovery and development of CCR5 antagonists, SAR and clinical status are reviewed. Finally, modeling-based structure of CCR5 is discussed in the context of a small-molecule antagonism of the CCR5 receptor.

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Introduction

HIV-1 infects millions of people worldwide to cause the lethal disease Acquired Immune Deficiency Syndrome (AIDS). An asymptomatic period precedes AIDS in which the immune system becomes progressively compromised and unable to fight opportunistic infections and certain cancers. To inhibit HIV-1 replication and slow the destruction of the immune system, current therapies utilize a combination of protease and reverse transcriptase inhibitors. While suppression of viral replication through combination therapy[1,2] delays progression to AIDS, the virus is not eradicated and the immune system eventually succumbs to infection.[3,4]

A third molecular target would be desirable in view of compliance problems due to side effects, high cost and viral rebound effects with current Highly Active Anti-Retroviral Therapy (HAART).[5] A therapeutic agent which inhibits viral entry would be particularly desirable. A proof of concept for this approach has been provided by T20, a gp41-based entry inhibitor that blocks the fusion process,[6] as well as Sch-C, a CCR5 receptor antagonist (vide infra). Although several chemokine receptors have been implicated in HIV entry in various experimental systems,[7–17] CCR5 is the most important mediator of R5 virus entry because of its role in transmission early in the disease.[8,10,18–22]

Validation of CCR5 as a Target for HIV-1 Infection

CCR5 is a highly validated target for the treatment of HIV-1 infection. In vitro, cells cannot become infected with certain strains of HIV-1, designated R5-tropic, if they do not express CCR5 in conjunction with the primary receptor CD4.[23–28] Natural and synthetic ligands for CCR5 such as RANTES,[22,29] AOP-RANTES,[30,31] Mip-1α, Mip-1β, Met-RANTES[30,32] and LD78b (MIP-1α isoform),[33] protect against R5 virus infection in vitro. This extends to in vivo where it has been observed that individuals with high levels of MIP-1β have decreased risk of HIV progression.[34,35]

Conditions whereby CCR5 expression is compromised lead to resistance to HIV R5 infection. For example, when newly synthesized CCR5 is prevented from trafficking from the endoplasmic reticulum to the cell surface through gene targeting, the cells are resistant to HIV infection.[36] Similarly, reduction in vitro in CCR5 mRNA makes HOS cells resistant to R5-tropic, also known as macrophage or M-tropic HIV.[37] Powerful in vivo data that demonstrates that CCR5 is a critical factor is the resistance to HIV infection in individuals homozygous for the Δ32 CCR5 allele (leading to inoperative CCR5).[38–45] These individuals are otherwise healthy suggesting that the blockade of this target may not lead to secondary side effects. Similarly, CCR5 knockout mice show no untoward effects.[46] Individuals heterozygous for Δ32 CCR5 (one Δ32 and one normal CCR5 gene) show retention of CCR5 in the endoplasmic reticulum[47] and thus have lower cell surface levels of CCR5.[48] These individuals have slower progression to AIDS and longer survival periods.[41,49–51] As with in vitro data, the converse appears to be true in that individuals with allelic variants that cause increased CCR5 expression have accelerated progression to AIDS.[52,53] Similarly, tuberculosis patients, who show upregulation of CCR5, progress more quickly to AIDS.[54] In general, there are in vitro and in vivo data linking the presence of CCR5 to increased risk of HIV infection and subsequent progression to AIDS.

Blocking HIV-1 Infection

HIV-1 binds to cellular membrane protein CD4 through an HIV envelope protein gp120. This binding induces a conformational change in gp120 to expose binding sites for CCR5.[55–58] This process, through a second conformational change in the HIV protein gp41, leads to the formation of a hairpin configuration that subsequently leads to membrane fusion. Two obvious approaches to the blockade of this process is through interference with protein binding to CCR5 or through removal of CCR5 from the cell surface.[59] First to be considered is the interference of protein binding to CCR5.

Blockade of Protein Binding to CCR5

Multiple domains of CCR5 appear to be involved in the infection process.[60–64] Single point mutations fail to affect the process.[65] Similarly, mutations of gp120 indicate that multiple domains of this protein are required for infection as well.[7,55,56,66,67] Mutational studies also indicate that the protein-protein interactions relevant to CD4, gp120 and CCR5 interaction involve all four extracellular domains of the CCR5 molecule.[7,65,67–72] In general, it is clear that numerous points of interaction are involved in CCR5-mediated HIV binding suggesting that a simple single point orthosteric interference of these interactions would not be sufficient to block the process. One method by which a small molecule could interfere with large protein–protein interactions is through the production of an allosteric change in the complete receptor conformation. CCR5, being a seven transmembrane receptor, is a naturally allosteric protein in that the interaction of small molecules such as neurotransmitters (i.e., acetylcholine) to this receptor family produce conformational changes in receptor to

affect receptor/G-protein interaction. In this manner, it might be suggested that a small allosteric modulator of CCR5 could induce a conformation that could affect numerous points of CCR5–gp120 interaction and thus go on to prevent membrane fusion and HIV infection. Seven transmembrane receptors are known to adopt numerous conformations according to thermal energy[73–78] and selective ligand binding to some of these can stabilize them to produce a bias in the conformational distribution. Therefore, a ligand that stabilizes a conformation that does not support interaction with gp120 could produce blockade of R5 virus entry. Such effects, referred to as allosteric modulation, transmit conformational changes throughout the protein to affect different regions of the receptor.

There are two features of allosterism that are found in CCR5 ligand-receptor interaction. The first is that allosteric ligands often bind to a domain separate from the one binding endogenous ligands. Experiments with chimeric CCR5 receptors indicate that there are separate binding domains for HIV gp-120 and the endogenous chemokine agonist macrophage inflammatory protein type 1α, MIP-1α.[79,80] Since this chemokine is able to block HIV infection, this strongly suggests that binding of the chemokine is allosterically linked to the gp120 domain through the receptor protein. Separate binding sites for HIV and chemokines also are suggested by the fact that the monoclonal antibody PRO 140 (PA14) is a potent inhibitor of HIV-1 but does not block chemokine activation of CCR5.[81] Another hallmark of allosteric interaction is that allosteric modulators alter the kinetics of binding of ligands to receptors.[82] In this regard, Staudinger et al.,[83] have reported direct evidence for the alteration of the kinetics of binding of ^{125}I-MIP-1β by gp120.

CCR5 Endocytosis as an Antiviral Mechanism

As well as physically interfering with the binding of viral envelope proteins to CCR5, another potential mechanism to prevent HIV infection is to capitalize on the natural proclivity of seven transmembrane receptors to internalize into the cell. This is part of a natural process involving receptor synthesis, transport to the cell surface, activation and/or phosphorylation, internalization and subsequent degradation or recycling. In general, agonists increase the rate of receptor phosphorylation and this can lead to internalization.[84–86] The factor relevant to HIV infectivity, namely the steady-state density of CCR5 receptors on the cell surface, depends upon the relative rates of receptor synthesis, transport and internalization.[87,88] If a ligand could alter this steady-state in favor of a decrease of cell surface CCR5, this could lead to a decrease HIV-1 infection.

CCR5 internalization, while not required for HIV-1 infection,[89] appears to be involved in the prevention of HIV-1 infection by chemokines.[31,90–93] Receptor internalization historically is linked to receptor agonism[87,94,95] although there are antagonists that are known to not induce response but cause rapid internalization of receptors.[96] Agonists have differing capabilities for producing internalization. While enkephalins and morphine both stimulate δ and μ opioid receptors, only the latter class of agonist induces receptor internalization.[97] These data suggest that receptor internalization, a process therapeutically favorable for protection against HIV-1 infection, is not inextricably linked to chemokine receptor agonism. This disparity also can be found in ligands for CCR5. For example, the chemokine antagonist RANTES (9–68)[98] produces CCR5 internalization[31,91] but no overt CCR5 agonism. Therefore, the link between CCR5 agonism and therapeutically useful CCR5 internalization should be considered tenuous leaving open the possibility that a molecule can be found that possesses the ability to internalize CCR5, and thus block HIV-1 infection, but not activate CCR5 signalling pathways.

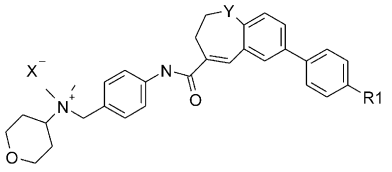
Seven transmembrane receptors dimerize and it is known that dimerization is essential for CCR5-mediated calcium mobilization, chemotaxis and internalization.[47,99,100] Interestingly, there also are data to suggest that CCR5 clustering is involved in HIV-1 infection.[101,102] For example, CCR5-02 mAb, a CCR5 specific antibody which does not block gp120 or chemokine binding and which is not a CCR5 agonist, has been shown to be an effective inhibitor of HIV infection.[103] However, this antibody does promote CCR5 dimerization thereby implicating this mechanism in the inhibition of viral infection.[103] These data suggest also that drugs that interfere with CCR5 dimerization may also be useful inhibitors of HIV-1 infection.

CCR5 Receptor Agonism

It is clear that chemotaxis and HIV-1 blockade can be dissociated.[30,32,72,104–106] What is not clear is the relationship between CCR5 agonism and HIV-1 blockade. While CCR5 agonism might promote CCR5 receptor internalization, chemotaxis per se may be undesirable to the host.[99,107–109] CCR5 agonism also may promote enhancement of T-tropic viral replication[110] and stimulation of post-entry events involved in viral replication.[111–113] It is interesting to note that gp120 induces CCR5 mediated elevation of calcium,[114–117] an activity which could promote viral replication once infection occurs. Finally, it is clear that CCR5 agonism is not necessary for inhibition of HIV infection since small molecule antagonists lacking CCR5 efficacy are potent inhibitors of HIV entry (vide infra).

Resistance and CCR5-Induced Changes in Viral Tropism

One theoretical concern for the long-term protection against R5 infection is the possible selective pressure this may provide for a change of viral tropism to enable the virus to utilize CXCR4 receptor, which occurs naturally by evolution of the virus during disease, or perhaps another novel co-receptor. These changes occur because of the highly variable nature of the viral envelope glycoprotein,[8,67,118–124] and such mutations could



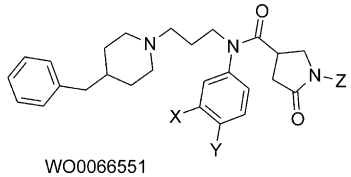
				IC50 (nM) for:		
Compound	R1	Y	X	Membrane Fusion	HIV-1 replication	^{125}I-RANTES binding
1 (TAK779)	Me	CH_2	Cl	0.87	1.4	1.4
2	EtO	O	I	0.69	1.8	1.8
3	CF_3	O	I	1.4	1.2	1.5
4		CH_2	I	1.0	1.1	3.8
5		CH_2	I	0.96	1.3	2.2
6	Me	CH_2	I	0.71	1.2	1.8

Figure 1. Structure of TAK-779 and related analogues.

be selected for by prolonged blockade of R5 virus.[125–127] On the other hand, individuals exposed to both R5 and X4-tropic virus appear to control the proliferation of the X4-virus for prolonged periods of time. In addition, recent studies of Trkola et al.[128] using a clinical candidate Sch-C (vide infra) failed to generate X4 (also known as T-tropic) virus. In general, the concern that CCR5 therapy will promote early changes in HIV tropism is still theoretical and much debated in the field and, as new data emerges with new drugs, a better understanding of the scope of this problem will emerge.[129]

Small Molecule CCR5 Antagonists

Until recently, all development-stage CCR5-targeting ligands, such as AOP-RANTES[99,130] and PRO140,[81] were protein-based. While these proteins proved invaluable as research tools, it remains to be seen if they can be further developed into effective therapeutic agents. Only recently have small molecule CCR5 antagonists been disclosed in the patent and scientific literature.



WO0066551

Compound	X	Y	Z	% inhibition @ 1 uM
7	H	H	Me	57%
8	Cl	H	Me	82%
9	Cl	Cl	Me	95%
10	H	H	CH2-CF3	90%
11	H	H	CH2-C6H5	92%
12	H	H	2-Cl-Bn	91%
13	H	H	Ph	76%

Figure 2. Takeda *N*-alkylaniline based chemokine inhibitors (WO 200066551).

The first small-molecule CCR5 antagonist published in the scientific literature was TAK-779 (Fig. 1), discovered in a chemical optimization program based on hits from a high-throughput screen.[131] TAK-779 is a potent (IC_{50} 1.4 nM in CHO/CCR5 cells) and selective (>1000-fold over other chemokine receptors, except for CCR2b, $IC_{50}=27$ nM) CCR5 antagonist, which blocks CCR5-mediated Ca^{2+} signaling.

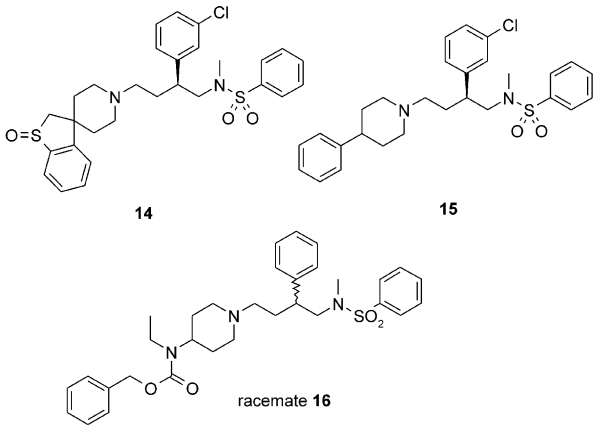
A subsequent publication by Shiraishi et al.[132] described a number of potent R1-alkyl,-ether and -amine derivatives of TAK-779 (**4**, **5**, **6**, Fig. 1). In addition, oxo-substitutions (Y = O) in the benzosuberene ring resulted in potent inhibitors **2** and **3**, (Fig. 1). Additional publications detailed synthetic progress towards the large-scale synthesis of TAK-779.[133,134] TAK-779 was scheduled to enter phase I clinical trials in 1999 with subcutaneous administration, consistent with low oral bioavailability generally expected from a quaternary salt.

More recently, Takeda disclosed another class of anti-HIV compounds which act via the CCR5 chemokine receptor (WO200066551, Fig. 2) and active at sub-micromolar concentrations as HIV-1 inhibitors in cell-based HIV assays. The SAR in this class seems to favor the 3-Cl-substitution in **8,** which was substantially more potent than the unsubstituted **7**. Addition of the 4-chloro substituent in **9,** resulted in only a marginal improvement of potency over **8**. The Z-substituent on the pyrrolidone moiety had a substantial effect on potency, with both alkyl and benzyl (**10**–**12**) preferred over phenyl in **13** and methyl in **7**.

In a series of papers, Merck Research Laboratories[135–140] reported a series of novel CCR5 antagonists developed from the 1-amino-(2*S*)-2-aryl-4-(piperidin-1-yl)-butane scaffold, as exemplified by compounds **14**–**16** (Fig. 3).

Although compounds **14**–**16** (Fig. 3) possessed strong affinity for the CCR5 receptor, **16** exhibited only weak antiviral potency ($CIC_{95}=12{,}500$ nM). A dramatic improvement in the antiviral potency ($IC_{50}=0.8$ nM, $CIC_{95}=94$–188 nM) was observed in the enantiomerically pure **17**,[139,141a] a quaternary hydroxymethylene derivative of **16** (Fig. 4).

Derivatives **17** and **18** recognized the CCR5 receptor enantiospecifically, as evidenced by poor binding of enantiomer **18**. Further optimization in this class of compounds resulted in a potent allyl-derivative **19** ($CIC_{95}<8$ nM) with an excellent selectivity profile versus other chemokine and neurokinine receptors (CCR1, CCR2, CCR3, CCR4, CXCR3, CXCR4, NK1, NK2, and NK3). In addition, **19** had moderate oral bioavailability ($F=29\%$ in rats) and a good serum half-life ($t_{1/2}=2$ h, rat serum). However, **19** was not orally bioavailable in dogs ($F<1\%$) despite the long plasma half-life ($t_{1/2}=10$ h). Speculation that **19** was undergoing *N*-demethylation in vivo, led to the design of sulfone **20**,[141b] which was found to be a potent CCR5 inhibitor in the MIP-1α assay, but only a moderately active antiviral agent in the HeLa cells assay.



compound	IC_{50} (nM) MIP-1α
14	10
15	30
16	20

Figure 3. 1-Amino-(2*S*)-2-aryl-4-(piperidin-1-yl)-butane based inhibitors from Merck with high receptor potency but moderate antiviral activity.

Substitution of the sulfone in **20** by the hydantoine moiety as in **21** also resulted in potent CCR5 inhibitors, but these possessed poor antiviral activity (Fig. 5).[142] Further modification of the sulfone with heterocycles also yielded potent inhibitors, but these results again did not translate into potent antiviral activity.[142] The discontinuity observed between the MIP-1α binding assay and the HeLa cells antiviral assay is not unusual and can be attributed to different allosteric probes used in both assays; MIP-1α and gp120 from the live HIV-1, respectively. Since these probes interact differently with CCR5, these assays can result in a divergent SAR.

Piperidine-based CCR5 modulators homologous to **14**–**16** were recently reported by Pfizer (WO200039125, EP 1013276), however no detailed biological data was disclosed (Fig. 6).

Merck has reported a series of CCR5 antagonists based on 1,3,4-trisubstituted pyrrolidines in a number of patents and publications (Fig. 7).[139,143,144] In particular, inhibitors **22** and **23** were CCR5 receptor-selective (relative to other chemokine receptors, such as CCR1, CCR2, CCR3, CCR5 and CXCR4) and potent antiviral agents, endowed with acceptable pharmacokinetic properties.

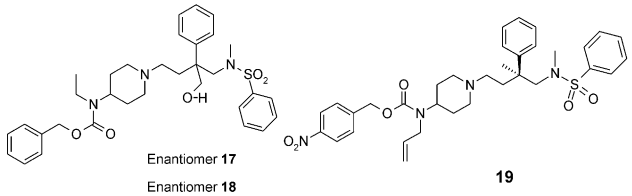
Recognition of the CCR5 receptor by the pyrrolidine series was diasteroselective, as evidenced by high potency of **24** versus diastereomer **25** (Fig. 8).

A more recent series of patent applications from Merck Laboratories features cyclopentane-based CCR5 inhibitors (Fig. 9). While no specific biological data was provided, cyclopentane-based amines, carba- and oxo-scaffolds were claimed potent against the CCR5 receptor.

In a recent study, Lynch et al.[145] conformationally restricted the rotation around the tertiary amide in **26** with an isoxazolidine scaffold, resulting in derivatives **27** and **28** (Fig. 10). Interestingly, in the cyclohexyl series, there was a definitive preference for isomer **28** over **27**, possibly suggesting that this isomer strongly mimics the receptor bound conformation of **26**.

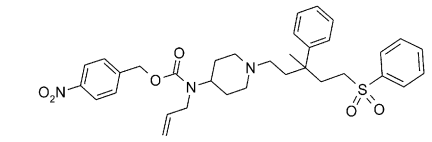
In another development, Schering has recently reported the development of piperidine- and piperazine-based CCR5 antagonists, which has culminated in the discovery of Sch-C **32** (Fig. 11).[146–149] Both series were discovered in the lead optimization program starting from submicromolar CCR5, High Throughput Synthesis hits in 125I-labeled RANTES SPA assay. Since these hits (such as **29**, Fig. 11) have been designed and synthesized for the muscarinic M2 program, a substantial effort had to be undertaken to eliminate the M2 activity from CCR5-selective compounds such as **30**.

Compound **30** was reported oral bioavailability of 33% in dog and 27.5% in monkey. Subsequent identification of the major metabolite of **30** as M + 16, suggested a fast oxidation of the pyridyl nitrogen, and led to Sch-350634, **31**. Although slightly less potent and selective than **30**, Sch-350634 exhibited improved plasma exposure following oral administration in rats, potentially due to improved serum stability. Subsequent addition of the ethyl oxime moiety and elimination of the methyl substituent in the piperidine ring in 31 resulted in the discovery of Sch-C (**32**, Fig. 11), currently in phase I clinical trials.

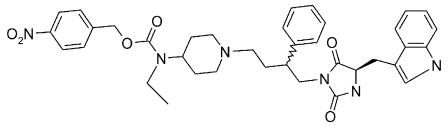


	enantiomer 17	enantiomer 18	19
Inhibition of [^{125}I]- MIP-1α binding, IC_{50} [nM]	0.8	>1000	0.1
HeLa (Bal) CIC_{90} [nM]			3
PBMC (YU-2) CIC_{95} [nM]	94-188		<8

Figure 4. Quaternary CCR5 inhibitors from Merck Laboratories.



20



21

	20	21
Inhibition of [^{125}I]- MIP-1α binding, IC_{50} [nM]	4.4	13
HeLa (Bal) CIC_{90} [nM]	>300	n/a
PBMC (YU-2) CIC_{95} [nM]	n/a	25,000

Figure 5. Sulfonamide substitutions in **19** produced compounds with good affinity for the CCR5 receptor, but poor antiviral activity.

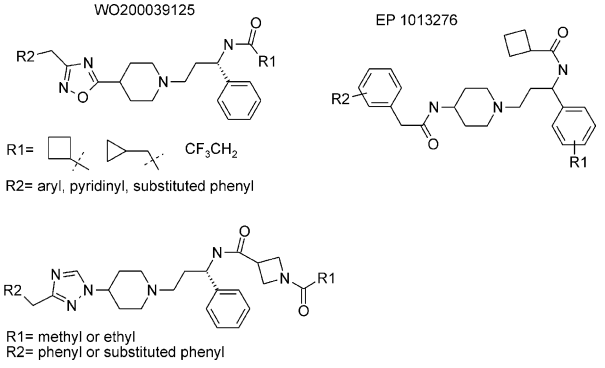
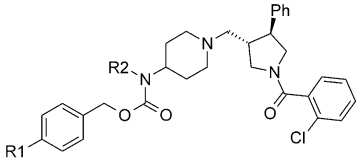


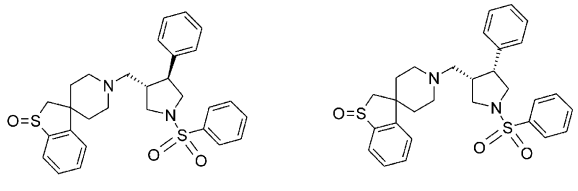
Figure 6. Examples of 1,3-propanediamine-based CCR5 inhibitors.



22 R1=H, R2=Et **23** R1= NO_2, R2= Allyl

	22	23
Inhibition of [^{125}I]- MIP-1α binding, IC_{50} [nM]	2	0.8
HeLa (Bal) CIC_{90} [nM]	50	1
PBMC (YU-2) CIC_{95} [nM]	1500	31 (Bal)
Rat %F	n/a	39%

Figure 7. 1,3,4-Trisubstituted pyrrolidine-based CCR5 inhibitors.



24 **25**

	24, trans	25, cis
Inhibition of [^{125}I]- MIP-1α binding, IC_{50} [nM]	26	3500

Figure 8. *trans* Configuration (**24**) is preferred over *cis*-(**25**) towards high affinity to the CCR5 receptor.

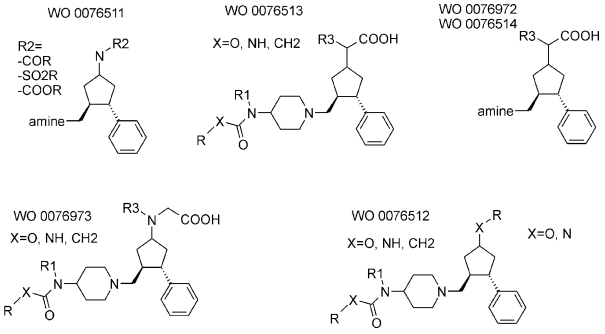


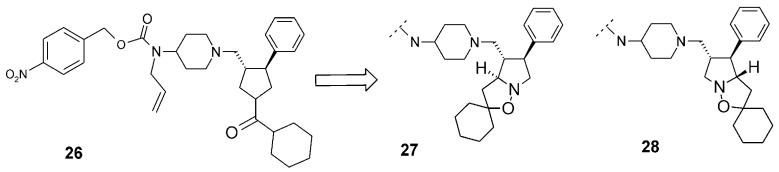
Figure 9. Cyclopentane-based CCR5 inhibitors from Merck Laboratories.

Sch-C antagonised MIP-1β- stimulated cell migration in a dose–response fashion, while not activating CCR5. In addition, in functional assays such as calcium flux, GTPγS binding, and chemotaxis, Sch-C was also demonstrated to be an antagonist.

Sch-C did not inhibit CYP 3A4, 2D6, 2C9, and 2C19 enzymes and did not interact with chemokine receptors CCR1, CCR2, CCR3 and CCR7. On the other hand, Sch-C inhibited infection of PBMC against many diverse R5HIV-1 isolates with single-digit nM to sub-nM IC_{50}s. Sch-C exhibited a strong synergy in combination with other anti-HIV agents. Co-application of Sch-C with AZT or Crixivan potentiated the antiviral potency, resulting in 6–12-fold decrease in the IC_{50}. A ternary mixture of all three inhibitors resulted in >80-fold increase of antiviral potency.[150]

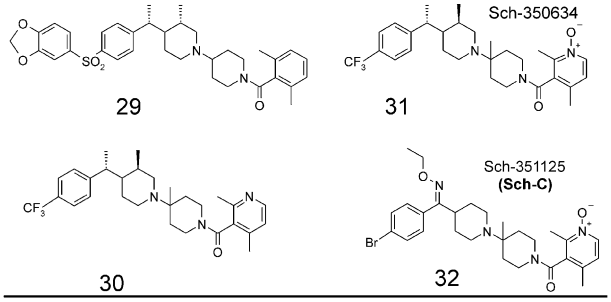
Sch-C shows only moderate 85–89% protein binding in either human or monkey plasma. In several species (rat, dog, monkey), Sch-C exhibited 52–92% oral bioavailability with an in vivo $t_{1/2}$ of 5–10 h. AUC 0→ infinity ranged between 3.9 and 8.2 μgh/mL for doses 2–10 mg/kg in these species, and was generally dose-proportional.

Taken together, it is not surprising that plasma levels of Sch-C exceeding the in vitro IC_{90} inhibitory concentrations could be observed 12–24 h after oral administration in several species. Investigation of Sch-C



	26	27	28
Inhibition of [^{125}I]- MIP-1α binding, IC_{50} [nM]	0.8	14	0.13
HeLa (Bal) IC_{90} [nM]	4	n/a	33

Figure 10. Isoxazolidine-based CCR5 inhibitors.



	29	30	31	32
RANTES CCR5 IC_{50} [nM]	440	2.3	7	2.1
K_i M2 IC_{50} [nM]	0.8	2,500	250	n/a
AUC uh.hr/mL	n/a	3.9	9.2	6.5

Figure 11. Piperidine-based CCR5 inhibitors from Schering Plough.

in the in vivo SCID-hu Thy/liv mice model by twice/day oral doses of 3, 10 and 30 mg/kg/day in the presence of R5HIV-1 isolate Bal, revealed a dose-dependent antiviral activity for Sch-C, with no p24 antigen detectable at the highest concentration.

Sch-C has entered phase I clinical studies, with daily PO doses in humans in the range of 25–600 mg. Even at the lowest dose of 25 mg, the level of Sch-C in the plasma exceeded its in vitro IC_{90} after 12 h. Multiples of IC_{90} were achieved for higher doses at 12 h, except that QTc prolongation was observed at the highest dose of 600 mg.

Significantly, in a 10-day monotherapy of a cohort of 12 patients, which were administered 25 mg of Sch-C BID, no discontinuations from adverse effects were observed and 10/12 patients exhibited >0.5 log decrease of viral RNA concentration in the plasma (with 4/12 experiencing >1 log decrease).

An important study designed to evaluate if the use of CCR5 ligands could accelerate viral switch to strains utilizing the CXCR4 receptor was recently described by Trkola et al.[128] This work demonstrated that the HIV-1 escape strains from Sch-C were M-tropic and continued to utilize CCR5 for cellular entry.

Recently, Schering announced the discovery of Sch-D (structure undisclosed), a 10-fold more potent analogue of Sch-C. It remains to be seen if the improvement in potency of Sch-D translates into a superior safety window versus the unwanted QTc prolongation.

GlaxoSmithKline has disclosed heteroanilides and benzanilides claimed for treating COPD, asthma and atopic disorders, rheumatoid arthritis, atherosclerosis, sarcoidosis and other fibrotic disease, psoriasis, autoimmune diseases such as multiple sclerosis, inflammatory bowel disease and HIV, [151] (Fig. 12). No specific information about receptor binding has been disclosed.

An important new class of spiroketopiperazine-based CCR5 inhibitors, exemplified by **33** (E913, Fig. 13) has been disclosed by Ono Pharmaceuticals.[152] The lead compound E913 was highly potent (IC_{50} 2 nM) in the MIP-1α binding assay, and inhibited replication of R5HIV-1 strains in the cell assay with $IC_{50} = 30$–60 nM. E913 did not inhibit T-tropic strains of HIV-1, which utilize CXCR4 receptors for entry, confirming its selectivity for the CCR5 receptor.

E913 did not downregulate CCR5 receptors and when co-dosed with CXCR4 antagonist AMD-3100, it synergistically inhibited the replication of dualtropic HIV-1 and a 50:50 mixture of R5 and X4HIV-1. E913 was highly CCR5 receptor specific, but was characterized by only a modest bioavailability in rodents (3–30%).

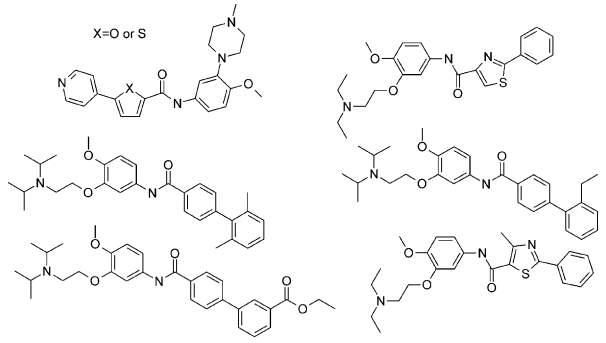


Figure 12. Selected structures from GlaxoSmithKline CCR5 patents.

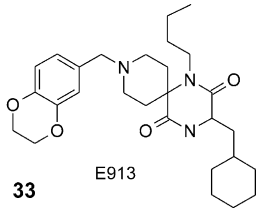


Figure 13. Structure of ONO spiroketopiperazine CCR5 inhibitor E913 (**33**).

Molecular Modeling and Mutagenesis: Mapping the TAK-779 Binding Site

TAK-779 (Fig. 1) interferes with HIV-1 replication by inhibiting the interaction between gp120 and CCR5, thus preventing HIV-1 entry into target cells (Fig. 14). Development of a reliable CCR5 structural model and the delineation of its interaction with TAK-779 (as well as other CCR5 antagonists) would provide considerable insight into the structural determinants responsible for ligand-receptor affinity. Such modeling efforts would also provide insight into molecular modifications for TAK-779 (as well as other CCR5 antagonists) that could lead to more potent drug candidates.

To date, only two structural models of CCR5 have appeared in the literature. The first structural model of CCR5[153] was constructed in two stages, based on Herzyk and Hubbard's structural model of frog rhodopsin.[154] The first stage involved construction of the seven-transmembrane (TM) α-helices. The second stage involved construction of the three extracellular loops

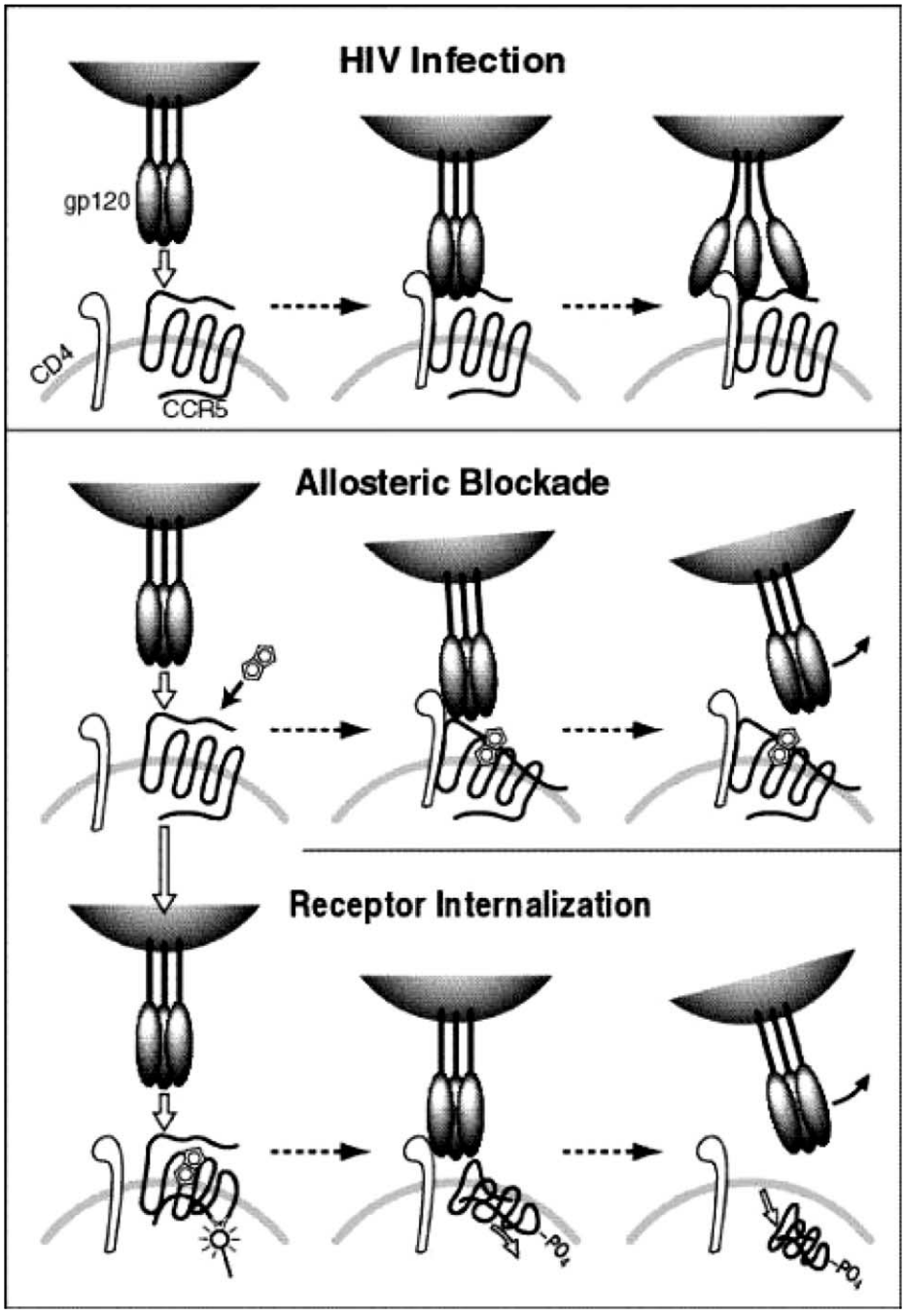


Figure 14. Schematic diagram of HIV-1 infection through interaction of viral coat protein gp-120, and the cellular membrane proteins CD4 and CCR5. Two possible methods to block these interactions are the allosteric modification of CCR5 tertiary conformation (reducing the affinity of gp120 for CCR5) and the removal of CCR5 from the cell surface through internalization mediated by phosphorylation of the carboxy-terminus of CCR5.

ECL-1, ECL-2 and ECL-3 onto the CCR5 TM domain; the N-terminal domain was not modeled. Huang et al. were able to identify a binding pocket for TAK-779 using their model, presumably by visual inspection of the molecular surface although this was not made clear by the authors.[153] The putative binding pocket, composed of conserved residues Tyr^{108}, Gly^{111}, Ser^{114}, Glu^{283}, Gly^{286} and Cys^{290} and non-conserved residues Thr^{105}, Leu^{107}, Phe^{112}, Gly^{115}, Lys^{197} and Met^{287}, was located between TM helices 3, 5, 6 and 7. The pocket was enclosed by the extracellular loops, in particular, ECL-2. Huang et al. speculate that ECL-2 is responsible for controlling the entrance of small molecules and endogenous peptides into the pocket.[153]

Huang et al. docked the inhibitor into the putative binding pocket using the anchor-first docking algorithm of DOCK 4.0 to determine the binding mode of TAK-779.[153] For docking purposes, it was assumed that the aminium group of TAK-779 interacts with the negatively charged side chain of Glu^{283}. A series of ligand orientations within the binding pocket were calculated and subsequently evaluated based on the electrostatic and van der Waals components of the binding energy. The top scoring (i.e., most energetically favored) ligand orientation within the binding pocket was assumed to be the most probable binding mode for TAK-779.

From the docking model, the tetrahydropyran group of TAK-779 was found to lie at the innermost part of the binding pocket with the oxygen atom forming an H-bond with the side chain of Ser^{114}. Huang et al. suggested that this H-bond is an important factor in determining the overall orientation of TAK-779 within the binding pocket.[153] Other amino acid residues found to interact with the tetrahydropyran group, in this case through hydrophobic interactions, included Gly^{111}, Ser^{114}, and Gly^{115} from TM3, and Gly^{286}, Met^{287} and Cys^{290} from TM7. As indicated, the aminium group was assumed to interact with the side chain of Glu^{283}. However, the distance between the charged atoms was 'longer than the normal length' of an H-bond. The reason for this according to Huang et al. is the steric hindrance from both methyl groups attached to the quaternary nitrogen.[153] The phenyl group adjacent to the amido nitrogen formed a π-stacking interaction with the phenyl groups of residues Tyr^{108} and Phe^{122}. It was also found to interact with the side chains of residues Leu^{104}, Leu^{107} and Met^{279}. The amido group itself formed an H-bond with the side chain of Thr^{105}. Lastly, the methylphenyl–benzocycloheptenyl group was positioned at the extracellular domain of CCR5 where it formed hydrophobic interactions with the side chains of residues Cys^{101} from ECL-1, Ser^{169}, Gln^{170}, Lys^{171}, Cys^{178}, Gln^{188}, and Phe^{189} from ECL-2, and Leu^{275} and Asp^{276} from ECL-3.

Although the docking model of Huang et al. may seem reasonable, there is no experimental evidence to support their proposed binding pocket as the binding site for TAK-779.[153] The structural model of CCR5 was built from a structural model of frog rhodopsin and the binding site for retinal is known to lie between TM helices 3, 5, 6 and 7 of rhodopsin. Thus, it could be that the binding pocket located by Huang et al. is a structural artifact that results from modeling the CCR5 receptor on the basis of the rhodopsin structure.[153] A recent mutagenesis study by Dragic et al. would seem to confirm this notion.[155]

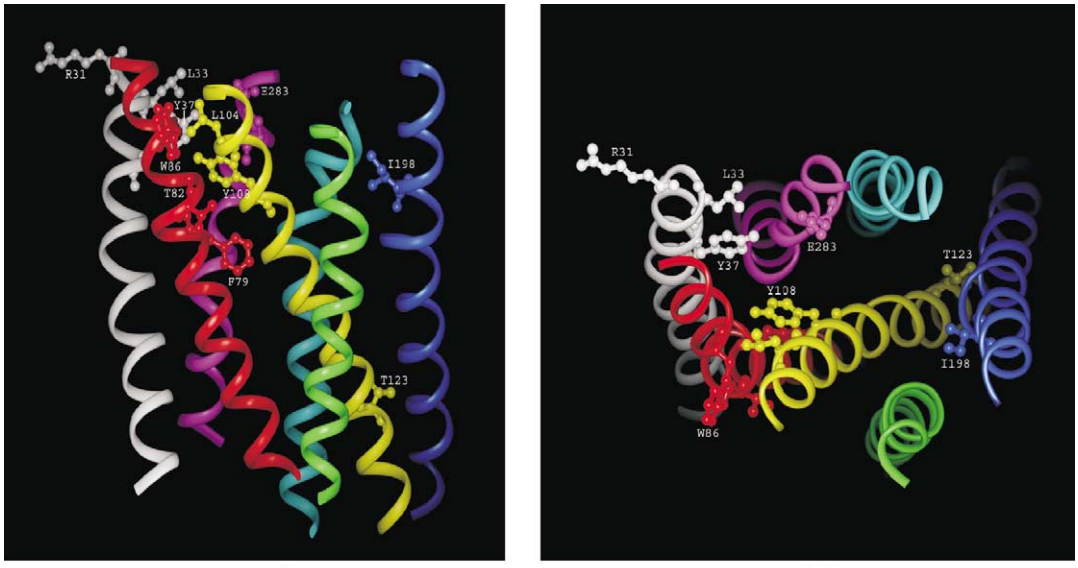


Figure 15. Transmembrane (TM) domain of our CCR5 homology model. The model was constructed in-house based on the 2.8 Å crystal structure of bovine rhopodsin. The TM helical segments are color-coded as follows: helix 1 is white, helix 2 is red, helix 3 is yellow, helix 4 is green, helix 5 is blue, helix 6 is cyan and helix 7 is magenta. The amino acid residues shown in ball-and-stick representation were those identified by Dragic et al. as having an effect (either strong, intermediate or weak) on the antiviral activity of TAK-779. (A) CCR5 viewed from within the plane of the membrane. The extracellular region is at the top of the figure; the cytoplasmic region is at the bottom of the figure. (B) CCR5 viewed from the extracellular surface. The model is rotated roughly 90° towards the viewer from the orientation in A.

Dragic et al. preformed extensive mutagenesis studies wherein selected CCR5 residues were substituted with alanine and the resulting mutants evaluated for their ability to prevent TAK-779 from inhibiting HIV-1 entry into the target cells.[155] Since the gp120-binding site maps to the extracellular domain of CCR5, in particular, the N-terminal tail, mutants for these regions of the receptor were evaluated first. No single-site mutation of the CCR5 extracellular domain had any significant effect on the antiviral activity of TAK-779; the CCR5 mutants were still susceptible to HIV-1 infection and to the antiviral activity of TAK-779.

Given the negligible effects of alanine substitution in the extracellular domain, Dragic et al. directed their attention to the TM segments of CCR5. A structural model of the CCR5 TM domain was constructed based on a low-resolution structure of bovine rhodopsin to help guide their mutagenesis strategy.[155] A putative ligand-binding site for TAK-779 was located within the TM helices using this model. A carefully selected set of residues surrounding the binding pocket were mutated first, followed by other sets of residues that were in close proximity to those residues found to have an effect on the antiviral activity of TAK-779. Nine CCR5 mutants were identified as having significant resistance to the antiviral activity of TAK-779. Five of these mutants were classified as strongly resistant: L33A and Y37A in TM1, W86A in TM2, and Y108A and T123A in TM3. The four remaining mutants were classified as moderately resistant: R31A in TM1, T82A in TM2, I198A in TM5, and E283A in TM7. Of note, two additional CCR5 mutants were found to be 'borderline' resistant to TAK-779: F79A in TM2 and L104A in TM3.

Dragic et al. mutagenesis and modeling data strongly suggest that the TAK-779 binding site is located near the extracellular surface of the receptor in a cavity surrounded by TM helices 1, 2, 3, and 7.[155] We have also constructed a CCR5 homology model somewhat similar to that of Dragic et al. which appears to agree with their assessment (Fig. 15).[155] The residues forming part of the putative binding site include Leu^{33}, Tyr^{37}, Thr^{82}, Trp^{86}, Tyr^{108}, and Glu^{283}. Of the residues having significant effects on TAK-779 activity, only Thr^{123} and Ile^{198} are not clustered with the aforementioned residues near the binding site. Dragic et al. speculate, however, based on their CCR5 model, that Thr^{123} and Ile^{198} help stabilize the TAK-779 binding site through inter-helical interactions.[155]

Although no docking studies were conducted by Dragic et al., they have speculated as to how TAK-779 might interact with the CCR5 receptor.[155] The depth of the binding pocket was roughly the same length as the methylphenyl–benzocycloheptenyl group of TAK-779. As to whether or not the volume associated with the binding pocket is sufficient to accommodate the methylphenyl–benzocycloheptenyl group was not discussed. At any rate, it is thought that the methylphenyl–benzocycloheptenyl group binds into the TM pocket, where the aromatic rings of TAK-779 form favorable interactions with the aromatic residues Tyr^{37}, Trp^{86} and Tyr^{108}. This ligand orientation then allows the positively charged end of TAK-779 to interact with the polar extracellular domain, where it may interfere with the interaction between the gp120–CD4 complex and CCR5.

Currently, Sakmar and coworkers (pers. commun.) are using the same mutagenesis strategy as outlined in ref 155 to map the binding site of several additional small molecule inhibitors of HIV-1 entry. They are also in the process of constructing an improved structural model of CCR5 based on the 2.8 Å crystal structure of bovine rhodopsin.[156] The additional mutagenesis data in combination with a more reliable structural model of CCR5 should help improve our understanding of the interactions between CCR5 inhibitors and the receptor itself.

Conclusions

Since 1996 when CCR5 was first identified as an HIV co-receptor, many structurally-diverse, small-molecule CCR5 inhibitors have been discovered and disclosed in the patent and scientific literature. Among the compounds mentioned in this review, Sch-C and Sch-D have progressed into phase I/II clinical trials, with several new compounds entering phase I trials as well. Although these inhibitors will antagonize the physiological function of CCR5, it is expected that the resulting effects will be either inconsequential or compensated for by known chemokine receptor redundancy. This expectation is supported by the good safety profile seen with Sch-C as well as by the lack of adverse effects seen in individuals homozygous for the Δ32 CCR5 allele.

On a more basic level, the identification of small-molecule CCR5 antagonists provides yet another example of the disruption of macromolecule complexes via small molecules. By analogy with many other 7TM GPCRs, such inhibition likely occurs via allosteric mechanisms and not a direct steric blockage of interaction. Successful drug development in this area will further validate the small molecule approach to drug discovery against diseases mediated by protein-protein interactions. Such targets have traditionally been considered very difficult, although they are an untapped source of medicinal chemistry targets.

Note Added in Proof

The structure and properties of Sch D have been disclosed while this manuscript was in proof: Tagat, Jayaram; McCombie, Stuart; Nazareno, Dennis; Steensma, Ruo; Labroli, Marc; Baroudy, Bahige; Strizki, Julie; Cox, Kathleen. Discovery of potent, selective, and orally active CCr5 antagonists for the potential treatment of HIV infection. *Abstracts of Papers*, 225th ACS National Meeting, New Orleans, LA, United States, 23–27 March, 2003 (2003); MEDI-015.

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