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Prospective CCR5 Small Molecule Antagonist Compound Design Using a Combined Mutagenesis/Modeling Approach

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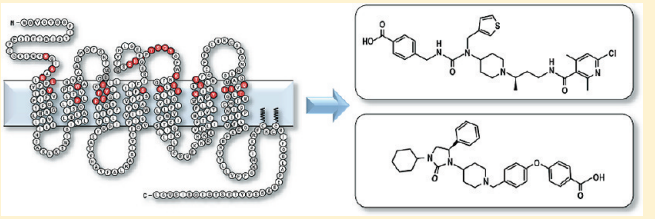
Supporting Information

ABSTRACT: The viral resistance of marketed antiviral drugs including the emergence of new viral resistance of the only marketed CCR5 entry inhibitor, maraviroc, makes it necessary to develop new CCR5 allosteric inhibitors. A mutagenesis/modeling approach was used (a) to remove the potential hERG liability in an otherwise very promising series of compounds and (b) to design a new class of compounds with an unique mutant fingerprint profile depending on residues in the N-terminus and the extracellular loop 2. On the basis of residues, which were identified by mutagenesis as key interaction sites, binding modes of compounds were derived and utilized for compound design in a prospective manner. The compounds were then synthesized, and in vitro evaluation not only showed that they had good antiviral potency but also fulfilled the requirement of low hERG inhibition, a criterion necessary because a potential approved drug would be administered chronically. This work utilized an interdisciplinary approach including medicinal chemistry, molecular biology, and computational chemistry merging the structural requirements for potency with the requirements of an acceptable in vitro profile for allosteric CCR5 inhibitors. The obtained mutant fingerprint profiles of CCR5 inhibitors were used to translate the CCR5 allosteric binding site into a general pharmacophore, which can be used for discovering new inhibitors.



INTRODUCTION

The entry of HIV-1 into T cells is preceded by the formation of a ternary complex between the gp120 viral envelope protein, the CD4 receptor, and one of two chemokine receptors: CCR5 or CXCR4. As a consequence, the gp41 viral protein is exposed and assists in cell membrane fusion.[1] Depending on the chemokine receptor selectivity of HIV, three main variants are known: CXCR4-tropic (X4), CCR5-tropic (R5), and dual-tropic (R5/X4).[2] While CCR5 is used as a coreceptor in the early stages of infection, the CXCR4 coreceptor using virus is linked to significant disease progression leading to AIDS.[3]

On the basis of the understanding of how HIV-1 enters the T cell, two strategies have been successfully applied to prevent HIV infection. Fuzeon is an approved fusion inhibitor interacting with gp41[4,5] and maraviroc a CCR5 allosteric inhibitor[6−8] suppressing ternary complex formation. Maraviroc is the only marketed CCR5 inhibitor. Several other CCR5 small molecule allosteric inhibitors have been reported in the literature,[9−18] but only very few were progressed into the clinic before studies were halted.[19−21] A similar fate occurred to AMD070, a CXCR4 allosteric inhibitor.[22] The value of new CCR5 inhibitors lies in the realization that there is not a general viral cross resistance among this class of inhibitors. Recent studies led to the development of a cross resistance model in which only N-terminus (N-ter) using viruses develop cross resistance among all CCR5 inhibitors investigated, while viruses using also the extracellular loop 2 (ECL 2) show distinct resistance profiles.[23]

In this work, we designed and developed two structurally diverse CCR5 inhibitors with a mutagenesis/modeling approach. Single site mutations were introduced into the chemokine receptor, and the effect of these mutations on compound/receptor interaction was interrogated using an antifusogenic assay in which the viral envelope protein on one cell binds to CD4 and CCR5 on a second cell triggering an enzyme reporter read-out. In this way, we determined a "mutant fingerprint", which describes a

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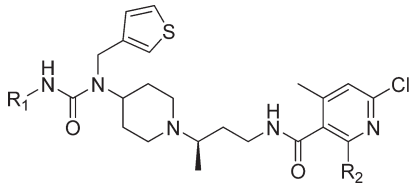
Table 1. Cell Fusion Inhibition, anti-HIV-1 (BaL) Activity, Cellular Cytotoxicity, and hERG Inhibition of Compounds of the Thiophen-3-yl-methyl-urea Series[a]



compd	R_1	R_2	log D^{39}	^{125}I RANTES binding IC_{50} (nM)	cell fusion IC_{50} (nM)	HIV-1 PBMC IC_{50} (nM)	PBMC CC_{50} (μM)	hERG IC_{50} (μM)
1	CH_3	CH_3	0.59	3.6	0.3 ($n = 4$)	16.0 ($n = 4$)	>40.6	2.8
2	CH_3O	CH_3	0.73	4.0	0.3 ($n = 4$)	15.0 ($n = 10$)	>35.2	4.0
3	CH_3O	Cl	1.29	2.2	0.08 ($n = 5$)	1.9 ($n = 7$)	>37.8	1.9
4	$HO_2C(CH_2)_2$	CH_3	−0.92	nd	26.1	nd	nd	>40
5	$HO_2C(CH_2)_3$	CH_3	−0.51	nd	2.3 ($n = 3$)	690.0 ($n = 3$)	>35.5	nd
6	$H_3CNHOC(CH_2)_3$	CH_3	−0.11	nd	1.0 ($n = 3$)	413.0 ($n = 4$)	>34.6	>50
7	$HO_2C(CH_2)_4$	CH_3	−0.06	nd	0.5 ($n = 3$)	125.0 ($n = 2$)	>34.6	>40
8	$H_3CO_2C(CH_2)_4$	CH_3	1.22	nd	0.3 ($n = 3$)	nd	nd	nd
9	HO_2C-4-$PhCH_2$	CH_3	0.48	12.6	0.48	6.9 ($n = 3$)	>32.2	>50

[a] Assays were performed in duplicate, and values represent the mean with standard deviations <30% of the mean. Bracketed values represent the number of experiments.

compound's dependency of cell fusion inhibition on a selected set of single site mutations. This has allowed us to identify key amino acids on the chemokine receptor, which are responsible for inhibitor binding and thus map the key components of the inhibitor binding site. This assay avoids the use of radioactivity and HIV infection assays and can be used in high throughput mode. Successful assay validation was accomplished by comparing the mutant fingerprint profiles of known CCR5 compounds such as maraviroc, aplaviroc, and vicriviroc with those obtained from natural ligand binding and HIV infection. Furthermore, the mutant fingerprint profiles of known CCR5 inhibitors obtained with these assays were used to identify crucial binding site residues for compound design.[24]

An important criterion for a compound to be put forward into lead optimization is that it should have little or no hERG inhibition as measured by the patch clamp assay. Inhibition of the hERG channel is a strong indicator of QT prolongation in vivo, and hence of cardiovascular toxicity. Given the continuous use of medication for treating HIV infection, this criterion seemed necessary. There are several strategies to overcome hERG inhibition.[25,26] One of these is the use of zwitterions (ZIs). Mutagenesis studies on aplaviroc identified potential extracellular interaction sites, which could be used for ZI compound design. The validity of this approach was confirmed after aplaviroc showed good exposure in animals and later in humans.[21]

We have explored the interaction of our investigational CCR5 inhibitors with the CCR5 receptor by introducing single site mutations in the chemokine receptor and interrogating the effect of these mutations on the inhibitory activity of the compounds using the antifusogenic assay. Utilizing an interdisciplinary approach including medicinal chemistry, molecular biology, and computational chemistry merging the structural requirements for potency with the requirements of an acceptable in vitro profile, we developed two structural classes of novel CCR5 inhibitors. In this article, we present two case studies: one example demonstrates the elimination of hERG channel inhibition while retaining potency against CCR5; the second example demonstrates the use of this approach for the rational optimization of potency from modestly active structural leads.

Table 2. Fold Differences[a] of Amino Acid Mutation on Fusion Inhibition[b]

		compound					
location[c]	mutant	**3**	**4**	**5**	**6**	**7**	**8**
N-ter	K26A	nd	1	2	1	5	1
TM1	L33A	57	35	17	25	55	22
TM1	Y37A	712	46	383	211	737	392
TM2	W86A	958	47	275	301	496	330
TM3	Y108A	73	12	11	21	64	36
ECL2	K191A	nd	5	12	1	10	2
TM5	Y251A	50	8	22	26	69	102
TM7	E283A	3846	47	411	2252	5833	6329

[a] Fold differences are defined as mutant IC_{50}/wild-type IC_{50}. [b] The following mutations are silent mutations (see Discussion for the explanation of silent mutations) for any compound: K197A, R31A, F109A, L255A, T284A. Wild-type fusion IC_{50}s in parentheses for compounds are: **3** (0.3 nM); **4** (216 nM); **5** (24 nM); **6** (4 nM); **7** (1 nM); **8** (2 nM). [c] N-ter = N-terminus, TM = transmembrane helix; ECL = extracellular loop.

RESULTS

Case Study 1: Thiophen-3-yl-methyl-urea Series and Elimination of hERG Activity. We have previously described our efforts in which we produced compounds with exceptional antiviral potency. Compounds **1**, **2**, and **3** have IC_{50} values below 20 nM in the HIV-1 PBMC assay (Table 1).[27] In addition, these compounds showed promising drug-like character with the exception

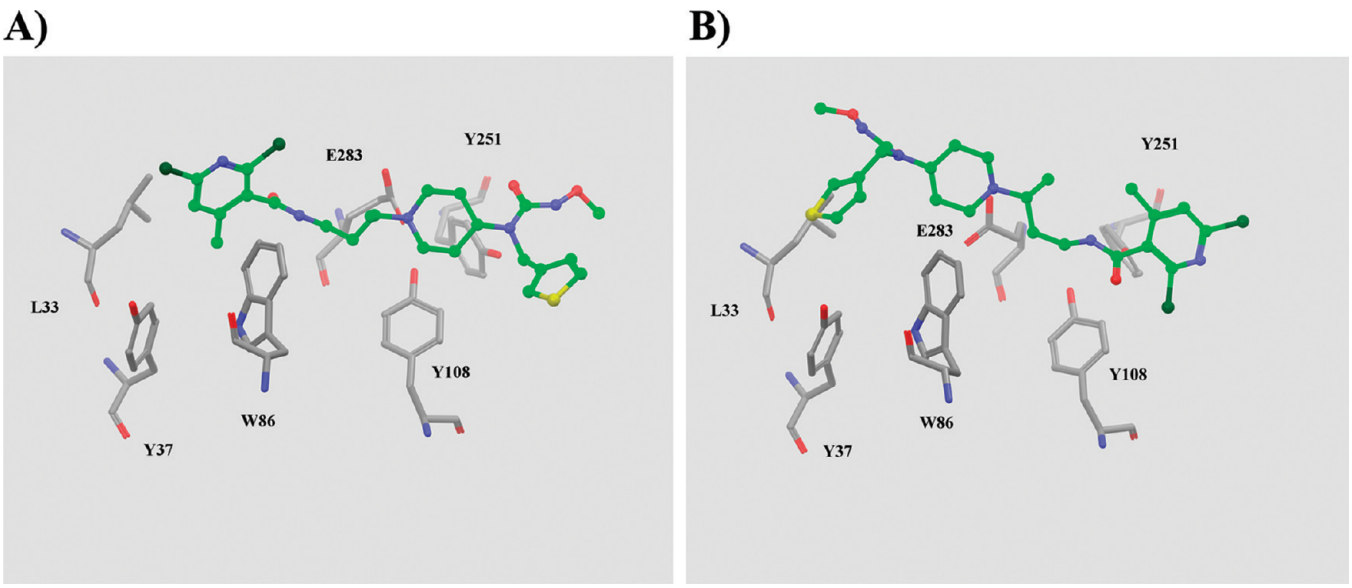


Figure 1. Proposed binding modes of **3**. (A) The 2,6-dichloro-4-methylnicotineamide interacts with residues in TM1 and TM2; the thiophene moiety interacts with residues of TM3 and TM6. (B) The 2,6-dichloro-4-methylnicotineamide interacts with residues in TM3 and TM6; the thiophene moiety interacts with residues of TM1 and TM2. Only residues are shown that have an impact on inhibition upon mutagenesis results obtained after the binding mode prediction.

of a rather high hERG inhibition. Because hERG inhibition in the low micromolar range together with prolongation of cardiac QTc interval in preclinical studies and phase I human trials was reported for Schering C,[28] efforts were undertaken to address this concern and find ways to remove this potential liability in the thiophen-3-yl-methyl-urea compound series. Literature data revealed that lysines in the N-ter (Lys^{26}) and in the ECL 2 (Lys^{191}) of CCR5 could serve as potential interaction sites.[24,29] Because compounds **1**, **2**, and **3** are structurally related, we only identified the binding mode for one of these (compound **3**). This was followed by synthesis of analogues to obtain SAR data both for biological activity, including hERG inhibition, and to further investigate the proposed binding mode. These data formed the basis for a hypothesis for lead compound design, which was tested through assessment of the predicted lead biological activity.

Mutant Study and Binding Mode Prediction for Compound 3. The binding mode of compound **3** was determined by obtaining its mutant fingerprint (Table 2). Similar to investigations on structurally diverse CCR5 compounds, compound **3** depended strongly on E283, which is at the center of the allosteric binding site of CCR5 defined by helices TM1, TM2, TM3, TM5, TM6, and TM7, and beneath ECL 2.[24,29−37] Mutation of the charge compensating potentially hydrogen-donating residues (Tyr^{108}, Tyr^{251}) reduced the inhibition 50- and 73-fold. Additionally, mutation of residues Leu^{33} and Tyr^{37} had a significant impact on the fusion inhibition of compound **3**. Other residues (Table 2) did not show any fold changes. Some of the fold changes of the mutations were large. However, as addressed during the discussion, these mutations do not severely impact the structural integrity or function of the mutated CCR5.

The obtained mutant fingerprint results were used to place compound **3** into the allosteric binding site of CCR5 with the requirement to explain these mutant data as much as possible by direct ligand−receptor contacts.

The hydrophobic nature of the remaining residues did not allow for differentiating among several binding modes represented by the two shown in Figure 1. As shown in Figure 1A, the thiophene-urea moiety can potentially interact with Tyr^{108} and Tyr^{251}, while the 2,6-dichloro-4-methylnicotineamide moiety reaches into the TM1 and TM2 region to interact with Leu^{33}, Tyr^{37}, and Trp^{86}. Alternatively, in Figure 1B, the thiophene−urea moiety interacts with residues in the TM1 and TM2 region, while the 2,6-dichloro-4-methylnicotineamide moiety approaches the Tyr^{108} and Tyr^{251} residues.

Prospective Binding Mode Prediction for Hypothetical Compounds. On the basis of the placement of compound **3** into the allosteric binding site of CCR5, structural modifications to target either Lys^{26} or Lys^{191} as interaction sites were made. Both proposed binding modes for compound **3** allowed the same structural modifications, such as attaching a carboxylic acid group by a linker with at least two methylene groups to the terminal urea nitrogen. In Figure 2, compound **7** with an *n*-butyl linker bound into the CCR5 allosteric binding site is shown.

Correlation of Biological Activity with Binding Mode Predictions. Compounds **4**, **5**, and **7** were synthesized, and their antiviral activity, hERG affinity, as well as their mutant fingerprints were determined (Tables 1 and 2). The antiviral activity correlates with the length of the linker, and introduction of a carboxylic group reduces the hERG affinity. The mutant fingerprints reveal a dependency on Lys^{191} for compounds **5** and **7**; in both cases, the inhibitory potency by mutating this lysine to alanine is reduced by at least 10-fold. This can be explained by the ionic interaction between the carboxylic group and Lys^{191}. Compound **4** did not show any Lys^{191} interaction, which is very likely due to being too short to form this interaction. The reduced inhibitory potency of compound **4** would also agree with this explanation. All other fold changes due to mutations reflect the observations obtained with compound **3** (vide supra). These data suggest that the proposed binding mode in which Lys^{191} interacts with the carboxylic group is the preferred one of the two shown in Figure 2A and B. Mutation of Lys^{26} seemed not to

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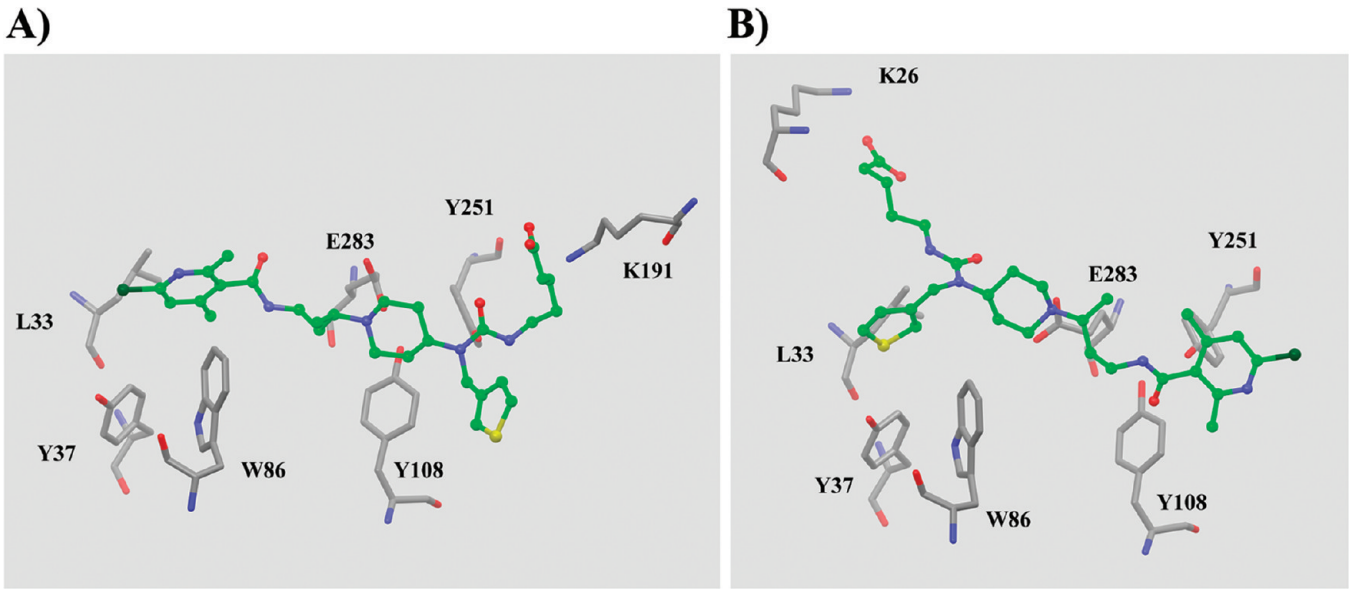

Figure 2. Proposed binding modes of **7**. (A) The carboxylic acid group interacts with Lys^{191}; (B) carboxylic acid interacts with Lys^{26}. With exception of Lys^{26}, which is used for binding mode prediction, only residues are shown that have an impact on inhibition upon mutagenesis results.

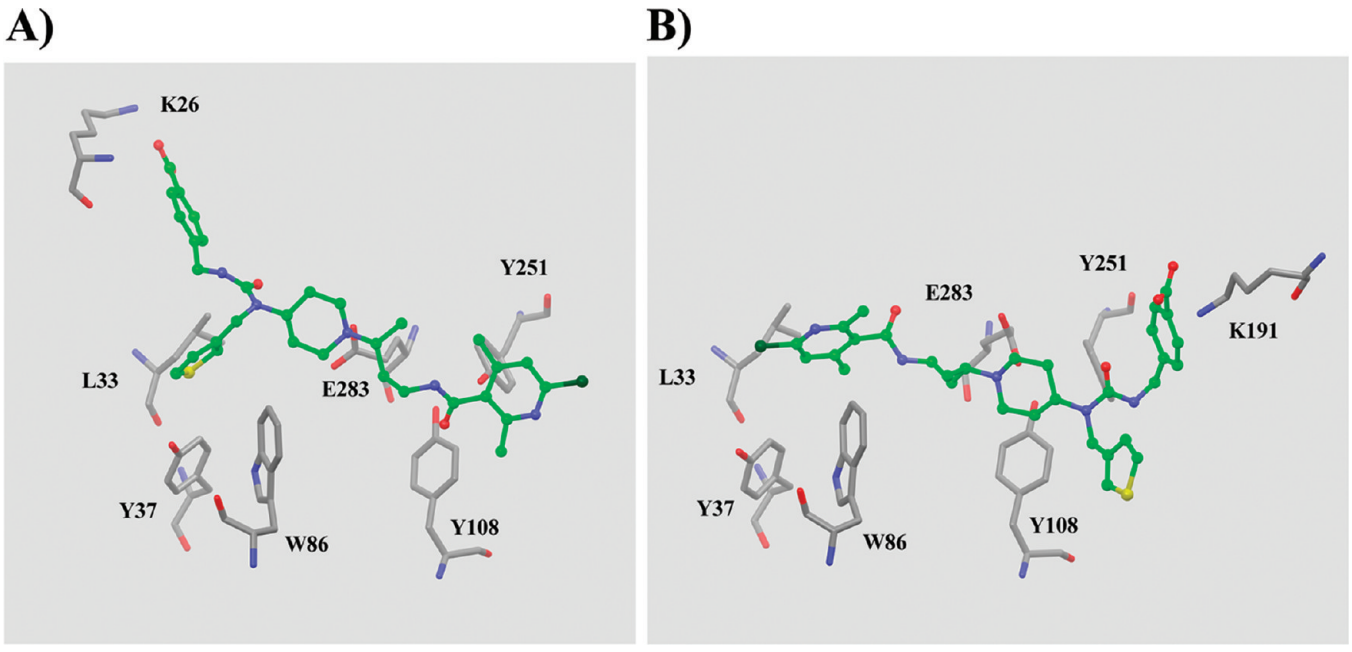

Figure 3. Proposed binding modes of **9**. (A) The carboxylic acid interacts with Lys^{191}; (B) carboxylic acid interacts with Lys^{26}. With exception of Lys^{26} and Lys^{191}, which are used for binding mode prediction, only residues are shown that have an impact on inhibition upon mutagenesis results for related compounds.

impact the small molecule inhibition for this series of compounds. The corresponding amide and ester compounds (compounds **6** and **8**) were also synthesized to emphasize the significance of the Lys^{191} interactions with the carboxylic groups in compounds **5** and **7**. The hydrogen-bond interactions of carboxylic acids with protonated amines are stronger due to an additional ionic contribution explaining the experimentally observed fold change upon lysine to alanine mutation. In addition, the presence of charge could prevent alternative binding interactions if the cationic side chain is removed.[38] Finally, in the patch clamp assay, compound **6** has a reduced hERG affinity comparable to compounds **4** and **7**, indicating that only a distal polar group is required.

Lead Compound Prediction. On the basis of the binding conformations of compound **7**, the flexible *n*-butyl linker was replaced by a benzyl group. While this modification increases the lipophilicity of compound **9** as indicated by the change of log *D* value, the antiviral potency as well as the low hERG inhibition is maintained (Table 1). Although the mutant data for compound **9** were not obtained, it can be hypothesized that this linker would

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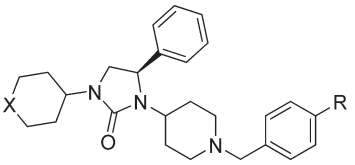
Table 3. Cell Fusion Inhibition, anti-HIV-1 (BaL) Activity, Cellular Cytotoxicity, and hERG Inhibition of Compounds of the Cyclic Urea Series[a]



compd	X	R	log D^{39}	^{125}I RANTES binding IC_{50} (nM)	cell fusion IC_{50} (nM)	HIV-1 PBMC IC_{50} (nM)	PBMC CC_{50} (μM)	hERG IC_{50} (μM)
10	CH_2	Br	6.83	63 (n = 2)	700 (n = 36)	nd	nd	2.7
11	CH_2	O-Ph-4-CO_2CH_3	7.78	30	65	nd	nd	15.8
12	CH_2	O-Ph-4-$CONH_2$	6.18	18	29	40 (n = 3)	14.4	2.8
13	CH_2	O-Ph-4-CO_2H	5.68	39	13 (n = 2)	34 (n = 4)	>30.6	4.0
14	O	O-Ph-4-CO_2H	3.90	25	16 (n = 6)	190 (n = 2)	>32.5	>40

[a] See footnotes in Table 1.

Table 4. Fold Differences of Amino Acid Mutation on Fusion Inhibition[a]

		compound				
location	mutant	**10**	**11**	**12**	**13**	**14**
N-ter	K26A	nd	4	1	16	14
TM2	W86A	18	212	458	920	346
TM3	Y108A	16	5	13	8	7
ECL2	K191A	nd	3	3	6	12
TM5	I198M	22	9	15	14	33
TM7	E283A	22	212	359	161	346

[a] See footnotes in Table 2. The following mutations do not significantly change the effect of any compound: L33A, Y37A, Y251A, K197A, R31A, F109A, L255A, T284A, F79A, F112A, L255A. Wild-type fusion IC_{50}'s in parentheses for compounds: **10** (458 nM); **11** (47 nM); **12** (22 nM); **13** (11 nM); **14** (28 nM).

maintain the ionic interaction with Lys^{191} as shown in Figure 3A. Interestingly, the same modification could utilize Lys^{26} as an interaction site (Figure 3B). Because the introduced linker modification could potentially change the binding mode, the Lys^{26} interaction remains an alternative.

Case Study 2: 4-Phenylimidazolidine-2-one Series and Optimization of Antiviral Activity. The experimental confirmation of Lys^{191} as well as Lys^{26} as potential interaction sites[24,29] offered the possibility to fine-tune the log D^{39} of new generations of CCR5 antagonists and as a consequence their molecular properties.

Prior work on *i*-butyl CCR5 antagonists identified the 4-phenylimidazolidine-2-one moiety as replacement for aniline- and phenyl-*O*-aromatic moieties.[27] Efforts focused on replacements for the *i*-butyl chain using the mutagenesis/docking approach. Simple replacements of the *i*-butyl chain based on substituted benzyl groups showed promise as compound **10** is a modest inhibitor of natural ligand binding as well as of fusion (Table 3). The mutant fingerprint of compound **10** was determined (Table 4) and used for binding mode prediction.

Mutant Study and Binding Mode Prediction for Compound 10. The antifusogenic behavior of compound **10** depends on Glu^{283}. Mutating this residue to alanine completely abrogated all inhibition. Similar observations were made with other compounds.[24,27−29] Compound **10** also depends on Tyr^{108}, a potential binding partner of Glu^{283}. Similarly, the I198A mutation completely abrogated inhibition. Another mutation impacting the small molecule binding is Trp^{86}. The potential impact of these mutations on receptor conformation and on the integrity of the viral envelope protein/CCR5 fusion is discussed below. The mutant fingerprints were used to dock compound **10** into the allosteric binding site. In Figure 4, the binding mode of compound **10** is shown. Again, the hydrophobic nature of the binding site did not allow for a single binding mode. As in the case of the thiophen-3-yl-methyl-urea series above, two different binding modes were used to explain the mutant data. One binding mode describes the interaction of the cyclic urea with Ile^{198} and the benzyl group with Trp^{86} (Figure 4A). In the alternative binding mode, which differs from the first by rotating the molecules around an imaginary vertical axis, the interactions of the molecules with the Ile^{198} and Trp^{86} residues are interchanged (Figure 4B).

Prospective Binding Mode Prediction for Hypothetical Compounds. The obtained binding modes for compound **10** were used to target the extracellular lysines by replacing the substituted benzyl group by a 4-(*p*-tolyloxy)benzoic acid moiety. This structural suggestion provides two distinct binding modes targeting either Lys^{26} or Lys^{191} (Figure 5A and B). Other structural modifications to compounds **10** also provided potent compounds.[40,41]

Correlation of Biological Activity with Binding Mode Predictions. Compounds **11**−**13** were synthesized and tested in the in vitro assays. The antifusogenic potency of these compounds increased, which also translated into good antiviral potency in the PBMC/Bal assay. As in the case for the thiophen-3-yl-methyl-urea series, modifications with a terminal carboxylate, ester, or amide provide potent compounds. The mutant fingerprint of compound **13** (Table 4) revealed that Lys^{26} can be used as an interaction site (Figure 5B). On the other hand, compounds **11** and **12** did not pick up any interactions in the extracellular regions, which could be explained by alternative interactions for the primary amide and ester as well as weaker hydrogen-bond interactions (vide supra). Because the hERG affinity of compound **13** is rather high, additional modifications were needed to make the compounds more polar.

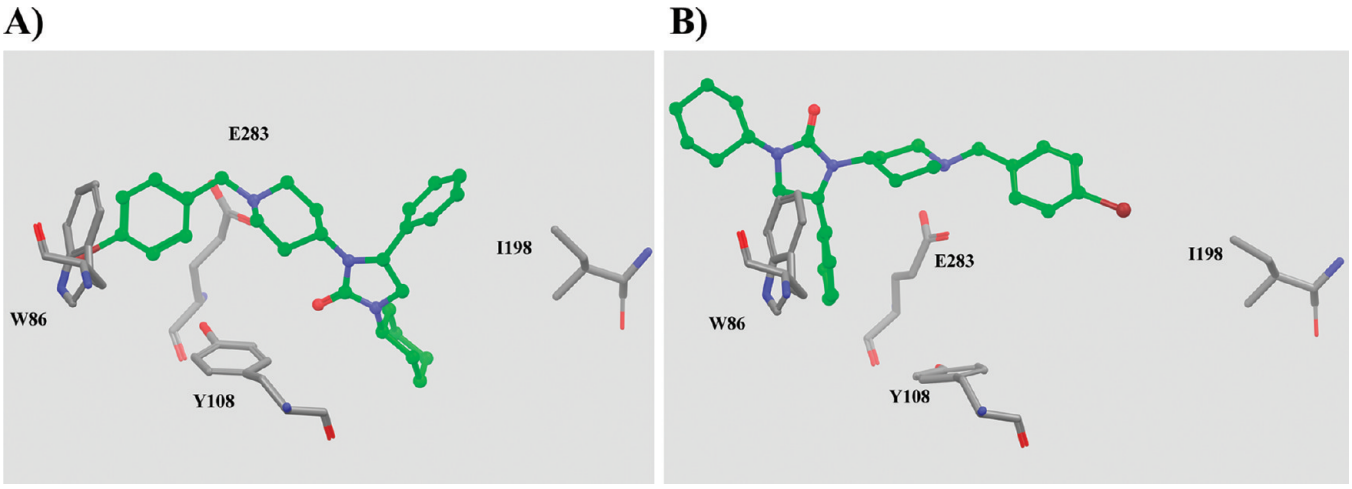


Figure 4. Proposed binding modes of **10**. (A) The 4-bromobenzyl group interacts with Trp^{86} and the cyclic urea with Ile^{198}; (B) the cyclic urea interacts with Trp^{86} and the 4-bromobenzyl group with Ile^{198}. Only residues are shown that have an impact on inhibition upon mutagenesis results obtained after the binding mode prediction.

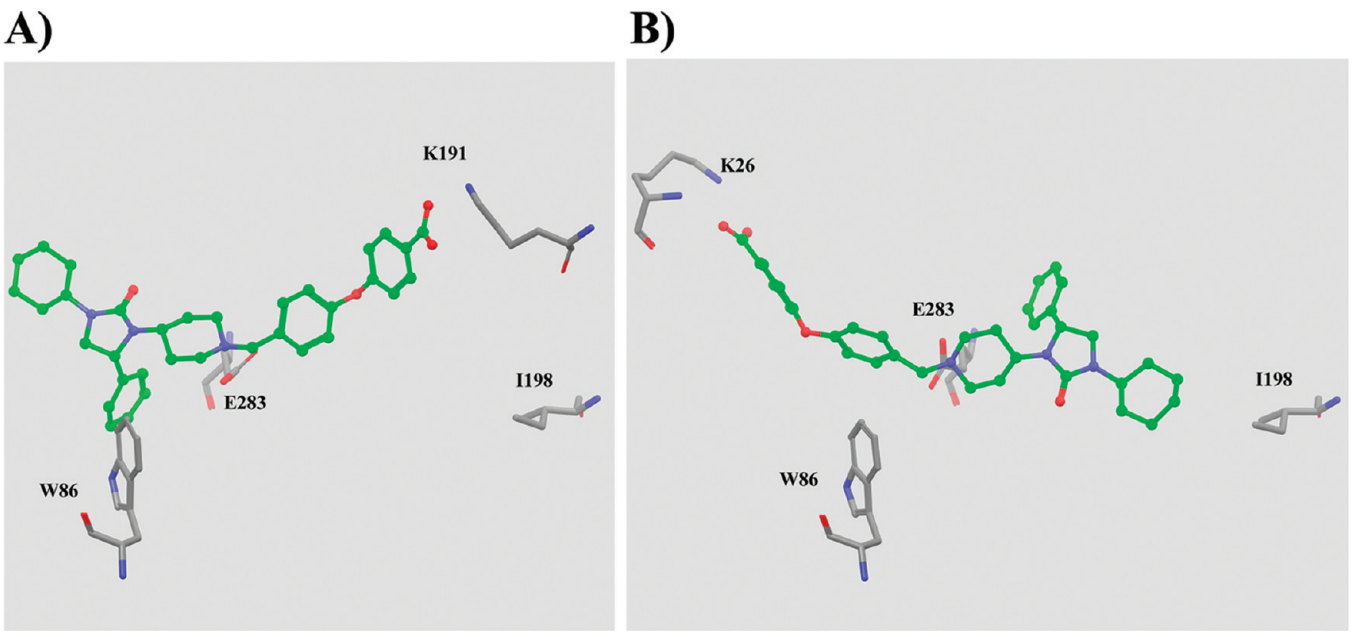


Figure 5. Proposed binding modes of **13**. (A) The carboxylic group interacts with Lys^{191}; (B) the carboxylic group interacts with Lys^{26}. With exception of Lys^{191}, only residues are shown that have an impact on inhibition upon mutagenesis results obtained after the binding mode prediction.

Lead Compound Prediction. The preferred binding mode explaining the mutant fingerprint for compound **13** (Table 4) is depicted in Figure 5B. Utilizing the results on the thiophen-3-ylmethyl-urea series, the replacement of the cyclohexyl group with a tetrahydropyran (THP) group could allow for an interaction with Lys^{191}. The preferred proposed binding mode for compound **14** is characterized by interactions with Lys^{26} and Lys^{191} simultaneously (Figure 6A). However, the binding mode in Figure 6B cannot be completely excluded, explaining the compound's mutant fingerprint equally well. Both binding modes cannot explain the fold change of the I198A mutation, suggesting alternative binding modes or binding site flexibility. The experimental evaluation of compound **14** provided the ideal lead compound. Despite that the introduction of a THP group decreased antiviral potency slightly, more importantly, the increased polarity completely abrogated any hERG interaction. Compound **14** was transitioned into lead optimization. To further test our binding model, the mutant fingerprint of compound **14** was determined, and the mutation of both lysines lowered the antifusogenic activity by more than 10-fold (Table 4).

DISCUSSION

The occurrence of viral resistance makes the continued development of new CCR5 antagonists necessary. Numerous publications and patents show that this is an active research field.[9−18]

The current study utilized a combined mutagenesis/modeling approach to generate new CCR5 antagonists. Two case studies

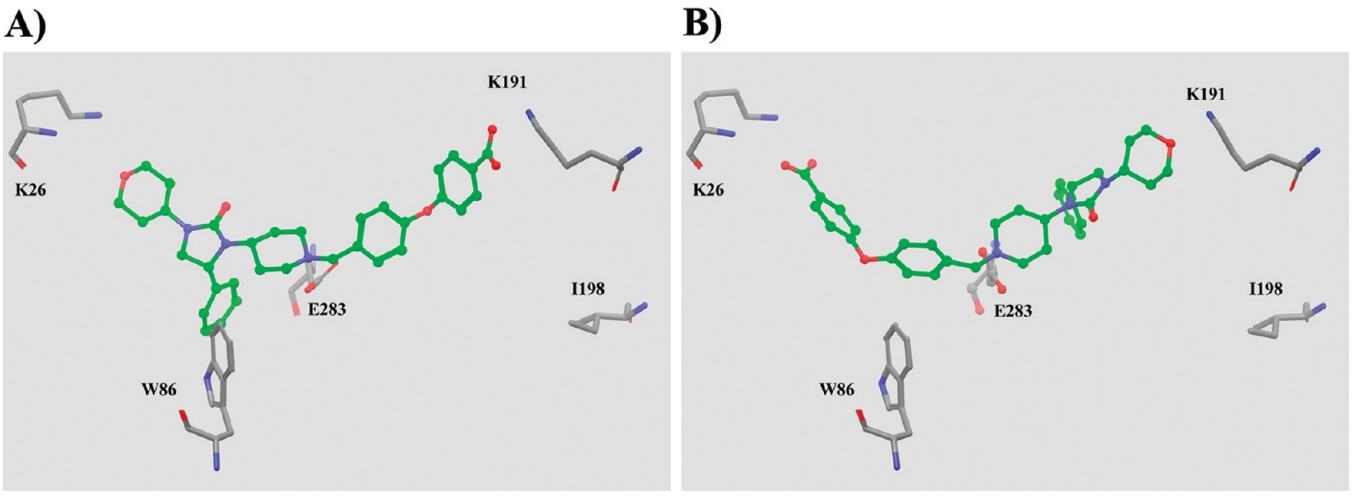


Figure 6. Proposed binding modes of **14**. (A) The carboxylic group interacts with Lys^{191}; (B) the carboxylic group interacts with Lys^{26}. Only residues are shown that have an impact on inhibition upon mutagenesis results obtained after the binding mode prediction.

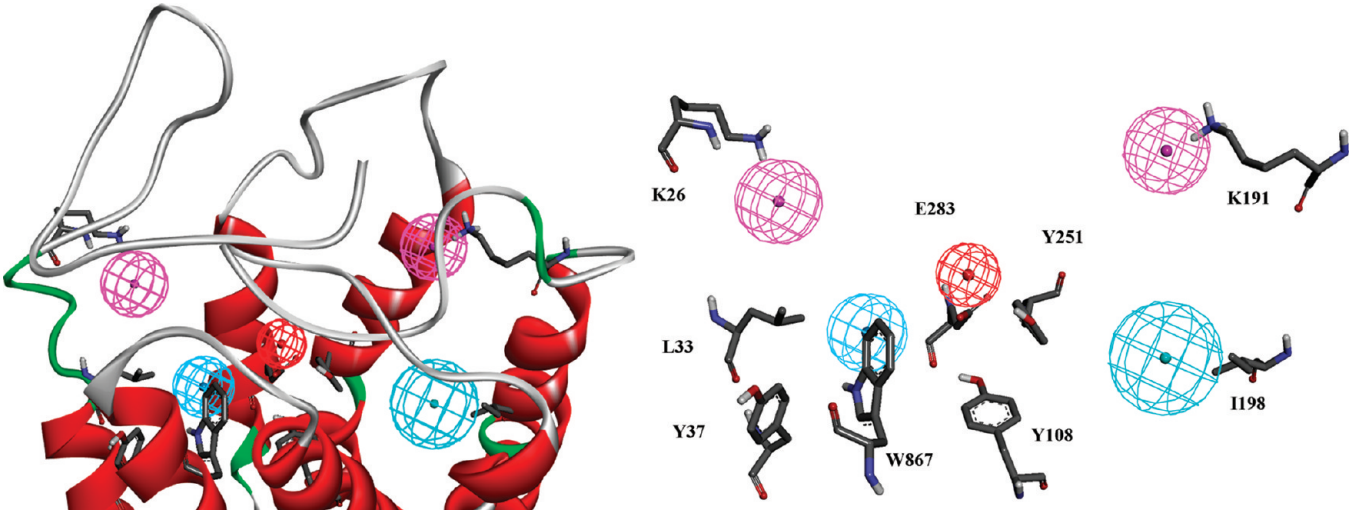


Figure 7. Extended description of CCR5 binding site shared by small molecule antagonists; for clarity, only the transmembrane region at the extracellular end is depicted together with important pharmacophore elements (left); same representation without secondary structure depiction (right); red and magenta spheres, polar binding site regions; cyan, hydrophobic regions.

are presented, which assisted medicinal chemistry efforts in different stages of the project. In the first example, the introduction of a distal polar group such as a carboxylic group eliminated interactions with the hERG channel, removing potential issues later on in development in an otherwise promising series of compounds. Potential binding modes for compounds were determined by explaining experimental mutant data as much as possible by direct ligand−receptor contacts. The determined binding conformation allowed the replacement of a flexible linker chain by a more rigid benzyl group, which decreased the overall polarity of the compound making it more drug-like. The second example describes the rational design of potent antagonists from modestly active small molecules. The lead compound, which also showed no hERG inhibition, was chosen for further optimization.

We used an antifusogenic assay with a reporter enzyme readout, which mimicked the interaction of the viral envelope protein with CCR5 while eliminating the need to use HIV or radioactivity, and could be run in a high throughput manner. This assay was validated with reference compounds against a panel of single site mutations of the CCR5 receptor. An important result of this study is that none of the mutations severely impacts receptor functionality. In addition, the mutagenesis results for these reference compounds provided important information on crucial interaction sites.[24] How this information was used is shown successfully in the current study.

Using a functional assay such as the antifusogenic assay or a HIV-1 infection assay represents an indirect method of investigating small molecule binding with the mutant CCR5 receptor. This can be used to explain why mutation of several residues had little or no apparent impact on small molecule inhibition, or only on a subgroup of compounds, although such interactions were predicted by the binding model to be important. These silent mutations can be explained either with competing binding modes or with flexibility of the binding site residues (Tables 2 and 4).

The comparison of mutant fingerprints for compounds **6** and **8** versus **5** and **7** or compounds **11** and **12** versus **13** and **14** reveals that the ester and amide compounds do not show any dependency on any of the two lysines. The charge of the functional group as well as alternative binding modes can be used to explain the experimentally observed fold changes. Binding site flexibility can also be used as an explanation for residues impacting small molecule inhibition upon mutagenesis but do not interact closely with the compound in the binding model (e.g., Ile^{198} in Figure 6).

Some of the residues important for small molecule binding are highly conserved among chemokine receptors. This raises the question as to how much the replacement of these residues by alanine changes the receptor conformation and therefore prevents the viral protein from binding. Using the Weinstein−Ballesteros nomenclature[42] to refer to the same residue in different chemokine receptors, GluVII:06 (CCR5: Glu^{283}) has been identified in other chemokine receptors such as CCR2b[43] and CXCR4[44,45] as important for small molecule binding. However, this residue is not present in some other chemokine receptors such as CXCR3 and thus is not a prerequisite for receptor integrity and function. An elegant study describes how mutated CXCR3 maintains function and can be inhibited by mozobil (AMD3100, plerixafor) or a metal-ion loaded version, while WT CXCR3 does not bind this or other bicyclam compounds. Among the CXCR3 binding site changes was the introduction of GluVII:06.[46] In addition, studies on structurally diverse small molecule inhibitors show that some of the compounds investigated inhibit mutated CCR5 with mutations such as Glu283A, W86A, and I198A CCR5. For example, CCR5 inhibition of TAK-779 is not impacted by I198A mutation and to a lesser extent by mutating Glu^{283}.[24] It is noteworthy that the same observations were made using a radioligand binding assay with ^{125}I-RANTES[33] or a saturation binding assay with tritiated inhibitors.[29] Trp^{86}, which is part of the extracellular ECL 2, was identified as important for signal transduction.[46,47] However, we have previously shown that this mutation had no impact on viral envelope protein/CCR5 fusion. All of these data imply that all of the mutations Glu^{283}, Trp^{86}, or Ile^{198} have a direct effect on compound binding.[24]

Recently, the crystal structure of human CXCR4 has been published.[48] However, even though CCR5 shares a higher sequence homology with CXCR4 as compared to bovine rhodopsin (30% vs 18%), the results obtained with CXCR4 as structural template agree very well with those obtained with bovine rhodopsin as template described here, implying that the two CCR5 homology models could represent different conformational states[49,50] (Supporting Information). The profile analysis of hundreds of GPCR sequences revealed conserved elements such as prolines in the transmembrane region, no sequence gaps in the seven helical regions, the DRY motif, or disulfide bonds justifying the use of structural templates with very low sequence identity.[51]

The current results can be used to extend the CCR5 small molecule binding site description[24] by two hydrogen-accepting features (Figure 7) intended for interaction with Lys^{26} in the N-ter and Lys^{191} in ECL 2. Upon mutation of these residues, selected compounds lose inhibitory activity by 10-fold (Tables 2 and 4). The locations of the two lysines are on either end of a binding channel consisting of a charged center defined by Glu^{283} sandwiched by two hydrophobic pockets. A general pharmacophore description evolves from these data, which can be used for the discovery of new drug-like CCR5 antagonists.

CONCLUSIONS

To our knowledge, this is the first published report on applying a combined approach of mutagenesis, molecular modeling, and medicinal chemistry to not only design new antivirally potent CCR5 antagonists but also to experimentally confirm their predicted extracellular interactions.

This approach allowed the proposal of potential binding modes of small molecules in the allosteric binding site of CCR5. Binding site residues identified as crucial for interacting with reference compounds were used to suggest structural modifications for existing CCR5 inhibitors to improve their in vitro profiles. Alternatively, this approach was used to obtain new inhibitors from promising small molecules with no antiviral potency.

For this study, a validated antifusogenic assay was used to mimic the interaction of CCR5 with the viral envelope protein gp120. By using mutant variants of the CCR5 receptor, a mutant fingerprint was obtained for the compounds tested. This work led to the abstraction of the small molecule CCR5 antagonist binding site, which consists of a polar center flanked by two hydrophobic pockets in the allosteric binding site followed by two polar hotspots in the extracellular regions, the N-ter and ECL 2. This general pharmacophore description can be used to design structurally diverse drug-like CCR5 inhibitors.

MATERIALS AND METHODS

Experimental Data for Compounds. The compounds described in this Article are prepared by standard organic chemistry procedures, which were described previously.[27] Details of compound characterization can be found in the Supporting Information.

Experimental Methods. Technical details of the antifusogenic, HIV, natural ligand binding, and patch clamp assays can be found in the Supporting Information.

Molecular Modeling. Details of the performed homology modeling studies and the docking calculations can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information. Complete list of authors for refs 11,14, 28, and 35. Experimental data for all compounds. Technical details of experimental methods. Details of computational studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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