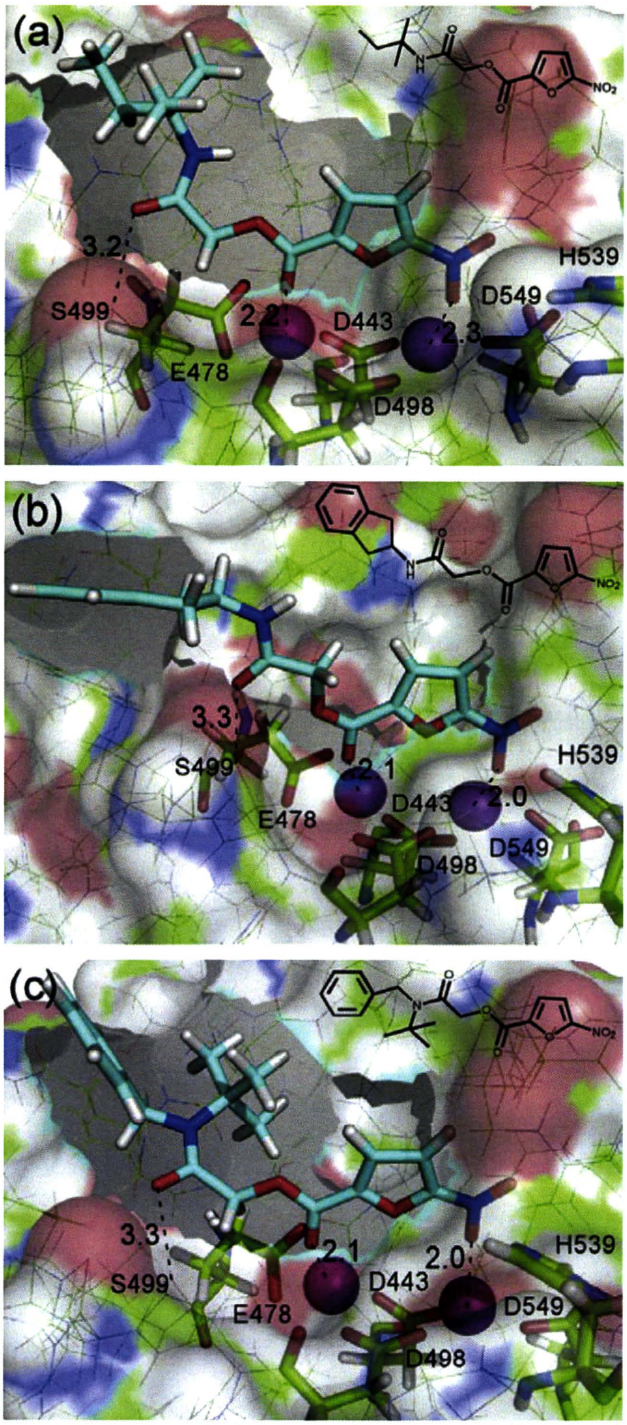

Figure 2. Optimized binding structures of the active compound to the RNase H domain, obtained by QM/MM calculation. (a), (b) and (c) correspond to compounds (**3**), (**7**) and (**10**), respectively. Chemical structures of the compounds are shown at the right top. Inhibitor compound and several polar residues are shown in stick representation. Two Mg^{2+} ions are denoted by spheres. Inter-atomic distances are shown in units of Å.

with coordinating to two divalent metal ions, while the interaction of other moiety is moderate.

Close observation of the optimized geometry by QM/MM calculation indicates that there is some space between the RNase H domain and inhibitory compounds around the ether oxygen at the ester linkage. This suggests that a few water molecules occupy the space when the compounds are bound to the RNase H domain. There exists a polar residue, Ser499, deep inside at this space on the RNase H domain. This residue would have little influence on the function of RNase H. Therefore, one of the designs to improve inhibitory activity is to modify the compound to bear some polar functional group that can interact with Ser499. Substitution of ether oxygen with nitrogen or carbon atom to enable the incorporation of a polar functional group is one of the possible conversions of our derivatives to enhance binding affinity to the RNase H domain.

The difficulty in developing an RNase H inhibitor for practical use lies in the specificity and toxicity. In spite of much effort to enhance the inhibitory potency, the 50% inhibitory concentrations of many compounds reported so far are still in the order of sub-micro molar and they often lack sufficient specificity for HIV-1 RT-associated RNase H activity. Most problematically, they sometime display cytotoxicity to mammalian cells. Many previous compounds have shown little inhibitory activity in an in vitro cell culture replication assay. The derivatives synthesized in this work have a scaffold different from that of the previously reported inhibitors. It was shown in our previous study[17] that the inhibitory potency of the hit chemical was highly specific to RNase H of retrovirus and the hit chemical further displayed an inhibitory activity in a cell culture replication assay. The present study showed that our derivatives had little cytotoxicity and that the chemical conversion at a part other than the 5-nitro-furan-2-carboxylic moiety increased the inhibitory activity. Moreover, there is still much room for modulation of the chemical structure. Accordingly, the analogues bearing the scaffold addressed in this study are good candidates for anti-HIV-1 drugs acting on RT-associated RNase H.

5. Summary

RNase H activity of reverse transcriptase is an attractive target of an antiviral agent for HIV-1 that is not yet addressed by currently approved drugs. A series of chemical compounds were synthesized on the basis of a hit chemical found in our previous in vitro screening. Inhibition of RNase H enzymatic activity was measured in a biochemical assay with a real-time fluorescence monitoring technique. Conversion of the nitro-furan group into other chemical structures drastically decreased the inhibitory activity except for nitro-thiophene. This means that the structural basis of nitro-furan is indispensable for inhibitory activity induced by analogues of the hit chemical. No notable change was observed in inhibitory potency when the hydrophobic moiety located at the opposite part of nitro-furan was modulated. This indicates that the modulated region has little interaction with the RNase H domain. Theoretical calculation with QM/MM method suggested the binding mode of the synthesized compounds to RNase H reaction active site. The characteristic property of the nitro-furan group is large electric polarity. Since oxygen atoms are negatively charged, these oxygen atoms will be strongly coordinated to divalent metal ions of the active site. The findings obtained in this work will be informative for designing potent inhibitors of RNase H enzymatic activity.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.011.

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Azamacrocyclic Metal Complexes as CXCR4 Antagonists

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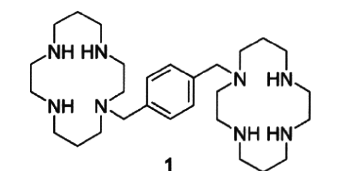
The chemokine receptor CXCR4 is a member of the seven transmembrane GPCR family, which is implicated in multiple diseases, including HIV infection, cancers, and rheumatoid arthritis. Low-molecular-weight nonpeptidic compounds, including AMD3100 and various pyridyl macrocyclic zinc(II) complexes, have been identified as selective antagonists of CXCR4. In the present study, structure–activity relationship studies were performed by combining the common structural features of alkylamino and pyridiyl macrocyclic antagonists. Several new zinc(II) or copper(II) complexes demonstrated potent anti-HIV activity, strong CXCR4-binding activity, and significant inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation. These results may prove useful in the design of novel CXCR4 antagonists, and the compounds described could potentially be developed as therapeutics against CXCR4-relevant diseases or chemical probes to study the biological activity of CXCR4.

Introduction

The chemokine receptor CXCR4, which transduces signals of its endogenous ligand, CXCL12/stromal cell-derived factor-1 (SDF-1),[1–4] is classified as a member of the seven transmembrane GPCR family, and plays a physiological role via its interaction with CXCL12 in chemotaxis,[5] angiogenesis,[6,7] and neurogenesis[8,9] in embryonic stages. CXCR4 is, however, relevant to multiple diseases including HIV infection/AIDS,[10,11] metastasis of several types of cancer,[12–14] leukemia cell progression,[15,16] and rheumatoid arthritis (RA),[17,18] and is considered an attractive drug target to combat these diseases. Thus, inhibitors targeting CXCR4 are expected to be useful for drug discovery.

Several CXCR4 antagonists have been reported,[19–35] including our discovery of the highly potent CXCR4 antagonist T140, a 14-mer peptide with a disulfide bridge, its smaller derivative, the 5-mer cyclic peptide FC131, and several other potent analogues.[19,24–26,28–30] Clinical development of these peptidic antagonists could be pursued using specific administration strategies involving biodegradable microcapsules.[14,36] However, herein we focus on novel nonpeptidic low-molecular-weight CXCR4 antagonists. To date, AMD3100 (**1**),[20,22] Dpa-Zn complex (**2**),[37] KRH-1636,[27] and other compounds[31–35] have been developed in this and other laboratories as low-molecular-weight nonpeptidic CXCR4 antagonists. The present study reports structure–activity relationship studies based on the combination of common structural motifs, such as xylene scaffolds and cationic moieties that are present in the aforementioned compounds.



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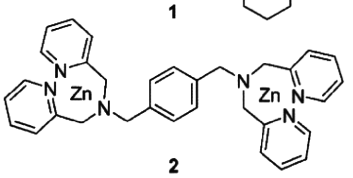

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Results and Discussion

In order to determine spatially suitable positioning of cationic moieties, *p*- and *m*-xylenes were utilized as spacers. Cationic moieties such as bis(pyridin-2-ylmethyl)amine (dipicolylamine), 1,4,7,10-tetraazacyclododecane (cyclen), and 1,4,8,11-tetraazacyclotetradecane (cyclam) were introduced as R^1 and R^2 (Figure 1). This combination of R^1, R^2, and spacer groups led to the design and synthesis of compounds **12–31**.

The CXCR4 binding activity of synthetic compounds was assessed based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells, which express CXCR4.[38] The percent inhibition of all compounds at 1 μM is shown in Table 1. Seven compounds (**16**, **17**, **20–22**, **28**, and **29**, Table 1) resulted in greater than 87% inhibition. The high activity of **16** is consistent with re-

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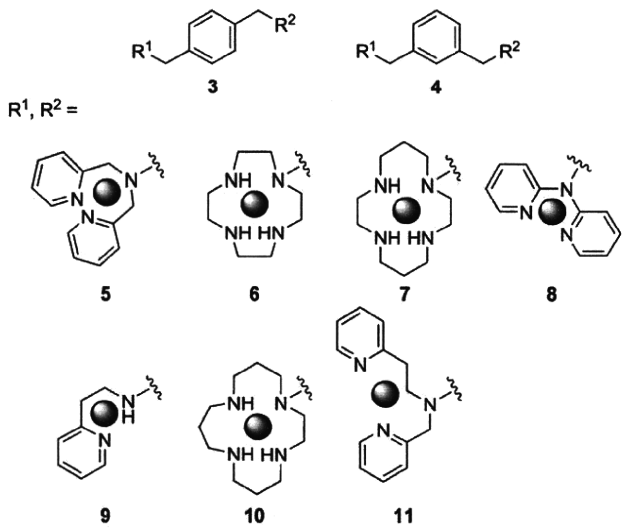


Figure 1. The structures of aromatic spacers (upper) and cationic moieties (R^1 and R^2). The shaded circle represents the position of the metal cation (Zn^{II} or Cu^{II}) in the chelate.

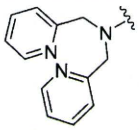
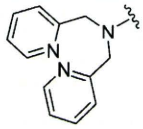
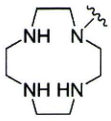
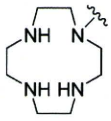
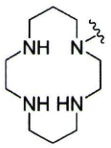
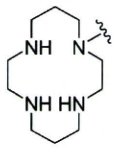
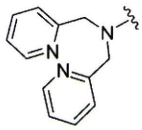
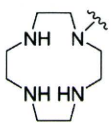
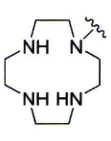
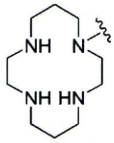
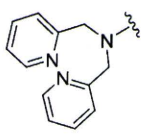
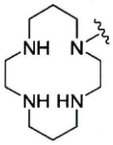
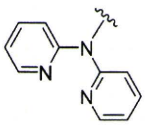
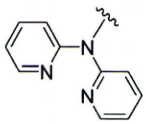
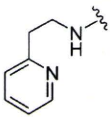
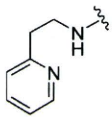
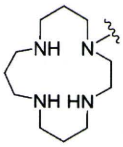
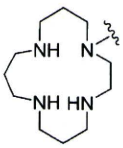
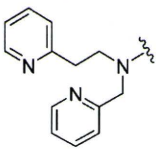
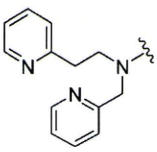
sults reported previously.[20,22] The anti-HIV activities of **17** and **29**, which contain only cyclam or cyclal rings, were reported by De Clercq et al.[39,40] Compounds with only pyridine and/or cyclen rings did not show any high binding activity. The presence of azamacrocyclic rings is presumably indispensable to the interaction of these compounds with CXCR4, and the size of rings appears to be important because not only compounds **16** and **17**, with two cyclam rings in the molecule, but also compounds **28** and **29**, with two cyclal rings, have remarkably more potent CXCR4 binding activity than compounds **14** and **15**, which have two cyclen rings. Compound **22**, with a *p*-xylene moiety, exhibited higher activity than compound **23**, which has an *m*-xylene moiety, indicating that *p*-xylene is more suitable than *m*-xylene as a spacer for approximate positioning of cationic moieties. At 0.1 μM, compound **22** resulted in 86% inhibition of [^{125}I]CXCL12 binding, while the other six compounds exhibited 37–66% inhibition. The IC_{50} value of compound **22** was estimated to be 37 nM.

$ZnCl_2$ was added to phosphate-buffered saline (PBS) solutions of these 20 compounds, **12–31**, to form zinc(II) complexes. The percent inhibition for each compound at 1 μM against [^{125}I]CXCL12 binding was determined and is given in Table 1. Zinc complexation of **12–15**, **18**, **19**, and **23** resulted in a remarkable increase in CXCR4 binding activity compared to the corresponding zinc-free compounds. These molecules contain dipicolylamine and/or cyclen moieties, suggesting that chelation of the nitrogen atoms with the zinc(II) ion significantly affects their interactions with CXCR4. The high activity of the zinc chelates of **12** and **13** is consistent with results provided in our previous paper.[37] Additionally, the anti-HIV activity of zinc complexes of **14** and **15** was reported by Kimura et al.[41] For compounds with only dipicolylamine and/or cyclen macrocycles as cationic moieties (**12–15**, **18**, and **19**), zinc complexation is critical to achieve high binding activity; the corresponding zinc-free compounds exhibit no significant activity. Compounds **16**, **17**, **20–22**, **28**, and **29** demonstrated high binding affinity in metal-free states as well as in zinc complexation states, indicating that zinc complexation of either of the macrocyclic rings in these compounds is not essential for high activity. The CXCR4 binding activity and anti-HIV activity of the zinc complex of **16** were reported previously.[42,43] Measured inhibition percentages for 0.1 μM of the zinc complexes of **12**, **14–23**, **28**, and **29** are given in Table 1. The zinc complexes of **20–22**, **28**, and **29** at 0.1 μM exhibited greater than 79% inhibition of [^{125}I]CXCL12 binding, and the other eight zinc complexes (of **12**, **14–19**, and **23**,) showed less than 55% inhibition. The IC_{50} values of zinc complexes of **20–22**, **28**, and **29** were estimated to be 11, 8.3, 22, 40, and 52 nM, respectively. Zinc complexes of compounds containing a combination of cyclen and cyclam moieties, **20** and **21**, had remarkably potent IC_{50} values.

To form chelates with a copper(II) cation, $CuCl_2$ was added to solutions in PBS of **12-31**. The inhibition percentages of all the compounds at 1 μM against [^{125}I]CXCL12 binding are shown in Table 1. Copper complexes of **14** and **15** exhibited a significant increase in CXCR4 binding activity as compared to the corresponding copper-free compounds, a phenomenon which is also seen in the zinc chelates. These compounds have two cyclen moieties in the molecules, suggesting that zinc or copper complexation is critical for high binding activity. Compounds **16**, **17**, and **20–22** showed high binding affinities in metal-free states and zinc- and copper-complexed states, indicating that metallic complexation of the cyclam rings in these compounds is not necessary for high activity. The CXCR4 binding activity of the copper complex of **16** was previously reported.[42] For compounds **17**, **22**, **23**, **28**, and **29**, copper complexation caused a significant decrease in binding activity compared to the corresponding copper-free compounds, whereas for compounds **14**, **15**, **18**, and **19**, copper complexation caused an increase in binding activity. This phenomenon may be due to the difference in ring sizes and structures of macrocycles, and was not observed upon zinc-complex formation. Inhibition at 0.1 μM of the copper complexes of **16** and **20–22**, which exhibited greater than 85% inhibition of [^{125}I]CXCL12 binding at 1 μM, are given in Table 1. The copper complexes of **16**, **20**, **21**, and **22** at 0.1 μM showed 39, 69, 88, and 39% inhibition, respectively, with the IC_{50} value of the copper complex of **21** estimated to be 16 nM.

Molecular modeling analysis of compound **21** and its zinc(II) and copper(II) complexes predicted that these complexes would form a stable coordinate conformation as shown in Figure 2. In general, zinc(II) complexes are predicted to adopt a tetrahedral conformation, while copper(II) complexes form a planar four coordinate/square conformation. The zinc(II) complex of **21** is predicted to have a tetrahedral conformation and the copper(II) complex a square planar conformation in both the cyclen and cyclam rings. The carboxyl group of either Asp 171 or Asp 262 in CXCR4 is thought to coordinate strongly with zinc ions but not copper ions in the complexes,[41–43] and as a consequence, the zinc complex of **21** would bind more strongly than **21** or its copper complex. This order of binding

Table 1. CXCR4 binding activity of compounds **12–31** in the metal ion-free form, the zinc complex, and the copper complex.

Compd	Spacer	R^1	R^2	Metal free			Zinc complex			Copper complex		
				Inhibition[a] [%]		IC_{50}[b]	Inhibition[a] [%]		IC_{50}[b]	Inhibition[a] [%]		IC_{50}[b]
				1 μM	0.1 μM	[nM]	1 μM	0.1 μM	[nM]	1 μM	0.1 μM	[nM]
12	*p*-xylene			0	n.d.	n.d.	83±2	24±5	n.d.	10±4	n.d.	n.d.
13	*m*-xylene			0	n.d.	n.d.	31±3	n.d.	n.d.	0	n.d.	n.d.
14	*p*-xylene			30±4	n.d.	n.d.	87±4	0	n.d.	60±2	n.d.	n.d.
15	*m*-xylene			33±2	n.d.	n.d.	94±1	13±6	n.d.	80±3	n.d.	n.d.
16	*p*-xylene			94±4	59±6	n.d.	97±5	28±3	n.d.	98±1	39±3	n.d.
17	*m*-xylene			95±3	49±9	n.d.	98±4	55±7	n.d.	75±1	n.d.	n.d.
18	*p*-xylene			32±0.7	n.d.	n.d.	97±6	0	n.d.	52±3	n.d.	n.d.
19	*m*-xylene			17±5	n.d.	n.d.	91±4	0	n.d.	22±6	n.d.	n.d.
20	*p*-xylene			89±3	62±3	n.d.	>100	79±1	11	>100	69±3	n.d.
21	*m*-xylene			89±3	66±3	n.d.	92±3	>100	8.3	>100	88±1	16
22	*p*-xylene			94±3	86±3	37	99±8	79±0.6	22	85±3	39±3	n.d.
23	*m*-xylene			58±8	n.d.	n.d.	90±17	37±0.3	n.d.	48±4	n.d.	n.d.
24	*p*-xylene			3±0.9	n.d.	n.d.	0	n.d.	n.d.	0	n.d.	n.d.
25	*m*-xylene			4±3	n.d.	n.d.	0	n.d.	n.d.	0	n.d.	n.d.
26	*p*-xylene			14±2	n.d.	n.d.	10±3	n.d.	n.d.	0	n.d.	n.d.
27	*m*-xylene			10±3	n.d.	n.d.	10±4	n.d.	n.d.	0	n.d.	n.d.
28	*p*-xylene			91±0.4	37±0.9	n.d.	97±4	>100	40	57±4	n.d.	n.d.
29	*m*-xylene			87±2	50±1	n.d.	>100	91±4	52	55±1	n.d.	n.d.
30	*p*-xylene			0	n.d.	n.d.	14±3	n.d.	n.d.	14±3	n.d.	n.d.
31	*m*-xylene			24±2	n.d.	n.d.	20±3	n.d.	n.d.	0	n.d.	n.d.
FC-131	*cyclo*-[D-Tyr-Arg-Arg-Nal-Gly-]			100	100	1.8	–	–	–	–	–	–

[a] CXCR4 binding activity was assessed based on inhibition of [^{125}I]CXCL12 binding to Jurkat cells. Percent inhibition for all compounds at 1 and 0.1 μM were calculated relative to the percent inhibition by FC131 (100%). [b] IC_{50} values are the concentrations which correspond to 50% inhibition of [^{125}I]CXCL12 binding to Jurkat cells. All data are mean values ±SEM of at least three independent experiments. n.d. = not determined.

affinities is commonly seen for these compounds and their zinc(II) or copper(II) complexes.

We investigated the CXCR4 antagonistic activity of compound **22** and the zinc complexes of **20**, **21**, **22**, and **28**, all of which possess strong CXCR4 binding activity. The CXCR4 antagonistic activity was assessed based on the inhibitory activity of the compounds against Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4 (figure S1 in the Support-

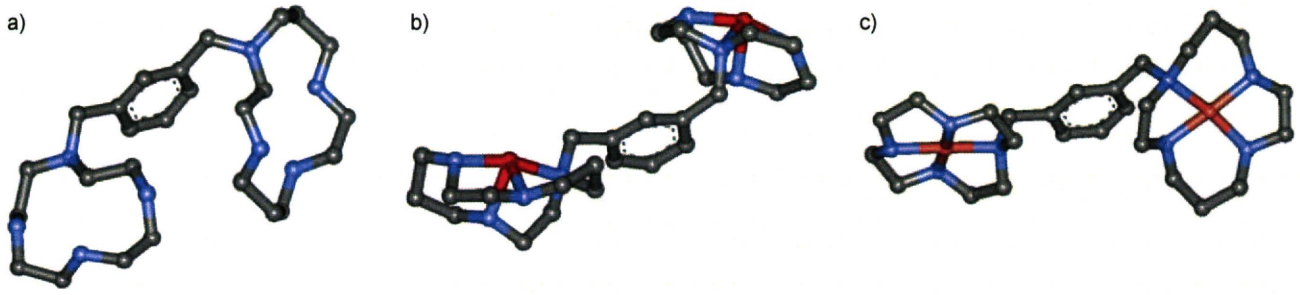


Figure 2. Structures calculated by molecular modeling of a) compound **21**, and its b) zinc and c) copper complexes. Atom color code: nitrogen = blue, carbon = gray, zinc = red, copper = light red.

ing Information). All of the tested compounds showed significant antagonistic activity at 1 μM.

The representative compounds **14**, **16**, **20–23**, **28**, and **29**, as well as their zinc chelates, were evaluated for anti-HIV activity. CXCR4 is the major co-receptor for the entry of T-cell-line-tropic (X4) HIV-1.[10,11] Inhibitory activity against X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells was assessed and is shown in Table 2.[38] A correlation between CXCR4 binding activity and anti-HIV activity was observed. For compound **16** and its zinc complex, anti-HIV activity was significantly stronger than CXCR4 binding activity, and for the zinc complexes of compounds **20–22**, the CXCR4 binding activity is two to four-times stronger than the anti-HIV activity. The anti-HIV activity of the zinc complex of **16** was the most potent (EC_{50} = 8.2 nM). This is comparable to the anti-HIV activities of **16** and its zinc complex that were reported previously.[20,22,42,43] The zinc complex of **21**, which was the most active compound in terms of CXCR4 binding activity, also exhibited potent anti-HIV activity (EC_{50} = 36 nM).

Taken together, these results show that all of the compounds exhibiting CXCR4 binding activity also showed significant anti-HIV activity (EC_{50} values < 300 nM), and none of the tested compounds exhibited significant cytotoxicity (CC_{50} values > 10 μM; Table 2). Conversely, zinc complexes of **20**, **21**, **22**, and **28** did not exhibit significant anti-HIV activity against macrophage-tropic (R5) HIV-1 (NL(AD8) strain)-induced cytopathogenicity in PM-1 cells at concentrations below 10 μM. Since R5-HIV-1 strains use CCR5 instead of CXCR4 as the major co-receptor for entry, this suggests that these compounds do not bind CCR5 but rather are highly selective for CXCR4.

Table 2. Anti-HIV activity and cytotoxicity of representative compounds in the metal ion-free and zinc chelates.

Compd	Metal ion-free		Zinc chelate	
	EC_{50}[a] [nM]	CC_{50}[b] [μM]	EC_{50}[a] [nM]	CC_{50}[b] [μM]
14	200	> 10	200	> 10
16	21	> 10	8.2	> 10
20	38	> 10	39	> 10
21	50	> 10	36	> 10
22	93	> 10	48	> 10
23	290	> 10	220	> 10
28	36	> 10	56	> 10
29	130	> 10	42	> 10
FC131	93	> 10		
AZT	69	> 100		

[a] EC_{50} values are the concentrations corresponding to 50% protection from X4-HIV-1 (NL4–3 strain)-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are the concentrations at which the viability of MT-4 cells is reduced by 50%. All data are mean values from at least three independent experiments.

Conclusions

The present study introduces a new class of low-molecular-weight CXCR4 antagonists and their zinc(II) or copper(II) complexes, which contain pyridyl or azamacrocycle moieties with *p*-xylene or *m*-xylene spacers. These compounds demonstrated strong CXCR4 binding activity. Zinc complexes of **20** and **21**, which were the two most active compounds, contain cyclen and cyclam rings with *p*- and *m*-xylene spacers and exhibited remarkably potent IC_{50} values (11 and 8.3 nM, respectively). These compounds showed significant CXCR4 antagonistic activity, based on inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4, as well as potent anti-HIV activity, as assessed by protection from X4-HIV-1-induced cytopathogenicity in MT-4 cells. These results provide useful insights into the future design of novel CXCR4 antagonists, complementing information from other CXCR4 antagonists such as T140, FC131, and KRH-1636. Furthermore, these new compounds are useful for the development of therapeutic strategies for CXCR4-relevant diseases and chemical probes to study the biological activity of CXCR4.

Experimental Section

Chemistry

Compounds **12–17**, **20**, **21**, **24**, **25**, **27–29**, and **31** were synthesized as previously reported.[20,22,37,40,41,44–47] Compounds **18**, **19**, **22**, **23**, **26**, and **30** were synthesized in the present study; details are provided in the Supporting Information. A representative compound, **18**, was synthesized by coupling *p*-dibromoxylene (1,4-bis-(bromomethyl)benzene) with tri-Boc-protected 1,4,7,10-tetraazacyclododecane, followed by treatment with trifluoroacetic acid and subsequent coupling with bis(pyridin-2-ylmethyl)amine. All crude compounds were purified by RP-HPLC and identified by FAB/ESI-

HRMS. Zinc(II) or copper(II) complex formation was accomplished by treatment of the above compounds with 10 equiv of $ZnCl_2$ or $CuCl_2$ in PBS. All zinc(II) or copper(II) complexes were characterized by chemical shifts of their methylene protons in ^1H NMR analysis. The pyridyl zinc(II) complex was characterized previously,[37] and zinc(II) or copper(II) complex formation with these macrocyclic compounds has been reported elsewhere.[41,42,48,49] Detailed procedures and data are provided in the Supporting Information.

Biological assays

A CXCR4 binding assay for compounds, based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells, was performed as reported by Tanaka et al.[38] CXCR4 antagonistic activity was evaluated as described by Ichiyama et al[27], measuring inhibitory activity against Ca^{2+} mobilization induced by CXCL12 stimulation in HOS cells expressing CXCR4. Anti-HIV activity was determined by inhibitory activity against X4-HIV-1(NL4–3)-induced cytopathogenicity in MT-4 cells as reported by Tanaka et al.[38] An X4 HIV-1 infectious molecular clone (pNL4–3) was obtained from the AIDS Research and Reference Reagent Program. The virus NL4–3 was obtained from the culture supernatant of 293T cells transfected with pNL4–3.

Molecular modeling

Molecular modeling calculations were performed using Sybyl (version 7.0, Tripos). Energy minimization was performed using the Tripos force field and Gasteiger–Hückel charge parameters. The lowest energy conformation was obtained by random search methods.

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Keywords: azamacrocycles · Ca^{2+} mobilization · CXCR4 · HIV · structure–activity relationships

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