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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.

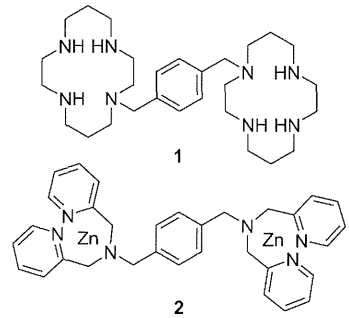
Introduction

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.

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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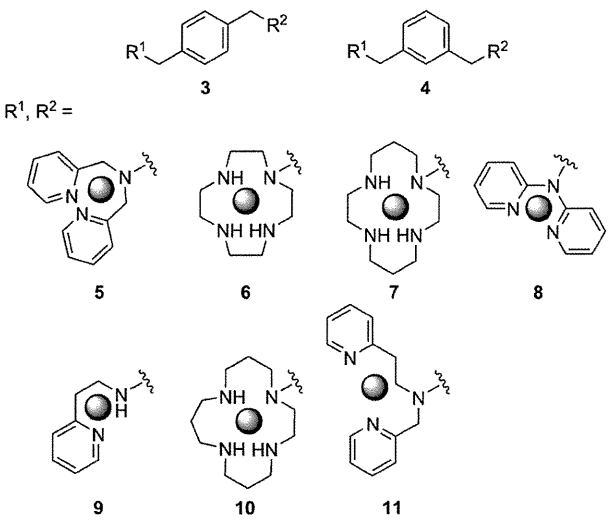


Figure 1. The structures of aromatic spacers (upper) and cationic moieties (R^1 and R^2). The shaded circle represents the position of the metal cation (Zn^{II} or Cu^{II}) in the chelate.

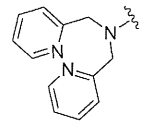
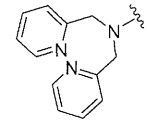
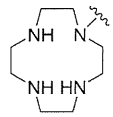
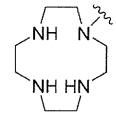
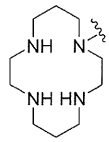
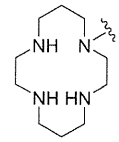
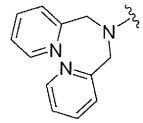
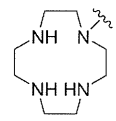
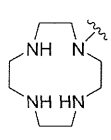
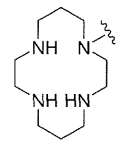
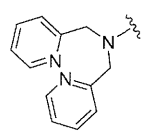
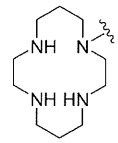
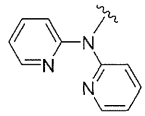
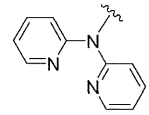
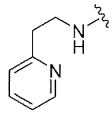
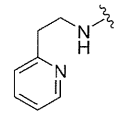
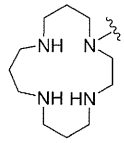
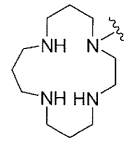
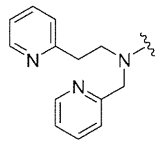
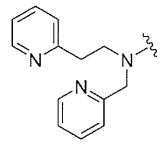
sults reported previously.[20,22] The anti-HIV activities of **17** and **29**, which contain only cyclam or cyclal rings, were reported by De Clercq et al.[39,40] Compounds with only pyridine and/or cyclen rings did not show any high binding activity. The presence of azamacrocyclic rings is presumably 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 Asp171 or Asp262 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-

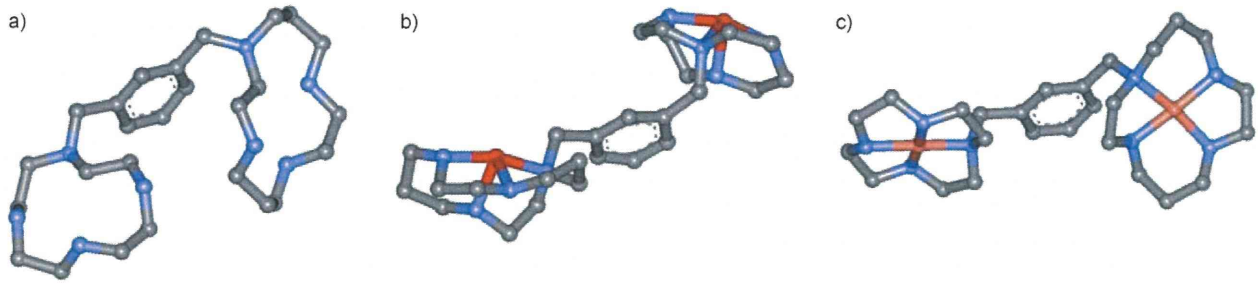


Figure 2. Structures calculated by molecular modeling of a) compound **21**, and its b) zinc and c) copper complexes. Atom color code: nitrogen = blue, carbon = gray, zinc = red, copper = light red.

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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Low-Molecular-Weight CXCR4 Ligands with Variable Spacers

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Low-molecular-weight CXCR4 ligands based on known lead compounds including the 14-mer peptide T140, the cyclic pentapeptide FC131, peptide mimetics, and dipicolylamine-containing compounds were designed and synthesized. Three types of aromatic spacers, 1,4-phenylenedimethanamine, naphthalene-2,6-diyldimethanamine, and [1,1′-biphenyl]-4,4′-diyldimethanamine, were used to build four pharmacophore groups. As pharmacophore groups, 2-pyridylmethyl and 1-naphthylmethyl are present in all of the compounds, and several aromatic groups and a cationic group from 1-propylguanidine and 1,1,3,3-tetramethyl-2-propylguanidine were also used. Several compounds showed significant CXCR4 binding affinity, and zinc(II) complexation of bis(pyridin-2-ylmethyl)amine moieties resulted in a remarkable increase in CXCR4 binding affinity.

Introduction

CXCR4 is a chemokine receptor that transduces signals of its endogenous ligand, CXCL12/stromal cell-derived factor-1 (SDF-1).[1–4] This receptor is a member of the seven-transmembrane GPCR family, and has been reported to exist and function as an oligomer,[5] which was elucidated by our molecular ruler approach.[6] The CXCR4–CXCL12 axis plays a physiological role in embryonic stages in chemotaxis,[7] angiogenesis,[8,9] and neurogenesis.[10,11] CXCR4 is associated with many disorders including cancer cell metastasis,[12–14] leukemia cell progression,[15,16] HIV infection/AIDS,[17,18] and rheumatoid arthritis;[19,20] it is therefore a major target in the discovery of chemotherapeutic treatments for these diseases. To date, many researchers, including ourselves, have developed potent CXCR4 antagonists. A 14-mer peptide, T140, and a cyclic pentapeptide, FC131, have been found to be potent CXCR4 antagonists.[21–27] In addition, downsizing of these peptides has led to the development of active small-molecular peptide mimetics.[28] Another peptide mimetic, KRH-1636,[29] and a bicyclam, AMD3100,[30,31] have also been reported. Furthermore, several compounds based on monocyclams[32] and noncyclams[33,34] have been reported. Other aza-macrocyclic compounds such as the Dpa–Zn complex **1**[35] and the Dpa–cyclam compound **2**[36] have been developed as non-peptide leads (Figure 1). These lead compounds have 1,4-phenylenedimethanamine structures with amino groups presenting basic/aromatic moieties. We recently developed small-molecular peptide mimetics containing benzyl and 2-pyridylmethyl amino groups, such as compound **3**[37] and cyclic pentapeptide FC131 derivatives containing two naphthalene moieties (e.g., **4**).[38] In the study presented herein, we tried to develop more effective small mole-

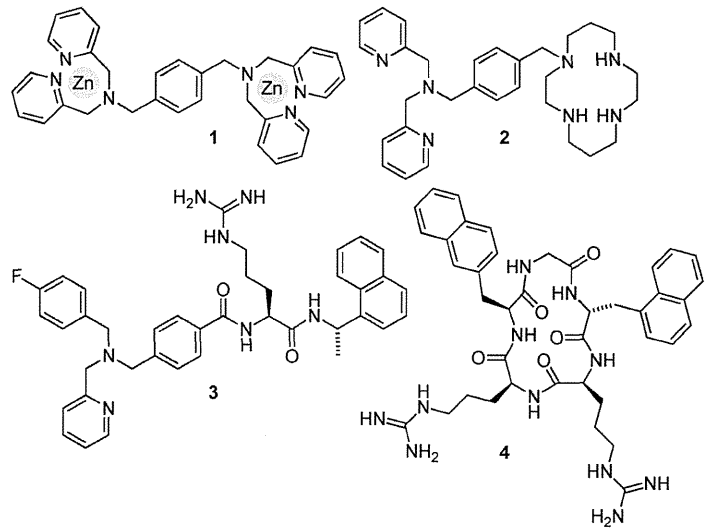

Figure 1. Reported low-molecular-weight CXCR4 antagonists.

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cules based on these lead compounds and to perform appropriate structure–activity relationship studies.

Results and Discussion

Design

We initially designed compounds that contain 1,4-phenylenedimethanamine, one amino group of which is linked to guanidine and naphthalene moieties, and the other to 2-pyridylmethyl and naphthalene analogues, as shown in Figure 2. The

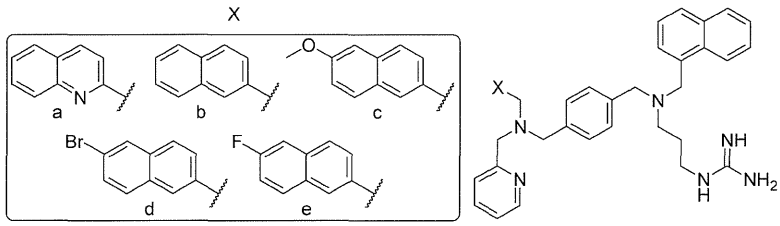


Figure 2. New compounds containing the 1,4-phenylenedimethanamine structure.

adoption of these functional moieties is based on structures of compound **3**, which contains 4-fluorobenzyl and 2-pyridylmethyl amino groups, and compound **4**, which contains two naphthalene moieties. Thus, 2-methylquinoline, 2-methylnaphthalene, 2-methoxy-6-methylnaphthalene, 2-bromo-6-methylnaphthalene, and 2-fluoro-6-methylnaphthalene (X-CH_2) moieties were introduced on a nitrogen atom of the 1,4-phenylenedimethanamino group in compounds **19a–c** and **23d,e**. Furthermore, compounds with 1,4-phenylenedimethanamine, naphthalene-2,6-diyldimethanamine, and [1,1′-biphenyl]-4,4′-diyldimethanamine structures as spacer templates (H_2N-Y^2-NH_2) were designed as shown in Figure 3 to refine the spacers. Monocyclic aromatic groups, 4- or 2-pyridylmethyl, 4-fluorobenzyl, and 4-trifluoromethylbenzyl groups (Y^1-CH_2) were introduced on a nitrogen atom of the above spacer templates, and guanidino and tetramethylguanidino groups were used as substituents for Y^3 in compounds **37a–42d**.

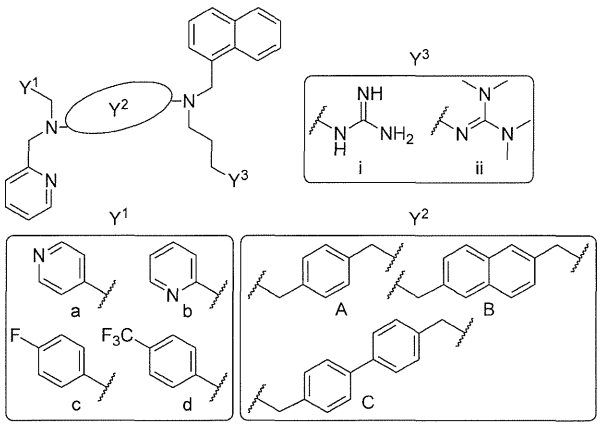


Figure 3. New compounds containing the 1,4-phenylenedimethanamine, naphthalene-2,6-diyldimethanamine, and [1,1′-biphenyl]-4,4′-diyldimethanamine structures.

Chemistry

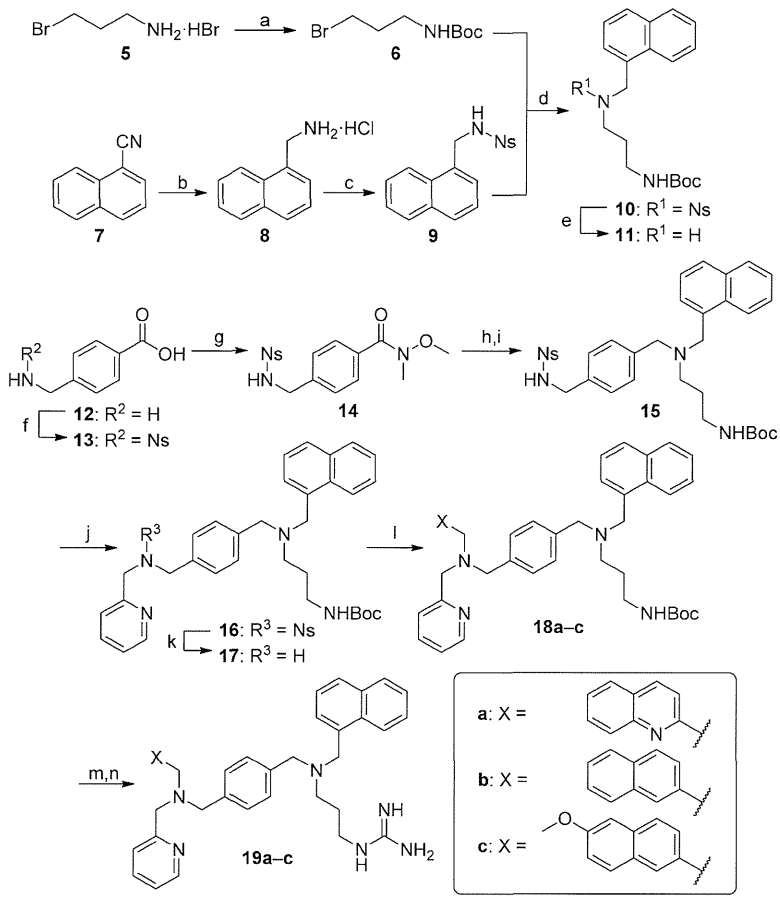
The synthesis of compounds **19a–c** is shown in Scheme 1. Condensation of *N*-Boc-3-aminopropylbromide (**6**) and *N*-Ns-aminonaphthalen-1-yl-methane (**9**; Ns = 2-nitrobenzenesulfonyl) followed by removal of the Ns group produced the amine **11**. The *N*-Ns-4-aminomethylbenzoic acid derived Weinreb amide **14** was treated with DIBAL to afford the corresponding aldehyde, the reductive amination of which was performed by treatment with amine **11** to afford the tertiary amine **15**. Introduction of a 2-pyridinylmethyl group into **15** by means of Mitsunobu reaction followed by removal of the Ns group yielded amine **17**. Introduction of 2-methylquinoline, 2-methylnaphthalene, and 2-methoxy-6-methylnaphthalene groups by reductive amination of **17** produced amines **18a–c**, respectively, and subsequent removal of the Boc group followed by N-guanylation yielded the desired compounds **19a–c**.

As shown in Scheme 2, introduction of 2-bromo-6-methylnaphthalene and 2-fluoro-6-methylnaphthalene moieties into **15** by Mitsunobu reaction followed by removal of the Ns group yielded amines **21d** and **21e**, respectively. Introduction of a 2-pyridinylmethyl group by reductive amination of **21d** and **21e** produced amines **22d** and **22e**, respectively, and subsequent removal of the Boc group followed by N-guanylation yielded the desired compounds **23d** and **23e**.

Scheme 3 shows the synthesis of **37a–39d** and **40a–42d**. Introduction of 4-pyridylmethyl, 2-pyridylmethyl, or 4-fluorobenzyl and 4-trifluoromethylbenzyl groups into *N*-Ns-(pyridin-2-ylmethyl)amide **25** by Mitsunobu reaction followed by removal of the Ns group yielded amines **27a–d**, respectively. Treatment of 1,4-phenylenedimethane, naphthalene-2,6-diyldimethane, and [1,1′-biphenyl]-4,4′-diyldimethane-derived dibromides **28–30** with amine **11** afforded the tertiary amines **31–33**, respectively. Subsequent treatment of **31–33** with amines **27a–d** yielded amines **34a–36d**. Subsequent removal of the Boc group followed by N-guanylation and N-tetramethylguanylation yielded the desired compounds **37a–39d** and **40a–42d**, respectively.

Biological studies

The CXCR4 binding affinity of the synthesized compounds was assessed through inhibition of [^{125}I]CXCL12 binding to Jurkat cells, which express CXCR4.[38] The activity was evaluated for compounds **19a–c** containing 2-methylquinoline, 2-methylnaphthalene, 2-methoxy-6-methylnaphthalene, and **23d,e**,



Scheme 1. *Reagents and conditions*: a) Boc_2O, Et_3N, MeOH/MeCN (1:1), 98%; b) $LiAlH_4$, THF, 0 °C, 89%; c) NsCl, Et_3N, THF, 78%; d) K_2CO_3, DMF, 60 °C, 96%; e) PhSH, K_2CO_3, DMF, 95%; f) NsCl, Et_3N, THF, 88%; g) EDCI·HCl, HOBt·H_2O, $NHCH_3(OCH_3)$·HCl, Et_3N, DMF, 88%; h) DIBAL/*n*-hexane, CH_2Cl_2, −78 °C; i) $NaBH(OAc)_3$, AcOH, amine **11**, 1,2-dichloroethane, 43% (two steps); j) PPh_3, DEAD, 2-pyridinemethanol, THF, 76%; k) PhSH, K_2CO_3, DMF, 87%; l) $NaBH(OAc)_3$, AcOH, X-CHO, 1,2-dichloroethane, 70% (**18a**), 69% (**18b**), 49% (**18c**); m) 4 M HCl/dioxane; n) *N*,*N*-diisopropylethylamine, 1-amidinopyrazole·HCl, DMF, 28% (**19a**), 58% (**19b**), 46% (**19c**) (two steps). Ns = 2-nitrobenzenesulfonyl.

with 2-bromo-6-methylnaphthalene and 2-fluoro-6-methylnaphthalene moieties, respectively (X-CH_2), introduced on a nitrogen atom of the 1,4-phenylenedimethanamino group. The percent inhibition data for all compounds at 10 μM are listed in Table 1. With the exception of **19c**, which contains a 2-methoxynaphthalene group, the compounds showed significant but very weak binding affinity. With an electron-donating methoxy group, the 2-methoxynaphthalene moiety is an electron-rich aromatic group. The quinoline, 2-bromonaphthalene, and 2-fluoronaphthalene moieties are electron-deficient aromatic groups because of the electron-deficient pyridine ring and electron-withdrawing fluorine and bromine atoms. It is suggested that when X represents bicyclic or electron-rich aromatic groups, the compounds are unlikely to be potent ligands.

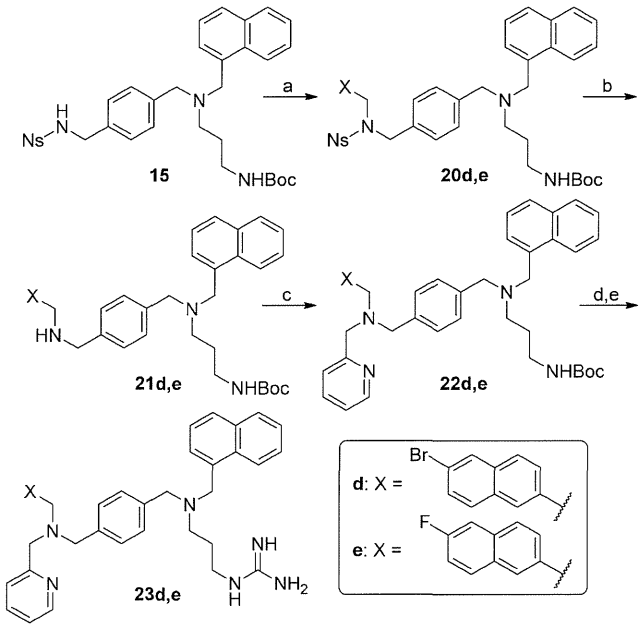
Table 1. CXCR4 binding affinities of compounds **19a–c** and **23d,e**.

Compd	X[a]	Inhibition [%][b]
19a	a	14.4 ± 1.0
19b	b	7.0 ± 0.6
19c	c	0
23d	d	9.0 ± 2.2
23e	e	9.5 ± 1.3
FC131	–	100

[a] The structures of X (a–e) are shown in Figure 2. [b] CXCR4 binding affinity was assessed based on inhibition of [^{125}I]CXCL12 binding to Jurkat cells; percent inhibition values for all compounds at 10 μM were calculated relative to that of FC131 (100%).

Because some compounds containing bicyclic or electron-rich aromatic groups at the group X position in Figure 2 do not have high binding affinity for CXCR4, compounds in Figure 3 in which Y^1 is a monocyclic and electron-deficient aromatic group were designed: 4-pyridylmethyl, 2-pyridylmethyl, 4-fluorobenzyl, and 4-trifluoromethylbenzyl groups (Y^1-CH_2) were introduced onto the nitrogen atom. In addition, as spacer templates (H_2N-Y^2-NH_2) 1,4-phenylenedimethanamine, naphthalene-2,6-diyldimethanamine, and [1,1′-biphenyl]-4,4′-diyldimethanamine structures were introduced to refine the spacer structures, and guanidino and tetramethylguanidino groups were used as Y^3 substituents. The CXCR4 binding affinities of compounds **37a–42d** were evaluated (Table 2). None of these compounds showed more than 50% inhibition at 10 μM. In general, 4-trifluoromethylbenzyl, [1,1′-biphenyl]-4,4′-diyldimethanamine, and tetramethylguanidino moieties seem to be more suitable as candidates for Y^1-CH_2, H_2N-Y^2-NH_2, and Y^3, respectively. Among these synthetic compounds, **40b**, containing 2-pyridylmethyl, 1,4-phenylenedimethanamine and tetramethylguanidino groups, and **42d** containing 4-trifluoromethylbenzyl, [1,1′-biphenyl]-4,4′-diyldimethanamine and tetramethylguanidino groups, have the highest binding affinity for CXCR4.

As described above in the Introduction, aza-macrocyclic compounds such as the Dpa–Zn complex **1**[35] and the Dpa–cyclam compound **2**[36] have high binding affinities toward CXCR4. The zinc complex of **2** also has a higher CXCR4 binding affinity. Thus, the CXCR4 binding affinities of the zinc complexes of **19a**, containing 2-pyridylmethyl and 2-methylquino-



Scheme 2. *Reagents and conditions*: a) PPh_3, DEAD, X-CH_2OH, THF, RT, 97% (**20d**), 59% (**20e**); b) PhSH, K_2CO_3, DMF, RT, 42% (**21d**), 64% (**21e**); c) NaBH-$(OAc)_3$, AcOH, 2-pyridinecarbaldehyde, 1,2-dichloroethane, RT, 78% (**22d**), 85% (**22e**); d) 4 M HCl/dioxane, RT; e) DIPEA, 1-amidinopyrazole·HCl, DMF, RT, 24% (**23d**), 18% (**23e**) (two steps).

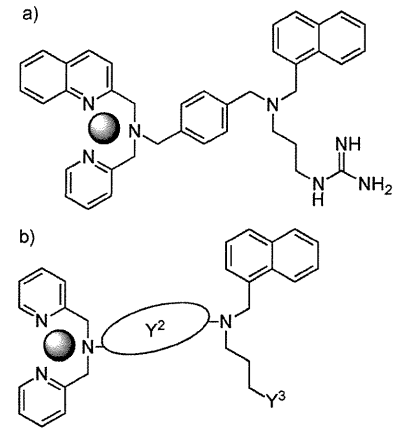


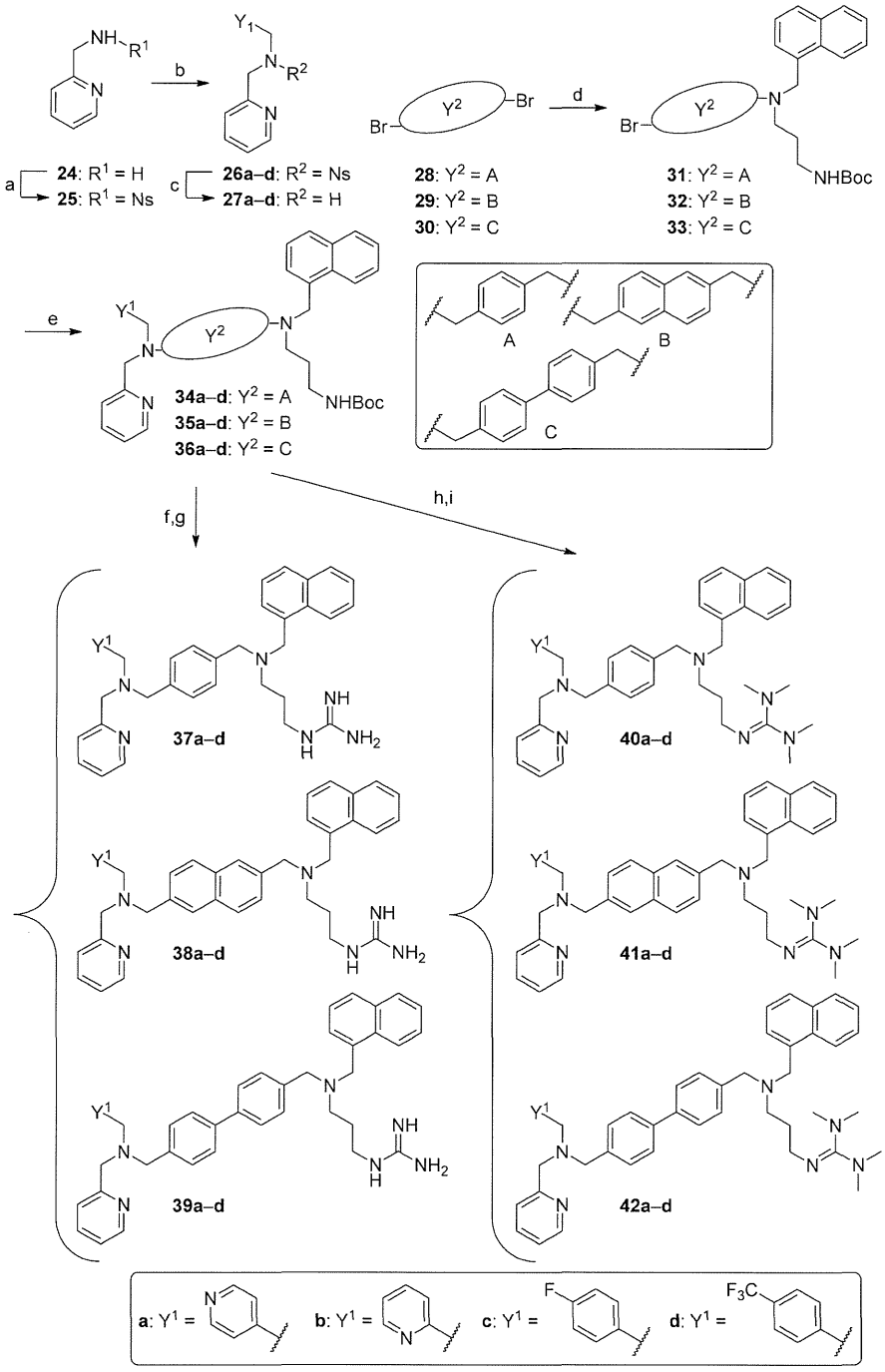
Figure 4. Zinc complexes of a) **19a** and b) **37b**, **38b**, **39b**, **40b**, **41b**, and **42b**. The shaded circle represents the position of the zinc cation in the chelate. The structures of Y^2 and Y^3 are shown in Figure 3 as A–C and i–ii, respectively.

Table 2. CXCR4 binding affinities of compounds **37a**–**42d**.

Compd	$Y^{1[a]}$	$Y^{2[b]}$	$Y^{3[c]}$	Inhibition [%][d]	Compd	$Y^{1[a]}$	$Y^{2[b]}$	$Y^{3[c]}$	Inhibition [%][d]
37a	a	A	i	9.6±1.9	**40a**	a	A	ii	0
37b	b	A	i	21.4±2.8	**40b**	b	A	ii	41.5±4.8
37c	c	A	i	8.5±1.8	**40c**	c	A	ii	12.7±4.0
37d	d	A	i	22.3±1.4	**40d**	d	A	ii	23.8±6.0
38a	a	B	i	0	**41a**	a	B	ii	3.2±2.2
38b	b	B	i	4.7±1.3	**41b**	b	B	ii	21.6±2.6
38c	c	B	i	4.2±6.0	**41c**	c	B	ii	13.2±1.5
38d	d	B	i	4.1±4.1	**41d**	d	B	ii	18.4±1.2
39a	a	C	i	8.1±1.1	**42a**	a	C	ii	8.8±1.0
39b	b	C	i	18.0±1.1	**42b**	b	C	ii	0
39c	c	C	i	26.0±3.0	**42c**	c	C	ii	26.6±4.4
39d	d	C	i	27.9±5.2	**42d**	d	C	ii	45.0±3.0

[a–c] The structures of Y^1, Y^2, and Y^3 are shown in Figure 3 as a–d, A–C, and i–ii, respectively. [d] CXCR4 binding affinity was assessed based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells; percent inhibition values for all compounds at 10 μM were calculated relative to that of FC131 (100%).

line groups, and **37b**, **38b**, **39b**, **40b**, **41b**, and **42b**, containing the Dpa group, were evaluated (Figure 4). $ZnCl_2$ (10 equiv relative to each compound) was added to phosphate-buffered saline (PBS) solutions of these compounds to form zinc(II) complexes. Chelation of the nitrogen atoms of **37b** and **40b** with the zinc(II) ion has been demonstrated by changes in NMR chemical shifts upon $ZnCl_2$ titration as zinc chelates as described in our previous studies.[35,36] The percent inhibition of the zinc complexes at 5 μM is listed in Table 3. A remarkable increase in CXCR4 binding affinity of all the zinc complexes except **39b** is observed if the inhibitory activities of the zinc complexes at 5 μM (Table 3) are compared with those of the corresponding metal-free compounds at 10 μM (Tables 1 and 2). The high activity of the zinc complexes is consistent with results reported in our previous work,[35,36] and suggests that the formation of chelates of the nitrogen atoms in the compounds with the zinc(II) ion might enhance their interaction with CXCR4. Fixation of the functional moieties by zinc(II) chelation, progression of electron deficiency of the aromatic moieties, interaction of the zinc(II) ion with residues on CXCR4, etc., might be considered as reasons for the enhanced CXCR4 binding affinity of the zinc complexes. According to previous reports,[39,40] in the case of chelation of the zinc complexes of AMD3100, a divalent metal ion such as zinc(II) in one of the bicyclam rings increased this compound's affinity for CXCR4 through a specific interaction with the carboxylate of Asp262 of CXCR4. A similar phenomenon could be occurring in the zinc complexes of the present compounds. The IC_{50} values of the zinc complexes of **37b** and **40b** containing 1,4-phenylenedimethanamine were evaluated to be 2.1 μM. In comparing the CXCR4 binding affinity of the zinc complexes of **37b**, **38b**, **39b**, **40b**, **41b**, and **42b**, 1,4-phenylenedimethanamine is the most suitable spacer template (H_2N-Y^2-NH_2), and naphthalene-2,6-diyldimethanamine is the second most effective. As substituents for Y^3, the tetramethylguanidino group is more appropriate than guanidine. The reason for this property has not been clarified yet; however, the tetramethyl group might stabilize a positively charged nitrogen atom, or might enhance a hy-



Scheme 3. *Reagents and conditions*: a) NsCl, Et_3N, THF, 84%; b) Y^1-CH_2OH, DEAD, PPh_3, THF, 53% (**26a**), 92% (**26b**), 70% (**26c**), 97% (**26d**); c) PhSH, K_2CO_3, DMF, 97% (**27a**), 74% (**27b**), 91% (**27c**), 91% (**27d**); d) KI, K_2CO_3, **11**, MeCN, 78% (**31**), 53% (**32**), 71% (**33**); e) KI, K_2CO_3, amine **27a–d**, MeCN, 25% (**34a**), 78% (**34b**), 80% (**34c**), 90% (**34d**), 38% (**35a**), 75% (**35b**), 67% (**35c**), 55% (**35d**), 23% (**36a**), 59% (**36b**), 80% (**36c**), 80% (**36d**); f) 4 M HCl/dioxane; g) DIPEA, 1-amidinopyrazole·HCl, DMF, 19% (**37a**), 49% (**37b**), 52% (**37c**), 30% (**37d**), 42% (**38a**), 56% (**38b**), 62% (**38c**), 44% (**38d**), 39% (**39a**), 48% (**39b**), 87% (**39c**), 50% (**39d**) (two steps); h) 4 M HCl/dioxane; i) DIPEA, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DMF, 24% (**40a**), 36% (**40b**), 31% (**40c**), 32% (**40d**), 31% (**41a**), 14% (**41b**), 47% (**41c**), 27% (**41d**), 37% (**42a**), 25% (**42b**), 27% (**42c**), 44% (**42d**) (two steps).

drophobic interaction with residues on CXCR4. Comparison of the CXCR4 binding affinity of the zinc complexes of **19a** and **37b** shows that the 2-pyridylmethyl group is more suitable than the 2-methylquinoline group as X-CH_2 or Y^1-CH_2 introduced on the nitrogen atom.

Conclusions

New low-molecular-weight CXCR4 ligands were designed and synthesized. The most potent compounds are **37b** and **40b**, zinc complexes with a Dpa group on the 1,4-phenylenedimethanamine spacer template. The distances between all the functional moieties of the compounds linked by the 1,4-phenylenedimethanamine spacer might be appropriate for interaction with CXCR4. These compounds exhibited IC_{50} values at micromolar levels in CXCR4 binding affinity. Zinc complexation of Dpa-containing compounds resulted in a remarkable increase in CXCR4 binding affinity relative to the corresponding zinc-free compounds. The results reported herein might provide useful insight into the design of novel CXCR4 ligands, complementing information from other compounds such as T140, FC131, and KRH-1636. These compounds will be useful for the development of future therapeutic strategies for CXCR4-relevant diseases.

Experimental Section

Chemistry

Synthetic strategies of compounds reported in the present study are described in Results and Discussion above, and details are provided in the Supporting Information. Zn^{II} complex formation was performed by treatment of the compounds with 10 equiv $ZnCl_2$ in PBS. The Zn^{II} complexes were characterized by the chemical shifts of their methylene protons in ^1H NMR spectroscopic analysis. The Dpa–Zn^{II} complex was characterized previously.[35] Detailed data are provided in the Supporting Information.

Table 3. CXCR4 binding affinities of compounds **19a**, **37b**, **38b**, **39b**, **40b**, **41b**, and **42b** in zinc(II) complex.

Compd	Inhibition [%][a]	IC_{50} [nM][b]
19a	34.5±6.5	ND
37b	93.4±6.4	2100
38b	25.6±2.4	ND
39b	0	ND
40b	98.0±1.0	2100
41b	80.7±0.8	ND
42b	35.9±0.9	ND
FC131[c]	100	15.9

[a] CXCR4 binding affinity was assessed based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells; percent inhibition values for all zinc complexes at 5 μM were calculated relative to that of FC131 (100%). [b] IC_{50}: zinc complex concentration required for 50% inhibition of [^{125}I]CXCL12 binding to Jurkat cells; all data are the mean values from at least three independent experiments; ND: not determined. [c] Metal free.

Biological assays

CXCR4 binding assays of compounds based on the inhibition of [^{125}I]CXCL12 binding to Jurkat cells were performed as reported by Tanaka et al.[38]

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Keywords: aza-macrocycles · chemokine receptors · CXCR4 · low-molecular-weight ligands · zinc complexes

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RAPID COMMUNICATION

Single oral administration of the novel CXCR4 antagonist, KRH-3955, induces an efficient and long-lasting increase of white blood cell count in normal macaques, and prevents CD4 depletion in SHIV-infected macaques: a preliminary study

Tadashi Nakasone · Sei Kumakura · Michiko Yamamoto · Tsutomu Murakami · Naoki Yamamoto

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Abstract We evaluated the long-term effects of the single oral administration of a new CXCR4 antagonist, KRH-3955, on elevation of white blood cell (WBC), neutrophil and lymphocyte counts in normal cynomolgus monkeys. In the monkeys treated with 0, 2, 20, 200 mg/kg of the compound, WBC, neutrophil and lymphocyte counts increased dramatically at 2 days after treatment. This effect was dose-dependent, and these cell counts remained elevated 15 days after drug treatment. Since neutrophils are the most abundant WBCs in circulation and bone marrow neutrophil exhaustion impairs the response to bacterial infections, it is intriguing to exploit this pharmacological increase of neutrophils as a tool to address its influence on viral infections in vivo. The SHIV infection studies using the SHIV-KS661c/cynomolgus monkey model showed that a single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before virus exposure did not prevent infection, although it did prevent CD4 cell depletion in 3/3 monkeys. Furthermore, single oral administration of the drug 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys. This prevention of CD4 cell depletion was observed in both blood and lymphoid tissues. These results show that natural course of the SHIV infection is modulated by artificial increase of neutrophils and lymphocytes caused by KRH-3955 in the cynomolgus monkey model.

Keywords CXCR4 antagonist · WBC mobilization · SHIV · HIV

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Abbreviations

CXCR4	C-X-C motif receptor 4
CCR5	C–C motif receptor 5
SDF-1	Stromal-derived factor-1
CBC	Complete blood cell count
PrEP	Pre-exposure prophylaxis
SHIV	Simian human immunodeficiency virus
PBL	Peripheral blood lymphocytes
PB	Peripheral blood
LT	Lymphoid tissue
LN	Lymph node
WBC	White blood cell
AZT	Zidovudine
IC50	Half maximal inhibitory concentration

Background

Leukocytopenia places individuals at increased risk of infection. Among various leukocytes, neutrophils play an

important early defensive role against infection. Neutropenia—a decrease in the neutrophils count—is the most important indicator of infection risk [1]. Neutrophils occupy about 70 % of total leukocyte population. Under normal settings, only a small fraction less than 2 % of the neutrophil pool is circulating in the periphery, whereas most are found to be stored in the bone marrow [2, 3]. Upon exposure to infection, neutrophils are mobilized from the bone marrow and epithelium, and control invading pathogens in the periphery through its specialized innate immune activities such as phagocytosis, release of soluble anti-microbials including granule proteins, and generation of neutrophil extracellular traps [4]. Indeed, it is reported that interruption of the neutrophil supply is detrimental to the control of bacterial infections [5, 6]. After engagement of neutrophils in the process of bacterial killing, they tend to die, while granulocyte colony-stimulating factor is up-regulated to induce granulopoiesis and to replenish the reservoir in bone marrow [7]. The average lifespan of non-activated neutrophils in the circulation is about 5 days but once activated, they survive only for 1–2 days [8]. Through its interaction with CXCR4, SDF-1 retains the leukocyte and stem cells in the bone marrow by a process referred to as homing [9]. AMD3100, a novel antagonist of CXCR4, has been shown to specifically antagonize this interaction and recruit WBC, including neutrophils and CD34 + hematopoietic stem cell to the peripheral blood (PB) in human studies [10].

We have previously reported that unique CXCR 4 antagonists, the KRH series of compounds, exhibit potent and selective anti-HIV-1 activity [11, 12]. One of the derivatives, KRH-3955 is orally bioavailable (25.6 % bioavailability) and has high anti-HIV activity in vitro compared with AMD3100 [12, 13]. Furthermore, KRH-3955 efficiently inhibits the replication of HIV in a human peripheral blood lymphocyte-severe combined immunodeficiency mouse model after a single oral administration, even when given 2 weeks before HIV challenge [12]. Since this long elimination half-life of KRH-3955 (99.0 ± 13.1 h) after a single administration to animals suggests the long-term accumulation of the compound within tissues, it is intriguing to address whether KRH-3955 can also increase the WBC counts in normal monkeys by single oral treatment regimen and modulate the SHIV infection in the SHIV-KS661c/cynomolgus monkey (*Maccaca fascicularis*) model developed as a non-human primate (NHP) AIDS model [14–17].

In the present work, we evaluated initially the long-term efficacy of KRH-3955 on increase of WBC, neutrophils and lymphocytes in normal monkeys through single oral challenge of the drug. Having confirmed that all these WBC populations were efficiently mobilized to PB, we conducted further experiments using our cynomolgus monkey model.

Methods

Animal care

Drug toxicity study with monkeys was carried out in Medicinal Safety Research Laboratories, Sankyo Co. Ltd. (717 Horikoshi, Fukuroi, Shizuoka, 437-0065 Japan). The monkey infection experiments were conducted at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBIO), Japan, in accordance with requirements specifically stated in the laboratory biosafety manual of the World Health Organization. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals and were maintained in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of NIBIO, Japan. Both IACUCs of NIBIO and the National Institute of Infectious Diseases (NIID), Japan, approved the study. Both guidelines are in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research". The use of SHIV was also approved by both Institutional Advisory Committees for the Biosafety of Living Modified Organisms of NIBIO and NIID with the recognition (Dai17-17) of the Japanese Minister of Education, Culture, Sports, Science and Technology, 2005. Ethical standards incorporated into these guidelines and into our routine laboratory procedures include a maximum reduction in the number of animals, a psychological enrichment program, frequent contact with other animals (visual, auditory, and olfactory), regular veterinary supervision and care.

Drugs

The CXCR4 antagonist, KRH-3955 (*N*, *N*-dipropyl-*N*-[4-({[(1*H*-imidazol-2-yl) methyl][(1-methyl-1*H*-imidazol-2-yl) methyl] amino} methyl) benzyl]-*N*-methylbutane-1, 4-diamine tri-(2*R*, 3R)-tartrate), and the CCR5 antagonist, SCH-D, were synthesized and purified by Kureha Corporation [12].

Viruses

SHIV-KS661c, molecularly cloned from SHIV-C2/1, was used in this study. The SHIV-C2/1 stock comprised plasma obtained by serum passages of p-SHIV (derived from SHIV-89.6) in cynomolgus monkeys [14–17]. SHIV-KS661c, SHIV-C2/1, and SHIV-89.6 were CXCR4-tropic viruses. SHIV-KS661c was propagated in CEMx174 cells and was confirmed to be genetically identical to the major sequences of the parent virus. SHIV-KS661c has been

shown to infect cynomolgus monkeys both intravenously and intra-rectally and to induce high-peak viremia and drastic CD4 cell depletion within 2 weeks of inoculation [18–23]. Another SHIV-89.6P used in this study was brother strain of SHIV-KS661c and CXCR4-tropic [24]. Both virus stocks were kept at −80 °C and were thawed immediately prior to use.

In vitro drug susceptibility testing

The susceptibility of SHIV-KS661c to KRH-3955 was determined as follows: Peripheral blood lymphocytes (PBL) obtained from naïve cynomolgus monkeys were stimulated with concanavalin-A (5 μg/ml, Con-A, SIGMA) for 2 days. Con-A-activated PBL (1×10^7 cells/well) were exposed to 100 $TCID_{50}$ of SHIV-KS661c or SHIV-89.6P for 4 h. After extensive washing, cells (2×10^5 cells/well) were incubated with various concentrations of the drugs. The amount of SIV p27 antigen produced in the culture supernatants was measured using an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY) 12 days after infection. The IC50 was calculated using XLfit analysis software (ID Business Solutions). Zidovudine (AZT, SIGMA) and Vicrovic (SCH-D, Schering-Plough) were used for comparison. All assays were carried out in duplicate in 96-well culture plates.

Drug toxicity study design

A single-dose toxicity study was conducted on male and female cynomolgus monkeys over a 15-day observation period. KRH-3955 was administered at dose levels of 0, 20, 200, and 2,000 mg/ml. Compound was formulated in water and was administered intragastrically (i.g.) via a nasal feeding tube under anesthesia, followed by additional water to wash out any compound remaining in the tube. All groups consisted of two animals/sex. The general condition of the animals including appetite, activity, and body weight was carefully observed. In the toxicity study, blood chemistry, complete blood cell counts (CBC), and WBC populations were automatically measured during the observation period. Phospholipidosis was observed as vacuolation in hematoxylin-eosin staining, and the tissues with vacuolation were characterized using Sudan Black B staining at 200 mg/kg in monkey.

Animal infection study design

Nine cynomolgus monkeys were enrolled and divided into three groups, each containing three animals. Group 1 (G1) was given a single dose of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure; Group 2 (G2) was treated with a single dose of KRH-3955 (100 mg/kg) 2 weeks before viral exposure; and Group 3 (G3, naïve control group) was not treated with any drugs. The study was divided into two research reports: this report and another [25]; group 3 was the same in both papers. KRH-3955 in water was administered i.g. with additional washing same as the above. All monkeys were then intra-rectally challenged with 10 times the AID_{50} (50 % animal infectious dose) of a highly pathogenic SHIV-KS661c. The general condition of the animals including appetite, activity, and body weight was carefully observed. Blood chemistry, CBC, absolute CD4 cell counts, and plasma virus RNA copy number were measured frequently for more than 12 weeks. Finally, the monkeys were euthanized for virological analysis and analysis of the CD4 population in lymphoid tissues.

Real-time RT-PCR quantification of SHIV RNA in plasma

Plasma viral loads were evaluated using real-time reverse transcriptase polymerase chain reaction (RT-PCR) with a TaqMan probe as previously reported [18–20, 25]. Briefly, viral RNA was extracted from the plasma and purified using the QIAamp Viral RNA Mini Kit (Qiagen). For quantitative analysis of the RNA, the TaqMan system (Applied Biosystems) was used with primers and probes targeting the SIVmac239 gag region. The viral RNA was amplified using a QuantiFast Probe RT-PCR Vial Kit (Qiagen) with primers and TaqMan probes. The fluorescence intensity of the RT-PCR product was quantitatively monitored using an Opticon 2 (former MJ Research). The plasma viral load, measured in duplicate, was calculated based on the standard curve for control RNA and on the RNA recovery rate. To obtain the RNA recovery rate, 10^5 copies of SHIV-KS661c were extracted and purified using the same kit in parallel with the sample treatment. The recovered RNA was also amplified at the same time as that of the samples. The limit of detection was approximately 500 RNA copies/ml.

CD4 cells in blood and lymphoid tissues

The absolute CD4 cell count in the PB was measured as previously described [18–20, 25]. Briefly, 50 μl of whole blood was incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource), Phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton–Dickinson), or peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton–Dickinson). After red blood cell lysis using FACS lysis solution (Becton–Dickinson), the cells were analyzed along with reference beads (Beckman Coulter) using a FACS Calibur (Becton–Dickinson) and Cell Quest software (Becton–Dickinson). The monkey lymphoid cells used for

flow cytometric analysis were prepared from thymus, spleen, and lymph node (LN) tissues obtained at necropsy. The cells were stained with the same three antibodies described above and the CD4 population analyzed.

Results

Dramatic and persistent increase of WBC count upon treatment with KRH-3955 of normal monkeys

In the hematological examination in normal monkeys, WBC, neutrophils, and lymphocytes counts increased dramatically in each treatment group (Fig. 1), whereas RBC counts were unchanged with a slight decrease at 2 days of treatment. The peak effects were observed at 2 days after dosing between 20 and 2,000 mg/kg. Strikingly, this enhancing effect of WBC was seen even 15 days after drug treatment. Additional lymphocyte subset analysis indicated that the increase in count was dependent on increases in the T, B, and NK cells (data not shown). There was no increase in immature band forms based on a differential analysis performed in all cases of leukocytosis. The hematological changes are considered to be related to the pharmacological effect of KRH-3955. All the above changes recovered or tended to recover in the 15-day recovery period.

In addition to the above changes, hemoglobin and hematocrit counts decreased in the group receiving 200 mg/kg (data not shown). In the blood chemical examination, aspartate aminotransferase slightly increased in the group receiving 200 mg/kg. In the histo-pathological examination, systemic phospholipidosis related to the pharmacological effect of KRH-3955 was observed in many organs as vacuolar change in the groups receiving >20 mg/kg, which is considered to be toxicologically insignificant. In addition, skeletal muscle injury and renal damage were observed in the 200 mg/kg group.

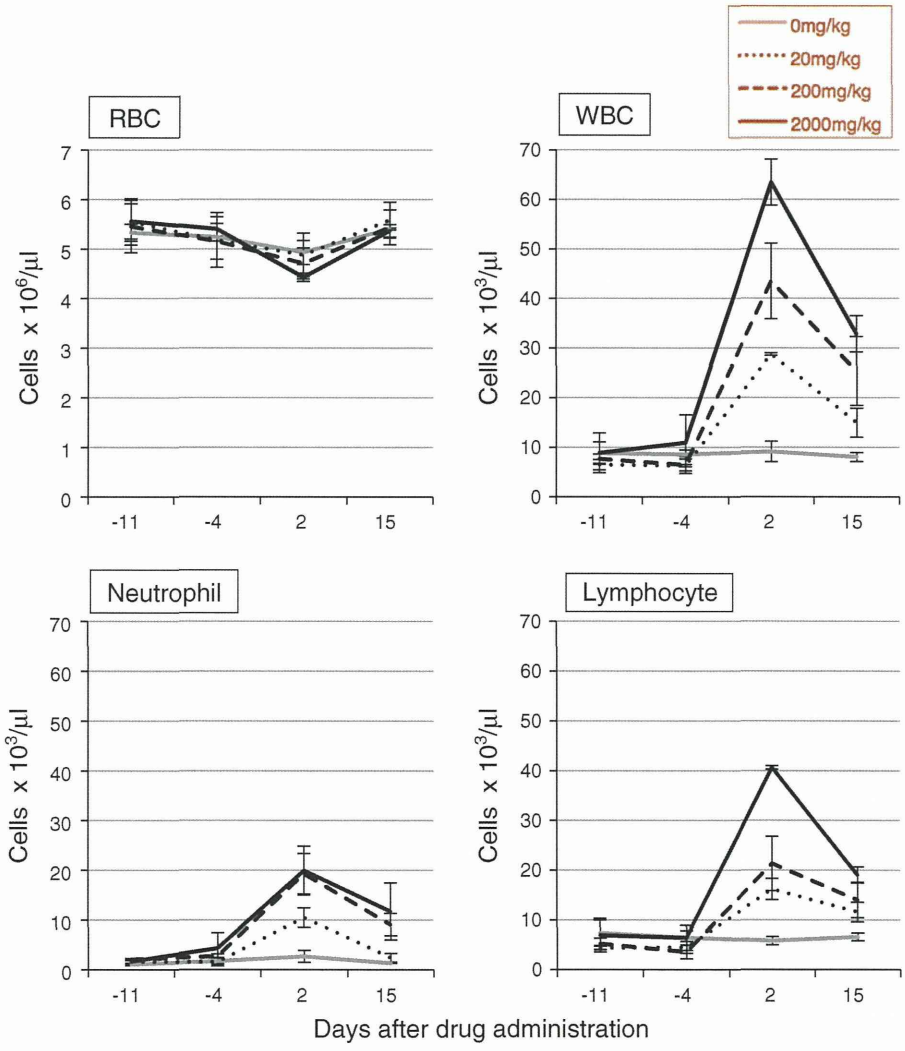

Fig. 1 Hematological examination of monkeys after treatment with KRH-3955. A single-dose study was conducted on male and female cynomolgus monkeys by a 15-day observation period. KRH-3955 dissolved in water was intragastrically administered at day 0 at dose levels of 0 (*gray*), 20 (*dot*), 200 (*dash*), and 2,000 (*black*) mg/ml. The number of red blood cells (RBC), white blood cells (WBC), neutrophils, and lymphocytes were monitored at the indicated time points. *Bars* indicate standard deviation

Anti-viral and CD4 protecting activities of KRH-3955 in SHIV-infected monkeys

In vitro effects of KRH-3955

The inhibitory activity of KRH-3955 against SHIV-Ks661c and SHIV-89.6P was examined in activated cynomolgus PBL from two different donors (Table 1). KRH-3955 inhibited the replication of SHIV-KS661c with an IC50 of 3.1–77.7 nM. Although SCH-D did not inhibit SHIV-KS661c in activated PBL isolated from monkey B (even at a concentration of up to 100 nM), it inhibited the virus in cells from monkey A with an IC50 of 37.9 nM. On the contrary, KRH-3955 inhibited the replication of SHIV-89.6P in activated PBL with an IC50 of 3.6–11.0 nM. SHIV-89.6P did replicate in the presence of SCH-D, even at concentrations up to 100 nM. SHIV-89.6P is highly pathogenic and CXCR4-tropic and has been used extensively in various experiments. AZT, used as a positive control, inhibited both viruses with an IC50 of 8.4–16.2 nM.

In vivo effect of KRH-3955 on PB

The efficacy of KRH-3955 was evaluated in the SHIV-KS661c/cynomolgus monkey model. Single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure did prevent CD4 cell depletion in 3/3 monkeys (Fig. 2, G1). But the drug did not protect monkeys from infection. In this group, two monkeys had an undetectable viral load after peak viremia. Furthermore, single administration of KRH-3955 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys (Fig. 2, G2). In this monkey, the virus did not replicate well during the course of the experiment. All six monkeys in Group 1 and Group 2 showed transient CD4 lymphocytosis (2,077–6,347 cells/μl at peak) for 4 weeks after KRH-3955 administration. We did not observe any abnormal findings in any monkeys during the course of the experiment.

Table 1 KRH-3955 inhibits SHIV replication in simian PBLs in vitro

Conc. (nM)	SHIV-KS661c (% replication)			SHIV-89.6P (% replication)		
	AZT	KRH-3955	SCH-D	AZT	KRH-3955	SCH-D
Monkey A						
100	13.1	49.0	37.1	24.3	15.8	59.5
20	30.7	68.5	51.2	42.4	28.0	74.0
4	65.2	91.8	98.4	63.2	76.7	82.3
0.8	82.7	147.9	173.5	148.6	123.7	134.6
IC50 (nM)	8.4	77.7	37.9	16.2	11.0	>100
Monkey B						
100	8.6	1.1	252.1	5.1	0.5	90.2
20	35.7	1.1	224.0	33.7	0.6	135.7
4	98.2	7.5	249.7	96.2	8.1	128.7
0.8	131.9	153.9	252.3	180.2	175.4	211.7
IC50 (nM)	15.9	3.1	>100	15.5	3.6	>100

Con-A-activated simian PBLs from two cynomolgus monkeys were exposed to SHIVs

Exposed cells were cultivated in the presence of various concentrations of the drugs for 12 days

Infection was confirmed by the presence of SIV p27 antigen and expressed as % replication

IC50 (50 % inhibitory concentration) was calculated using XLfit analysis software (ID Business Solutions)

Assays were carried out in duplicate in 96-well culture plates

The HIV RT inhibitor AZT (zidovudine) and the CCR5 antagonist, SCH-D (vicrovic), were used for comparison

In vivo effect of KRH-3955 on various lymphoid tissues

After more than 12 weeks of observation, all monkeys were killed, and the CD4 cell population in the lymphoid tissues (LTs) was analyzed. Macroscopic findings generally suggested that thymus and any LN were atrophic in those monkeys showing severe CD4 lymphocytopenia (<100 cells/μl) at necropsy. However, no atrophic tissues were found in monkeys not presenting with severe CD4 lymphocytopenia.

The CD4 cell population in the LTs of the monkeys was analyzed by flow cytometry and was expressed as ratio of CD4 cells/CD3 cells (%) (Fig. 3). In Group 1, CD4 cells were well conserved with LTs (>25 %) other than spleen (12–30 %), similar to the results seen for PB. In Group 2, CD4 cells were well conserved within the LTs of two monkeys (>20 %), except in the spleen (13 and 17 %). The CD4 cells of another monkey, which showed moderate CD4 cell depletion in PB, were not rescued in any of the LTs (2–20 %). Thus, the preventive effects of KRH-3955 on CD4 cell depletion were seen not only in PB, but also in several LTs. The total ratio of CD4/CD3, including that in the PB of animals in Group 1 and Group 2, was significantly higher ($p < 0.001$ and $p < 0.05$, respectively) than that in Group 3 (naïve control). In contrast, two monkeys in Group 3 showed severe CD4 cell depletion in all LTs (0–8 %), although the CD4 cells of another monkey were well conserved in all LTs (15–48 %).

Discussion

The increase of WBC, neutrophils, and lymphocytes counts noted in all treatment groups in monkeys receiving parenteral KRH-3955 was dramatic. At 2 days after treatment, there were 3.2–10-, 4–7.5-, and 2.8–7-fold increases for WBC, neutrophils, and lymphocytes, respectively, compared to untreated controls. Though the activity of

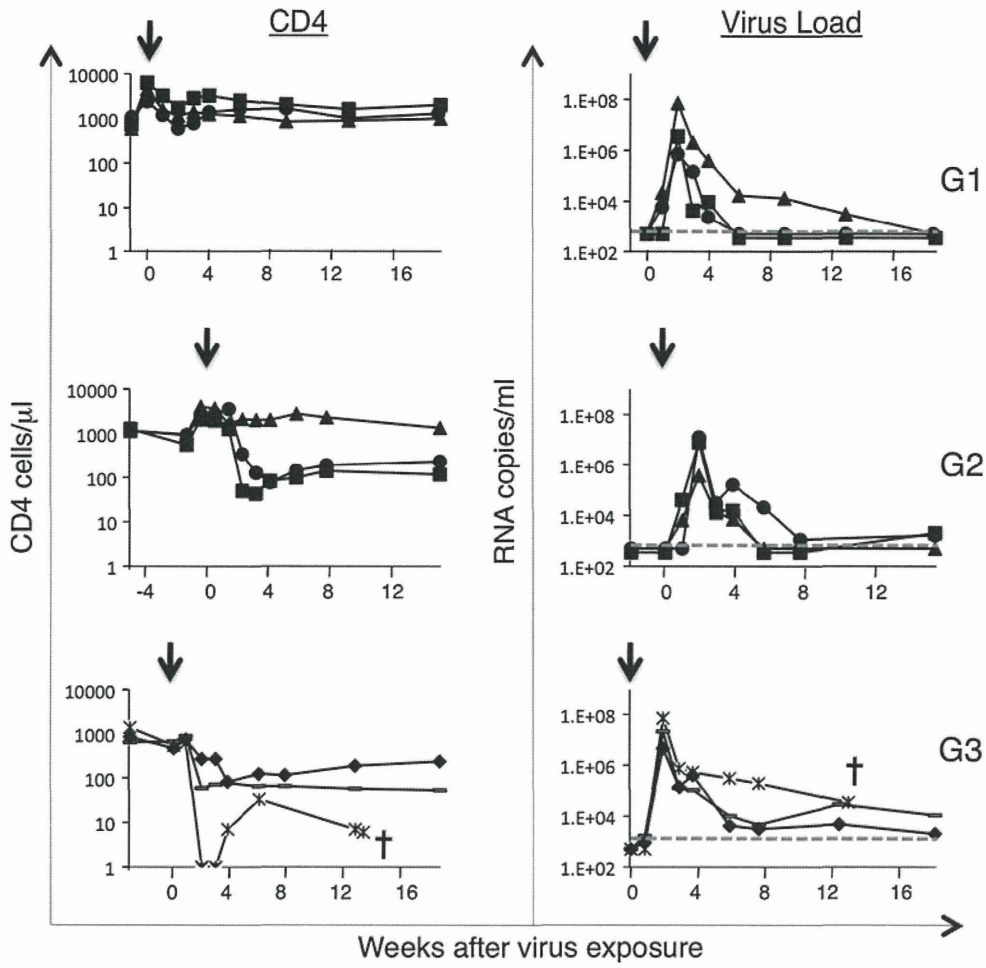

Fig. 2 Effect of PrEP on CD4 cells and virus in peripheral blood. Absolute CD4 cell counts (cells/μl) and virus RNA copy numbers (copies/ml) are shown for monkey peripheral blood and plasma, respectively, during the experiments. Each *symbol* indicates each individual monkey. *Arrows* indicate intrarectal viral challenges. *Dash* indicates threshold (<500 copies/ml) in this system. One naïve control monkey (cross in G3) showing severe CD4 cell depletion and high set-point viremia was euthanized due to AIDS. *G1* Group 1, *G2* Group 2, *G3* Group 3

KRH-3955 was reversible, its long-lasting activity was also striking because even 15 days after treatment with this compound, significant higher counts were seen (1.8–4.1-, 1.9–9-, and 1.8–2.9-fold increases for WBC, neutrophils, and lymphocytes, respectively, compared to untreated controls). The effect was clearly dose-responsive. It has been already shown that AMD3100, a novel antagonist of CXCR4, specifically antagonizes CXCR4-SDF-1 interaction and mobilizes WBC including neutrophils and CD34 + hematopoietic stem cell counts in the PB in phase I clinical trials [10]. When administered to healthy volunteers, AMD3100 produced an increase in WBC count of 1.5–3.1 times the baseline, peaking at 6 h following the intravenous infusion and largely returning to the baseline at 24 h. We believe that an increase in WBCs induced by KRH-3955 is also CXCR4-mediated since KRH-3955 inhibits both SDF-1 binding to CXCR4 and Ca2++ signaling through the receptor in consistency with the observation with AMD3100. These data suggest that binding of KRH-3955 to CXCR4 may inhibit the chemotactic effects of SDF-1, causing mobilization of WBCs from bone marrow [1, 26].

AMD3100 is now approved as a hematopoietic stem cell mobilizer by FDA (renamed plerixafor or Mozobil™). Since KRH-3955 and AMD3100 show significant difference in terms of anti-CXCR4 efficiency and their long half-life, KRH-3955 could also be considered to use in some disease conditions, especially those where chronic treatment of the drug is needed such as warts, hypogammaglobulinemia, infections, and myelokathexis syndrome [27]. On the other hand, long-term effect of KRH-3955 may complicate the therapy, and in general, a prolonged effect of drugs in elevating WBC count is viewed as a disadvantage in selecting a new drug for the development. However, the effect inherent to this compound provides us with a unique opportunity to study a significance of neutrophils in viral and bacterial infections. Turning this to our own advantage, we attempted to address two issues using the same species of cynomolgus monkey: (1) the possible modulation of natural course of SHIV infection through artificially increasing neutrophils and lymphocytes counts and (2) possibility for this compound as a drug for PrEP using our SHIV-KS661c model. Before starting our in vivo experiments, we confirmed the inhibitory effect of

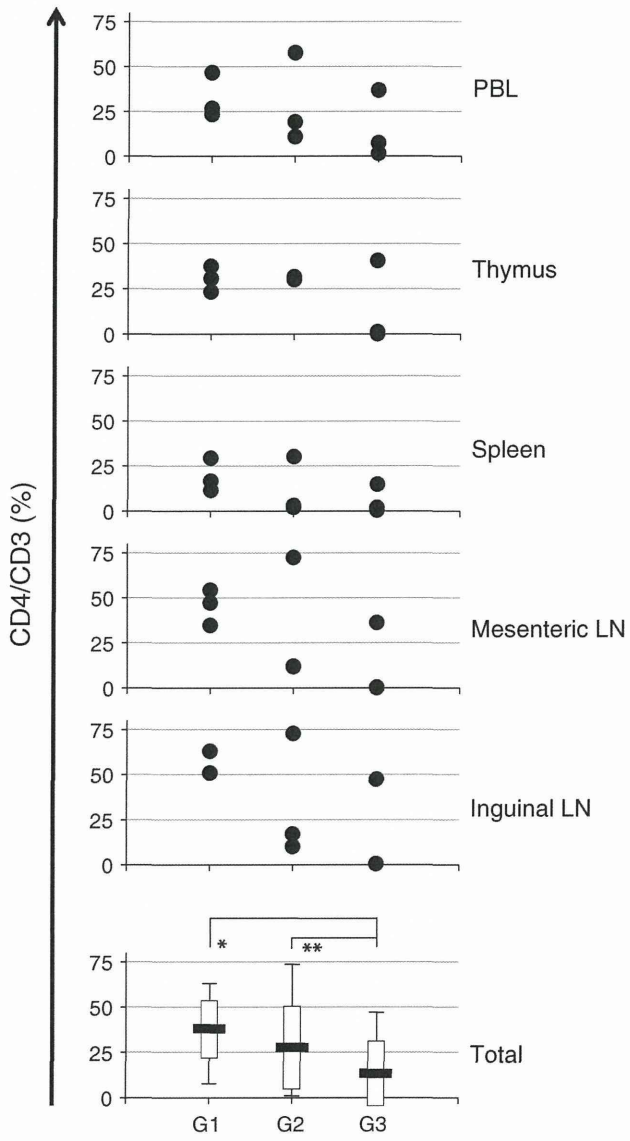

Fig. 3 Effect of pre-exposure prophylaxis against SHIV on various lymphoid tissues of monkeys. The population of CD4 cells in the various lymphoid tissues at necropsy is shown as a ratio of CD4 cells/CD3 cells (%). The ratios of CD4/CD3 in peripheral blood at necropsy are also shown. The total CD4/CD3 ratios assembled from three compartments for analysis using a one-sided Student's *t* test are shown at the *bottom*. *Narrow bars* indicate ranges from minimum to maximum. *Boxes* indicate the standard deviation. *Bold bars* indicate mean values. $*p < 0.001$, $**p < 0.05$. *PBL* peripheral blood lymphocytes, *LN* lymph node, *G1* Group 1, *G2* Group 2, *G3* Group 3

KRH-3955 against in vitro infection by predominantly CXCR4-tropic SHIV-KS661c. The results revealed that single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure effectively prevented CD4 cell depletion in 3/3 monkeys. Furthermore, single administration of the drug 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys. Prevention of CD4 cell depletion was seen not only in PB, but also in several LTs. The characteristically long half-life of KRH-3955 in LTs may contribute to its long-term preventive effects. Thus, artificial increase of WBC induced by KRH-3955 could indeed modulate natural infection process of SHIV significantly. This long half-life of KRH-3955 may contribute to the studies to address the active role of innate immunity through neutrophils in various viral infections, especially acute infection models such as influenza and dengue.

However, KRH-3955 did not protect monkeys from SHIV infection under the same experimental condition. Why did single oral administration of KRH-3955 (100 mg/kg) fail to protect from infection? One possible explanation is that SHIV-KS66c may not be "absolutely CXCR-4-tropic" but is simply "predominantly CXCR4-tropic". This could not be predicted at the stage of our in vitro studies but might be possible to determine in animal experiments where multiple types of cells are present. It is highly likely that some types of cells other than activated cynomolgus PBL which we used in vitro should be susceptible to SHIV infection in monkeys. Another possibility could be due to the experimental system we choose, that is, the single high-dose intra-rectal viral challenge. Under this condition, multiple low-dose intra-vaginal (IVAG) viral challenges would be more likely to result in HIV infection, and KRH-3955 may "miss" an opportunity to block viruses administered via single high-dose intra-rectal challenge. Future studies of multiple high-dose IVAG viral challenges might reveal whether a CXCR4 antagonist is a suitable drug for PrEP or KRH-3955 is useful for PrEP.

In conclusion, single oral administration of KRH-3955 induced long-lasting increase of neutrophils and lymphocytes very efficiently in normal monkeys and prevented CD4 depletion in SHIV-infected monkeys. This efficient and long-lasting effect of KRH-3955 may give us unique opportunity to study an active role of innate immunity, especially that of neutrophils, in various viral infection models. In terms of the usefulness of KRH-3955 as a possible drug for the future long-term intermittent PrEP, further extensive studies are needed.

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Conflict of interest The authors have no conflicts of interest to declare.