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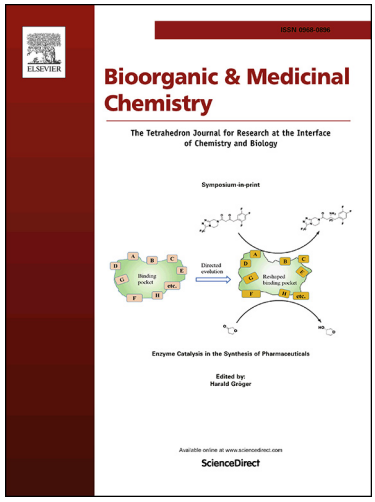

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PII: S0968-0896(18)32183-7
DOI: https://doi.org/10.1016/j.bmc.2019.02.013
Reference: BMC 14749

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 31 December 2018
Revised Date: 1 February 2019
Accepted Date: 5 February 2019

Please cite this article as: Sakyiamah, M.M., Kobayakawa, T., Fujino, M., Konno, M., Narumi, T., Tanaka, T., Nomura, W., Yamamoto, N., Murakami, T., Tamamura, H., Design, synthesis and biological evaluation of low molecular weight CXCR4 ligands, *Bioorganic & Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.bmc.2019.02.013

Design, synthesis and biological evaluation of low molecular weight CXCR4 ligands

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Abstract

The chemokine receptor CXCR4/stromal cell-derived factor-1 (SDF-1: CXCL12) signaling axis represents a crucial drug target due to its relevance to several diseases such as HIV-1 infection, cancer, leukemia, and rheumatoid arthritis. With the aim of enhancing the binding affinity and anti-HIV activity of a potent CXCR4 ligand as a lead, 23 low molecular weight compounds containing dipicolylamine (Dpa) and cyclam cationic moieties with varying spacers and spatial positioning were designed, synthesized and biologically evaluated. All of the synthesized compounds screened at 1.0 μM in the NanoBRET assay system exhibited more than 70 % inhibition of the binding of a competitive probe TAMRA-Ac-TZ14011 (10 nM) to CXCR4 in the presence of zinc (II) ion. Furthermore, selected compounds **3**, **8**, **9**, **19** and **21** with spatial distances between the next carbon to Dpa and the next carbon to cyclam within the range of 6.5-7.5 Å showed potent binding affinity selective for CXCR4 with IC_{50} values of 1.6, 7.9, 5.7, 3.5 and 4.5 nM, respectively, with corresponding high anti-HIV activity with EC_{50}s of 28, 13, 21, 28 and 61 nM, respectively, in the presence of zinc (II) ion. Some compounds with remarkably more potent CXCR4-binding affinity than that of an initial lead were obtained. These compounds interact with different but overlapping amino acid residues of CXCR4. The present studies have developed new low molecular weight CXCR4 ligands with high CXCR4-binding and anti-HIV activities, which open avenue into the development of more potent CXCR4 ligands.

Keywords

anti-HIV

azamacrocyclic compound

CXCR4-binding

dipicolylamine

NanoBRET assay

1. Introduction

CXC chemokine receptor type 4 (CXCR4) is a seven transmembrane (7TM) G-protein coupled receptor (GPCR), which is expressed mainly in immune cells and in hematopoietic stem cells. In response to its natural ligand stromal cell-derived factor-1 (SDF-1: CXCL12), it transmits signals to enhance development and chemotaxis of these cells.[1] However, the CXCR4/CXCL12 signaling axis plays relevant roles in a multiple of diseases such as HIV-1 infection, metastasis of various cancers and inflammatory diseases including rheumatoid arthritis.[2] Thus, this axis represents a prospective target for therapeutic interventions. To this aim, drugs and their candidates ranging from peptides such as T140 and 4F-benzoyl-TN14003 (LL-8040/BKT140), through to low molecular weight non-peptide compounds such as AMD3100 (plerixafor) and its analogs have been developed.[3] Our previous studies have developed a new class of low molecular weight CXCR4 antagonists such as SKA028-*p* (Fig. 1)[4] with bis(pyridin-2-ylmethyl)amine (dipicolylamine: Dpa) and 1,4,8,11-tetraazacyclotetradecane (cyclam) moieties attached to a *p*-xylene spacer, based on modification of the parent compounds, AMD3100 and Dpa-zinc(II) complex.[5] According to these studies, it is deduced that the positioning of the cationic moieties and the length of the spacer could influence the binding affinity of the compound for CXCR4 as well as its anti-HIV activity. In addition, the information might be useful that linker optimization was a key contributing factor leading to the synthesis of AMD3100.[6] Based on these backdrops, in this study we tried the design, synthesis and structure-activity relationship studies of a series of compounds with emphasis on varying spacers and positioning of the cationic moieties aimed at development of potent low molecular weight CXCR4 ligands.

2. Results and Discussion

2.1. Design and synthesis of the desired compounds

Compounds **1-23** were designed and synthesized employing spacers which would increase the spatial distance between the next carbon to Dpa and the next carbon to cyclam based on a lead compound

SKA028-*p* as shown in Fig. 1. In our previous study, SKA028-*p*, having the *p*-xylene spacer with a length of 5.9 Å, showed remarkably higher anti-HIV activity than the derivative having the *m*-xylene spacer with a length of 5.0 Å, in particular, in the presence of zinc (II) ion,[4] suggesting that longer distances between the cationic moieties could enhance binding affinity for CXCR4 and HIV-inhibitory activity. In place of benzene, naphthalene was therefore introduced to achieve a longer spatial length with a degree of angular restrain in compounds **1-3**. To further increase the length, a biphenyl spacer was utilized in compounds **4-12**. The biphenyl spacer was modified by introducing crosslinks to hinder the rotation around the single bond of the biphenyl spacer in compounds **15-18**. From our previous studies, the azamacrocyclic compounds showed enhanced activity in the presence of a bivalent cation, zinc (II).[4,5] Therefore, we modified the biphenyl and napththalene structures by introducing nitrogen atoms to derive bipyridine (compounds **13**, **14**), phenylpyridine (compounds **19-22**) and quinoline (compound **23**) (Fig. 1), to possibly enhance the chelation effects of the resulting compounds. In addition to the difference in spacer types, the positioning of the cationic moieties was varied with the aim of achieving the optimal spatial orientation of the Dpa and cyclam moieties for potent CXCR4-binding affinity and anti-HIV activity (Table 1).

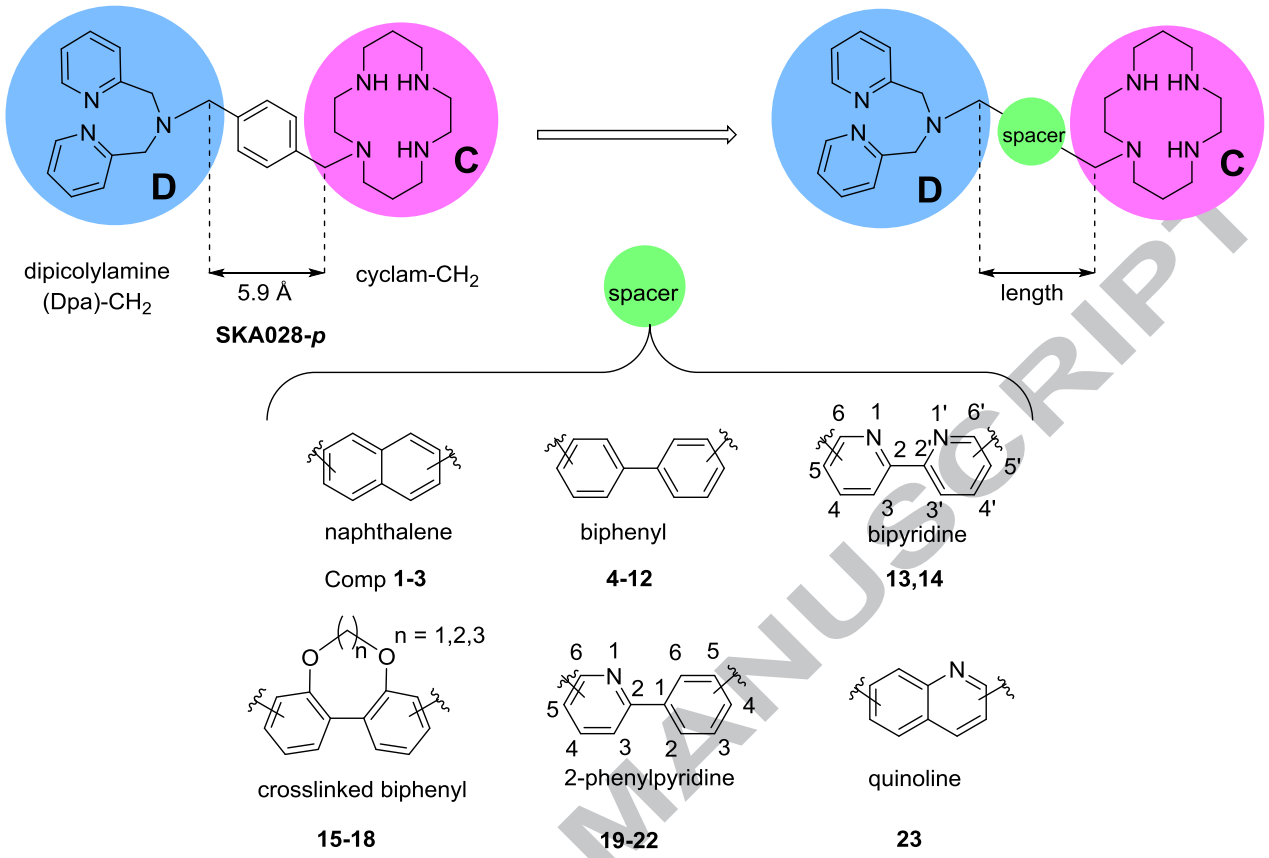
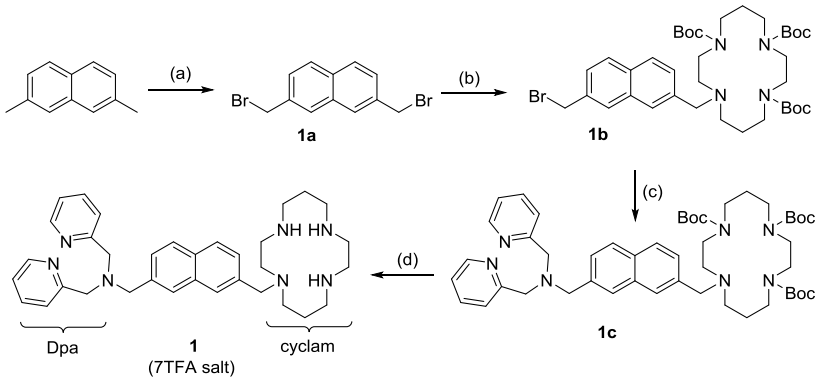


Figure 1. Design of the desired compounds with Dpa-CH_2 and cyclam-CH_2 moieties *via* various spacers based on our previous lead compound SKA028-*p*.

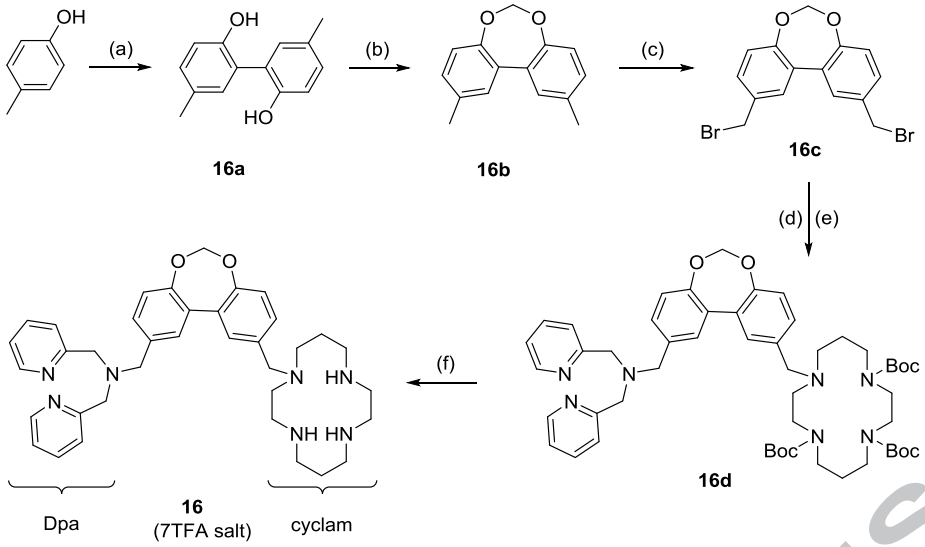
In the compound synthesis, depending on spacer types, synthetic schemes were changed. Schemes 1-3 are representative of the synthetic schemes for compounds **1-23**. Detail synthetic procedures and characterization data of all of the compounds **1-23** are described in Supplementary data. In the synthesis of a naphthalene spacer-containing compound **1**, 2,7-bis(bromomethyl)naphthalene **1a**, which was obtained by treatment of 2,7-dimethylnaphthalene with *N*-bromosuccinimide (NBS) and benzoyl peroxide (BPO), was reacted with tri-*tert*-butyl 1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate ($(Boc)_3$-cyclam) in the presence of KI and K_2CO_3, and then reacted with bis(pyridin-2-ylmethyl)amine

(Dpa) in the presence of KI and K_2CO_3 to obtain a tri-*N*-Boc-protected amine **1c**, followed by TFA treatment to yield the desired compound **1** (Scheme 1).



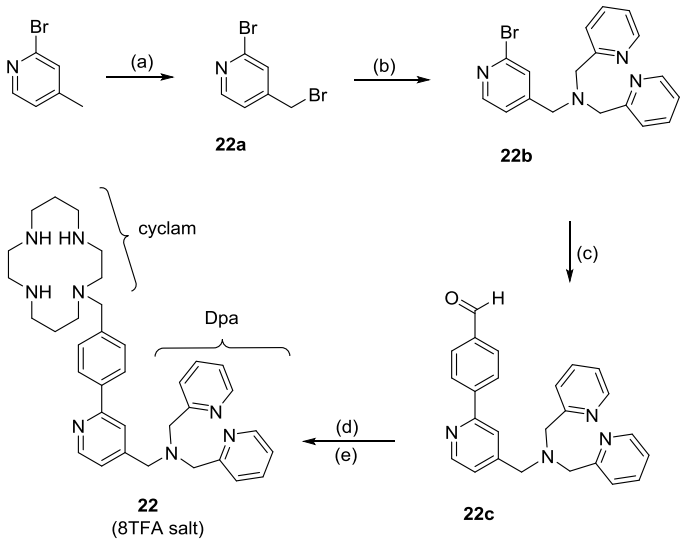
Scheme 1. Synthesis of a naphthalene spacer-containing compound **1**. Reagents and conditions: (a) *N*-bromosuccinimide (NBS), benzoyl peroxide (BPO), CCl_4, reflux, 67%; (b) tri-*tert*-butyl 1,4,8,11-tetraazacyclotetradecane-1,4,8-tricarboxylate ($(Boc)_3$-cyclam), KI, K_2CO_3, CH_3CN, rt; (c) bis(pyridin-2-ylmethyl)amine (Dpa), KI, K_2CO_3, CH_3CN, 80 °C; (d) TFA/$CHCl_3$/H_2O (5 : 47.5 : 0.25) (v/v), 0 °C to rt, 11% from **1a**.

In the synthesis of a crosslinked biphenyl spacer-containing compound **16**, 5,5'-dimethyl-[1,1'-biphenyl]-2,2'-diol **16a**, which was obtained by treatment of *p*-cresol with copper(II) chloride and cyclohexylamine, was reacted with bromochloromethane in the presence of sodium hydride to obtain the dioxepine derivative **16b**, followed by treatment with NBS and BPO to obtain the corresponding dibromide **16c**. The dibromide **16c** was reacted with $(Boc)_3$-cyclam in the presence of KI and K_2CO_3, and then reacted with Dpa in the presence of KI and K_2CO_3 to obtain a tri-*N*-Boc-protected amine **16d**, followed by TFA treatment to yield the desired compound **16** (Scheme 2).



Scheme 2. Synthesis of a crosslinked biphenyl spacer-containing compound **16**. Reagents and conditions: (a) $CuCl_2$•$2H_2O$, cyclohexylamine, MeOH, 30 °C, 30%; (b) NaH, CH_2BrCl, dry DMF, rt, 49%; (c) NBS, BPO, CCl_4, reflux; (d) $(Boc)_3$-cyclam, KI, M K_2CO_3, CH_3CN, rt; (e) Dpa, KI, K_2CO_3, CH_3CN, 80 °C; (f) TFA/$CHCl_3$/H_2O (5 : 47.5 : 0.25) (v/v), 0 °C to rt, 29 % from **16b**.

In the synthesis of a 2-phenylpyridine spacer-containing compound **22**, 2-bromo-4-(bromomethyl)pyridine **22a**, which was obtained by treatment of 2-bromo-4-methylpyridine with NBS and BPO, was reacted with Dpa in the presence of K_2CO_3 to yield 1-(2-bromopyridin-4-yl)-*N*,*N*-bis(pyridin-2-ylmethyl)methanamine **22b**, followed by treatment with (4-formylphenyl)boronic acid, tetrakis(triphenylphosphine)palladium(0) and cesium carbonate to obtain the corresponding 4-carbaldehyde **22c**. The 4-carbaldehyde **22c** was reacted with $(Boc)_3$-cyclam and sodium cyanoborohydride in the presence of acetic acid, and then treated with TFA to yield the desired compound **22** (Scheme 3).



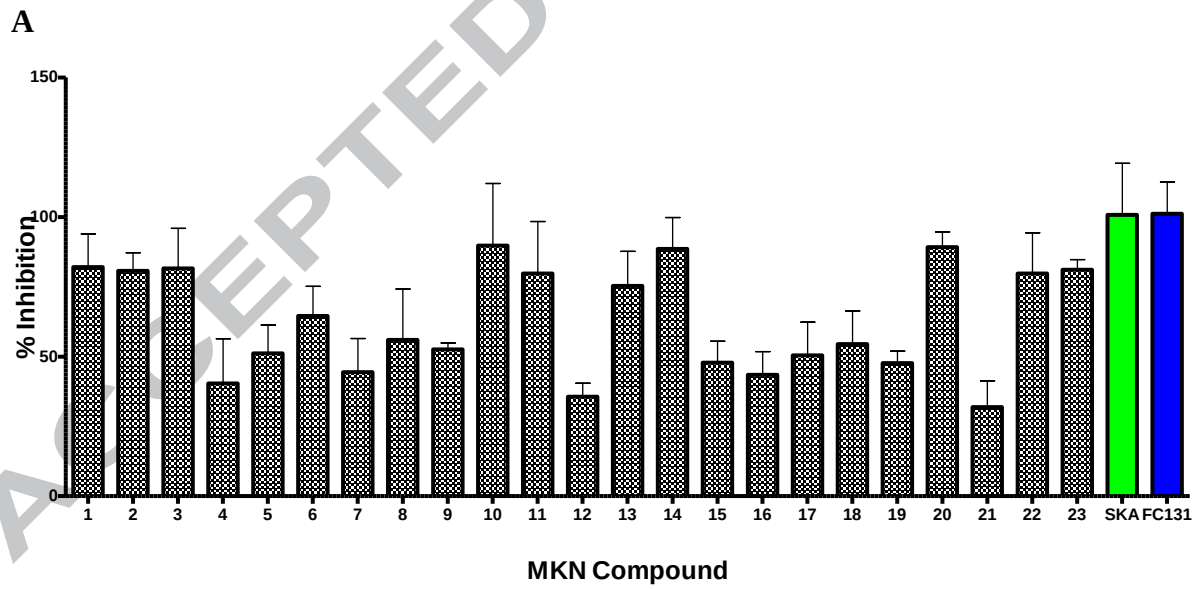
Scheme 3. Synthesis of a 2-phenylpyridine spacer-containing compound **22**. Reagents and conditions: (a) NBS, BPO, CCl_4, reflux, 31%; (b) Dpa, K_2CO_3 CH_3CN, rt, 99%; (c) (4-formylphenyl)boronic acid, 4 mol% $Pd(PPh_3)_4$, Cs_2CO_3, MeOH, 150 °C, MW; (d) $(Boc)_3$-cyclam, $NaBH_3CN$, AcOH, MeOH, rt; (e) TFA/$CHCl_3$/H_2O (47.5 : 47.5 : 0.25) 0 °C to rt, 9 % from **22b**.

2.2. Evaluation of CXCR4-binding affinity of the synthesized compounds

The binding affinity of the synthesized compounds was evaluated using the NanoBRET assay system, which was recently established by us,[7] based on their ability to compete for the binding of the fluorescent labeled ligand (TAMRA-Ac-TZ14011) in CHO cells stably expressing CXCR4 with Nanoluc tagged to its *N*-terminus. The NanoBRET assay system, which has recently gained wide application, is a non-radioactive assay system based on the transfer of energy from a bioluminescent donor (Nanoluc luciferase) to a fluorescent acceptor (5(6)-carboxy-tetramethylrhodamine; TAMRA) in close proximity (approximately 10 nm) upon binding of TAMRA-Ac-TZ14011 to CXCR4. We used the NanoBRET assay system due to its enhanced sensitivity and usefulness in high throughput screening of GPCR ligands.[8]

The present synthesized compounds, as well as the parent compounds AMD3100 and Dpa-Zn complex, have the propensity to coordinate with bivalent cations such as zinc, copper and nickel resulting

in highly stable metal complexes with enhanced binding affinity for CXCR4.[4,5,9] Both Dpa and cyclam moieties have the propensity to coordinate with zinc (II) ions.[4,5] Thus, the synthesized compounds **1-23** were evaluated at a predetermined concentration of 1.0 μM in the presence and absence of 10 equivalent of zinc (II) ion,[4] and the results were comparable to values obtained by radioisotope (RI)-based binding assay using [^{125}I]-CXCL12 as a competitive probe[10] (Fig. 2, Supplementary data Table S1). All of the compounds **1-23** screened showed more than 70 % inhibition of TAMRA-Ac-TZ14011 binding in the presence of zinc (II) ion (Fig. 2) whereas in the absence of zinc (II) ion, compounds **1-3** with a naphthalene spacer, **10** and **11** with a biphenyl spacer, **13** and **14** with a bipyridine spacer, **20** and **22** with a 2-phenylpyridine spacer, and **23** with a quinoline spacer showed more than 70 % inhibition. This observation corroborates with the aforementioned assertion that binding of the azamacrocyclic compounds is generally improved in the presence of bivalent ions. In the presence of zinc (II) ion, these compounds are predicted to assume more stable octahedral conformation upon interaction with the zinc (II) ions through the nitrogen atoms, and this enhances their affinity for CXCR4.[11]



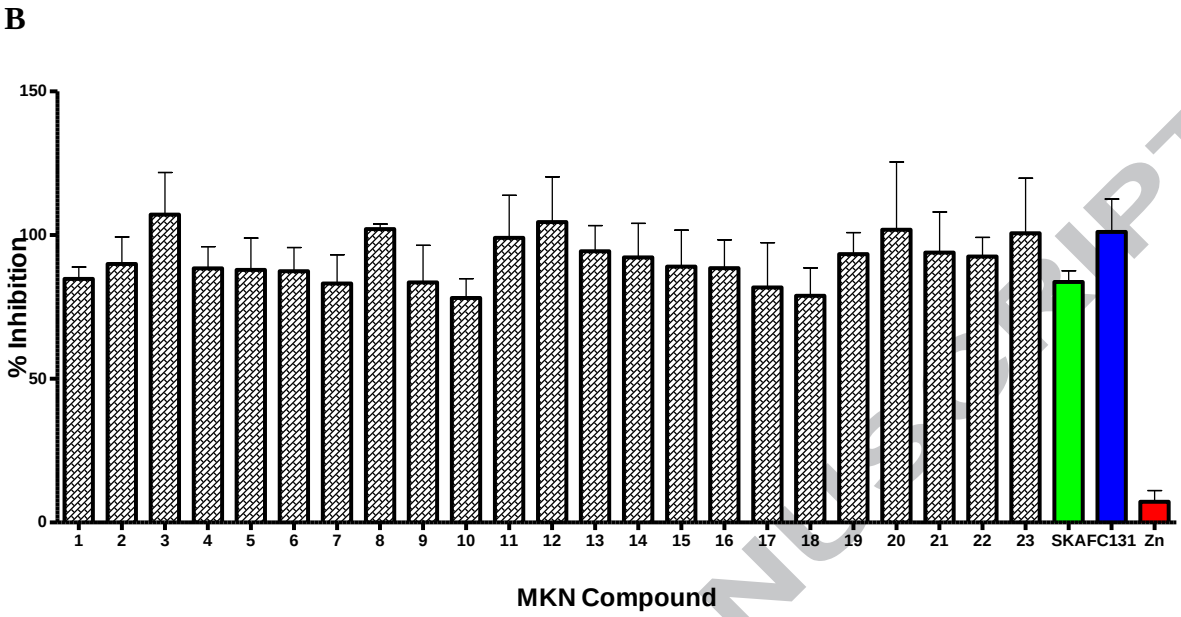


Figure 2. Single point percentage inhibition of the synthesized compounds using the NanoBRET assay system. CHO cells stably expressing Nluc-CXCR4 (5 x10^4) were seeded 24 h prior to experiments. Cells were incubated with 10 nM TAMRA-Ac-TZ14011 in the presence of 1.0 μM of the synthesized compounds without (**A**) or with 10 μM $ZnCl_2$ (**B**) for 1 h at 25°C. Values are mean ± SEM, n = 3. Green: SKA (SKA028-*p*).[4] Blue: CXCR4 antagonist, FC131.[3h] Red: 10 μM $ZnCl_2$ without compounds.

2.3. Evaluation of anti-HIV activity of the synthesized compounds

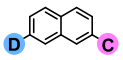
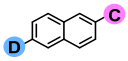
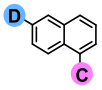
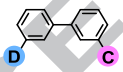
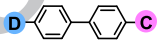
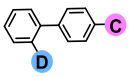
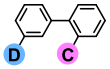
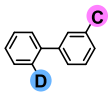
The inhibitory activity of the synthesized compounds against T-cell-line tropic (X4-) HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells was evaluated. CXCR4 is a major co-receptor for the entry of X4-HIV-1,[1,2] therefore a potential target for anti-HIV drug development.

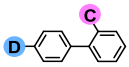
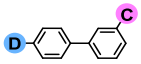
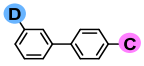
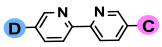
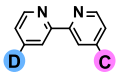
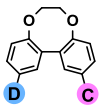
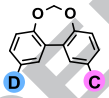
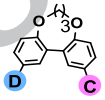
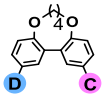
As a result, the compounds with 2-phenylpyridine as a spacer **19**-**22**, in particular **19** and **20**, appeared to show higher anti-HIV activity which increased by the presence of zinc (II) ion (Table 1), and **19** with zinc (II) ion is the most potent (EC_{50} = 28 nM). A similar increase by the presence of zinc (II) ion

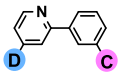
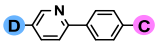
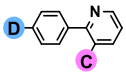
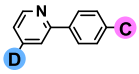
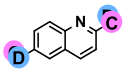
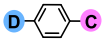
was observed in binding affinity of **19** and **21** for CXCR4 (Fig. 2). The Dpa/cyclam-CH_2 moieties at the 3/4-positions on pyridine/phenyl rings of the 2-phenylpyridine spacer of **19**-**22** seem to favor high CXCR4-binding affinity which possibly leads to high anti-HIV activity in the presence of zinc (II) ion. The additional nitrogen atom in the 2-phenylpyrine spacer might contribute to the interaction with the zinc (II) ion and thus, its increased affinity for CXCR4 in the presence of zinc (II) ion. However, contrary to what was expected, **13** or **14** with Dpa/cyclam-CH_2 moieties at the 5/5' or 4/4'-position, respectively, on two pyridine rings of the bipyridine spacer, did not show higher anti-HIV activity compared to **19** or **20** with the 2-phenylpyridine spacer. Furthermore, the presence of zinc (II) ion did not improve the CXCR4-binding affinity of **13** or **14** in spite of existence of the two nitrogen atoms (Fig. 2), suggesting the coordinative interaction with zinc (II) ion might cause some conformational change. Compounds **1-3** with the naphthalene spacer showed high CXCR4-binding activity in the presence and absence of zinc (II) ion (Fig. 2), and **3** showed potent anti-HIV activity in the presence of zinc (II) ion (EC_{50} = 28 nM). All of the compounds with the biphenyl spacer **4-12**, with the exception of **8** and **9**, did not show high anti-HIV activity in the presence or absence of zinc (II) ion whereas some showed potent binding affinity in the presence of zinc (II) ion. Similarly, **15-18** having the crosslinked biphenyl spacer did not show high anti-HIV activity in the presence or absence of zinc (II) ion although **15-18** exhibited enhanced binding affinity for CXCR4 in the presence of zinc (II) ion (Fig. 2). This suggest that the introduced angular restrain did not necessarily contribute to the anti-HIV activity of **15-18.** Compound **23**, with a mixture of isomers **23AB**, having the quinoline spacer did not show high anti-HIV activity in the presence or absence of zinc (II) ion, and did not exhibit increased binding affinity for CXCR4 in the presence of zinc (II) ion (Fig. 2). All 23 compounds, with the exception of **5**, did not show significant cytotoxicity in the absence of zinc (II) ion (CC_{50} > 10 μM, Table 1). Three selected compounds, **2**, **13** and SKA028-*p* did not show significant cytotoxicity in the presence of zinc (II) ion (CC_{50} > 10 μM, data not shown). All 23 compounds, with the exception of **9** in the presence of zinc (II) ion (58% inhibition at 10 μM), did not show significant anti-HIV activity against macrophage-tropic (R5-) HIV-1 (NL(AD8) strain) in the

presence or absence of zinc (II) ion (< 30% inhibition at 10 μM) (data not shown), suggesting that these compounds selectively bind for CXCR4.

Table 1. Anti-HIV and CXCR4-binding activities of the synthesized compounds.

compd. No.	structure	spacer length (Å)	anti-HIV activity		cytotoxicity	CXCR4-binding activity
			EC_{50} (μM) (- Zn(II))[a]	EC_{50} (μM) (+ Zn(II))[a]	CC_{50} (μM) (- Zn(II))[b]	IC_{50} (nM) (+ Zn(II))[c]
naphthalene spacer-containing compounds						
1		7.5	0.79	1.1	>10	
2		8.1	0.47	0.24	>10	10
3		6.7	0.22	0.028	>10	1.6
biphenyl spacer-containing compounds						
4		8.7	0.75	0.98	>10	
5		10.2	0.78	1.0	4.8	
6		6.8	2.6	0.89	>10	
7		5.4	4.2	1.1	>10	
8		6.5	0.040	0.013	>10	7.9

9		6.6	0.040	0.021	>10	5.7
10		9.1	0.80	1.1	>10	
11		4.7	1.0	5.8	>10	
12		9.1	3.1	1.0	>10	
bipyridine spacer-containing compounds						
13		10.0	0.68	0.90	>10	
14		8.8	0.92	0.20	>10	
crosslinked biphenyl spacer-containing compounds						
15		7.5	1.9	0.97	>10	
16		7.2	0.80	0.87	>10	
17		7.8	0.58	0.30	>10	
18		7.3	0.67	0.28	>10	
2-phenylpyridine spacer-containing compounds						

19		7.5	0.17	0.028	>10	3.5
20		10.1	0.59	0.13	>10	
21		6.7	0.11	0.061	>10	4.5
22		9.1	0.14	0.045	>10	
quinoline spacer-containing compounds						
23AB		8.0	>0.97	5.5	>10	
control						
SKA028-*p*		5.9	0.0065	0.0027	>10	32

[a]EC_{50} values are the concentrations of test compounds in the presence and absence of $ZnCl_2$ (10 eq) corresponding to 50 % protection from X4-HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells. [b]CC_{50} values are the concentrations of test compounds in the presence of $ZnCl_2$ (10 eq) which correspond to 50 % reduction in the viability of MT-4 cells. All data are mean values of at least three independent experiments. [c]IC_{50} values are the concentrations of test compounds in the presence of $ZnCl_2$ (10 eq) based on the inhibition of TAMRA-Ac-TZ14011 binding to CXCR4 in Chinese hamster ovary (CHO) stable cells using the NanoBRET assay system. D: Dpa-CH_2 moiety C: cyclam-CH_2 moiety. Spacer length: spatial distance between the next carbon to Dpa and the next carbon to cyclam as shown in Fig. 1.

Contrary to our initial hypothesis, the synthesized compounds did not show the expected trend of increasing CXCR4-binding and anti-HIV activities with increasing spatial distances between the next carbon to Dpa and the next carbon to cyclam (Fig. 3), and rather an undulating pattern was observed.

Compounds with shorter spatial distances such as **11** with a spacer length of 4.7 Å exhibited weaker anti-HIV activity (EC_{50} = 5.8 μM, Table 1) compared to the control SKA028-*p* (EC_{50} = 0.0027 μM, Table 1) with a length of 5.9 Å, whereas compounds with longer spatial distances such as compounds **1**, **4**, **5** and **23** with lengths of 7.5 Å, 8.7 Å, 10.2 Å, and 8.0 Å, respectively, showed weaker anti-HIV activity than SKA028-*p*, in spite of the presence or absence of zinc (II) ion. Compounds **19** and **22**, however, exhibited high anti-HIV activity despite having longer spatial lengths, 7.5 Å and 9.1 Å, respectively. Compounds **3**, **8**, **9** and **21** with spatial distances within the range of 6.5-6.7 Å showed higher anti-HIV activity in the presence and absence of zinc (II) ion (Table 1). This implies that a spatial distance within the range of 6.5-6.7Å could be the most suitable length for optimal interaction between the synthetic compounds and the corresponding amino acid residues in the binding pocket of CXCR4. However, the spatial orientations and the presence of a basic group such as a nitrogen atom for the zinc (II) ion interaction could partly or altogether complement the contribution of the spatial distance between the cationic moieties to enhance the CXCR4-binding and anti-HIV activities of the compounds (Compounds **13**,**14** and **19-22**, Fig. 2 and Table 1).

In addition to binding affinity, the selectivity of candidates is imperative in the drug discovery targeting GPCRs. The present control compound, SKA028-*p*-Zn(II) complex, as well as AMD3100, have been reported in previous studies to show high selectivity for CXCR4 over CCR5.[3j,10,12] Similarly, all of the 23 compounds synthesized here, with the exception of **9** in the presence of zinc (II) ion did not show significant anti-HIV activity against R5-HIV-1 (NL(AD8) strain) in the presence or absence of zinc (II) ion (< 30% inhibition at 10 μM). Compound **9** with zinc (II) ion exhibited quite slightly activity against R5-HIV-1 (58% inhibition at 10 μM). This suggests that the present compounds are also highly selective for CXCR4 because R5-HIV-1 strains prefer CCR5 to CXCR4 as the main co-receptor for viral entry. Taken together, the present compounds such as **3**, **8**, **9**, **19** and **21** are potential CXCR4 ligands.

A **B**

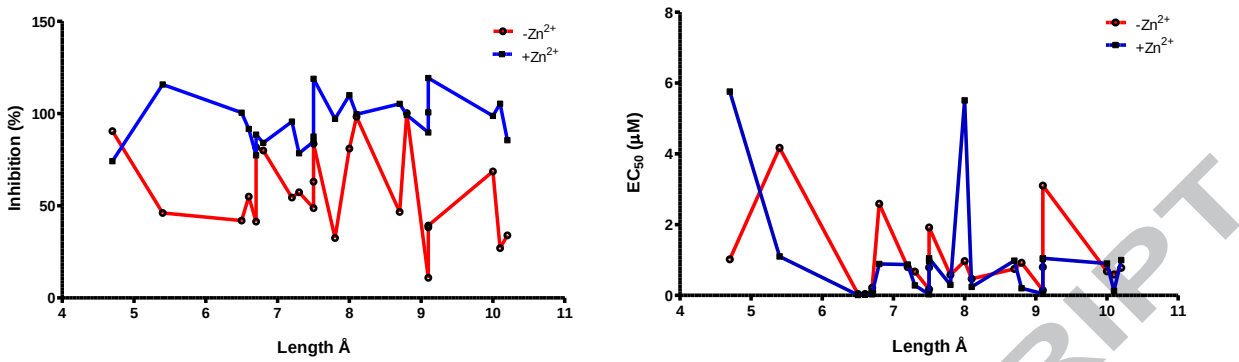


Figure 3. Correlation of CXCR4-binding affinity (competitive inhibition (%) of binding of TAMRA-Ac-TZ14011 (10 nM) in CHO cells stably expressing CXCR4 by the synthesized compounds (1.0 µM) in the presence/absence of 10 µM $ZnCl_2$) **(A)** and anti-HIV activity (EC_{50} (µM)) **(B)** of the synthesized compounds with increasing spatial distances (length (Å)) between the next carbon to Dpa and the next carbon to cyclam (Fig. 1).

2.4. Structure-activity relationship studies (SARs) of selected potent CXCR4 ligands

Based on the anti-HIV activity (Table 1) and the initial data for the CXCR4-binding affinity (Fig. 2) of the synthesized compounds, the IC_{50} values of six selected compounds: **2** and **3** with the naphthalene spacer; **8** and **9** with the biphenyl spacer; **19** and **21** with the 2-phenylpyrindine spacer, were evaluated through a competitive binding assay using the NanoBRET assay system in the presence of zinc (II) ion. All the six compounds showed high binding affinity for CXCR4 compared to the control SKA028-*p*, with **3** showing the highest activity (IC_{50} = 1.6 nM, Table 1, Supplementary data Fig. S2). This observation could be attributed to the spacer distances and/or spatial positioning of the cationic moieties of these six compounds, suggesting that positioning of Dpa and cyclam moieties orient these compounds to achieve suitable spatial length for the interaction with CXCR4 leading to higher binding affinity. As indicated above, a spacer within the range of 6.5-6.7 Å appear to enhance the binding characteristics of the compounds to CXCR4.[4,13] Compounds **3**, **8**, **9** and **21** possessing spatial lengths within the 6.5 - 6.7 Å range (that is, 6.7Å, 6.5Å, 6.6Å, and 6.5Å, respectively) showed high CXCR4-binding affinity (IC_{50} = 1.6,

7.9, 5.7 and 4.5 nM, Table 1, Supplementary data Fig. S2). Compound **19** with a spatial distance of 7.5Å, slightly over the 6.5- 6.7 Å range, showed high affinity (IC_{50} = 3.5 nM) whereas **2** with 8.1Å, certainly over the 6.5- 6.7 Å range, showed slightly weaker affinity (IC_{50} = 10 nM) than other five compounds. Furthermore, among the three group of spacers and spatial positioning of cationic moieties, the rigidity of the naphthalene spacer and the 1, 6-positioning of compound **3** probably contributed largely to the stability of its zinc (II) ion complex resulting in the highest binding affinity of **3** for CXCR4.

There was, however, no defined correlation between the CXCR4-binding and ant-HIV activities of the six compounds analyzed (Table 1). In other words, strong binding to CXCR4 did not necessarily translate into high anti-HIV activity. Compound **3**, for example, does not have the highest anti-HIV activity in the presence of zinc (II) ion (EC_{50} = 28 nM) while it possesses the highest binding affinity for CXCR4 (IC_{50} = 1.6 nM) among the six compounds analyzed. Similarly, compound **8** with the highest anti-HIV activity (EC_{50} = 13 nM) exhibited lower binding affinity (IC_{50} = 7.9 nM) compared to compound **3** in the presence of zinc (II) ion. These differences could be attributed to possible variations in the CXCR4 amino acid residues responsible for TAMRA-Ac-TZ14011 and HIV-1 binding to CXCR4.

2.5. Analysis of binding mode to CXCR4

The plausible binding modes of compound **3** and SKA028-*p* to CXCR4 (PDB ID: 3ODU) were analyzed using 2D Molecular Operating Environment (MOE) software in order to expatiate the SARs of the synthesized compounds on binding to CXCR4 (Fig. 4). In this analysis zinc (II) ions cannot be included. AMD3100 on binding to CXCR4 is predicted to interact with three main residues, Asp171 (transmembrane (TM) IV), Asp262 (TM VI), and Glu288 (TM VII) of CXCR4, whereby one of its two cyclam moieties interacts with Asp171 while the other cyclam moiety is sandwiched by Asp262 and Glu288.[14] Similarly, in the present molecular docking simulation studies, SKA028-*p* and compound **3** showed interactions with Asp171, Asp262 and Glu288, whereby the cyclam moiety of SKA028-*p* and a picolyamine moiety of compound **3** are sandwiched by Asp262 and Glu288. In addition, both SKA028-*p*

and compound **3** showed hydrogen bond interactions with Arg188 and Tyr116. In addition, compound **3** interacts with 1*H*-imidazole of His203 using a lone pair of a nitrogen atom of the cyclam moiety as an H-bond acceptor, and is located close to Val197, Gln200, Ser263, Ile265, Leu266, Gln277 and Ile 284 with non-bonded interactions. Furthermore, the naphthalene spacer of compound **3** was seated in the hydrophobic pocket of CXCR4 interacting with Val196, F199 and Ile259 to enhance its stability. SKA028-*p*, on the other hand, interacts with His281 (through H-bonding) and with Asp187 (through ionic interaction), and is located close to Arg30, Trp94, Trp102, Val112, His113, Cys186 and Ile259 with non-bonded interactions. Taken together, the variation in spacer lengths and/or orientation of cationic moieties appear to influence the CXCR4 amino acid residues available for ligand-receptor interaction, and thus the binding affinity. This observation could be the cause of the disparities in the binding affinities of the six selected compounds and the inconsistency in the trends of the binding affinity and anti-HIV activity. For instance, in addition to Asp262, HIV-1 entry involves Asp193 of the extracellular loop (ECL) 2, mostly *N*-terminal residues of CXCR4 and the upper region of TM II such as Tyr 97,[10,12,14] which are different from the residues observed to the interaction of compound **3**. SKA028-*p* was observed to interact with Arg30 (*N*-terminal region) and Trp94 (TM II), which could account for the higher anti-HIV activity observed despite the weaker CXCR4-binding affinity compared to compound **3**.

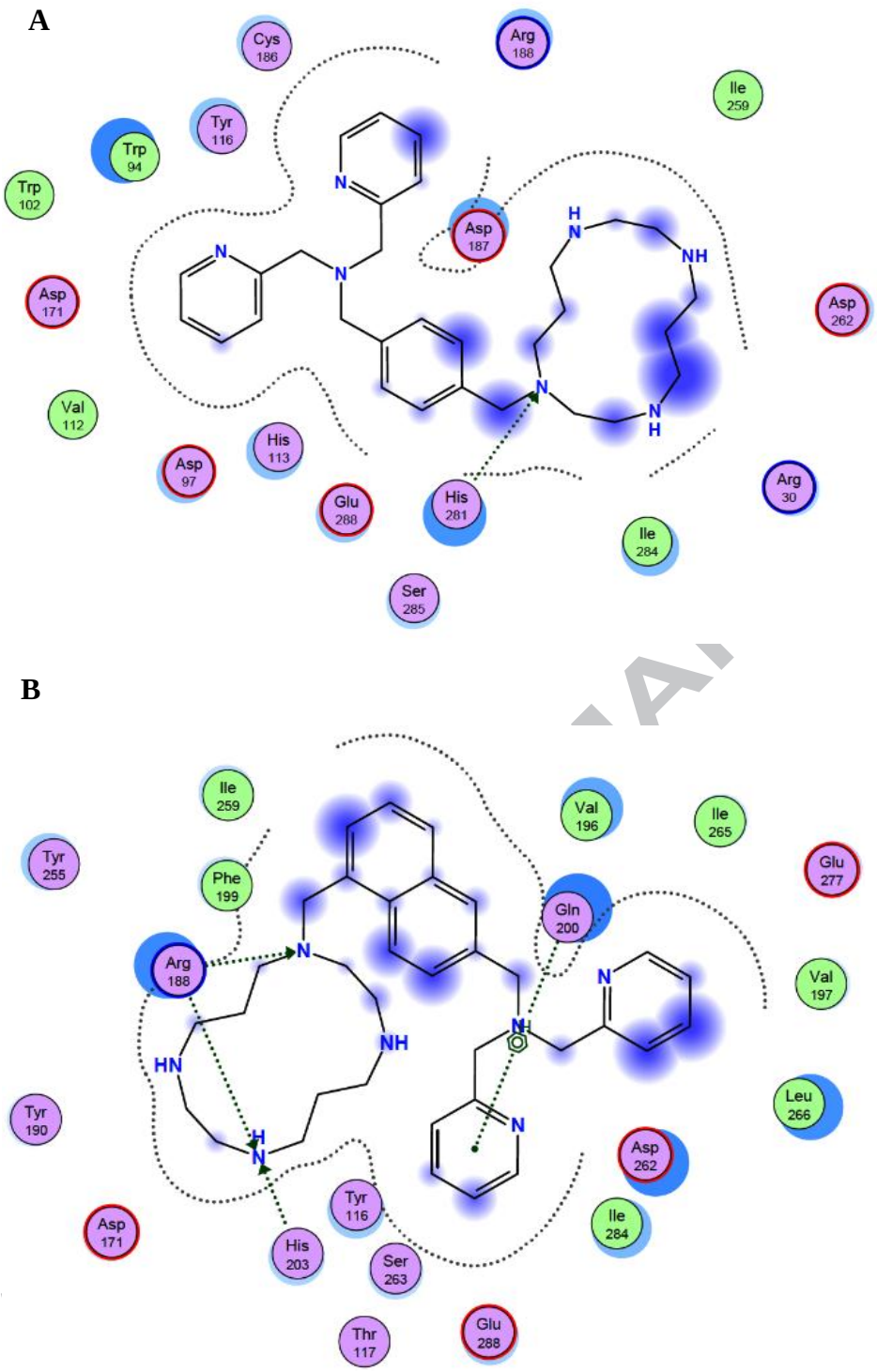


Figure 4. Binding models of (A) SKA028-*p* and (B) compound **3** to CXCR4 (PDB: 3ODU). Green circle: hydrophobic residues without Tyr, purple circle: hydrophilic residues including Tyr (blue outline: basic

residues, red outline: acidic residues), indigo shade: ligand exposure, cyano blue shadow: receptor exposure.

3. Conclusion

In summary, a series of low molecular weight CXCR4 ligands have been designed and synthesized based on a lead compound SKA028-*p* by introduction of spacers which provide longer lengths between the cationic moieties, Dpa-CH_2 and cyclam-CH_2, with emphasis on the spatial positioning of these moieties. All of the synthesized compounds at 1.0 μM with zinc (II) ion exhibited more than 70% inhibition of the binding of a competitive probe TAMRA-Ac-TZ14011 (10 nM) to CXCR4 in the NanoBRET assay. Compounds **3**, **8**, **9, 19** and **21** with spacers within the range of 6.5 Å-7.5Å were identified to exhibit high binding affinity selective for CXCR4 as well as high anti-HIV activity in the presence of zinc (II) ion. The selected compounds interact with varying but overlapping amino acid residues of CXCR4 due to the differences in the spacer lengths and/or orientation of the cationic moieties, accounting for disparities in CXCR4-binding and anti-HIV activities. At least, concerning CXCR4-binding affinity compounds **3**, **8**, **9, 19** and **21** are remarkably more potent than an initial lead SKA028-*p* in the presence of zinc (II) ion whereas the present study has not produced a higher anti-HIV compound compared to that of SKA028-*p*. The present data provide a new window for exploration into the development of more potent CXCR4 ligands, especially low molecular weight compounds, which can be considered in the future drug development for chemotherapy of diseases including cancer.

4. Experimental

4.1. Chemistry

Three representative compounds were synthesized as shown in Schemes 1-3. Detailed procedures and characterization data are described in Supplementary data.

4.2. Biological assay methods

NanoBRET assay

The NanoBRET assay system for CXCR4 ligands was developed in our laboratory.[7] Briefly, post transfected CHO cells stably expressing Nluc-CXCR4 (5 x 10^4) were seeded into a white Thermo Scientific Matrix 96 well microplate and incubated for 24 h at 37 °C under 5% CO_2 prior to experiments. The medium in each well was removed and replaced with OptiMEM (reduced serum). Single point competitive binding assay was performed by incubating seeded cells with TAMRA-Ac-TZ14011 (10 nM) in the presence of a test compound (1 μM) with and without $ZnCl_2$ (10 eq). For IC_{50} value evaluation, the seeded cells were incubated with TAMRA-Ac-TZ14011 (10 nM) in the presence of increasing concentrations of a test compound with $ZnCl_2$ (10 eq). The substrate, furimazine (Promega corperation, USA), reconstituted in the LCS dilution buffer (provided by the manufacturer) was then added to each well (25 μL) according to the manufacturer's protocol and BRET was read immediately using the Wallac 1420 ARVO MX plate reader (fitted with 100 nm filter) at room temperature. The donor emission of Nluc was measured at 460 nm (80 nm bandpass) and the acceptor emission of TAMRA was measured at >610 nm (longpass). Raw BRET ratio was given as;

$$\text{Raw BRET ratio} = \frac{\text{Acceptor emission}}{\text{Donor emission}}$$

The percentage inhibition was calculated with equation below;

$$\text{Inhibition (\%)} = (E0 - Et)/(E0 - E100)\ X100$$

Where E0, Et and E100 represent the BRET ratio without a test compound, BRET ratio in the presence of a test compound, and BRET ratio with excess amount of Ac-TZ14011 as a competitor, respectively.

Anti-HIV assay

Anti-HIV-1 activity was determined based on the protection against X4-HIV-1(NL4-3 stain)-induced cytopathogenicity in MT-4 cells. Various concentrations of a test compound were added to HIV-1-infected MT-4 cells at multiplicity of infection (MOI) of 0.001 and placed in wells of a flat-bottomed microtiter tray (2.0×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of

viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[10]

Data presentation and statistical analysis

The data were presented as mean of independent experiments conducted in triplicate and analyzed using GraphPad Prism software (version 5.0). The IC_{50} and EC_{50} values were generated from non-linear regression analysis.

4.3. Molecular modeling

Molecular modeling calculations were performed using compounds docked into CXCR4 (PDB: 3ODU) with MOE (version 2018.0101, Chemical Computing Group Inc., Montreal, QC, Canada, 2018). In the molecular modeling, global energy minimization of compounds docked into CXCR4 was searched.

Declaration of interest

Conflicts of interest: none.

Acknowledgments

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (JSPS KAKENHI Grant Number JP15H04652). It was also supported in part by the Platform for Drug Discovery, Informatics, and Structural Life Science of MEXT, Japan, and the Cooperative Research Project of Research Center for Biomedical Engineering. M.S. was also supported by MEXT through the award of a scholarship. Our profound gratitude also goes to Prof. Shoji Yamaoka for his immense support.

Supplementary data

Supplementary data (synthetic procedures and characterization data of compounds, an RI-based binding assay method and CXCR4-binding assay data) associated with this article can be found, in the online version, at doi: .bmc. .

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