Table 2 CXCR4 ligands derived from the SDF-1 sequence

Peptide	Sequence	Agonist/antagonist	Ref.
SDF-1(2-67)		Antagonist	62
SDF-1(3-67)		Antagonist	62
SDF K1R		Antagonist	62
SDF P2G		Antagonist	62
SDF-1β P2G		Antagonist	65
SDF-1(1-13)	KPVSLSYRCPCRF	Agonist	64
L5H	KPVSHSYRCPCRF	Antagonist	64
RSVM	RSVMLSYRCPCRFFESH	Partial agonist	66
ASLW	ASLWLSYRCPCRFFESH	Superagonist	66
SD-2	KPVSLSYRCPCRFF-AAAA-RARLKAKLHK	—[a]	67
SD-4	KPVSLSYRCPCRFF-GGGG-RRRRRRRRR	Agonist	67
NCT-tide	LSYRCPCRFF-GGGG-LKWIQEYLEKALN	Agonist	68
CTCE0021 [cyclo(Lys^{20}-Glu^{24})-sdf-(1–31)-NH_2]	KPVSLSYRCPCRFF-GGGG-L**K**WIQ**E**YLEKALN[b]	Agonist	69,70
cyclo(Glu^{24}-Lys^{28})-sdf-(1-31)-NH_2	KPVSLSYRCPCRFF-GGGG-LKWIQ**E**YLE**K**ALN[b]	Agonist	69
CTCE0214	KPVSLSYRAPFRFF-GGGG-L**K**WIQ**E**YLEKALN[b]	Agonist	70
SDF-1(1-9)	KPVSLSYRC	Agonist	71
SDF-1(1-9) dimer	$(KPVSLSYRC)_2$	Agonist	71
SDF-1(1-9) P2G dimer	$(KGVSLSYRC)_2$	Antagonist	71
CTCE-9908	$(KGVSLSYR)_2K$	Antagonist	73

[a] No information on the receptor activation. [b] Cyclic peptide by lactamization between Lys and Glu shown in bold.

provided several potent peptidomimetics analogues such as **10**, including an *N*-alkylated Gly at the D-Pro residue.[60] A recent patent reveals the SAR of the side-chain functional groups.[61] Potent derivatives such as **11** were obtained by substitution of Gln with a modified Lys residue.

3. Small peptide analogues of SDF-1

Since it was demonstrated that SDF-1 prevents infection of T-cell line tropic HIV strains,[1] a number of SDF-1 small peptide analogues have been designed as CXCR4 agonists and antagonists (Table 2). The structure of SDF-1 was determined by NMR spectroscopy[62] and X-ray crystallography.[63] SDF-1 had three structural motifs, including an N-terminal unstructured sequence, a central β-sheet structure and a C-terminal α-helix structure. SAR studies of SDF-1 analogues demonstrated that the disordered N-terminal region takes on a critical role in receptor binding and activation.[62,64] Crump *et al.* revealed that the N-terminal sequence (KPVSLSYR) and secondary region (RFFESH motif) comprise interactive residues for CXCR4, and that the N-terminal Lys1 and Pro2 residues in SDF-1 were involved in receptor activation and function.[62] Substitution of Pro2 with Gly in SDF-1α and SDF-1β prevented the chemotactic effect with potent receptor binding and the receptor internalization effect.[62,65] Heveker *et al.* identified several anti-HIV peptides by the screening and subsequent optimization of SDF-1 peptide fragments.[64] For example, SDF-1(1-13), an N-terminal peptide of SDF-1, represents a CXCR4 agonist that induced an intracellular Ca^{2+} response and receptor desensitization of HeLa cells. In contrast, the analogous peptide L5H, in which the Leu5 of SDF-1(1-13) was substituted with His, did not elicit the same Ca^{2+} response or desensitization with efficient inhibition of HIV-1 entry. Sachpatzidis *et al.* reported two 17-residue allosteric peptide agonists for CXCR4 identified from 160 000 SDF-1-derived peptides using a yeast screening system.[66] RSVM was a weak partial agonist, whilst ASLW showed superagonistic activity with high chemotactic effects. It is of interest that the biological effects of RSVM and ASLW peptides were not prevented by AMD3100 and T140, suggesting binding to alternative sites on CXCR4. However, the potencies of these SDF-1-derived short peptides were significantly lower in comparison with the native SDF-1.

To improve the bioactivity of SDF-1 N-terminal peptides, two approaches were developed to append positively charged sequences to an SDF-1 small peptide.[67] On the basis of the common characteristics between the β-sheet region of SDF-1 and CXCR4 antagonists, a highly positive sequence was conjugated to the C-terminus of SDF-1(1-14). SD-2 containing the β-sheet region of SDF-1 exerted greater binding activity for CXCR4 and inhibitory activity of HIV-1 gp120-mediated cell–cell fusion than SDF(1-14). SD-4 with a nine-arginine sequence at the C-terminus is a distinct agonist that induces intracellular Ca^{2+} flux showing a slow leakage in Sup T1 cells. The mechanism of the Ca^{2+} flux by SD-4 may be different from those in SDF-1 and SDF-1(1-14).

Luo *et al.* demonstrated the contribution of the C-terminal α-helix region of SDF-1 to the bioactivity.[68] NCT-tide was designed by direct conjunction of SDF(5-14) and SDF(55-67) through a four glycine linker in place of the central β-sheet region. This linker mimicked the distance between the N- and C-terminal regions of SDF-1. NCT-tide induced dose-dependent migration of sup T1 cells and intracellular Ca^{2+} flux. More recently, researchers at Chemokine Therapeutics have reported more potent analogues of NCT-tide.[69] The C-terminal α-helix structure in an NCT-tide derivative was stabilized by lactamization between Glu and Lys at the polar surface to provide CTCE0021 with enhanced receptor binding and potent Ca^{2+} mobilization. CTCE0214 is an analogous cyclic peptide, in which the two Cys residues of CTCE0021 were substituted to improve the plasma stability.[70] Dose-dependent chemotaxis migration of $CD34^+$ cells was observed in *in vitro* assays. Evaluation of CTCE0214 for *in vivo* effects in a mouse study also demonstrated increased migration of hematopoietic cells.

Loetscher *et al.* reported that symmetric dimer peptides of the SDF-1 N-terminus bind to CXCR4 as agonists and antagonists.[71] For example, dimerization of SDF-1(1-9) at Cys9 using a disulfide bond provided SDF-1(1-9) dimer, which exhibited 10-fold more potent receptor binding for CXCR4 compared with the monomeric SDF-1(1-9). SDF-1(1-9) dimer can desensitize only CXCR4 and no other chemokine receptor including CXCR3, CCR1, CCR2 and CCR5 as native SDF-1, indicating the specificity for CXCR4. Modification of Pro2 to Gly in the SDF-1(1-9) dimer converted the ligand into an antagonist [SDF-1(1-9) P2G dimer], which did not trigger detectable chemotactic activity. Comparative NMR studies of the SDF(1-17) and SDF-1(1-9) dimers demonstrated both peptide molecules contained two β-αR turn motifs.[72]

CTCE-9908 is an alternative CXCR4 antagonist with a dimerized sequence of SDF-1(1-8).[73] Two peptides having P2G were loaded on α- and ε-amino groups of the C-terminal Lys. CTCE-9908 did not exert significant effects on migration and adhesion of $CD34^+$ cells, whilst increased podia formation of $CD34^+$ cells was observed.[73] In contrast, there have been a number of reports on the inhibition of metastasis of osteosarcoma,[74] melanoma,[74] and breast cancer cells[75] towards secondary organs such as the lung and bone. Additionally, direct effects of CXCR4 inhibition by CTCE-9908 on cancer cell proliferation have also been demonstrated.[75b,d,76] For example, Kwong *et al.* reported the cell death by mitotic catastrophe of ovarian cancer cells by CTCE-9908.[76a]

4. Miscellaneous peptide and peptidomimetic CXCR4 ligands

Although it is well known that SDF-1 is the sole endogenous ligand for CXCR4, the viral macrophage inflammatory protein-II (vMIP-II) encoded by Kaposi's sarcoma-associated herpes virus is a chemokine-like protein capable of binding with CXCR4 (Table 3).[77] CXCR4-selective peptides were designed from the vMIP-II sequence, because vMIP-II moderately inhibits HIV infection through CCR5 and CXCR4. Zhou *et al.* reported that the N-terminal 21-residue peptide of vMIP-II, named V1, blocked SDF-1-mediated chemotaxis and HIV-mediated cell–cell fusion.[78] A subsequent structure–function analysis of the V1 peptide revealed that Leu1, Arg7 and Lys10 were indispensable for CXCR4 binding. V1 derivatives with substitution at Cys11 in V1 with Ala (V1-C11A) or Phe (V1-C11F) significantly improved the receptor binding.[79] A vMIP-II(1-11) dimer was also designed on the basis of the success of the SDF-1(1-9) dimer peptides as CXCR4 ligands.[80] Two vMIP-II(1-11) sequences were dimerized at the C-terminal Cys residue to provide vMIP-II(1-11) dimer, which exhibited slightly more potent receptor binding in comparison with the SDF-1(1-9) dimer. Interestingly, the mirror image peptide DV1 exhibited more potent CXCR4 antagonistic activity than V1.[81] The short sequence analogue DV3 is also a CXCR4 antagonist with less receptor binding activity.[81] NMR studies demonstrated that DV3 displayed partially structured turn conformations,[82] which was consistent with the mirror image conformations of vMIP-II (1-10).[80] When a part of the DV3 sequence was appended onto SDF-1(9-68), the hybrid peptide RCP222 showed comparable receptor binding to SDF-1α but no Ca^{2+} flux induction.[83] Ligand binding site mapping using a panel of CXCR4 mutants revealed that RCP222 shared the interactive residues on CXCR4 with HIV-1 gp120 rather than the parent ligand SDF-1α.[84]

Pepducins are synthetic lipopeptides of the intracellular loop sequence in GPCRs.[85] Following penetration through the cell membrane, the pepducins interact with the intracellular component including signaling molecules to regulate signal transduction. Tchernychev *et al.* reported that ATI-2341 is a potent CXCR4 agonist to induce Ca^{2+} flux, chemotaxis and receptor internalization.[86] The palmitic acid-conjugated 16-residue peptide in the intracellular loop 1 of CXCR4 induced the peritoneal recruitment of polymorphonuclear neutrophils *in vivo*. Recently, mechanistic insights for ATI-2341 bioactivity were reported by photochemical crosslinking experiments using a photoaffinity probe ATI-2766.[87] The binding site of ATI-2766 on CXCR4 was distinct from the ones of SDF-1 and T140, suggesting ATI-2341 is an allosteric agonist for CXCR4.

In a separate study, several positively charged peptides were reported to inhibit HIV-1 replication through competitive binding with CXCR4. ALX40-4C is an anti-HIV peptide, which was originally designed from the basic domain of the HIV-1 transactivation domain for an inhibitor of the Tat–TAR interaction.[88] Subsequent investigations demonstrated that the anti-HIV effects of ALX40-4C against X4 and dual-tropic HIV-1

Table 3 Miscellaneous CXCR4 ligands

Peptide	Sequence	Agonist/antagonist	Ref.
vMIP-II		Antagonist	77
V1 [vMIP-II(1-21)]	LGASWHRPDKCCLGYQKRPLP	Antagonist	78
V1-C11A	LGASWHRPDKACLGYQKRPLP	—[a]	79
V1-C11F	LGASWHRPDKFCLGYQKRPLP	—[a]	79
vMIP-II(1-11) dimer	$(LGASWHRPDKC)_2$		80
DV1	lgaswhrpdkcclgyqkrplp	Antagonist	81
DV3	lgaswhrpdk	Antagonist	81
RCP222 [D-vMIP-II(1-10)-SDF-1(9-68)]		Antagonist	83
ATI-2341	palmitoyl-MGYQKKLRSMTDKYRL	Agonist	86
ALX40-4C	Ac-rrrrrrrrr	Antagonist	88
DC13	Ac-rrmyrriyrr	Antagonist	91
β-Defensin 3 (hBD3)		Antagonist	92

[a] No information of the receptor activation.

Table 4 Imaging probes for CXCR4

Peptide	Sequence[a]	Ref.
In-DTPA-Ac-TZ14011	*Ac*-RRX^{1}CYX^{2}R-k(DTPA)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	93,94
In-DTPA-TN14003	*Ac*-RRX^{1}CYX^{2}K-k(DTPA)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	94
In-DTPA-TF14016	*4FB*-RRX^{1}CYX^{2}K-k(DTPA)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	94
Ac-TZ14011-MSAP	*Ac*-RRX^{1}CYX^{2}R-k(linker-DTPA/CyAL-5.5$_b$)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	95
T140-2D	*4FB*-RRX^{1}CYX^2-K(DOTA)-k(DOTA)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	97
DOTA-NFB	DOTA-RRX^{1}CYX^{2}KkPYRX^{2}CR-*NH$_2$* (C–C bridged)	98
NOTA-NFB	NOTA-RRX^{1}CYX^{2}KkPYRX^{2}CR-*NH$_2$* (C–C bridged)	98
4-^{18}F-T140	(*^{18}F-4FB*)-RRX^{1}CYX^{2}KkPYRX^{2}CR-*NH$_2$* (C–C bridged)	99
^{68}Ga-CPCR4-2	see Fig. 6	100,101
^{68}Ga-25	see Fig. 6	52*b*
FITC-SDF-1		102
CXCL12^{AF647}		103
TY14003	*Ac*-RRX^{1}CYX^{2}R-k(FL)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	105
TAMRA-Ac-TZ14011	*Ac*-RRX^{1}CYX^{2}R-k(Acp-TAMRA)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	107
Ac-TZ14011-FITC	*Ac*-RRX^{1}CYX^{2}R-k(FITC)-PYRX^{2}CR-*NH$_2$* (C–C bridged)	108

[a] X^1: L-3-(2-naphthyl)alanine; X^2: L-citrulline; 4FB: 4-fluorobenzoyl; FL: carboxyfluorescein; Acp: 6-aminohexanoic acid; TAMRA: tetramethylrhodamine; FITC: fluorescein isothiocyanate; DTPA: diethylenetriaminepentaacetic acid; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; NOTA: 1,4,7-triazacyclononane-1,4,7-triacetic acid.

strains (but not against R5 strains) were derived from the selective binding to CXCR4.[89] The binding sites of ALX40-4C for CXCR4 were overlapping with those of SDF-1 and 12G5. It is worthy of note that Zhou *et al.* revealed that the APJ receptor is an alternative target of ALX40-4C to block HIV gp120 binding to the cell membrane.[90] On the basis of the successes in ALX40-4C and SDF-1-derived D-peptides, a library of D-amino acid decapeptides was prepared for the development of novel HIV entry inhibitors.[91] Among the D-peptides tested, DC13 showed the most potent inhibitory activity against both X4 and R5 HIV-1 strains. The mechanism of anti-HIV activity against X4 strains was verified by the inhibitory effects of SDF-1 and 12G5 binding to CXCR4, whilst the mechanism of action for R5 strains has not been determined. β-Defensin 3 (hBD3) also inhibits SDF-1-mediated Ca^{2+} mobilization and ERK phosphorylation.[92] The anti-HIV activity of hBD3 is derived from the receptor internalization of CXCR4 and direct interaction with HIV-1 virions.[92a]

5. Imaging probes for CXCR4

5.1. Radiolabeled CXCR4 probes

The first report of radiolabeled imaging probes for CXCR4 was the ^{111}In-labeled T140 derivative [^{111}In-DTPA-Ac-TZ14011 (DTPA: diethylenetriaminepentaacetic acid)] (Table 4, Fig. 5).[93] Ac-TZ14011 with a single Lys residue was designed for the site-selective conjugation of radiolabels. In biodistribution experiments, high accumulation of ^{111}In-DTPA-Ac-TZ14011 was observed in the CXCR4-expressing tumors compared with the blood and muscle. Recently, a more concise synthetic protocol for In-DTPA-Ac-TZ14011 was reported.[94] In the article, a more potent analogue, In-DTPA-TF14016, with an N-terminal 4-fluorobenzoyl group was also described. Kuil *et al.* reported bimodal antagonist probes having a DTPA and a fluorescent CyAL-5.5$_b$.[95] Ac-TZ14011-MSAP was prepared using a multifunctional single-attachment-point (MSAP) reagent[96] and was used for SPECT/CT and fluorescent imaging. Dimeric and

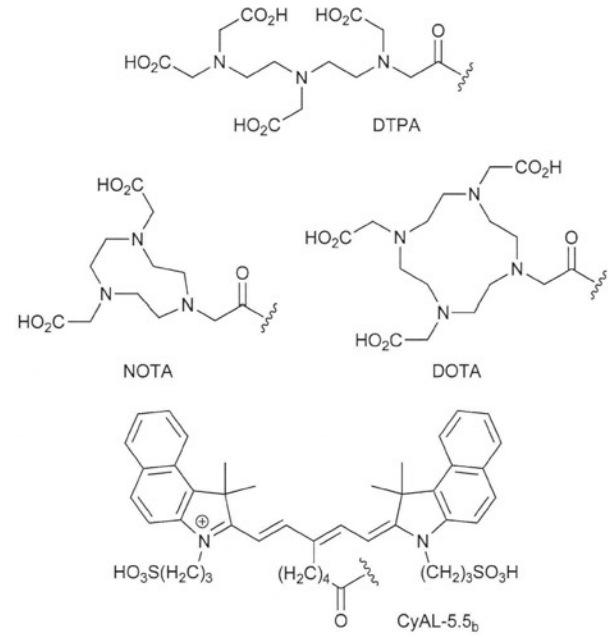

Fig. 5 Structures of metal-chelating and labeling functional groups for imaging probes.

tetrameric Ac-TZ14011 units, which were conjugated to a DTPA/CyAL-5.5$_b$ MSAP reagent, were also designed to improve the receptor binding and biodistribution.[95b]

Jacobson *et al.* reported a CXCR4 antagonist probe, designated T140-2D, in which two DOTA groups were appended onto Lys^7 and D-Lys^8 in TF14016 (DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).[97] Using ^{64}Cu and ^{68}Ga as radionuclei for PET imaging and biodistribution analysis, CXCR4-expressing tumors were clearly visualized, although significant accumulation in the liver and kidneys was also observed. Recently, the same group reported DOTA- and NOTA-labeled CXCR4 tracers (NOTA: 1,4,7-triazacyclononane-1,4,7-triacetic acid), which do not bind to undesired targets such as red blood cells.[98]

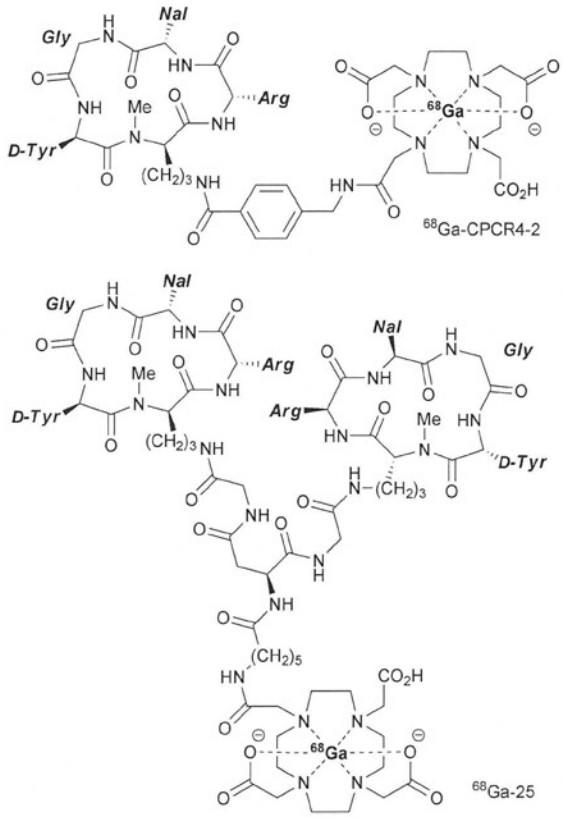

Fig. 6 Structures of FC131-based imaging probes.

4-^{18}F-T140 is an alternative CXCR4 probe for PET imaging, which was designed based on the structure of TF14016.[99] An ^{18}F-fluoride labeling was appended to the N-terminal 4-fluorobenzoyl group by using an *N*-succimidyl 4-^{18}F-fluorobenzoate (^{18}F-SFB) reagent. For the site-selective labeling and short-time treatment after ^{18}F-SFB-mediated labeling, *N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl] (Dde) groups were used as protecting groups for two Lys side-chains. In PET studies and biodistribution experiments using 4-^{18}F-T140, clear visualization of CXCR4-transfected CHO cells with high tumor-to-muscle and tumor-to-blood ratios were observed.

Cyclic pentapeptide-based antagonist FC131 provided an alternative series of CXCR4-specific probe molecules (Fig. 6). Demmer *et al.* reported a DOTA-conjugated cyclic peptide ^{68}Ga-CPCR4-2, in which the labeling group was appended onto the side-chain of D-N^{α}-methylornithine (D-MeOrn) through a (4-aminomethyl)benzoic acid (Amb) linker.[100] This tracer peptide showed equipotent receptor binding to the parent FC131 and high tumor accumulation.[100,101] The ^{68}Ga-labeled dimeric peptide (^{68}Ga-25) of the FC131 derivative was also designed on the basis of the known antagonists with a C_2 symmetry element, allowing visualization of an OH1 h-SCLC xenograft by PET imaging.[52b]

5.2. Fluorescent CXCR4 probes

There have been several reports on fluorescent SDF-1 derivatives, which were used to detect CXCR4 expression and localization (Table 4). Dar *et al.* studied the internalization of SDF-1 by bone marrow endothelial cells using fluorescein isothiocyanate (FITC)-labeled SDF-1 (FITC-SDF-1).[102] In confocal microscopy analysis, co-localization of the internalized FITC-SDF-1 with α-adapton was observed, suggesting that the internalization was mediated by clathrin-coated pits. Hatse *et al.* evaluated the expression level of CXCR4 in peripheral blood mononuclear cells using CXCL12^{AF647}, in which AlexaFluor 647 was conjugated at the second residue to the C-terminus.[103] The biological activities of CXCL12^{AF647} including intracellular Ca^{2+} mobilization and MAPK phosphorylation were compatible to those of unlabeled SDF-1. Strong *et al.* also utilized CXCL12^{AF647} for flow cytometry and binding inhibition experiments.[104]

Development and application of antagonist-based probes for CXCR4 have also been reported (Table 4). TY14003 is a fluorescent T140 derivative, in which the Lys ε-amino group at the β-turn of Ac-TZ14011 was modified with carboxyfluorescein.[105] CXCR4 expression on migrated cells in the chemotaxis experiments were clearly characterized by flow cytometry analysis using TY14003. TY14003 also labeled a CXCR4-expressing bladder cancer cell line as well as urothelial cells in urinary sediments from patients with invasive bladder cancer.[106] In *in vivo* fluorescent labeling experiments, mouse *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced bladder cancer in the focal areas was detected by intravesical administration of TY14003.[106]

Fluorescent labeling with TAMRA and FITC at the β-turn position of Ac-TZ14011 was also tolerated. TAMRA-Ac-TZ14011 was employed for receptor staining of NP-2 CXCR4-GFP and CCR-CD4-HeLa cells.[107] When fluorescent immunohistochemistry of tumor tissue using Ac-TZ14011-FITC was performed, a different staining pattern between MDAMB231 and MDAMB231^{CXCR4+} cells was observed.[108] Luminescent iridium dye complexes of Ac-TZ14011 for CXCR4 imaging were also reported recently, in which the cyclometalated octahedral iridium(III) 2-phenylpyridine complex was modified with one or multiple Ac-TZ14011 peptides. These probes detect CXCR4 expression efficiently by confocal microscopy and flow cytometry.[109]

6. Conclusions and future perspectives

In this review article, the medicinal chemistry processes of peptide and peptidomimetic CXCR4 ligands have been described. Descriptions included polyphemusin II-derived antagonists, chemokine-derived agonists/antagonists and receptor fragment pepducin. Several potent substances have also been employed for the design of CXCR4 imaging probes with a variety of labeling groups for cancer diagnostics. In the last decade, considerable progress has been made in the cancer biology of CXCR4-expressing cells. These peptide and peptidomimetic ligands have served as selective inhibitors and probe molecules for basic biological and biophysical research on the SDF-1/CXCR4 axis.

Although it had been believed that SDF-1 mediated biological process *via* a sole receptor CXCR4, RDC1/CXCR7, an orphan GPCR, was identified as another receptor of SDF-1 in 2005.[110] CXCR7 recognizes SDF-1 and interferon-inducible T cell

α chemoattractant (I-TAC)/CXCL11 as the endogenous agonists and CXCR7 expression provides tumor cells with cell growth and adhesion properties.[111,112] Future investigations using selective CXCR4 and CXCR7 ligands will reveal the distinct pivotal roles of SDF-1/CXCR4 and SDF-1/CXCR7 axes in physiological and pathogenetical processes.

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Paradoxical Downregulation of CXC Chemokine Receptor 4 Induced by Polyphemusin II-Derived Antagonists

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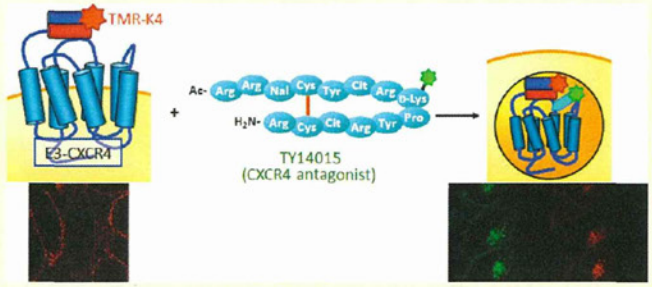
Supporting Information

ABSTRACT: CXC chemokine receptor 4 (CXCR4) is a G protein-coupled receptor implicated in cell entry of T-cell line-tropic HIV-1 strains. CXCR4 and its ligand stromal cell derived factor-1 (SDF-1)/CXCL12 play pivotal parts in many physiological processes and pathogenetic conditions (e.g., immune cell-homing and cancer metastasis). We previously developed the potent CXCR4 antagonist T140 from structure–activity relationship studies of the antimicrobial peptide polyphemusin II. T140 and its derivatives have been exploited in biological and biomedical studies for the SDF-1/CXCR4 axis. We investigated receptor localization upon ligand stimulation using fluorescent SDF-1 and T140 derivatives as well as a specific labeling technique for cellular-membrane CXCR4. Fluorescent T140 derivatives induced translocation of CXCR4 into the perinuclear region as observed by treatment with fluorescent SDF-1. T140 derivative-mediated internalization of CXCR4 was also monitored by the coiled-coil tag-probe system. These findings demonstrated that the CXCR4 antagonistic activity and anti-HIV activity of T140 derivatives were derived (at least in part) from antagonist-mediated receptor internalization.



INTRODUCTION

CXC chemokine receptor 4 (CXCR4) is a G-protein-coupled receptor. It is widely expressed in leukocytes such as T-cells, B-cells, and monocytes.[1,2] Under physiological conditions, the endogenous ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12, is secreted by bone marrow stromal cells for expansion and development of precursor B-cells.[3] High concentrations of SDF-1 are present at inflammatory sites, so the migration of CXCR4-expressing stem cells toward an SDF-1 gradient promotes repair of injured tissues.[4] There have been many reports on the pathology of CXCR4-related cancer, including CXCR4 overexpression and organ-specific metastasis among various types of cancer cells.[5,6] During metastasis, SDF-1 from secondary lesions functions as a chemoattractant for directional migration of CXCR4-expressing malignant cells.[5,6]

The activation process of CXCR4 by SDF-1 has been well documented.[7] Upon SDF-1 binding, CXCR4 evokes downstream signaling via dissociation of heterotrimeric G proteins, followed by decrease in intracellular cyclic adenosine monophosphate (cAMP) concentrations, upregulation of Ca^{2+} release, and increase in extracellular-signal-regulated kinase (ERK) 1/2 phosphorylations.[8−10] Furthermore, CXCR4 internalization in response to SDF-1 occurs in early endosomes through β-arrestin recruitment, just like other GPCRs,[11] in which phosphorylated serine residues and a dileucine motif at the CXCR4 C-terminus have critical roles.[12,13] The complex is sorted into late endosomes/lysosomes for the degradation pathway or for recycling endosomes.[11,14] In addition, CXCR4 is used as a major co-receptor for the entry of T-cell line tropic human immunodeficiency virus type 1 (HIV-1) into target host cells.[15,16] The inhibitory effect of SDF-1 on HIV infection is thought to be by competitive binding to CXCR4 as well as CXCR4 downregulation.[17,18] CXCR4 is a promising molecular target for potential anti-metastatic agents and anti-HIV agents, so several CXCR4 ligands have been developed.[5,19−22]

We previously developed the potent anti-HIV peptide T140. This was designed from the structure–activity relationship studies of a self-defense peptide of horseshoe crabs, polyphemusin II (Figure 1).[19] Inhibition of HIV-induced cytopathogenicity by T140 and its derivatives was derived from selective CXCR4 antagonistic and/or inverse agonistic activity (in which the basal signal levels were decreased in the guanosine triphosphate (GTP) binding and intracellular calcium flux assay using a constitutively active mutant).[23] A recent report on the crystal structure of CXCR4 in complex with the T140 derivative CVX15 revealed that arginine residues in CVX15 made polar interactions with Asp171 and Asp187 in CXCR4.[24] Point-mutation experiments of CXCR4 revealed that additional residues on the extracellular domain (Arg188, Gly207, and Asp262) are necessary for the interaction of

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H-Arg-Arg-Nal-Cys-Tyr-Arg-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-OH T140
H-Arg-Arg-Nal-Cys-Tyr-Gln-Lys-D-Pro-Pro-Tyr-Arg-Cit-Cys-Arg-Gly-D-Pro-OH CVX15
4FBz-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2 TF14016
R
Ac-Arg-Arg-Nal-Cys-Tyr-Cit-Arg-D-Lys-Pro-Tyr-Arg-Cit-Cys-Arg-NH_2 TY14015 (R = AF488)
TR14011 (R = TMR)

Figure 1. Amino acid sequences of CXCR4 antagonist peptides. The disulfide bonds between cysteine residues are shown by solid lines. D-Amino acids are not first-letter capitalized. Abbreviations: Nal, L-3-(2-naphthyl)alanine; Cit, L-citrulline; 4FBz, 4-fluorobenzoyl; AF488, AlexaFluor 488; TMR, tetramethylrhodamine.

T140, which is independent of the interaction with SDF-1.[25,26] These residues contribute to stabilization of the CXCR4 structure via formation of hydrogen bonds with adjacent residues, so T140 binding to these residues may impair SDF-1 binding via conformational changes of CXCR4. ALX-40C is an alternative CXCR4 antagonist that inhibits HIV-1 infection into T-cells.[27] The anti-HIV activity of ALX-40C with a sequence of nine D-Arg is also reported to be derived from binding to acidic residues in the second extracellular loop of CXCR4.[27]

It was demonstrated that highly basic peptides such as ALX-40C and HIV-1 Tat translocate into the intracellular compartment through endocytosis.[28−31] On the basis of the biological properties of these highly basic peptides, we assumed that polyphemusin II-derived CXCR4 antagonists could induce the internalization and/or translocation of receptors into the intracellular compartment, resulting in the apparent antagonistic or inverse agonistic activity for CXCR4.

In the present study, we investigated the mechanism of action of T140 derivatives using novel fluorescent probes. The uptake and localization of CXCR4 was monitored by analyses of flow cytometry and confocal microscopy using the coiled-coil tag-probe system[32] for the labeling of cell-surface CXCR4.

■ EXPERIMENTAL SECTION

Quantitative Analyses of Cell-Surface E3-CXCR4 Using a Fluorescent K4-Peptide by Flow Cytometry. E3-CXCR4 CHO cells were detached using versene and incubated with 100 nM FL-K4 in F-12 medium (500 μL) at 0 °C for 15 min in the absence or presence of unlabeled K4-peptide or SDF-1. The intensity of cell staining was analyzed using a FACScalibur system (BD Biosciences, San Jose, CA, USA). Ten thousand events per sample were analyzed, and the data collected from FL1 in log mode. Fluorescent intensity was calculated as a geometric mean of cellular fluorescence. Data were analyzed using *CellQuest Pro* software (BD Biosciences). Data were analyzed using a two-tailed Student's *t* test with significance set at $p \leq 0.05$.

Quantitative Analyses of Ligand-Mediated CXCR4 Internalization by Flow Cytometry. E3-CXCR4 CHO cells were detached using versene and resuspended in F-12 medium (100 μL) containing each ligand. After incubation at 37 °C for 30 min, ice-cold F-12 medium (400 μL) was added to the mixture, and cells centrifuged at 500 × *g* for 5 min at 4 °C. Cell pellets were then incubated with 100 nM FL-K4 in F-12 (100 μL) at 0 °C for 15 min. The mixture was diluted with ice-cold F-12 medium (400 μL), and analyzed using a FACScalibur flow cytometer.

Binding and Displacement of [^{125}I]-SDF-1. A membrane fraction of cells expressing CXCR4 was incubated with 0.5 nM of [^{125}I]-SDF-1 and FL-K4 in binding buffer [50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA) in H_2O] for 1 h at room temperature. Reaction mixtures were filtered through GF/B filters (PerkinElmer, Waltham, MA, USA) pretreated with 0.1% polyethyleneimine. The filter plate was washed with wash buffer [50 mM HEPES (pH 7.4), 500 mM NaCl, and 0.1% BSA in H_2O] and bound radioactivity measured by TopCount (PerkinElmer).

Confocal Microscopy Analyses of Ligand and CXCR4 Internalization. E3-CXCR4 CHO cells were plated on 35 mm glass-bottomed dishes and cultured in F-12 medium containing 10% heat-inactivated fetal bovine serum supplemented with penicillin/streptomycin and hygromycin. Cells were washed once with cold F-12 medium, and incubated with fluorescent ligands [SDF-1^{AF488} (100 nM) or TY14015 (1 μM)] in F-12 medium (100 μL) at 30 °C for 30 min. After rinsing once with cold F-12 medium, cells were observed by confocal microscopy (Eclipse Ti-E: Nikon, Tokyo, Japan). To monitor CXCR4 localization, E3-CXCR4 CHO cells were pretreated with 100 nM fluorescent K4-peptide in F-12 medium (100 μL) at 0 °C for 15 min before treatment with CXCR4 ligands.

For staining of cellular compartments, after incubation with fluorescent ligands or K4-peptide, cells were rinsed once with cold F-12 medium and treated with the marker (FM 4−64 for the cell membrane; LysoTracker Red DND-99 for lysosomes; ER-tracker Red for the endoplasmic reticulum; or AlexaFluor 568-conjugated transferrin for endosomes) according to the manufacturer (Invitrogen, Carlsbad, CA, USA, for all markers) protocol. The green (AlexaFluor 488 and ATTO488) channel was excited by a 488 nm laser and detected through a BP 500−550 nm emission filter. The red (TMR and AlexaFluor 568) channel was excited by a 568 nm laser, and detected through a BP 575−605 nm emission filter. The blue (FM 4−64) channel was excited by a 568 nm laser, and detected through a LP 665 nm emission filter. Data were analyzed using *EZ-C1 Viewer* software (Nikon).

■ RESULTS

Labeling of Cell-Membrane CXCR4 by the Coiled-Coil Tag-Probe System. A stable CXCR4-expressing cell line was established to monitor the internalization of CXCR4. The surface-exposed tag sequence E3 $(EIAALEK)_3$ was appended at the N-terminus of CXCR4 for detection using the peptide probe K4 $(KIAALKE)_4$ with an appropriate tracer group.[32] This coiled-coil tag-probe system provides several distinct advantages to visualize cell-surface CXCR4. For example, K4-peptide is much smaller than anti-epitope antibodies, so ligand binding to the receptor is hardly disturbed. In addition, specific labeling of E3-tagged receptors on cell membranes with fluorescent K4-peptides can distinguish the internalized receptor from the receptor that is originally present in the cytosolic compartment. This is in contrast to receptors fused with fluorescent proteins, which have usually been employed for monitoring receptor localization.[14,33]

CHO cells stably expressing E3-tagged CXCR4 (E3-CXCR4) were generated by the Flp-In expression system, and were studied by flow cytometric analyses. E3-CXCR4

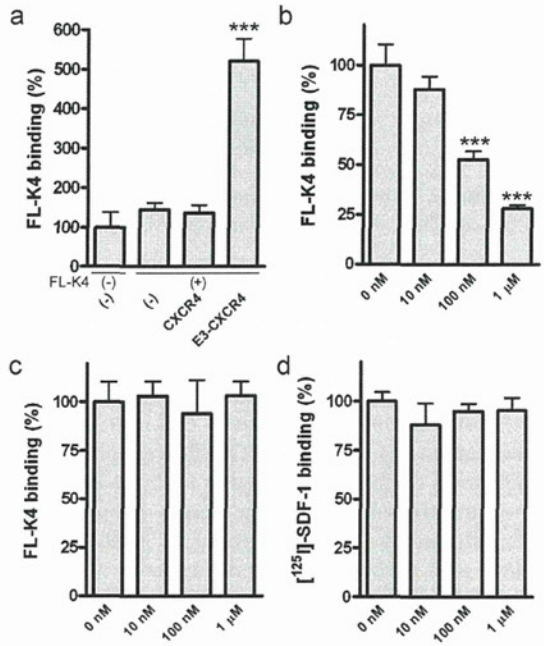

Figure 2. Labeling of E3-CXCR4 cells with fluorescein-K4-peptide (FL-K4). (a) FL-K4 binding to CXCR4 cell lines. After cells were treated with FL-K4 (100 nM) at 0 °C for 15 min, bound FL-K4 was measured by flow cytometry. (b) Inhibition of FL-K4 binding to E3-CXCR4 by unlabeled K4-peptide. After E3-CXCR4 CHO cells were labeled with FL-K4 (100 nM) in the presence of various concentrations of unlabeled K4-peptide at 0 °C for 15 min, bound FL-K4 was measured by flow cytometry. (c) Effect of SDF-1 on FL-K4 binding to E3-CXCR4. After E3-CXCR4 CHO cells were labeled with FL-K4 (100 nM) in the presence of various concentrations of SDF-1 at 0 °C for 15 min, bound various concentrations of FL-K4 was measured by flow cytometry. (d) Effect of K4-peptide on SDF-1 binding to E3-CXCR4. [^{125}I]-SDF-1 (0.5 nM) binding to E3-CXCR4. (±S.D., $n = 3$; *** $p \leq 0.005$).

CHO cells were clearly seen to be stained by fluorescein-conjugated K4-peptide (FL-K4) (Figure 2a). This staining was inhibited by unlabeled K4-peptide in a dose-dependent manner, suggesting specific labeling of E3-CXCR4 by interaction between E3-tag and K4-peptide (Figure 2b). FL-K4 binding to E3-CXCR4 was not disturbed by SDF-1 even at 1 μM, which demonstrated that FL-K4-mediated staining was independent of SDF-1 binding to CXCR4 (Figure 2c). SDF-1 binding to E3-CXCR4 was also unaffected by K4-peptide, which was verified by the binding inhibition assay using [^{125}I]-SDF-1 (Figure 2d). Taken together, specific fluorescent labeling of CXCR4 was accomplished by the coiled-coil tag-probe system without mutual competitive inhibition of SDF-1 and K4-peptide binding to the receptor.

Monitoring and Quantitative Analyses of SDF-1-Induced CXCR4 Internalization. The level of residual CXCR4 on the cell membrane after SDF-1 stimulation has been measured by flow cytometry using a CXCR4-specific antibody.[34,35] For example, Honczarenko et al. assessed SDF-1-induced internalization of CXCR4 by staining cell-surface CXCR4 by the monoclonal antibody 12G5.[34] However, the possible competitive binding of SDF-1 and antibody to cell-surface CXCR4 may impair receptor detection. To overcome this potential disadvantage, the coiled-coil tag-probe pair system could be an alternative to quantify cell-surface CXCR4.

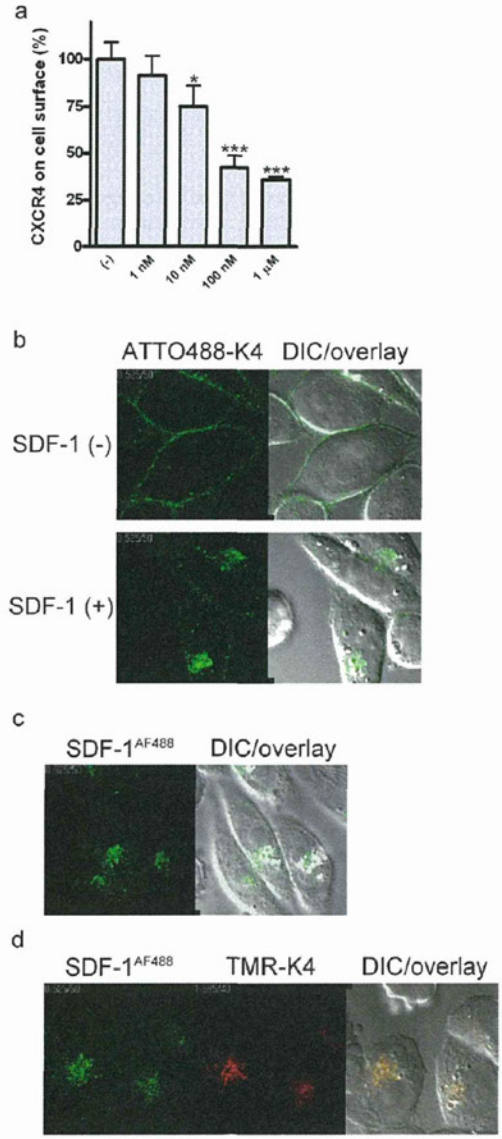

Figure 3. CXCR4 internalization induced by SDF-1 derivatives. (a) Quantitative analyses of E3-CXCR4 internalization by flow cytometry. After E3-CXCR4 CHO cells were treated with various concentrations of SDF-1 at 37 °C for 30 min, cells were labeled by FL-K4 (100 nM) at 0 °C for 15 min (±S.D., $n = 3$; * $p \leq 0.05$; *** $p \leq 0.005$). (b) Confocal microscopy images of SDF-1-mediated CXCR4 internalization. After E3-CXCR4-expressing cells were labeled with ATTO488-K4 (100 nM) at 0 °C for 15 min, cells were treated with SDF-1 (100 nM) at 30 °C for 30 min. (c) Confocal microscopy images of internalized fluorescent SDF-1. E3-CXCR4-expressing cells were treated with SDF-1^{AF488} (100 nM) for 30 min. (d) Confocal microscopy images of internalized SDF-1 (green) and CXCR4 (red). After E3-CXCR4-expressing cells were labeled with TMR-K4 (100 nM) at 0 °C for 15 min, and cells were treated with SDF-1^{AF488} (100 nM) at 30 °C for 30 min. Representative z-slice confocal microscopy images are shown.

SDF-1-mediated CXCR4 internalization was investigated using the established system. Initially, quantitative analyses of residual cell-surface E3-CXCR4 after SDF-1 stimulation were undertaken by flow cytometry. Cells were stained with FL-K4 after E3-CXCR4 CHO cells were treated with SDF-1 over a range of concentrations. The fluorescent intensity of FL-K4 was significantly decreased in a dose-dependent manner (Figure 3a). Confocal microscopy studies using ATTO488-conjugated

K4-peptide (ATTO488-K4) confirmed the translocation of E3-CXCR4 to the intracellular compartment by treatment with SDF-1, whereas ATTO488-K4/E3-CXCR4 remained on the cell surface without SDF-1 treatment (Figure 3b). Both results suggested that SDF-1 stimulation induced FL-K4 labeled E3-CXCR4 internalization, and that the E3 sequence on the N-terminus of CXCR4 could work as a functional tag to detect receptor localization without disturbing receptor internalization.

CXCR4 internalization was also monitored by confocal microscopy using a fluorescent SDF-1 derivative.[36] Translocation of SDF-1^{AF488} into the intracellular compartment was observed by treatment of E3-CXCR4 cells (without labeling by fluorescent K4-peptide) (Figure 3c). This was consistent with the SDF-1-mediated internalization of FL-K4-labeled E3-CXCR4 (Figure 3b). The intracellular localization of SDF-1^{AF488} was not observed in CHO cells without CXCR4 expression (see Supporting Information, Supplementary Figure 1). Hence, it could be concluded that this translocation was mediated by the interaction with CXCR4. To confirm the colocalization of SDF-1 and CXCR4, the same experiment was conducted using E3-CXCR4-expressing cells labeled with TMR-conjugated K4-peptide (TMR-K4). Incubation of cells with SDF-1^{AF488} induced translocation of TMR-K4/E3-CXCR4 complexes into the intracellular compartment, which was verified by colocalization of SDF-1^{AF488} and TMR-K4/E3-CXCR4 (Figure 3d). An identical phenotype was observed in an experiment using SDF-1^{TMR} and ATTO488-K4/E3-CXCR4, indicating that the fluorophore functional groups on the ligand and receptor did not influence the translocation (see Supporting Information, Supplementary Figure 2).

Polyphemusin-Derived CXCR4 Antagonists Induce Receptor Internalization. Next, we investigated receptor internalization by CXCR4 antagonists using the coiled-coil tag-probe system. E3-CXCR4 CHO cells were treated with polyphemusin II and CXCR4 antagonists (TF14016[37] or FC131[38]) at 37 °C. The proportion of residual receptors on the cell membrane was subsequently determined by flow cytometry in the presence of FL-K4 (Figure 4a). The fluorescence intensity of FL-K4 was reduced by 20−25% by the antagonists.[39] In contrast, a decrease in FL-K4 fluorescence was not observed in the same experiment at 0 °C, in which receptor internalization does not occur,[40] suggesting that the E3-tag/K4-peptide interaction was not inhibited by the antagonists. Antagonist-induced internalization was also confirmed by fluorescence microscopy analyses of ATTO488-K4/E3-CXCR4 CHO cells. TF14016 induced the translocation of ATTO488-K4/E3-CXCR4 into the perinuclear region, just like that seen in SDF-1 stimulation (Figure 4b). As such, it was demonstrated that CXCR4 antagonists partially induced receptor internalization.

The fluorescent CXCR4 antagonist TY14015 was similarly accumulated in the cytosolic perinuclear domain in E3-CXCR4 CHO cells after 30 min incubation at 30 °C (Figure 4c). TY14015-mediated translocation was not observed in the same experiments using nontransfected CHO cells,[41] nor by incubation of E3-CXCR4 CHO cells with TY14015 at 0 °C (see Supporting Information, Supplementary Figure 1). These findings indicated that CXCR4 serves as an essential receptor for the translocation of CXCR4 antagonists by an active pathway such as endocytosis. In contrast to the experiment using SDF-1^{AF488} (Figure 3c), staining of the cell membrane by TY14015 was also observed (Figure 4c).

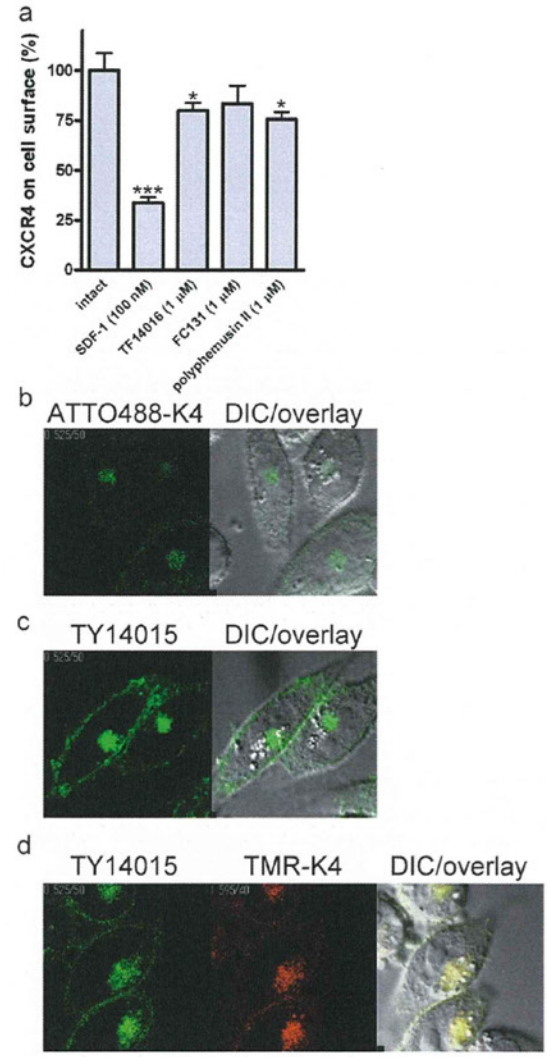

Figure 4. CXCR4 internalization upon stimulation by CXCR4 antagonists. (a) Quantitative analyses of E3-CXCR4 internalization by flow cytometry. After E3-CXCR4 CHO cells were treated with each antagonist (1 μM) at 37 °C for 30 min, and cells were labeled by FL-K4 (100 nM) at 0 °C for 15 min (±S.D., $n = 3$; * $p \leq 0.05$; *** $p \leq 0.005$). (b) Confocal microscopy images of TF14016-mediated CXCR4 internalization. After E3-CXCR4 CHO cells were labeled with ATTO488-K4 (100 nM) at 0 °C for 15 min, cells were treated with TF14016 (1 μM) at 30 °C for 30 min. (c) Confocal microscopy images of internalized TY14015. E3-CXCR4 CHO cells were treated with TY14015 (1 μM) at 30 °C for 30 min. (d) Confocal microscopy images of internalized TY14015 (green) and CXCR4 (red). After E3-CXCR4 CHO cells were labeled with TMR-K4 (100 nM) at 0 °C for 15 min, cells were treated with TY14015 (1 μM) at 30 °C for 30 min. Representative z-slice confocal microscopy images are shown.

The localization of fluorescent T140 derivatives and CXCR4 was simultaneously monitored by confocal microscopy using the coiled-coil tag-probe system. After preincubation with TMR-K4, E3-CXCR4 CHO cells were stimulated by TY14015. Merged confocal microscopy images revealed that TY14015 colocalized with TMR-K4/E3-CXCR4 (Figure 4d). This colocalization was not affected by the fluorophores, which was verified by experiments using ATTO488-K4/E3-CXCR4 and TR14011 (Figure 1, see also Supporting Information, Supplementary Figure 2). CXCR4-Venus CHO cells (in which a fluorescent Venus protein was fused at the C-terminus of CXCR4) showed an identical phenotype upon stimulation with

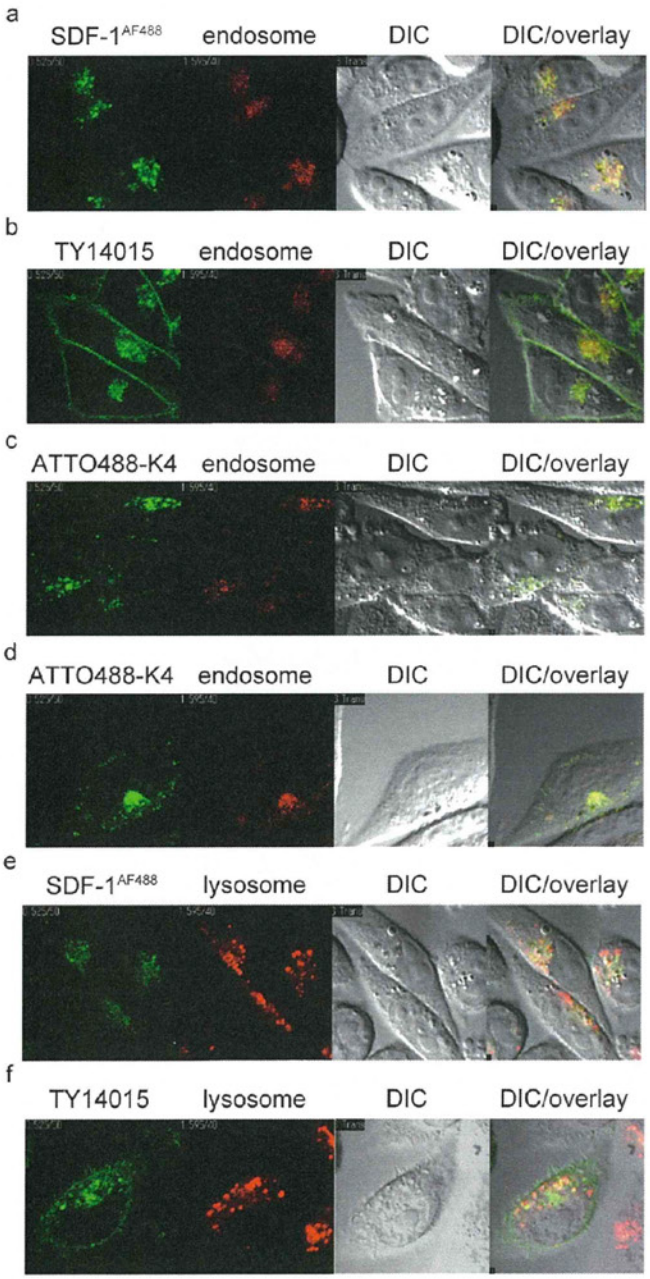

Figure 5. Translocation of fluorescent CXCR4 ligands and the receptor. (a,b) Confocal microscopy images of SDF-1^{AF488} (a) or TY14015 (b) and endosome. After E3-CXCR4 CHO cells were treated with fluorescent ligands at 30 °C for 30 min, cells were stained with AlexaFluor 568-transferrin (50 μg/mL) for 60 min. (c,d) Confocal microscopy images of internalized CXCR4 and endosome after stimulation with SDF-1 (c) or TF14016 (d). After E3-CXCR4 CHO cells were labeled with ATTO488-K4 (100 nM) at 0 °C for 15 min, cells were treated with ligand at 30 °C for 30 min and stained with AlexaFluor 568-transferrin (50 μg/mL) for 60 min. (e,f) Confocal microscopy images of SDF-1^{AF488} (e) or TY14015 (f) and lysosome. After E3-CXCR4 CHO cells were treated with fluorescent ligands at 30 °C for 30 min, cells were stained with LysoTracker (1 μM) for 30 min. Representative *z*-slice confocal microscopy images are shown.

SDF-1^{TMR} or TR14011 (see Supporting Information, Supplementary Figure 2). These data suggested that T140 derivatives translocated into the intracellular compartment with formation of ligand–receptor complexes.

Intracellular Translocation of CXCR4 Ligands and Receptor. The intracellular destination of CXCR4 ligand–receptor complexes after the binding of CXCR4 antagonists was investigated by confocal microscopy (Figure 5; see also Supporting Information, Supplementary Figure 3). After E3-CXCR4 CHO cells were treated with CXCR4 ligands, cells were stained with several organelle-specific fluorescent markers. SDF-1^{AF488} translocated to endosomal compartments in 30 min, which were stained by AlexaFluor 568-conjugated transferrin (Figure 5a). This result was in agreement with another report on SDF-1-mediated CXCR4 internalization.[17] Similarly, TY14015 accumulated in the same intracellular compartment (Figure 5b). Localization of ATTO-K4/E3-CXCR4 after treatment with SDF-1 and TF14016 was also similar to the distribution of transferrin, indicating that internalized CXCR4 ligand–receptor complexes translocate into endosomal compartments (Figure 5c,d). Meanwhile, localization of the fluorescent CXCR4 ligands in lysosomes was partial (Figure 5e,f). Agonists and antagonists of CXCR4 may indirectly affect the distributions of lysosomes. Staining with FM 4–64 or ER-trackers suggested that SDF-1^{AF488} existed neither on the cell membrane nor in the endoplasmic reticulum, whereas partial staining with TY14015 on cell membranes was observed (see Supporting Information, Supplementary Figure 3).

■ DISCUSSION

Agonist-mediated internalization of GPCRs induces the transduction of downstream signaling and desensitization to regulate cell homeostasis. In contrast, reports on antagonist-induced receptor internalization (e.g., cholecystokinin A, 5-HT_{2A}, and neuropeptide Y_1 receptors) are limited.[42−44] This is the first report on the antagonist-mediated internalization of CXCR4. A series of polyphemusin II-derived and other CXCR4 antagonists contain basic functional groups such as arginine and lysine residues, which are involved in the interactions with the extracellular domain of CXCR4-bearing negative charges. In the present study, using the coiled-coil tag-probe system to visualize cell-surface CXCR4, CXCR4-mediated translocation of T140 derivatives into intracellular compartments was demonstrated. Although the internalization effect of surface CXCR4 by T140 derivatives was partial (only 25%, much less than the agonist SDF-1), this CXCR4 internalization supports the apparent antagonistic activity of T140 derivatives against SDF-1 binding to CXCR4 as well as the induction of inverse agonistic activity signaling.[23] It was reported that treatment of CXCR4-expressing cells with HIV-1 gp120 peptide induces similar CXCR4 internalization without agonistic activity, which is closely related to HIV infection.[14] Although T140 derivatives have been thought to be competitive inhibitors against gp120 binding to CXCR4,[19] antagonist-mediated internalization of cell-surface CXCR4 could be an alternative mode of action for anti-HIV activity.[17,18] Further investigation of the mechanisms of this paradoxical antagonist-mediated down-regulation of CXCR4 could facilitate development of novel anti-metastatic and anti-HIV agents.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures as well as confocal microscopy images of ligand and receptor internalization in the control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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A simple, rapid, and sensitive system for the evaluation of anti-viral drugs in rats

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ABSTRACT

The lack of small animal models for the evaluation of anti-human immunodeficiency virus type 1 (HIV-1) agents hampers drug development. Here, we describe the establishment of a simple and rapid evaluation system in a rat model without animal infection facilities. After intraperitoneal administration of test drugs to rats, antiviral activity in the sera was examined by the MAGI assay. Recently developed inhibitors for HIV-1 entry, two CXCR4 antagonists, TF14016 and FC131, and four fusion inhibitors, T-20, T-20EK, SC29EK, and TRI-1144, were evaluated using HIV-1_{IIIB} and HIV-1_{BaL} as representative CXCR4- and CCR5-tropic HIV-1 strains, respectively. CXCR4 antagonists were shown to only possess anti-HIV-1_{IIIB} activity, whereas fusion inhibitors showed both anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities in rat sera. These results indicate that test drugs were successfully processed into the rat sera and could be detected by the MAGI assay. In this system, TRI-1144 showed the most potent and sustained antiviral activity. Sera from animals not administered drugs showed substantial anti-HIV-1 activity, indicating that relatively high dose or activity of the test drugs might be needed. In conclusion, the novel rat system established here, "phenotypic drug evaluation", may be applicable for the evaluation of various antiviral drugs *in vivo*.

1. Introduction

Numerous antiviral agents have been developed to suppress infection with viruses such as human immunodeficiency virus type 1 (HIV-1) [1], and have successfully provided excellent outcomes *in vivo*. However, the emergence of drug-resistant HIV-1 variants is a major concern in HIV therapy. Therefore, the development of novel drugs with sustained activity to resistant variants is desirable. Drugs, especially those targeting HIV-1 entry, have been recently developed and approved, such as a CCR5 antagonist, maraviroc [2], and a fusion inhibitor, enfuvirtide (T-20) [3], where both drugs effectively suppress HIV-1 in the patient even resistant to previous drugs [4,5].

In addition to CCR5, which is a main co-receptor for clinical HIV-1 strains, CXCR4 can also act as a co-receptor for HIV-1 (X4-tropic HIV-1), such as that seen for the vast majority of laboratory-adapted HIV-1 strains [6]. Thus, CXCR4 is also considered an important therapeutic target. We previously identified a β-sheet-like 14-residue peptide, T140 [7,8], and its down-sized analog, a cyclic pentapeptide FC131 (Fig. 1) [9], as potent and specific CXCR4 antagonists. Both T140 and FC131 were proved to inhibit X4-tropic HIV-1 infection *in vitro*. T140 has been further modified to TF14016 (4F-benzoyl-TN14003; BKT140) that shows more potent inhibitory effect [10].

The first fusion inhibitor, T-20, efficiently inhibits replication of HIV-1 resistant even to inhibitors for reverse transcriptase and protease [11,12]. However, the genetic barrier to overcome suppression by T-20 seems to not be high since a 1–2 amino acid(s) substitution in gp41 appears to be sufficient for resistance [13–15]. Therefore, we developed T-20EK [16] and SC29EK [17] as novel and potent fusion inhibitors that sustain their inhibitory effects on T-20 resistant HIV-1 stains. A series of systematic replacements with hydrophilic glutamic acid (E) or lysine (K) was introduced (EK motif) at the solvent-accessible site to enhance the α-helicity of the peptides by possible intrahelical electrostatic interactions [18]. T-20EK/S138A [16] was synthesized

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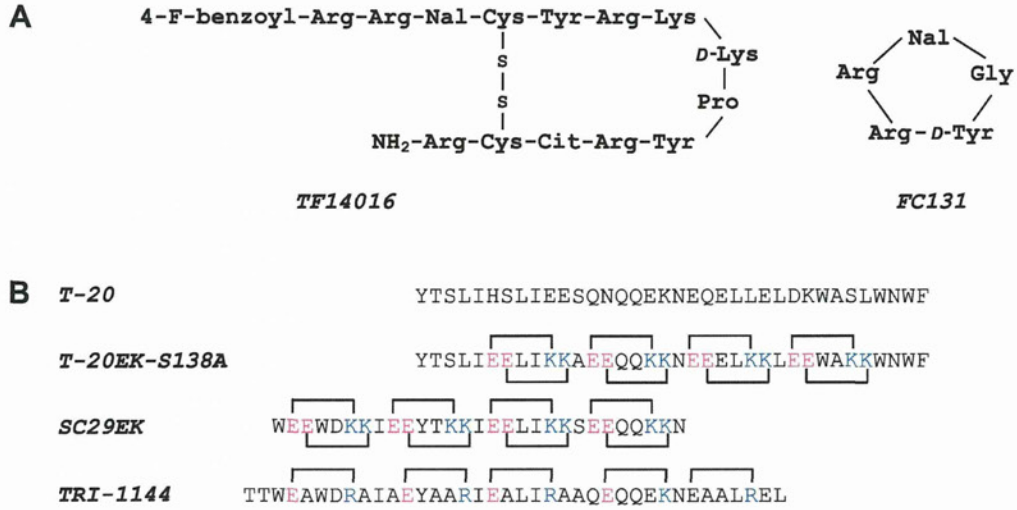

Fig. 1. Amino acid sequences of peptide-based drugs. (A) CXCR4 antagonists used in this study are shown. Nal: L-3-(2-naphthyl)alanine; Cit: L-citrulline. (B) Fusion inhibitors used are shown. T-20 is original sequenced of gp41 C-HR region. Electrostatic interactions are indicated by the linker. SC29EK and T-20EK/S138A, contain EExxKKx motif, while TRI-1144 does ExxxRxx motif. x indicates original and/or modified amino acids. Each motif creates 2 and 1 interaction(s) in each helical turn. T-20 resistance associated mutation, S138A, is introduced into T-20EK sequence (T-20EK/S138A). All peptides are N-terminally acetylated and C-terminally amidated.

with a combined rational design by the introduction of the EK motif for enhancement of α-helicity and increased affinity to mutated gp41 by S138A, a T-20 resistant associated mutation [19]. Dwyer et al. developed another fusion inhibitor, TRI-1144 (T-2635) that also exerted potent activity against T-20 resistant variants [20,21]. The amino acid sequence of TRI-1144 is also modified by substitutions with E and arginine (R), similar to the EK motif introduced into T-20EK and SC29EK.

Analyses of the efficacy and adverse effects of new drugs in animal models are important prior to their clinical application. Indeed, generally, the toxic effects, kinetics, and efficacy of new drugs are expected to be obtained by animal experiments. In the case of anti-HIV-1 drugs, the toxic effects of drug candidates can be determined by animal experiments. Furthermore, the kinetics of some drugs may be examined by some analytical methods such as liquid chromatography–mass spectrometry (LC–MS) [22] or bioimaging with labeled compounds. Unfortunately, these results may not be well-correlated with *in vivo* efficacy due to degradation and/or modification of drugs, and the detection of false positives of similar component(s) *in vivo* [23]. The efficacy of anti-HIV-1 drugs is, so far, hard to examine *in vivo* due to the lack of convenient animal infection models with low cost. One of the main obstacles to establish appropriate animal models is restricted infection of small animals with HIV-1, such as for mice, rats, and ferrets. An HIV-1 receptor-transgenic rat model has been developed for the analysis of HIV-1 infection *in vivo*; however, the levels of plasma viremia in infected rats were modest and not sustained [24,25]. Monkeys infected with simian immunodeficiency virus-HIV chimeric virus (SHIV) is the only model for the evaluation of HIV-1 replication [26], but comes at a high cost, especially for animal infection facilities. Taken together, novel rapid, simple, and sensitive HIV-1 infection models with low cost, such as those in small animals, are urgently needed to be established.

Here, we established a new system to evaluate the anti-HIV-1 activity of drugs and its kinetics in rats in addition to their toxic effects. The bioavailability of anti-HIV-1 drugs in sera was determined for the assessment of antiviral activity *in vitro*. The *in vivo* efficacy of various peptide-based entry inhibitors, such as TF14016, FC131, T-20EK/S138A, SC29EK, and TRI-1144, were assessed using this model and may be useful for the *in vivo* assessment of novel entry inhibitors.

2. Materials and methods

2.1. Drugs and cells

CXCR4 antagonists, TF14016 and FC131, and fusion inhibitors, T-20, T-20EK/S138A, SC29EK and TRI1144, were synthesized as previously described [7,9,16–18,20]. For *in vitro* drug susceptibility assays and *in vivo* administration, the test drugs were dissolved in 50% dimethyl sulfoxide (DMSO; 2 mM) and sterile water (3 or 10 mg/1.5 mL), respectively. MAGI CCR5 cells (HeLa CD4/CCR5/LTR-β-galactosidase cells) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: from Dr. Julie Overbaugh [27–29] and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum [30].

2.2. Administration of drugs

Animal experiments were performed in the Biotechnical Center of the Japan SLC, in accordance with the institutional ethical guidelines. To examine the pharmacological kinetics in sera, rats were used for collection of sera. Drugs were used at 3 mg/1.5 mL/kg of T-20, 3 mg/1.5 mL/kg of TF14016, 10 mg/1.5 mL/kg of FC131, 10 mg/1.5 mL/kg of SC29EK, 10 mg/1.5 mL/kg of T-20EK/S138A, and 3 mg/1.5 mL/kg of TRI1144, and were intraperitoneally administrated to six groups of six male SD rats (7 weeks). Sera were then harvested 0.5, 1, 2, 4, 8, and 12 h from the administrated rat, and stored at −80 °C.

2.3. MAGI assay

The anti-HIV-1 activity of drugs in rat sera after drug administration was detected by the MAGI assay, as previously described [31]. Briefly, MAGI CCR5 cells were transferred to 96-well plates at 1×10^4 cells per well. On the following day, serially-diluted drugs or rat sera were added to cells in triplicate with HIV-1 preparations ($HIV\text{-}1_{IIIB}$ or $HIV\text{-}1_{BaL}$). After 48 h, cultured cells were fixed with 1% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde in phosphate-buffered saline (PBS), and were stained with 0.4 mg/mL 5-bromo-4-chloro-3-indolyl-2-D-galactopyranoside (X-gal). Blue

cells were counted by observation under a light microscope. The 50% effective concentration was defined as the serum dilution fold or drug concentration that inhibited virus infection in 50% of the wells.

3. Results

3.1. Anti-HIV-1 activity of drugs in vitro

Prior to animal experiments, the anti-HIV-1 activity of test drugs *in vitro* was determined by the MAGI assay. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4- and R5-tropic HIV-1 strains, respectively. TF14016 exerted most potent anti-HIV-1 activity *in vitro* compared to other inhibitors as shown in Table 1. As expected, the two CXCR4 antagonists, TF14016 and FC131, inhibited replication of only HIV-1_{IIIB}, but not HIV-1_{BaL}, which uses CCR5 for its entry. All four fusion inhibitors, T-20EK/S138A, SC29EK, TRI-1144 and T-20, comparably inhibited replication of both HIV-1_{IIIB} and HIV-1_{BaL}. Among newly developed fusion inhibitors, T-20EK/S138A showed the strongest inhibitory effect both on HIV-1_{IIIB} and HIV-1_{BaL}. Our antiviral data are similar to previous observations for TF14016 and FC131 [7–10,32], T-20EK/S138A [16], SC29EK [17], and TRI-1144 [20,21].

3.2. Anti-HIV-1 activity of CXCR4 antagonists in rat

First, we examined background anti-HIV-1 activity in four PBS-injected rat sera as negative controls. In the control rat sera, anti-HIV-1_{IIIB} and HIV-1_{BaL} activities were detected (Fig. 2). Rat sera showed antiviral activity up to the 90- and 160-fold dilution for HIV-1_{IIIB} and HIV-1_{BaL} (Fig. 2; shown as a baseline activity).

The two CXCR4 antagonists, TF14016 and FC131, were intraperitoneally injected into six rats and sera were withdrawn at the indicated time as shown in Fig. 2. Drug activities were detected up to 4 h, with peak time point at 1 h after the administration. Surprisingly, sera from two rats injected with TF14016 and four rats with FC131 also weakly showed anti-HIV-1_{BaL} activity (data not shown). However, both CXCR4 antagonists were generally effective only against HIV-1_{IIIB}.

3.3. Anti-HIV-1 activity of fusion inhibitors in rat

Anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities were detected in four rat sera and all six rat sera, respectively, that were administered T-20. Anti-HIV-1 activity of T-20 in rats was detected up to 8 h with a peak time point 1–2 h after administration. Anti-HIV-1_{IIIB} activities were detected in sera of six rats injected with SC29EK, T-20EK/S138A, and TRI-1144, which were detected up to 3, 8, and 8 h, respectively, with serum peak levels at 1–2 h after administration. Anti-HIV-1_{BaL} activities were detected in sera with SC29EK, T-20EK/S138A, and TRI-1144 with similar extent with these for HIV-1_{IIIB}. These results indicate that in rats, intraperitoneally injected drug activities were present in sera and may exert anti-HIV-1 activity *in vivo*. Among these, TRI-1144 showed stable and relatively sustained activity.

3.4. Effect of heat inactivation

To identify component(s) for baseline anti-HIV-1 activity in rat sera, we examined heat inactivation. As expected, non-specific anti-HIV-1 activity in sera decreased in a time-dependent manner. At 1000-fold dilution of sera, non-specific activity was completely abolished (Fig. 3); unfortunately the drugs tested in the study were not heat stable and irreversible even at 56 °C (data not shown). However, when administered a physiological dose, anti-HIV-1 activity was detectable even without heat inactivation (Fig. 2). Therefore, the rat model system proved to be adequate to evaluate the efficacy of drugs.

3.5. Toxic effect of drugs in rats

All peptides tested showed no apparent lethal effect at the administered dosages, except for FC131, where one rat succumbed from unknown causes at a dose of 30 mg/kg.

4. Discussion

To develop effective and safe antiviral agents, *in vitro* screening systems are established for some viruses, while *in vivo* evaluation systems using small animals are hampered by limited infection efficiency and the need for specialized facilities. In the case of animal models for HIV-1, animal models are largely restricted [33]. In the present study, we describe the establishment of a novel evaluation system of anti-HIV-1 drugs through *in vitro* detection of anti-HIV-1 activity in the sera of rats administrated drugs using the MAGI assay. The *in vivo* efficacies of five potential entry inhibitors were evaluated. In this system, only TRI-1144 consistently showed potent and sustained activity compared with T-20. The glutamic acid–arginine (ER) modification, but not the glutamic acid–lysine (EK) modification and/or alanine substitutions to the peptide (Fig. 1), may have beneficial effects on stability and efficacy, resulting in sustained anti-HIV-1 activities. The simple and convenient *in vivo* efficacy evaluation system established in this study not only reveals whether drugs exert anti-HIV-1 activity *in vivo*, but also provides *in vivo* kinetics without the need for infectious animal facilities. Moreover, this system can be used for the evaluation of not only anti-HIV-1 drugs *in vivo*, but also of drugs against other viruses *in vivo*. Nonetheless, the sera produced by the rats can be also applied to resistant virus variants and clinical isolates resulting in a reduction of the number animal experiments required.

Other methods, such as a high performance liquid chromatography (HPLC), may provide accurate measurement of the drug concentration in sera and was performed in this study. Even after administration of FC131 at 30 mg/kg, we could only detect FC131 at the peak concentration (data not shown). In a case of small amount of agents with extremely high activity, it is possible to fail to detect by HPLC. For more sensitive detection by HPLC, further labeling, such as with radioisotopes, may be needed. In addition, HPLC analysis can detect drugs that have been modified and/or degraded by *in vivo* metabolism when they are spectrometrically indistinguishable. However, our system detects only the active

Table 1
Anti-HIV-1 activity of drugs *in vitro*.

Virus	EC_{50}[a] (nM)					
	TF14016	FC131	T-20	T-20EK/S138A	SC29EK	TRI-1144
HIV-1_{IIIB}	0.3 ± 0.0	17.4 ± 5.7	42.3 ± 7.6	2.0 ± 0.5	8.3 ± 1.3	4.6 ± 0.6
HIV-1_{BaL}	>10,000	>10,000	16.2 ± 4.9	0.4 ± 0.2	1.4 ± 0.2	0.4 ± 0.2

[a] Antiviral activity, shown as EC_{50}, was determined using the MAGI assay. Each EC_{50} represents the mean ± SD obtained from at least three independent experiments. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4 and R5 HIV-1 strains, respectively.

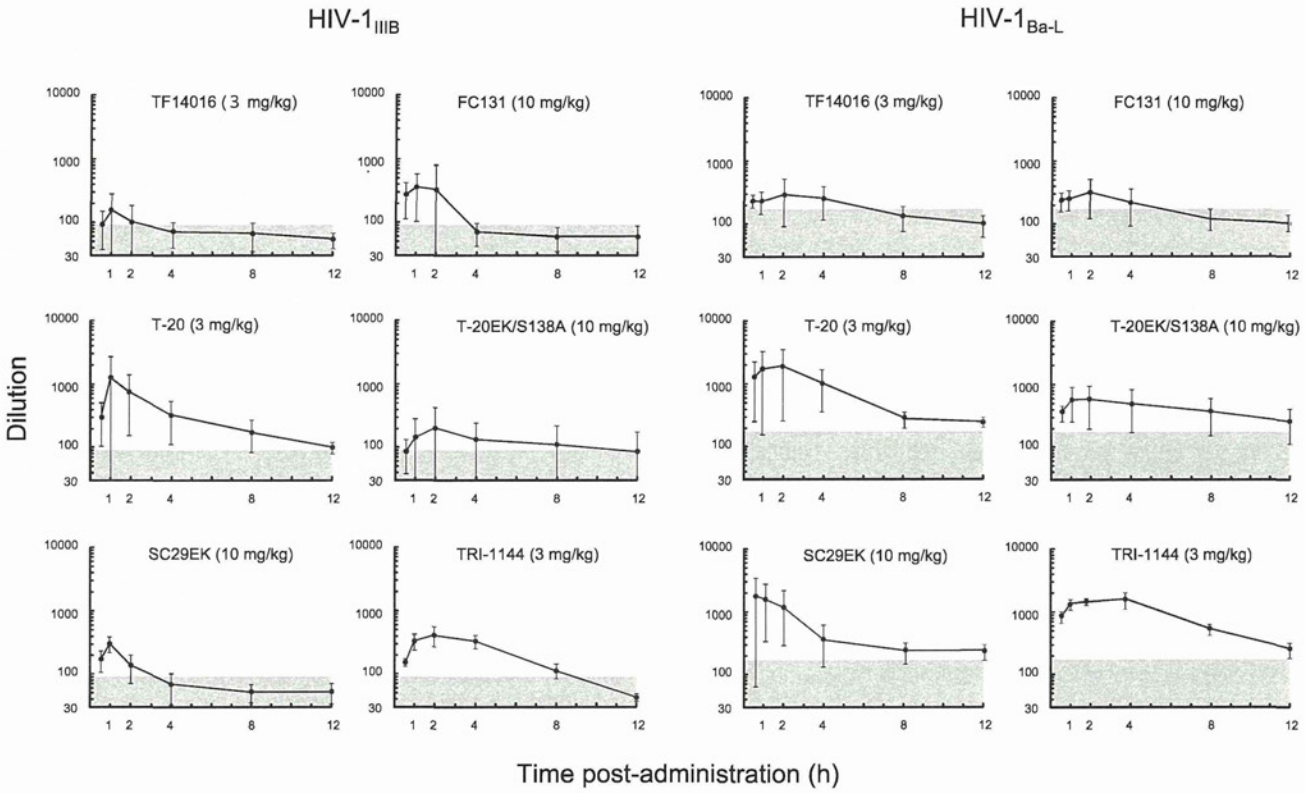

Fig. 2. Anti-HIV-1 activity of drugs *in vivo*. Six groups of six rats were administered each drug by intra-peritoneal injection and rat sera were harvested at different time points post-administration. All serum samples were analyzed by MAGI assay for 50% inhibition of infections of HIV-1_{IIIB} and HIV-1_{BaL}. This experiment was performed in triplicate for each rat. Data represent mean ± SD of from six rats. Gray shade indicates average results of age-matched rat sera as negative controls.

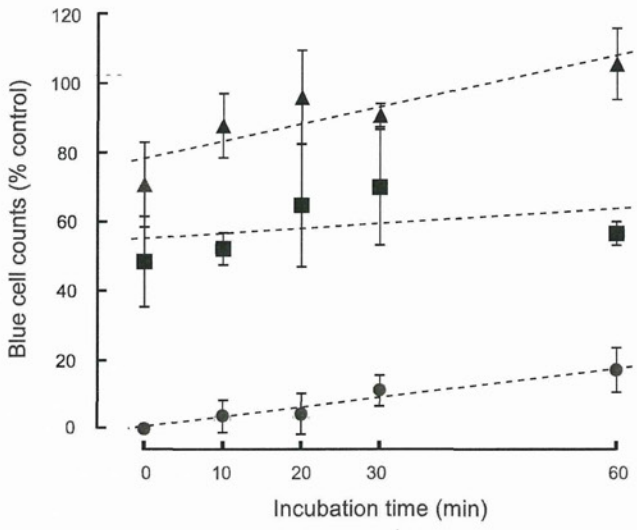

Fig. 3. Heat inactivation of sera. Rat sera without heat activation were examined using the MAGI assay. Heat inactivation was performed at 56 °C. Ten-fold dilutions of sera were resistant to heat inactivation even after 1 h inactivation (~20%). At the 1000-fold dilution, most of the inherent inhibitory effect was removed.

form of the agents, and in addition provides direct comparison of the tested drugs *in vitro* and *in vivo*, since the assay utilizes identical evaluation by the MAGI assay. In comparison, the rat *in vitro* system revealed that TRI-1144 showed strong and sustained activity compared with T-20EK/S138A and SC29EK. In this study, we only performed intraperitoneal injection that may have an effect on drug metabolism(s). Further experiments, such as subcutaneous injection, for which TF14016 shows greater efficacy [34,35], should be performed and compared with other administration roots.

The two CXCR4 antagonists analyzed in this study, TF14016 and FC131, showed moderate anti-HIV-1_{BaL} activity *in vivo*, and sera from two rats administered T-20 inhibited HIV-1 infection less efficiently (data not shown). These unexpected data might result from the relatively high background caused by non-specific inhibitory component(s) in sera. As shown in Fig. 2, sera from rats not administered drugs also showed moderate anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities. Therefore, the development of a reagent or method for removal of background activity in rat sera may improve the accuracy and sensitivity of this *in vivo* drug efficacy evaluation method. For instance, serum albumin [36], lactoferrin [37,38], and transferrin [39] may influence HIV replication. Unfortunately, the drugs used in this study were all peptide-derived agents, therefore, heat-inactivation may reduce antiviral activity. Therefore, administration of relatively high doses of drug may be required to overcome this inhibition.

In conclusion, we established a novel, simple and rapid system for the phenotypic evaluation of anti-HIV-1 drugs in a rat model. This system may also be applicable for the analysis of other antiviral drugs for viruses that do not have an appropriate infection model in rodents, and/or useful for the initial screening, such for dosing, administration root decision and other factors, prior to actual animal infection experiments. In this system for HIV infection, TRI-1144 displayed the most potent anti-HIV-1 activity *in vivo* of the six drugs analyzed.

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PAPER

Concise synthesis and anti-HIV activity of pyrimido[1,2-*c*][1,3]benzothiazin-6-imines and related tricyclic heterocycles†

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3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (PD 404182) is a virucidal heterocyclic compound active against various viruses, including HCV, HIV, and simian immunodeficiency virus. Using facile synthetic approaches that we developed for the synthesis of pyrimido[1,2-*c*][1,3]-benzothiazin-6-imines and related tricyclic derivatives, the parallel structural optimizations of the central 1,3-thiazin-2-imine core, the benzene part, and the cyclic amidine part of PD 404182 were investigated. Replacement of the 6-6-6 pyrimido[1,2-*c*][1,3]benzothiazin-6-imine framework with 5-6-6 or 6-6-5 derivatives led to a significant loss of anti-HIV activity, and introduction of a hydrophobic group at the 9- or 10-positions improved the potency. In addition, we demonstrated that the PD 404182 derivative exerts anti-HIV effects at an early stage of viral infection.

Introduction

Since azidothymidine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), was approved for the treatment of HIV infections, a number of anti-HIV drugs have been launched. For example, saquinavir and nevirapine were the first protease inhibitor and non-nucleoside reverse transcriptase inhibitor (NNRTI), respectively.[1] Highly active antiretroviral therapy (HAART) using a combination of these antiretrovirals is a standard treatment regimen for HIV infections. The HAART regimen significantly reduces viral load in infected patients, leading to significant therapeutic gains and reductions in morbidity and mortality.[2] However, long-term administration of multiple antiretrovirals to maintain life-long latent infection triggers the emergence of drug-resistant variants[3] and drug-related adverse effects.[4] For example, high-level viral resistance to NRTI such as AZT, stavudine, and didanosine is conferred by mutations frequently observed in patients with virologic failure on an NRTI-containing regimen.[5] In addition, lipodystrophy and metabolic disorders are often observed in patients receiving HIV protease inhibitors.[6] To overcome these problems, several antiretrovirals with new mechanisms of action have been developed in this decade. A peptide-based fusion inhibitor (enfuvirtide),[7] an integrase inhibitor (raltegravir),[8] and a CC chemokine receptor type 5 (CCR5) antagonist (maraviroc)[9] are examples of new molecular entities used as anti-HIV agents.

Recently, highly potent small-molecule anti-HIV agents have been reported, which bind to viral envelope proteins (Fig. 1). 2-Thioxo-1,3-thiazolidine derivative **1** shows potent inhibition of HIV-1 replication at nanomolar levels,[10] which are directed at the deep hydrophobic pocket in the N-terminal heptad repeat trimer of the viral gp41. Compound **1** blocks HIV-1-mediated cell–cell fusion and the formation of gp41 six-helix bundles, as does enfuvirtide.[10b] The bisindole derivative **2** also exhibits submicromolar inhibition of HIV-1 replication by interaction with the gp41 hydrophobic pocket in which compound **1** binds.[11] Small-molecule CD4 mimics with oxalamide and related substructures are another series of anti-HIV agents.[12] The representative BMS-448043 (**3**) exhibits subnanomolar anti-HIV activity by interaction with the CD4 binding pocket in gp120.[12d] These small-molecule entry inhibitors with potential oral bioavailability will provide alternative combination regimen(s) of anti-HIV agents for the treatment of drug-resistant variants.

In our efforts to develop novel anti-HIV compounds,[13] we have carried out the random screening of small molecules using multinuclear activation of a galactosidase indicator (MAGI) assay, in which the inhibitory activity of early-stage HIV infection, including virus attachment and membrane fusion to host cells, is evaluated. Among more than 30 000 compounds screened, 3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine **4** (PD 404182) was identified as a potent anti-HIV agent lead (Fig. 1). Compound **4** was reported to be an enzyme inhibitor against 3-deoxy-D-manno-octulosonic acid 8-phosphate synthase[14] and phosphopantetheinyl transferase,[15] exerting

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