However, there are limitations to the design of compound templates so far. Thus, in this study, we have attempted to develop the screening methods aimed at discovery of novel template structures for PKCδ ligands as drug leads.

■ EXPERIMENTAL PROCEDURES

General. ^{1}H NMR spectra were recorded using a Bruker AV500 spectrometer. Chemical shifts were reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as an internal standard. Low- and high-resolution mass spectra were recorded on a JMS-T1000LC AccuTOF and Bruker Daltonics microTOF-2focus in both positive and negative detection modes. Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) and silica gel 60 N (Kanto Chemical Co., Inc.) were employed for flash chromatography. Fluorescent spectra were recorded on a JASCO FP-6600 spectrofluorometer and JASCO V-650 spectrophotometer using a quartz cell with 1.0 cm path length. Fluorescent intensities of samples in 96-well plates were recorded on Wallac ARVO MX.

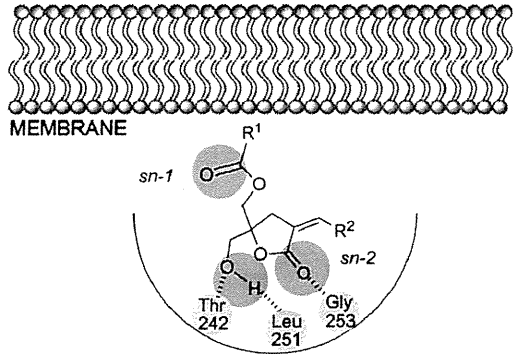


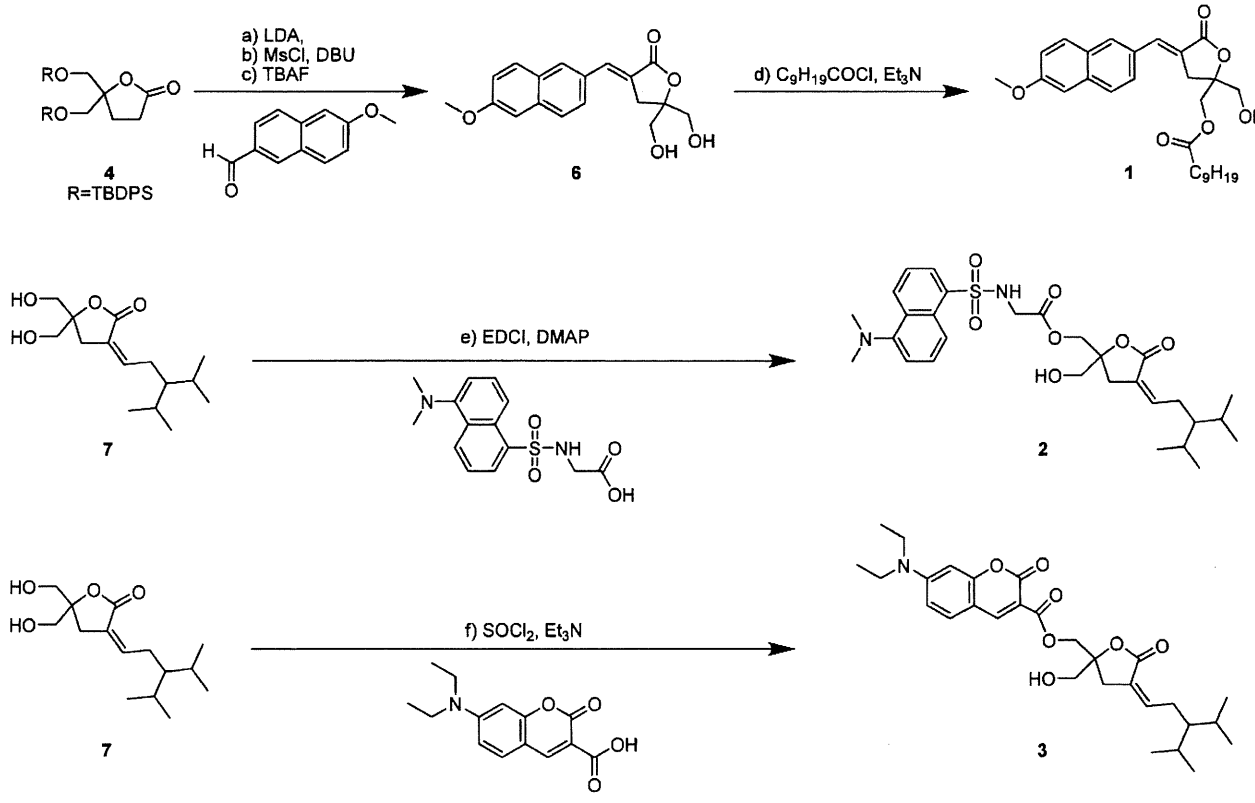
Figure 1. Binding mode of DAG-lactone derivatives to the δC1b domain. The *sn*-1 and *sn*-2 carbonyl groups are indicated. Three important pharmacophores are indicated by light blue spheres. Amino acid residues interacting with these pharmacophores (threonine at 242, leucine at 251, and glycine at 253) are indicated by red spheres.

Standard Monoacylation Procedure. Under argon, Et_3N (3 equiv) was added at 0 °C to a solution containing 5,5-bis(hydroxymethyl)oxalan-2-one in THF, and the mixture was stirred at 0 °C for 30 min. Then 0.2−1.1 equiv of a carboxylic acid derivative (an acyl chloride or carboxylic anhydride) was added. This mixture was stirred at 0 °C for a further 4 h. After evaporation of the solvent, the obtained residue was purified by flash column chromatography.

Preparation of Compounds 7, 10−13, 16, 17, and 19−21. Compounds **7**, **10**−**12**, and **16** were prepared as described in ref 17. Compounds **13** and **17** were prepared as described in refs 24 and 25. The synthesis of **19** is described elsewhere.[26] Compounds **20** and **21** are commercially available.

Expression and Purification of the δC1b Domain. DNA coding C1b domain of mouse PKCδ (231−280)[27] was subcloned into *Bam*H1 and *Eco*RI sites of pGEX-2tk (GE Healthcare) and expressed as gluthatione-*S*-transferase (GST) fusion protein in *Escherichia coli* C41 which contains extension sequences Gly-Ser-Arg-Arg-Ala-Ser-Val-Gly-Ser and Glu-Phe-Ile-Val-Thr-Asp at the N- and C-termini, respectively. The δC1b

Scheme 1. Synthesis of Fluorescent Compounds 1−3[a]



[a] (a) LDA, −78 °C; (b) MsCl, DBU, 0 °C to room temp; (c) TBAF, 0 °C to room temp; (d) $C_9H_{19}COCl$, Et_3N, 0 °C; (e) EDCI, DMAP, 0 °C; (f) $SOCl_2$, Et_3N, 0 °C.

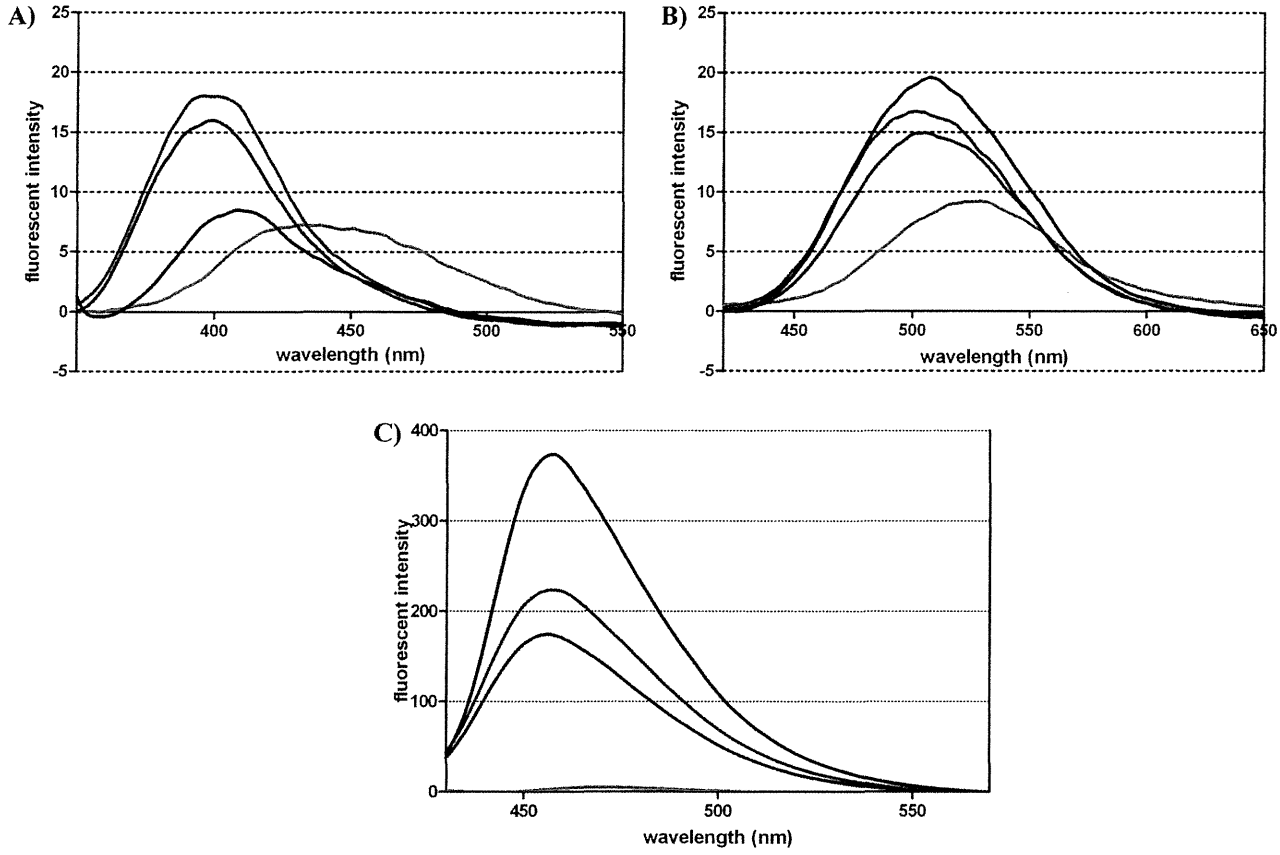


Figure 2. Fluorescent spectra of compounds **1** (A), **2** (B), and **3** (C) in different solvents. The spectra show fluorescence in the solvents color-coded as follows: red, MeOH; blue, THF; black, $CHCl_3$; green, EtOAc. Each ligand was prepared as 1 mM solution in DMSO. In UV measurement, 10 μL of ligand was added to 990 μL of solvents.

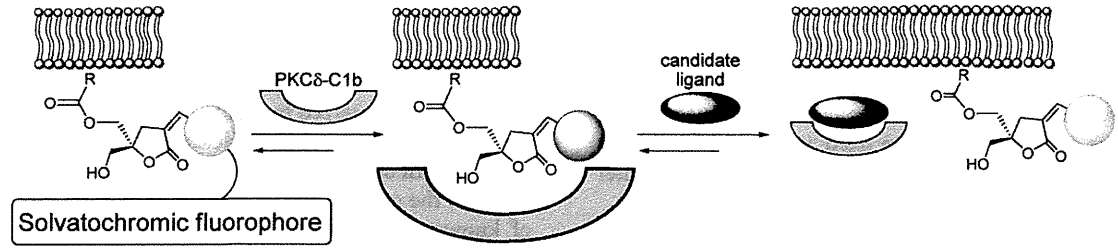


Figure 3. Schematic representation of a fluorescence-quenching screening system. In the presence of the δC1b domain, fluorescence of a solvatochromic fluorophore is enhanced. The replacement by candidate compounds results in fluorescence quenching.

Table 1. Changes of Fluorescent Intensity upon δC1b Binding and Inhibition Constants to δC1b Determined by Competition Assay against [^{3}H]PDBu

compd	ΔF[a]	K_i (nM)[b]
1	3.8	35.9
2	2.3	368
3	0.50	93.3

[a] Fluorescent change by binding of the δC1b domain. The values were determined by titration of the δC1b domain. The ΔF was determined by dividing F_1 by F_0 at the maximum of fluorescent emission in each spectrum. [b] Inhibitory constants determined by competition analysis against [^{3}H]PDBu.[28,36,37]

domain after cleavage contains 65 amino acid residues. Cells were grown at 37 °C in LB medium and induced with 0.3 mM IPTG at growth phase. Cells were cultured overnight at 20 °C after induction. Cells were collected and lysed in 50 mM Tris·HCl buffer (pH 8.0) containing 100 mM NaCl, 1 μg/mL leupeptine, 1 mM PMSF, and 1 mM DTT. Expressed protein was extracted by sonication, then purified by affinity chromatography utilizing glutathione-Sepharose 4B beads resin. GST moiety was cleaved by 300 units of thrombin at 4 °C overnight. The cleaved protein was eluted and further purified on a 2.6 cm × 60 cm Superdex S-75 gel filtration column. Purification was by FPLC system at a flow rate of 1 mL/min utilizing 0.1 M triethanolamine·HCl (pH 7.0) containing 0.5 M NaCl.

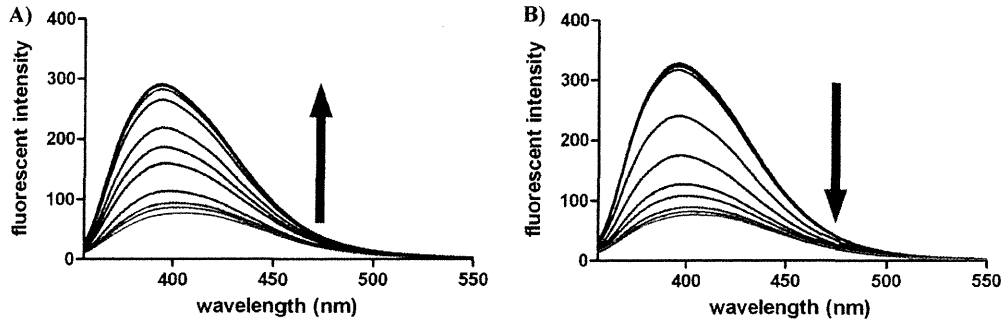


Figure 4. Changes of fluorescent spectra of compound **1** during titration of the δC1b domain (A) and during titration of PDBu (compound **8**) (B) after δC1b binding. The concentration of the δC1b domain was increased to 1.28 μM (6.4 equiv to compound **1**) by titration. The concentration of PDBu was increased to 10.2 μM (51.2 equiv to compound **1**) by titration.

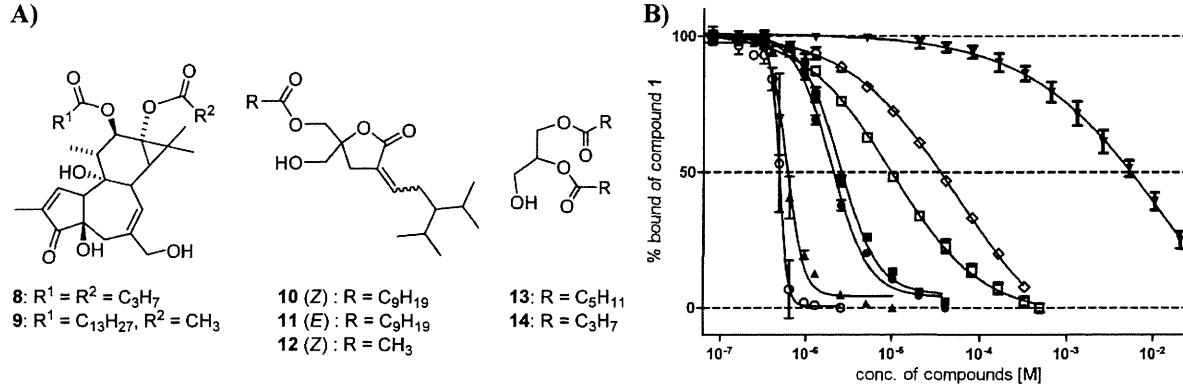


Figure 5. Competitive assay against compound **1** based on fluorescent intensity (spectrofluorometer). (A) Structures of test compounds. (B) Inhibition curves indicate results of compounds **8** (▲), **9** (○), **10** (●), **11** (■), **12** (□), **13** (◇), and **14** (▼).

Determination of K_i of Compounds for Full Length PKCδ. Enzyme–ligand interaction was assessed in vitro as the ability of the ligand to displace bound [20-^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) from recombinant human PKCδ in the presence of phosphatidylserine (PS) in an experimental procedure that was described previously.[28] In constant concentration analysis, the concentrations of compounds were fixed at 100 nM for known compounds and at 10 μM for library compounds. The other procedures were the same as those of the standard evaluation.

Fluorescent Titration of the δC1b Domain. An amount of 25 μL of a solution of PS in chloroform (10 mg/mL) was evaporated to dryness under nitrogen. Then 50 mM Tris·HCl buffer (pH 7.4) was added and the PS was sonicated with a Microtip for a total of 15 s. A stock solution of compound **1** was diluted with 50 mM Tris·HCl buffer (pH 7.4) containing 100 μg/mL PS to prepare a 0.2 μM solution. The recombinant δC1b domain was added to the 0.2 μM solution of compound **1**, and fluorescent spectra (λ_{ex} = 340 nm) were measured at 25 °C. The concentration of the recombinant δC1b domain started at 0.01 μM and increased to 1.28 μM by titration. The final concentrations of compounds **2** and **3** were 0.2 and 0.05 μM, respectively. Change of fluorescent intensity (ΔF) was calculated at the following wavelengths, which showed λ_{max} in each evaluation; compound **1**, 406 (F_0) and 394 (F_1) nm; compound **2**, 520 (F_0) and 480 (F_1) nm; compound **3**, 463 (F_0) and 461 (F_1) nm. Wavelengths recorded before and after the addition of the δC1b domain.

Fluorescent Titration of Candidate Ligands. Compound **1** was diluted with 50 mM Tris·HCl (pH 7.4) containing 100 μg/mL PS to obtain a solution with a 0.2 μM final concentration. The δC1b domain was added to the above solution of compound **1** to be 0.96 μM. A candidate ligand was added to the solution, and fluorescent spectra were recorded with a spectrofluorometer at 25 °C. Fluorescent titration curves (λ_{em} = 407 nm) were analyzed by nonlinear regression, and IC_{50} values were estimated by a nonlinear least-squares curve-fitting method using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, U.S.). In the constant concentration analysis, the concentrations of known compounds were fixed at 100 nM and of library compounds at 10 μM. The other procedures were the same as those in the standard evaluation.

Fluorescence-Quenching Analysis for Library Screening Recorded by Microplate Reader. Fluorescence intensity was recorded on Wallac ARVO MX (PerkinElmer) using a 96-well black plate. Compound **1** was diluted with 50 mM Tris·HCl (pH 7.4) containing 100 μg/mL PS to obtain a solution with a 0.2 μM final concentration. The δC1b domain was added to the above solution of compound **1** to be 0.96 μM. A candidate ligand (10 μM) was added to the compound **1**–δC1b domain complex solution, and fluorescent intensities (λ_{ex} = 355 nm, λ_{em} = 405 nm) were measured at 25 °C.

■ RESULTS AND DISCUSSION

Synthesis and Fluorescent Properties of Compounds 1–3. Three representative solvatochromic fluorophores, involving 6-methoxynaphthalene,[29] 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl),[6,30,31] and diethylaminocoumarin,[32–35] have

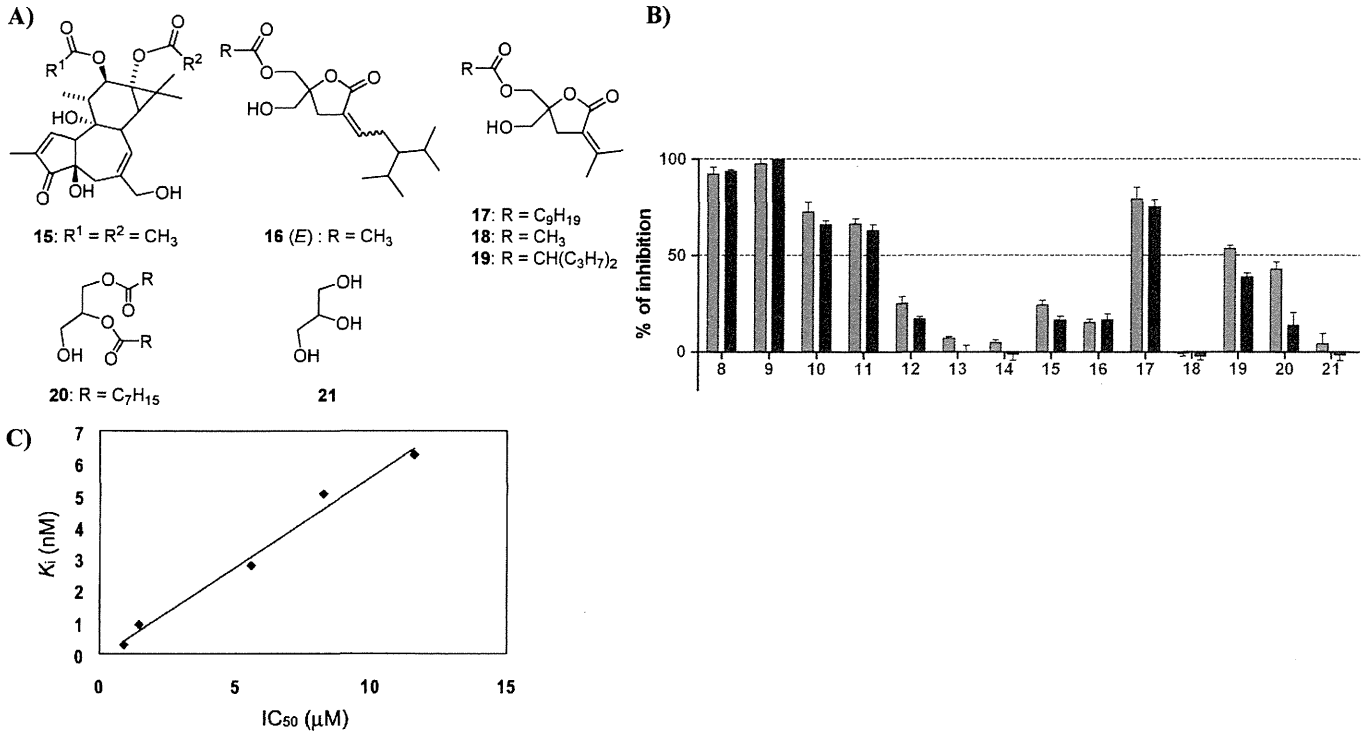


Figure 6. Comparison of inhibition percentages between the fluorescent assay (microplate reader) and the RI assay at a constant concentration of test compounds. (A) Structures of test compounds. (B) Inhibition of binding of compound **1** (fluorescent assay, left gray bars) or [^{3}H]PDBu (RI assay, right black bars) in the presence of test compounds (10 μM in the fluorescent assay and 100 nM in the RI assay). The final DMSO concentration is 4%. (C) Plots of IC_{50} values (μM) by the fluorescent assay versus K_i (nM) by the RI assay for compounds **8**, **9**, **10**, **11**, and **17**.

been utilized. The 6-methoxynaphthalene was incorporated at the α-carbon of the *sn*-1 carbonyl group of the DAG-lactone structure (Scheme 1). The dansyl and diethylaminocoumarin were incorporated into the *sn*-2 carbonyl group. In comparison with the fluorescent spectra of these compounds, obtained in various solvents, compound **1** showed the most variation with solvent polarity (Figure 2). It is known that solvatochromic fluorophores show higher fluorescent intensity in hydrophobic environments and that the fluorophores are sensitive to the environment of a binding pocket in target proteins. The fluorescent intensity of labeled DAG-lactones should reflect its binding to a target protein, PKCδ (Figure 3), and as expected, our reporter compounds **1** and **2** containing solvatochromic fluorophores exibited a remarkable increase of fluorescent intensity in the proximity of the C1b domain of PKCδ (δC1b), which is a specific target of DAG-lactone derivatives (Table 1 and Figure 4A). This indicates that in the binding of compounds **1** and **2** to the δC1b domain, the 6-methoxynaphthalene and dansyl moieties are located in the hydrophobic environment. However, for compound **3**, the fluorescent intensity was decreased as the concentration of δC1b increased. The spectra in various solvents showed dependence of the fluorescent intensity of the compound **3** on solvent polarity, and it was suggested that in the binding of compound **3** to the δC1b domain, the diethylaminocoumarin moiety could be located in the hydrophilic environment. The changes of fluorescent intensity of the synthetic compounds are summarized in Table 1. The binding constants of fluorescent compounds were evaluated by [^{3}H]PDBu competitive assay. Compound **1** showed the highest binding affinity for the δC1b domain. In the fluorescence-based analysis of binding, two factors are key to the sensitivity of the assay system: (i) the fluorescent change as a function of the ligand binding and (ii) the binding affinity of a reporter compound.

Evaluation of Binding to PKCδ by Fluorescent Change of Compound 1. The results obtained supported the selection of compound **1** as a reporter compound for further study. In competitive assays, when candidate ligands of PKCδ are present, they replace the fluorescent compounds, which are formerly bound to δC1b, and the fluorescent intensity should decrease. Practically, addition of PDBu to the complex of compound **1** and δC1b resulted in a remarkable decrease of fluorescent intensity (Figure 4B). By utilization of compound **1** as a reporter, binding analysis of known PKCδ ligands was performed to assess the reliability of the assay system. Compounds **8**−**14** were prepared as test compounds (Figure 5A), and their IC_{50} values, determined by the fluorescence-based competitive assay with a fluorospectrophotometer against compound **1**, were 0.60, 0.49, 1.95, 2.42, 10.0, 54.2, and 1.22 × 10^4 μM, respectively (Figure 5B). The order of the IC_{50} values of compounds **8**−**11** is identical to that of the K_i values (0.91, 0.26, 5.0, and 6.2 nM, respectively) determined by the [^{3}H]PDBu competitive assay.

The Binding Analysis by Competition with Compound 1 Showed Linearity with the Classical RI Assay. To further assess the collinearity of the fluorescent assay on microplates and the [^{3}H]PDBu assay, the inhibition percentages by compounds **8**−**21** in both assays (Figure 6A) at constant concentration were compared. The compound concentrations were set at 10 μM for the fluorescent assay and at 100 nM for the [^{3}H]PDBu assay. As shown in Figure 6B, the inhibition percentages of compounds **8**−**21** are similar in both assays. Comparison of the IC_{50} and K_i of compounds **8**, **9**, **10**, **11**, and **17**, possessing more than 60% inhibition percentages, showed acceptable linearity (Figure 6C).

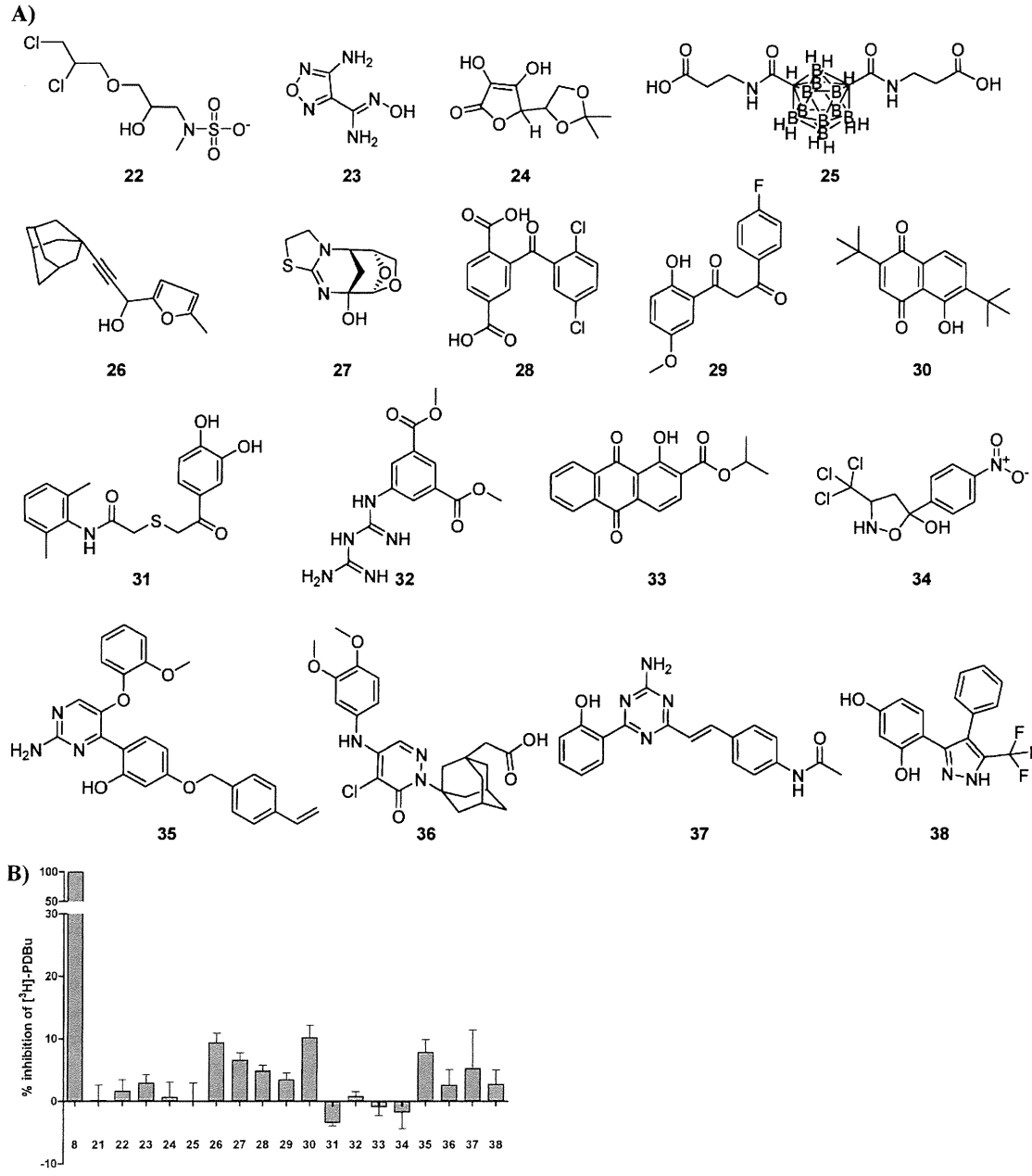


Figure 7. The RI competition assay of positive hit compounds found in fluorescent screening utilizing compound **1**. (A) Structures of test compounds obtained from screening. (B) Results of the inhibition assay based on RI methods. The concentration of compounds was fixed at 10 μM. The *X* axis indicates compound numbers. Columns show average inhibition obtained from triplicated experiments. Bars indicate standard errors.

In view of the above results, the present fluorescence-based evaluation of binding to the δC1b domain on microplates was deemed to be a promising alternative for the classical RI assay.

Expansion of the Fluorescence Evaluation to Screening of Library Compounds. As a proof-of-concept study, compound **1** was utilized in screening of a chemical library, supplied by the Screening Center at the Tokyo Medical and Dental University Screening Center. The structures of the library compounds were analyzed in advance of screening. For binding to the δC1b domain, pharmacophores including the carbonyl and hydroxy groups are necessary and only compounds including these functional groups were chosen. In the analysis of the library on microplates, compounds with more than 20% decrease of fluorescent intensity determined by triplicated assays were selected as candidates for further study. Compounds **22**−**38** were found from 2560 library compounds (Figure 7A) as candidate δC1b ligands. To further assess the binding affinity of these compounds, evaluation of binding by competition with the [^{3}H]PDBu was performed at a fixed concentration (10 μM) (Figure 7B). In this analysis, compounds **30** and **26**, with inhibition percentages at 10 μM of 10.2% and of 9.4%, respectively, showed the most and the second most potent binding affinities for the δC1b domain. The binding affinities of these compounds are still very low compared to that of DAG-lactones, but an SAR study of these compounds could lead to novel structure templates for synthetic PKC ligands. This analysis also revealed that compounds **24**, **25**, **31**, **32**, **33**, and **34** are false positive hits. The results obtained indicate that our screening system with a fluorescent

DAG-lactone derivative could be utilized for discovery of novel chemical leads for PKCδ.

■ CONCLUSION

A fluorescence-based method for chemical library screening has been developed. This method, which requires no washing steps, provides a linear response in relative binding affinity between compounds and is compatible with the classical RI assay for PKC ligands. By screening of more than 2500 library compounds, the method was proven to be a reliable means of discovery of compounds that bind to the C1b domain of PKCδ. The method would provide potent lead compounds that bind to the isozyme utilized in the assay. By utilization of the difference of the binding affinity of reporter compounds to different isozymes, it is possible that this screening system can provide an efficient selection of lead compounds highly specific to a target isozyme. This fluorescence-quenching method should be applicable to other known receptor−ligand combinations.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis procedures and NMR, HRMS, and IR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The authors thank Professor Masatoshi Hagiwara and Dr. Yukiko Okuno (Chemical Biology Screening Center, Tokyo Medical and Dental University) for their support in chemical library screening. This work was supported in part by Naito Foundation (W.N.) and Research Award of Graduate School of Biomedical Science, Tokyo Medical and Dental University (W.N. and T.I.)

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Expert Opinion

The successes and failures of HIV drug discovery

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Introduction: To date, several anti-human immunodeficiency virus (HIV) drugs, including reverse transcriptase inhibitors and protease inhibitors, have been developed and used clinically for the treatment of patients infected with HIV. Recently, novel drugs have been discovered which have different mechanisms of action from those of the above inhibitors, including entry inhibitors and integrase (IN) inhibitors; the clinical use of three of these inhibitors has been approved. Other inhibitors are still in development.

Areas covered: This review article summarizes the history of the development of anti-HIV drugs and also focuses on successes in the development of these entry and IN inhibitors, along with looking at exploratory approaches for the development of other inhibitors.

Expert opinion: Currently used highly active antiretroviral therapy can be subject to a loss of efficacy, due to the emergence of multi-drug resistant (MDR) strains; a change of regimens of the drug combination is required to combat this, along with careful monitoring of the virus and CD4 in the blood, by methods such as cellular tropism testing. In such a situation, entry inhibitors such as CCR5/CXCR4 antagonists, CD4 mimics, fusion inhibitors and IN inhibitors might be optional agents for an expansion of the drug repertoire available to patients at all stages of HIV infection.

Keywords: AIDS, CCR5, CD4 mimic, chemokine receptor, CXCR4, fusion, HIV, integrase

Expert Opin. Drug Discov. (2011) 6(10):1067-1090

1. Introduction

The human immunodeficiency virus (HIV) is the cause of acquired immunodeficiency syndrome (AIDS), which was discovered by Montagnier and colleagues in 1983 [1]. HIV infects human host cells and destroys immune systems, subsequently causing immunodeficiency. To date, the number of people worldwide infected with HIV is certainly in excess of 30 million.

Several anti-HIV drugs have been reported in the last 25 years (**Figure 1A**). HIV is a retrovirus, which is an RNA virus that is replicated in a host cell via the enzyme reverse transcriptase to produce DNA from its RNA genome. This DNA is then incorporated into the host genome by an integrase (IN) enzyme. These inhibitors block the action of reverse transcriptase, and include nucleoside reverse transcriptase inhibitors (NRTIs) such as azidothymidine (AZT) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs). The class of anti-HIV drugs that were initially approved for clinical use is reverse transcriptase inhibitors such as AZT [2]. The class of drugs that were subsequently approved is protease inhibitors, which prevent the cleavage of HIV precursor proteins into active proteins, a process that normally occurs in viral replication. This family of drugs includes Saquinavir (Invirase/Fortovase, Roche, Basel, Switzerland) and Ritonavir (Norvir, Abbott Laboratories, IL, USA), which have been used clinically in HIV/AIDS treatment. These drugs are usually administered as part of a two- or three-drug cocktail, accompanied by one or more

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10.1517/17460441.2011.611129 © 2011 Informa UK, Ltd. ISSN 1746-0441

Article highlights.

- The highly active anti-retroviral therapy (HAART) involving use of reverse transcriptase inhibitors and protease inhibitors has led to great success in clinical treatment of human immunodeficiency virus (HIV)-infected patients.
- Brand-new drugs with different action mechanisms have been discovered to date.
- Enfuvirtide, a fusion inhibitor, Maraviroc, a co-receptor CCR5 antagonist and Raltegravir, an integrase (IN) inhibitor have successively been approved for clinical use.
- The potential of new inhibitors from novel drug categories such as entry inhibitors including CCR5/CXCR4 antagonists and CD4 mimics, fusion inhibitors and IN inhibitors might be critical.
- Optional agents are valuable for an expansion of the drug repertoire available to patients because in case of loss of efficacy of HAART, change of regimens of the drug combination in HAART is required.

This box summarizes key points contained in the article.

NRTIs. Such cocktail therapies are known as highly active anti-retroviral therapy (HAART), which has brought great success and hope in the clinical treatment of HIV-infected patients [2]. HAART is capable of lowering the HIV level in the blood until it cannot be detected with current methods. Side effects associated with protease inhibitors include a lipodystrophy syndrome in which abnormal distribution of fat occurs and the face, arms and legs become thin but the therapy involves more serious clinical problems such as the emergence of multi-drug resistant (MDR) HIV-1 strains, and considerable expense. These drawbacks encouraged us to develop brand-new drugs with novel mechanisms of action.

Recently, the molecular mechanism of HIV-1 replication has been elucidated in detail. A dynamic supramolecular mechanism is associated with HIV entry/fusion steps. First, an HIV envelope protein gp120 interacts with a host-cell surface protein CD4, which causes gp120 to undergo a conformational change subsequently binding to the second cellular receptors, a chemokine receptor such as CCR5 [3-7] and CXCR4 [8]. The binding triggers the exposure of another envelope protein gp41 whose N-terminus penetrates the cell membrane. This is followed by the formation of the gp41 trimer-of-hairpins structure, which leads to fusion of HIV/cell membranes, completing the infection process [9]. The description of this dynamic molecular machinery has encouraged researchers to develop inhibitors which block the HIV entry/fusion steps targeting the receptors, CD4, CCR5 and CXCR4, and the viral protein gp41. In 2003, the Food and Drug Administration (FDA) approved Enfuvirtide (Fuzeon/T-20, Roche, Basel, Switzerland/Trimeris, Durham, NC, USA) as the first 'fusion inhibitor' for use in combination with other anti-HIV drugs to treat advanced HIV-1 infection [10]. In 2007, the FDA approved a CCR5 co-receptor antagonist, Maraviroc (Pfizer, New York, NY, USA), for use in combination with other anti-HIV drugs for the treatment of patients infected with CCR5-tropic HIV-1 [11]. In the same year, the FDA approved Raltegravir (Merck Sharp & Dohme Corp., NJ, USA) for use in combination with other antiretroviral agents in treatment-experienced adult patients who present with evidence of viral replication and HIV-1 strains resistant to multiple HAART agents [12,13]. Subsequently in 2009, the FDA granted expanded approval of Raltegravir for use in all patients. Numerous reviews exist concerning reverse transcriptase and protease inhibitors, and this review will focus on the success of a fusion inhibitor, Enfuvirtide, a CCR5 antagonist, Maraviroc and an IN inhibitor, Raltegravir, as well as the development of other anti-HIV agents including CXCR4 antagonists and CD4 mimics.

2. HIV fusion inhibitors such as Enfuvirtide

The binding of gp120 to CCR5 or CXCR4 triggers the formation of the trimer-of-hairpins structure of gp41 and subsequent fusion of the HIV/cell membranes, as described above. The trimer-of-hairpins structure is a six-helical bundle consisting of a central parallel trimer of the N-terminal helical region (HR1 region) surrounded by the C-terminal helical region (HR2 region) oriented in an antiparallel, hairpin fashion (**Figure 1B**) [9]. A subdomain is composed of two peptides, a 51-mer from the HR1 region and a 43-mer from the HR2 region, designated as N51 and C43, respectively [14]. There have been numerous reports that several HR2 region peptides inhibit bundle formation of six alpha-helices by the binding to the inner three-stranded coiled coils of the HR1 region thereby inhibiting membrane fusion (**Figure 2A**) [15]. An HR2 region peptide, C34, with 34 residues from the native sequence of gp41, has potent inhibitory activity against HIV-1 fusion [16]. In addition, a 36-residue peptide, T-20, which has the native sequence of gp41 and 24 residues in common with C34, shows potent anti-HIV activity, and its clinical use as the first entry/fusion inhibitor [10] in HIV/AIDS treatment was approved by the FDA in 2003 designated as Enfuvirtide. Enfuvirtide, in combination with other antiretroviral agents was approved for the treatment of advanced HIV-1 infection in adults and children aged 6 years or older with evidence of HIV-1 replication notwithstanding current therapy, and of resistance to current anti-HIV drugs. In view of the clinical use of Enfuvirtide, the dynamic supramolecular mechanism involving membrane fusion is a valid and rational target for inhibitors of HIV-1 replication. The success of Enfuvirtide has encouraged the development of entry/fusion inhibitors as a new class of anti-HIV drugs distinct from the first- and second-generation drugs. While reverse transcriptase inhibitors and protease inhibitors work internally in T cells to inhibit functions of viral enzymes, entry and fusion inhibitors work extracellularly, preventing HIV from invading cells. C34 has an exact interface that is capable of interacting with the inner three-stranded coiled coils of the gp41 HR1 region, compared with Enfuvirtide, which has a poorly delineated interface. However, a disadvantage

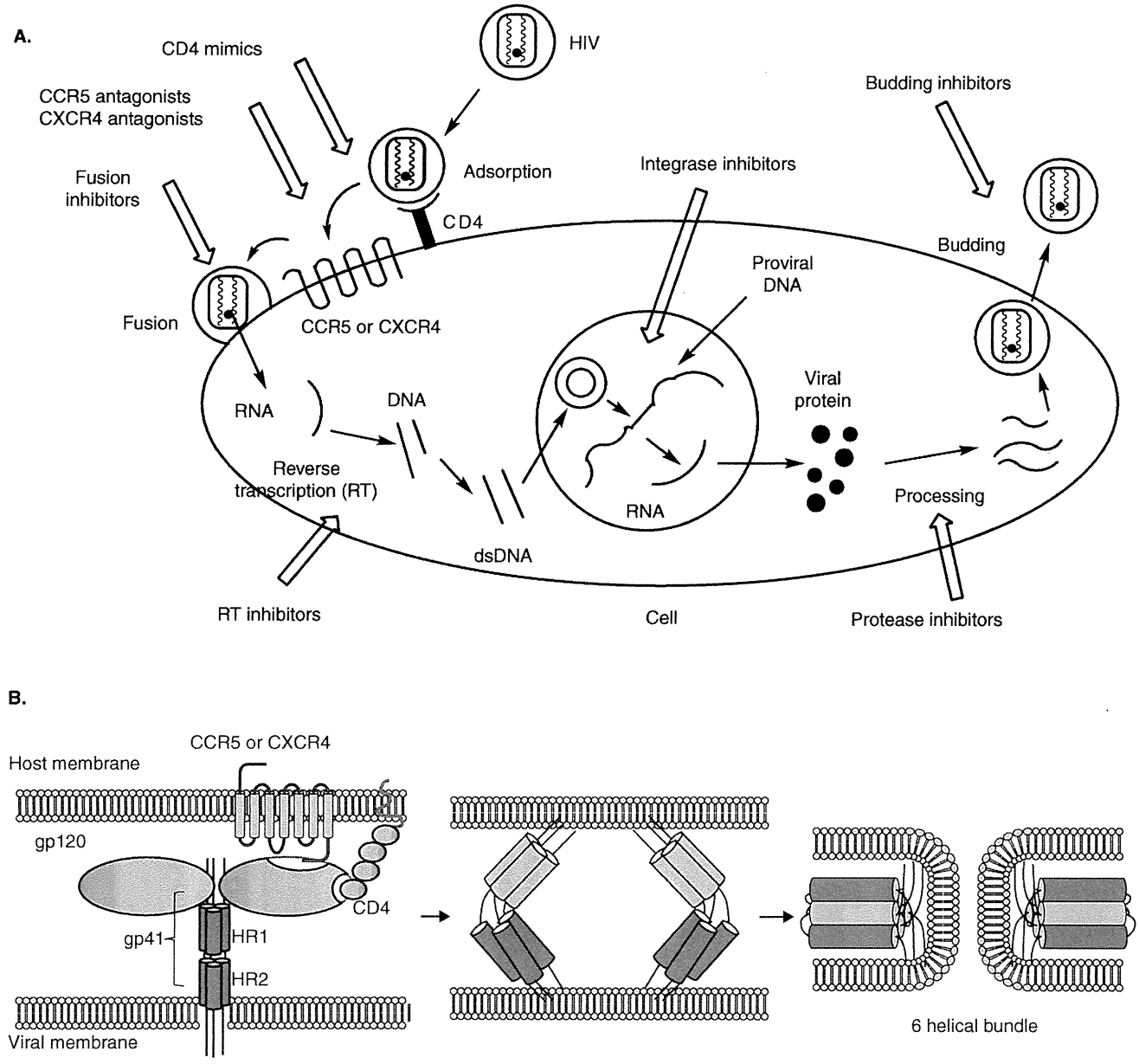


Figure 1. A. HIV-1 replication cycle and anti-HIV drugs that are effective at its various steps. **B.** Mechanisms of HIV-1 entry and fusion.

of C34 is its poor aqueous solubility and highly soluble C34 analogs, known as subcutaneous glucogon-like peptides (SC) peptides, were developed by artificial remodeling of C34 by Otaka *et al.* (Figure 2) [17]. In the design of SC peptides, the amino acid residues at the *a*, *d* and *e* positions of the helical wheel diagram of C34, which are essential for interaction with the inner coiled coils of HR1, were maintained with no substitution, while non-conserved residues at the *b*, *c*, *f* and *g* positions, which are located in the exterior region, were replaced by Glu or Lys. Several Glu-Lys side-chain ion charge pairs formed between i and i + 4 positions enhance solubility and alpha-helicity of the C34 analogs. The aqueous solubility of the SC peptides, SC34 and SC34EK, is more than three orders of magnitude greater than that of the original C34 peptide. Anti-HIV activities of these SC peptides were superior or comparable with that of the original C34 peptide, and an order of magnitude greater than that of Enfuvirtide [18]. Furthermore, SC peptides were even effective against an Enfuvirtide-resistant strain. The C34 and SC peptide sequences lack the C-terminal lipid binding domain of Enfuvirtide and it has been suggested that SC peptides have a mechanism of action distinct from that of Enfuvirtide [19]. Thus, these SC peptides are leads for further refinement and clinical development. C34, T-649 [20], Enfuvirtide and SC peptides are all 34- to 36-mer peptides derived from the HR2 region of

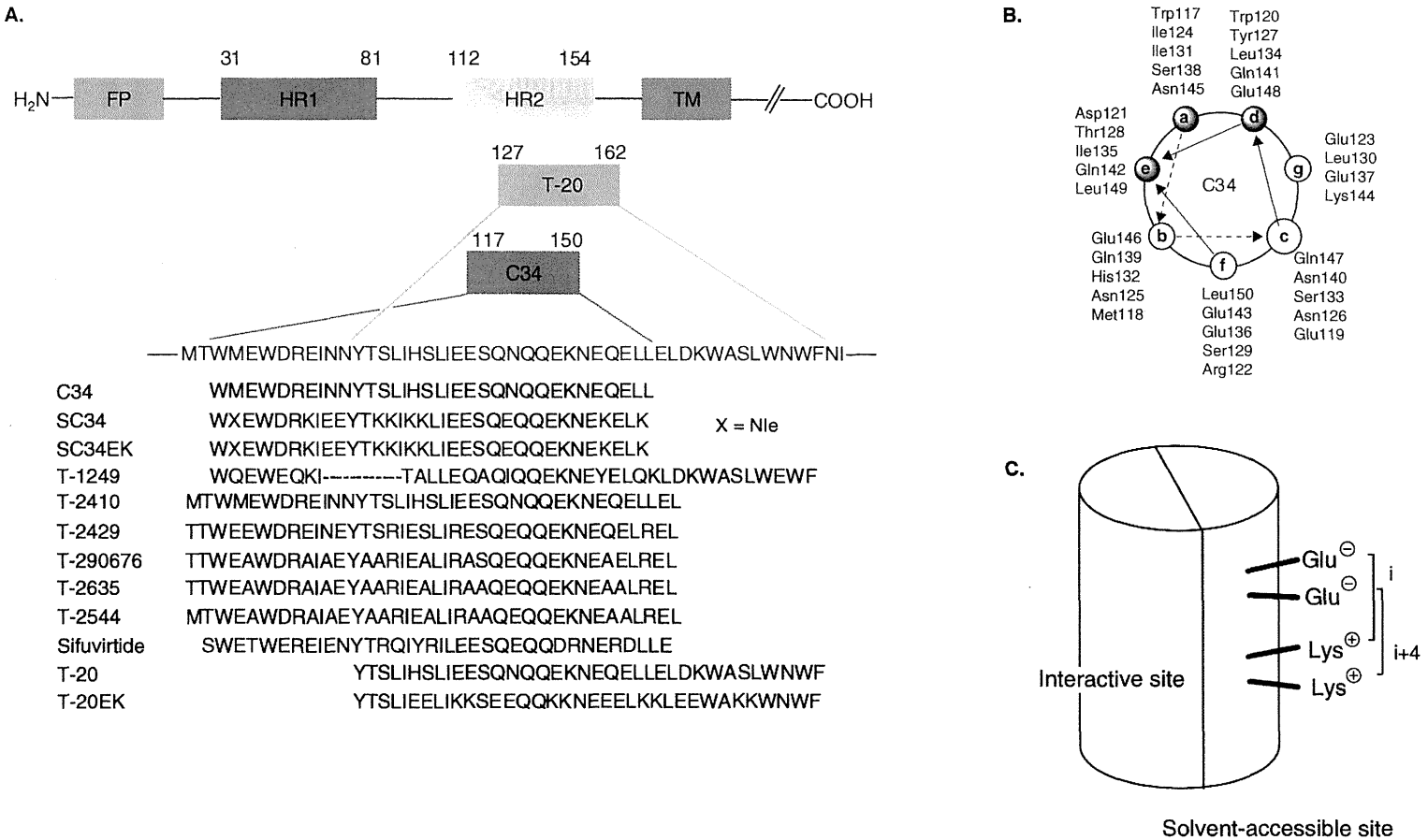


Figure 2. A. Schematic representation of gp41 and sequences of HR2 region peptides. **B.** Helical wheel representation of the C34 peptide. Amino acid residues are numbered according to gp41 of NL4-3 strain. **C.** The design concept of introduction of the Glu-Lys motif to the solvent-accessible site. **D.** Remodeling of dynamic structures of HR1 regions leads to synthetic antigen molecules inducing neutralizing antibodies.

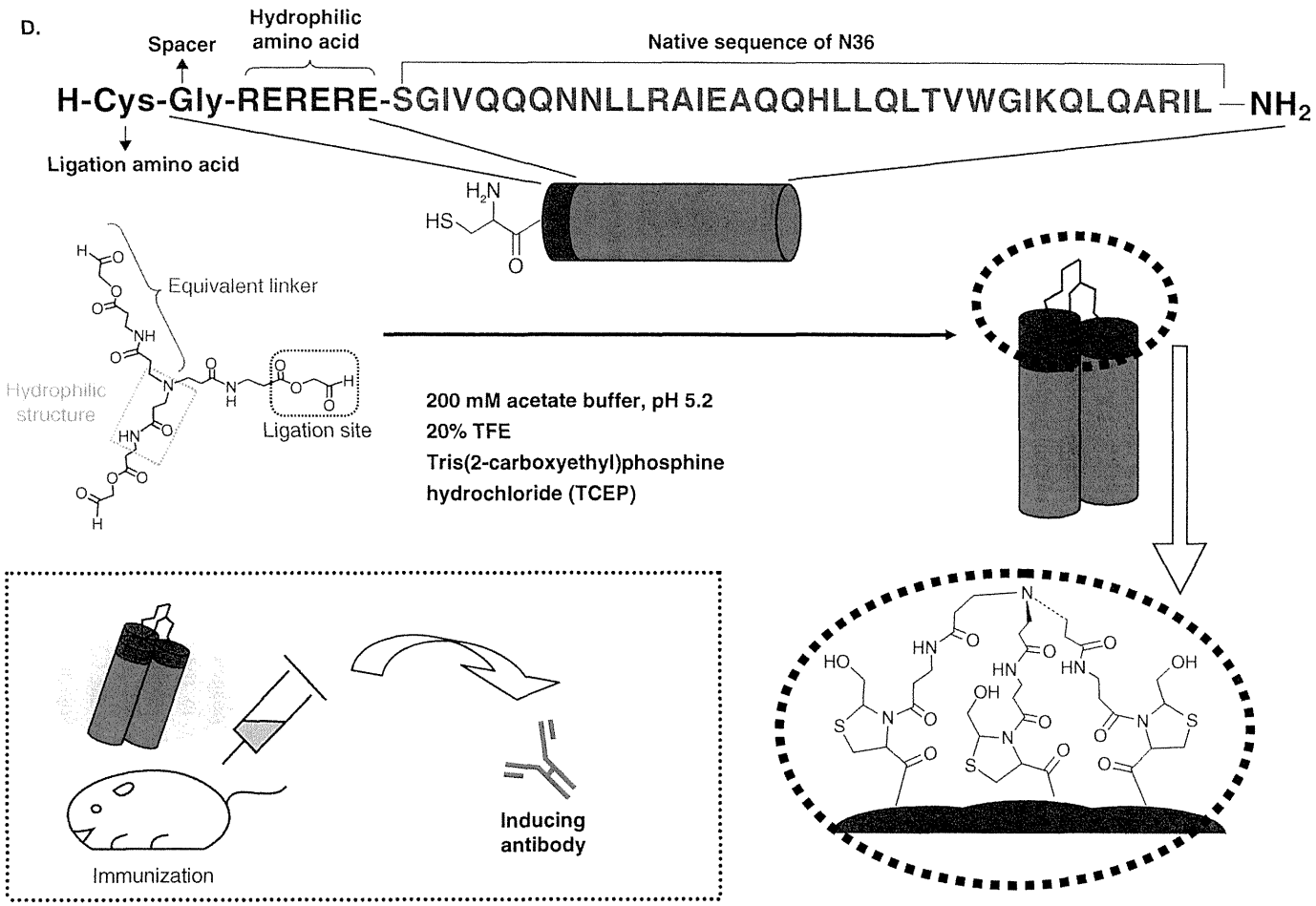


Figure 2. (continued). A. Schematic representation of gp41 and sequences of HR2 region peptides. **B.** Helical wheel representation of the C34 peptide. Amino acid residues are numbered according to gp41 of NL4-3 strain. **C.** The design concept of introduction of the Glu-Lys motif to the solvent-accessible site. **D.** Remodeling of dynamic structures of HR1 regions leads to synthetic antigen molecules inducing neutralizing antibodies.

gp41. T-1249 (Roche, Basel, Switzerland/Trimeris, Durham, NC, USA), a successor to Enfuvirtide [21], was much anticipated as its hydrophobic C-terminal sequence inhibits HIV-1 fusion by interacting with lipid bilayers in a manner similar to the interaction enjoyed by Enfuvirtide, but its clinical trial was discontinued in January 2005 because of formulation problems. In addition, attempts have been made to develop small non-peptide inhibitors that block gp41 activation [22-24]. To date, development of highly potent and useful low molecular weight inhibitors has been difficult, although non-natural binding elements that contribute to the formation of a stable complex with the inner coiled coils have been identified using a biased combinatorial chemistry library [25]. The development of small organic compounds as useful fusion inhibitors is continuing.

The HR1 region is critical for the development of AIDS vaccine antigens because the six-helical bundle structure is formed by interactive approach to the central trimer of the HR1 regions by the HR2 regions of three strands coiled coils. In the design of artificial antigens that induce broadly neutralizing antibodies, a useful strategy is to synthesize molecules that mimic the natural trimer on the virion surface. Using an original template with C3-symmetric linkers, we designed and synthesized a novel three-helical bundle mimetic corresponding to the trimeric form of N36 [26] (**Figure 2D**). The antiserum produced by immunization of the N36 trimeric form antigen in mice showed structural preference for binding to the N36 trimer and more potent neutralizing activity against HIV-1 infection than the N36 monomer. The exposed timing of epitopes was limited during HIV-1 entry, and carbohydrates, which could disturb accession of antibodies to epitopes, were not included in the amino acid sequences of the native protein [27]. These two advantages in the design based on the HR1 region of gp41 could further enhance the potential of a vaccine design based on the HR1 region.

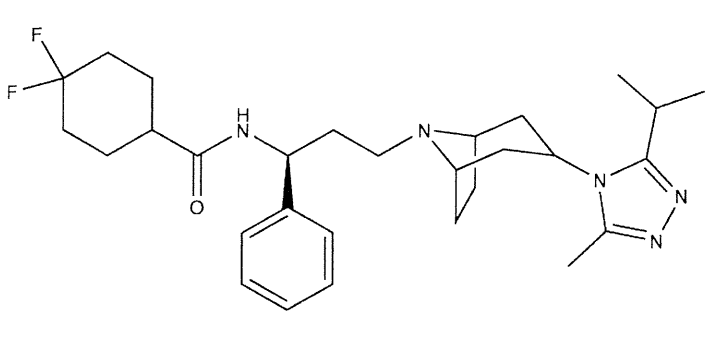
3. HIV co-receptor inhibitors such as Maraviroc

Interaction of CD4 with the HIV envelope protein gp120 causes a conformational change in the latter and its subsequent binding to the second cellular receptors, CCR5 [3-7] and CXCR4 [8] as described above, in Section 1. Macrophage-tropic (R5) HIV-1 strains, which constitute a majority in the early stage of HIV infection, use CCR5 as a co-receptor, whereas T cell line-tropic (X4) HIV-1 strains, which are the major species in the late stage of HIV infection and AIDS, use CXCR4 as a co-receptor. CCR5 and CXCR4 play physiological roles as the receptors for endogenous ligands, or chemokines. Several chemokine antagonists of CCR5 and CXCR4 have been developed as entry inhibitors. The validity of development of CCR5 antagonists is supported by the finding that individuals with the CCR5-Δ32 deletion mutation are healthy and strongly resistant to HIV-1 infection [28]. To date, several pharmaceutical companies have investigated novel CCR5 antagonists with suitable pharmaceutical properties. One CCR5-selective antagonist, Maraviroc (**1**) is the first CCR5 antagonist to be approved by the FDA as described above (**Figure 3A**) [11] and is used for the treatment of patients infected with CCR5-tropic HIV-1. Several other CCR5 antagonists are currently in clinical trials. Maraviroc has relatively high oral bioavailability (23%) with food effects in humans [29,30]. During the process of further improvement of Maraviroc's pharmacological profile, compound **2** was found to have potent fusion inhibitory activity (IC_{50} < 0.1 nM) and a pharmacological profile identical to that of Maraviroc [31]. Compound **2** showed high anti-HIV activity even against a Maraviroc-resistant mutant. Thus, compound **2** might be a desirable second-generation CCR5 antagonist. Takeda Pharmaceutical Co. Ltd., Osaka, Japan developed TAK-779 (**3**), a CCR5 antagonist with minimal bioavailability for i.v. use [32,33] but clinical development was discontinued because of local reactions at s.c. injection sites. High throughput screening has been used to develop an orally bioavailable CCR5 antagonist, and has led to the discovery of a novel lead compound **4** with a scaffold structure different from that of TAK-779 [34]. Subsequent optimization resulted in the development of TAK-220, a new CCR5 antagonist with a piperidine-4-carboxamide structure (**5**) (Takeda Pharmaceutical Co. Ltd., Osaka, Japan/Tobira Therapeutics, Inc.), which has high CCR5 binding activity and resistance to metabolic modification [35]. Merck/Schering-Plough Corp., which became Merck & Co., NJ, USA in a merger, reported a piperidinopiperazine series such as Vicriviroc (SCH-D/SCH417690) (**6**) [36]. In its Phase III studies, the safety and efficacy of Vicriviroc in the addition to optimized or HAART regimens was assessed based on established criteria. Vicriviroc, however, failed to show that its addition to the current regimens for the further treatment of HIV-1 infectious patients with evidence of HIV-1 replication is more effective than background regimens. As a result, Merck & Co. suspended its support of the FDA approval for Vicriviroc in treatment-experienced subjects [37,38]. A CCR5 antagonist with a spirodiketopiperazine scaffold, ONO-4128/873140 (**7**) (GSK, Middlesex, UK/Ono Pharmaceutical Co. Ltd., Osaka, Japan), has been developed by combinatorial chemistry utilizing solid-phase techniques [39]. ONO-4128/873140 has CCR5 binding activity in the nanomolar range and potent Ca^{2+} mobilization inhibitory activity. The spirodiketopiperazine is an attractive scaffold because it is likely to lead to more diverse derivatives. ONO-4128/873140 was advanced into the Phase II studies, but not into Phase III studies on account of its hepatotoxicity.

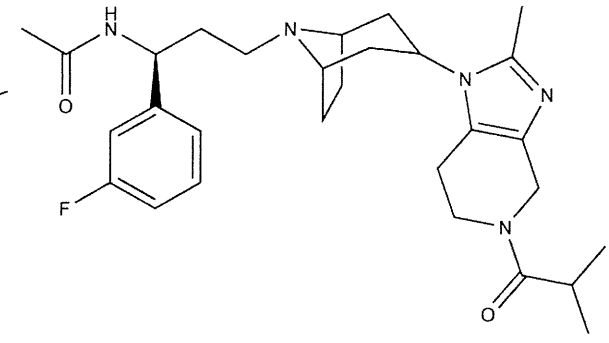
The other co-receptor for HIV-1 entry is CXCR4 [8], which is also relevant to mediation of the metastasis of a variety of cancer cells [40-42], leukemia [43,44] and rheumatoid arthritis [45,46]. Thus, CXCR4 is an important target for drug discovery, because CXCR4 antagonists might overcome these diseases. To date, several CXCR4 antagonists, peptidic and non-peptidic have been developed.

A 14-mer peptide T140 (**8**) derived from polyphemusin II, has been reported to be a potent CXCR4 antagonist (**Figure 3B**) [47] by us (Kyoto University, Kyoto, Japan). Biostable T140 analogs [48,49] (Biokine Therapeutics Ltd.,

A.



Maraviroc **1**



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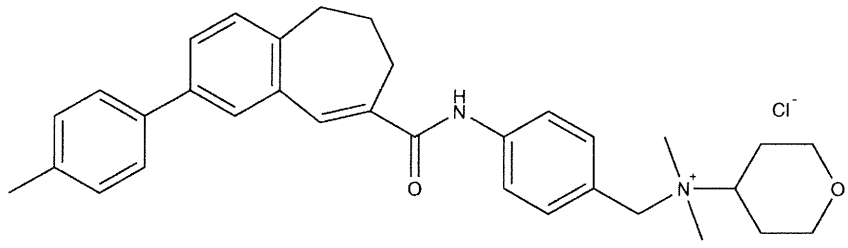

TAK-779 **3**

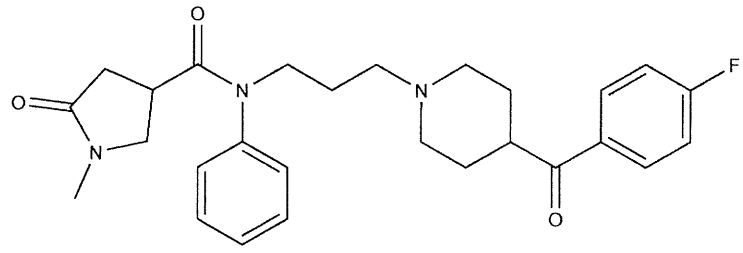

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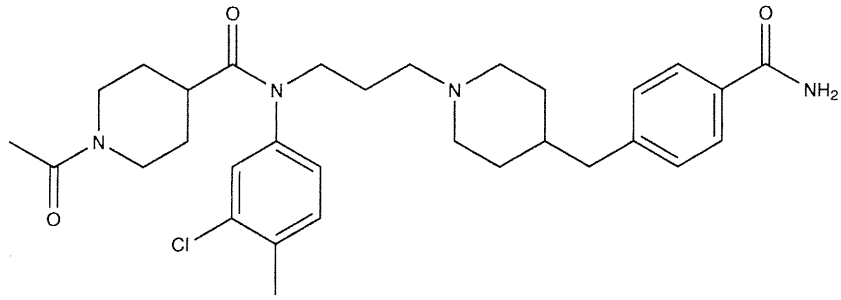
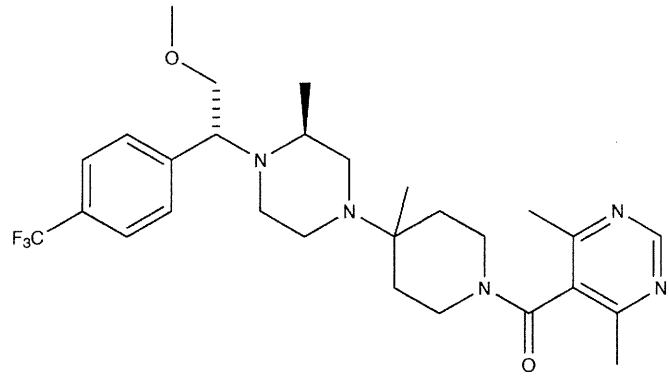
Figure 3. A. Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in **(25)**, n = 20 and **(26)**, m = 12.

Rehovot, Israel) have significant inhibitory activity not only against HIV infection *in vitro* but also against tumor metastasis *in vivo* [41,42]. FC131 (**9**) was developed by downsizing of **8** [50].

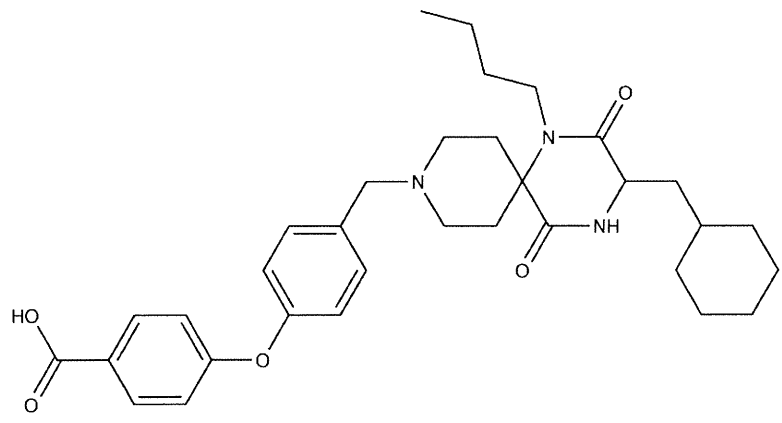
AMD3100 (**10**) (Genzyme Corp., Cambridge, MA, USA), a non-peptidic bicyclam-containing small molecular CXCR4 antagonist, was the first CXCR4 antagonist to enter clinical trials for the treatment of HIV-1-infected patients (**Figure 3B**) [51,52]. It was found, however, to have adverse cardiovascular effects and its use as an anti-AIDS drug was discontinued. AMD3100 has two cyclam moieties tethered by *p*-xylene templates. Teixidó and colleagues have constructed combinatorial libraries based on the structure of AMD3100 and containing: 1) at least two nitrogen atoms on each side of the *p*-xylene template; and 2) separation between these nitrogen atoms similar to that in cyclam [53]. As a result, the non-cyclam compound 11 with potent anti-HIV activity was found. Liotta and colleagues have screened various compounds in which two basic centers (e.g., guanidine, hydrazone or pyridine groups) were connected by a phenyl-containing bridge. A bisymmetric pyridine-containing compound 12 was found to have 10-nM potency [54] but further preclinical studies showed that compound 12 failed to exhibit any *in vivo*



TAK-220 **5**



Vicriviroc/SCH-D (SCH417690) **6**

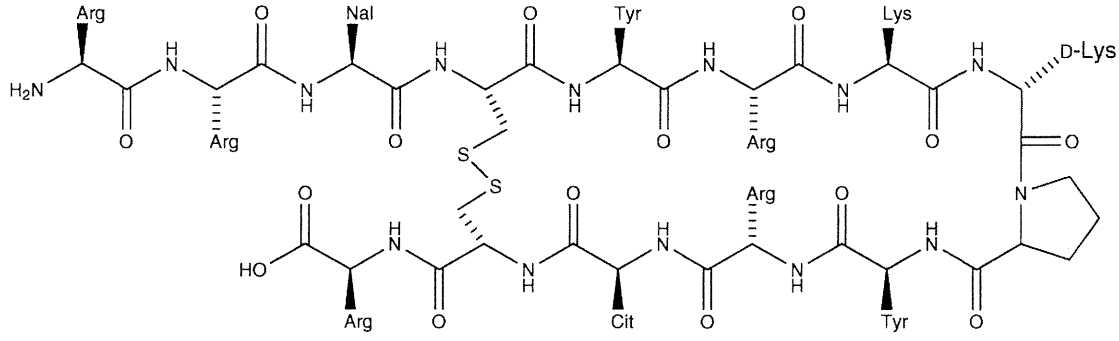


ONO-4128/873140 **7**

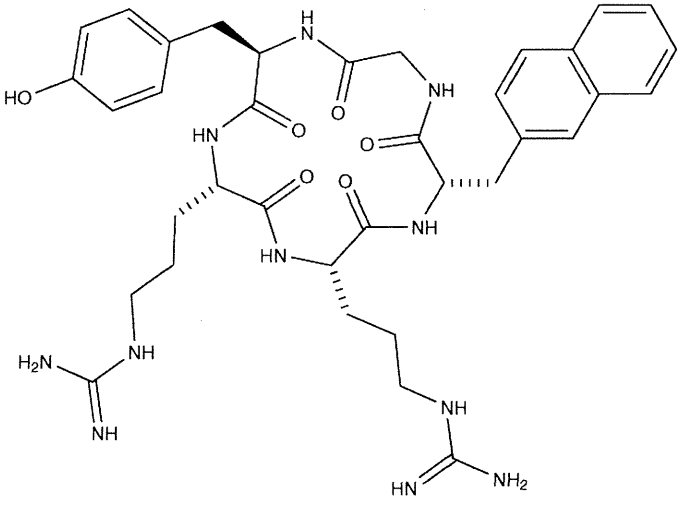
Figure 3. (continued). A. Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (25), n = 20 and (26), m = 12.

efficacy due to poor biostability. On the hypothesis that a poor pharmacokinetic profile of compound **12** might be a result of rapid oxidative metabolism, further structure–activity relationship studies of compound **12** were conducted and led to the development of compound **13** with a nanomolar level of CXCR4 antagonistic activity [55]. To develop non-cyclam CXCR4 antagonists, De Clercq and colleagues have carried out structure–activity relationship studies on AMD3100 (**10**) [56,57]. First, according to the hypothesis that both rings of **10** are not essential structural requirements, they synthesized and evaluated single cyclam analogs containing an aromatic ring instead of another cyclam ring. The

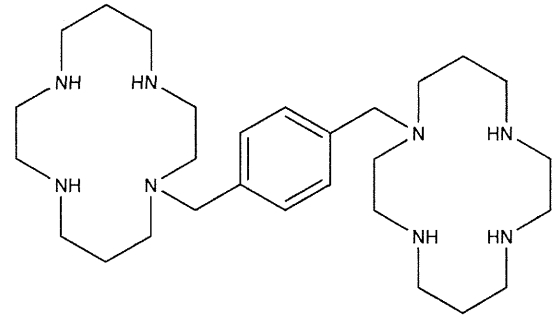
B.



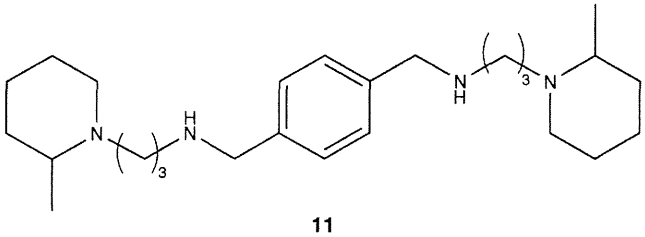
T140 **8**



FC131 **9**



AMD3100 **10**



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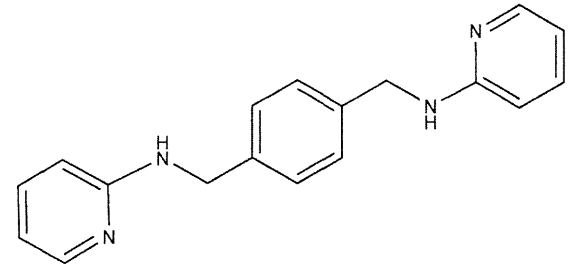

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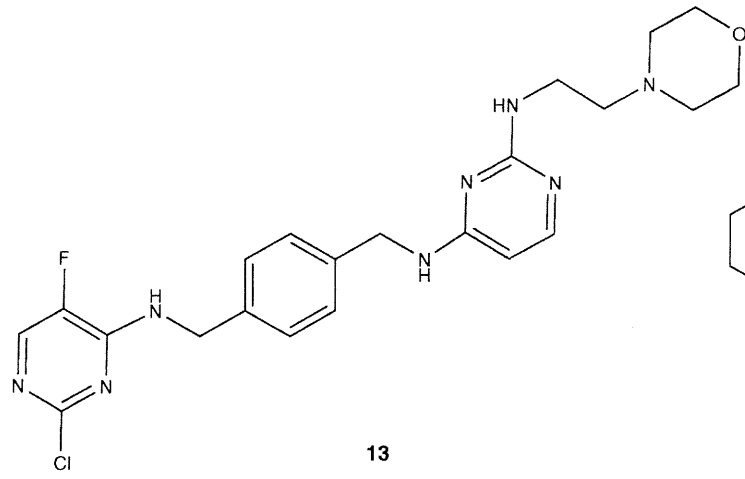
Figure 3. (continued). A. Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (25), n = 20 and (26), m = 12.



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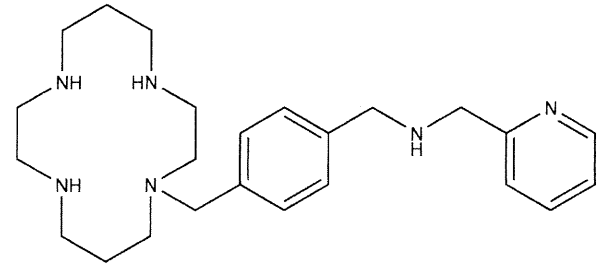
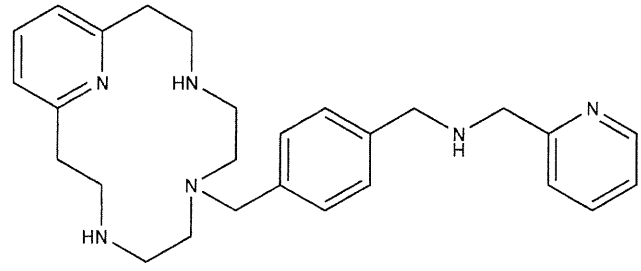


AMD3465 **14**



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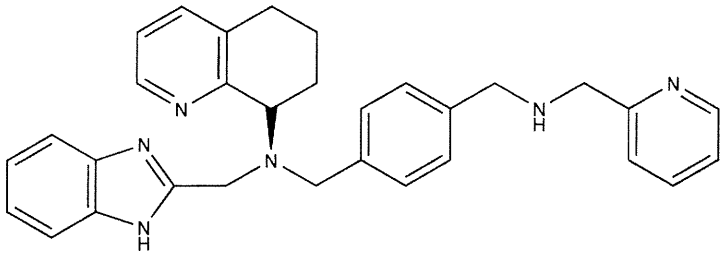

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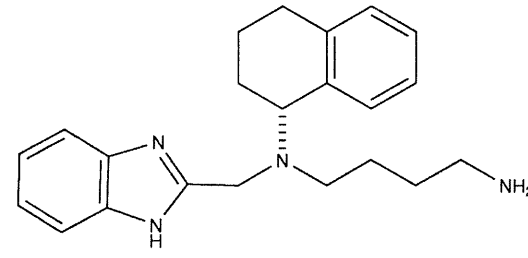
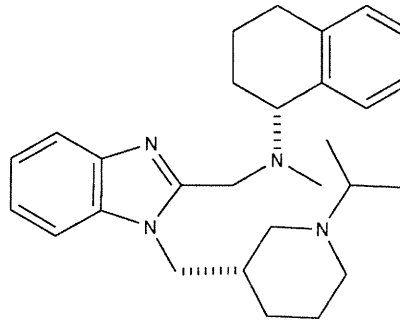


AMD070 **17**



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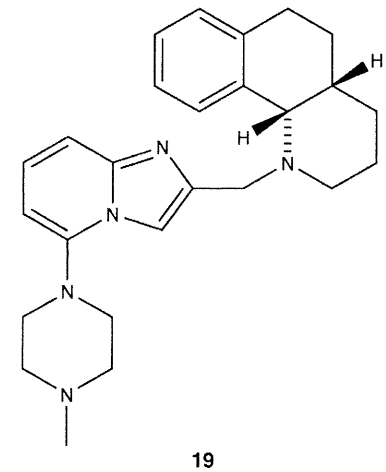

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Figure 3. (continued). A. Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (25), n = 20 and (26), m = 12.

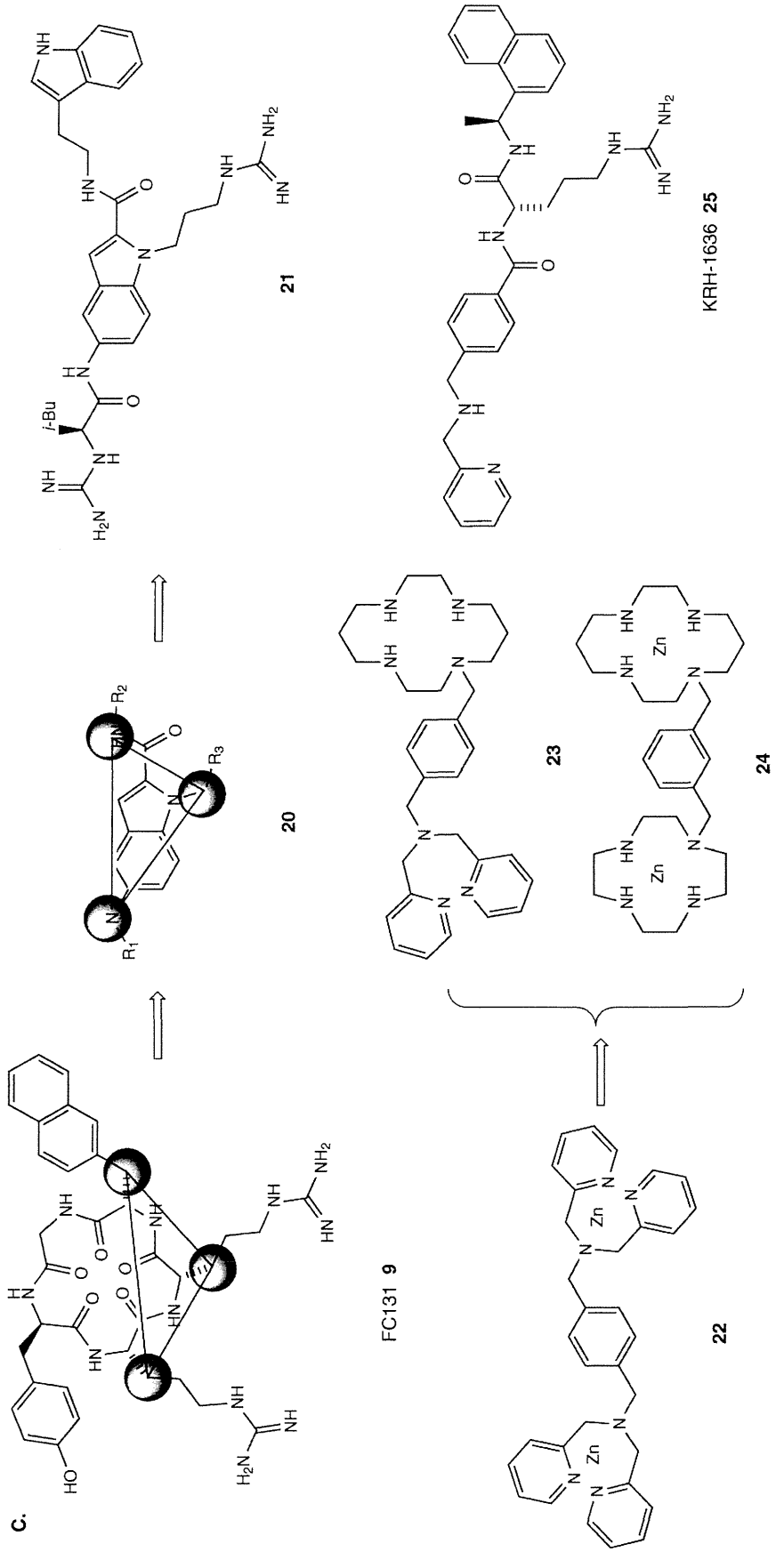

Figure 3. (continued). A. Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (25), n = 20 and (26), m = 12.

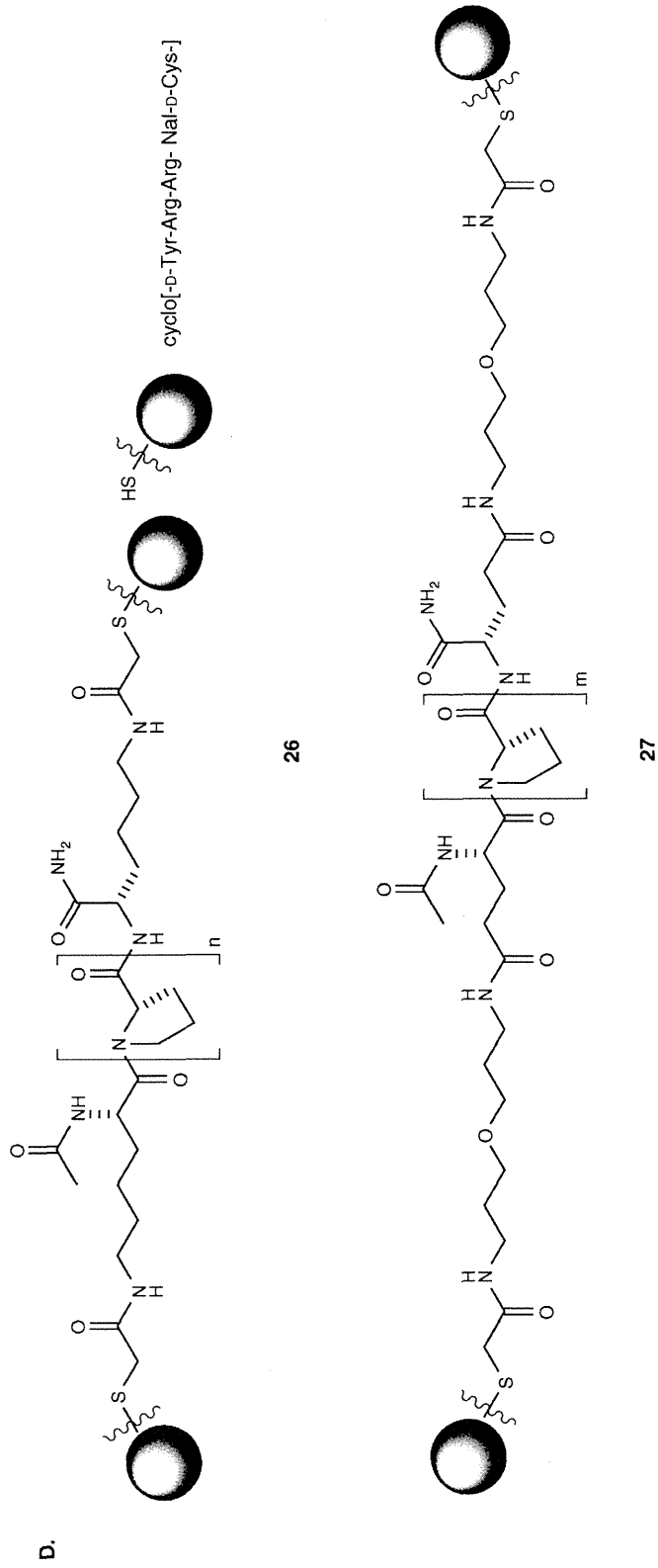

Figure 3. (continued). **A.** Structures of CCR5 antagonists. **B.** Structures of CXCR4 antagonists. **C.** Development of non-peptidic CXCR4 antagonists. **D.** Structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (25), n = 20 and (26), m = 12.

analogs bearing one cyclam ring and one 2-pyridyl ring such as AMD3465 (14) (Genzyme Corp., Cambridge, MA, USA) had CXCR4-binding activity at nanomolar levels [58]. Second, they have fixed the 2-pyridyl ring moiety and replaced the cyclam ring by various azamacrocyclic rings. Compound 15, which contains a pyr-[14]aneN$_4$ ring instead of the cyclam ring, showed potent nanomolar anti-HIV activity [56]. Compound 16, which contains a tetrahydroquinoline and benzoimidazole moiety, showed relatively high anti-HIV activity, although it does not have a cyclam ring [57]. A tetrahydroquinoline compound AMD070 (17) (Genzyme Corp., Cambridge, MA, USA) has been found to be a CXCR4 antagonist by recent antiviral evaluation and pharmacokinetic analysis [59,60]. This compound has a protein-adjusted EC_{50} value of 125 nM against HIV-1 in MT-4 cells and bioavailability of over 20% in rats and approximately 80% in dog and is now in Phase I/II clinical trials. Recently, compounds 18 and 19, which were designed based on the structure of AMD070 (17), have been reported by GlaxoSmithKline Co. Ltd. (GSK) [61,62]. Compound 19 has anti-HIV activity comparable with that of AMD070, relatively low bioavailability in rat (16%) and dog (30%) but a suitable cytochrome P450 profile. Screening against a panel of enzymes and receptors, suggests that compound 19 has little risk of unexpected enzyme and receptor inhibition and it has progressed into toxicology studies. Development of the cyclic pentapeptide FC131 has led to non-peptidic CXCR4 antagonists. In one case, the peptide backbone of FC131 (9) was entirely replaced by an indole template, which enabled reproduction of the disposition of the pharmacophore moieties in the original peptide (Figure 3C). A structure–activity relationship study using modified indoles, for example, 20 identified novel small-molecule antagonists with three appropriately linked pharmacophore moieties such as compound 21 which binds to CXCR4 with micromolar activity [63]. Non-peptide compounds having the dipicolylamine (DPA)–zinc(II) complex structure, utilized as chemosensors that can sense phosphorylated peptide surfaces, were identified as potent and selective antagonists against CXCR4 [64]. A DPA–Zn complex with a xylene scaffold 22 binds to CXCR4 with 50 nM activity and has micromolar anti-HIV activity. Structure–activity relationship studies performed by combining the common structural features of alkylamino and pyridiyl macrocyclic antagonists including DPA–Zn complex (22) and AMD3100 (10) led to new lead compounds 23 and 24 with 30 and 10 nM activity for binding to CXCR4, respectively [65]. Compounds 23 and 24 also have anti-HIV activity of 90 and 30 nM levels, respectively. These are attractive and useful leads for the future development of non-peptidic CXCR4 antagonists. A low molecular weight compound, KRH-1636 (25) (Kureha Chemical, Tokyo, Japan & Daiichi Sankyo Co. Ltd., Tokyo, Japan), derived by intensive modification of the N-terminal tripeptide of T140, Arg-Arg-Nal, was reported to be an orally bioavailable and duodenally absorbable CXCR4 antagonist and X4 HIV-1 inhibitor [66]. Continuous efforts to find more effective CXCR4 inhibitors have recently led to identification of KRH-2731, an orally bioavailable CXCR4 antagonist [67]. Finally, although their structures have not been disclosed yet, the KRH-1636 derivatives KRH-2731 and KRH-3955, which are in the preclinical stage, may be promising as novel inhibitory drugs for treatment of cancer metastasis as well as for HIV-1 infection.

CXCR4 belongs to the G protein-coupled receptor (GPCR) family, and several GPCRs can function *in vitro* as monomers, many of them, including the chemokine receptors, presumably existing *in vivo* as dimers and/or higher order oligomers. Chemokine receptors such as CXCR4 can form homodimers and/or heterodimers with other chemokine receptors [68,69]. Accordingly, we designed and synthesized CXCR4 bivalent ligands consisting of two molecules of an FC131 analog, (*cyclo*(-D-Tyr-Arg-Arg-Nal-D-Cys-)), connected by various lengths of poly(L-proline) or PEGylated poly(L-proline) linkers (26,27) (**Figure 3D**) [70]. A maximum increase in binding affinity for CXCR4 was observed for bivalent ligands of the two linker types with suitable lengths (5.5 – 6.5 nm). As a result, we have presented experimental results concerning the elucidation of the native state of the CXCR4 dimer as a function of the distance between the ligand-binding sites (5.5 – 6.5 nm). Fluorescent-labeled bivalent ligands have, however, been shown to be powerful tools for cancer diagnosis as a result of their ability to distinguish the density of CXCR4 on the surface of cancer cells.

4. HIV integrase inhibitors such as Raltegravir

The enzyme HIV-1-IN is critical to the stable infection of host cells since, by means of 3′-end processing and strand transfer reactions, it catalyzes the insertion of reverse-transcribed viral double-stranded DNA into the chromosomal genome of host cells. It is a 32-kDa protein consisting of 288 amino acid residues, and is divided into N-terminal, C-terminal and catalytic core domains [71,72]. The catalytic core domain has a triad of carboxylate residues, of Asp64, Asp116 and Glu152, which are critical for coordination of two magnesium ions to catalyze breaking and formation of DNA phosphodiester bonds (**Figure 4A**), and which are designated as 3′-end processing and strand transfer reactions, respectively [73-75]. Thus, several IN strand transfer inhibitors possessing a two magnesium-binding pharmacophore, which target the carboxylate triad, have been developed. Initially, diketo acids (DKAs) and their analogs, such as L-731,988 (28) and L-708,906 (29) (Merck & Co., NJ, USA), which have a two magnesium-binding pharmacophore, have been found as first-generation IN inhibitors (**Figure 4B**) [76]. This design is based on an interactive model of the binding of these inhibitors to the carboxylate triad through coordination of two magnesium ions. However, some DKA compounds lacked sufficient potency for binding to IN and pharmacokinetic properties. New heterocyclic DKA analogs with the two magnesium-binding pharmacophore including the naphthyridine