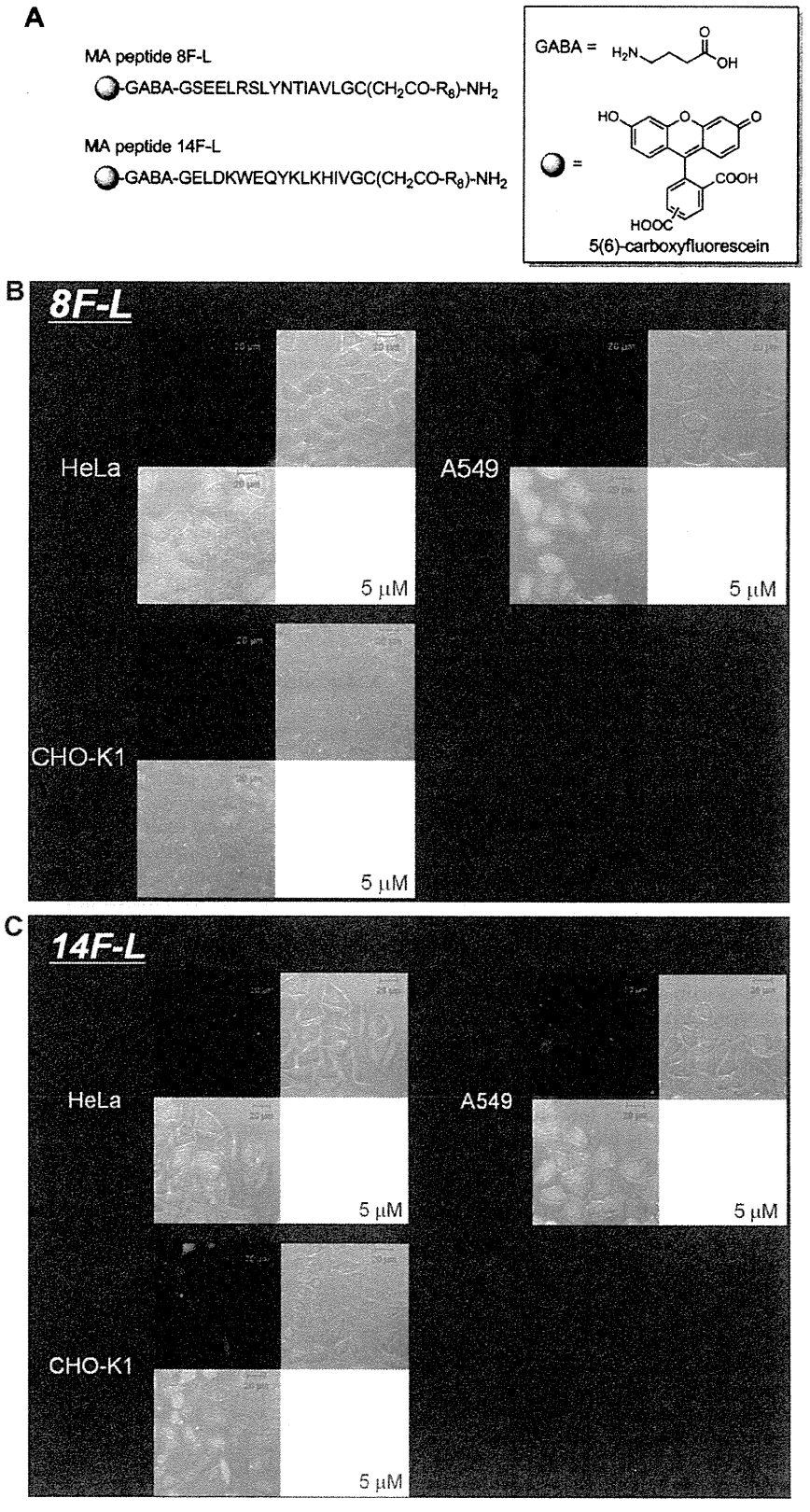

Figure 5. (A) The structures of fluorophore-labeled MA peptides 8F–L and 14F–L. (B) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 8F–L. (C) The fluorescent imaging of live cells HeLa, A549 and CHO-K1 by 14F–L.

expression of cytotoxicity and in future, a different effective strategy for cell penetration may be advisable.

In the present assay, the control MA peptides 6C and 9C, which cover MA(51–65) and MA(81–95), respectively, showed significant anti-HIV activity. This is consistent with the previous studies, in which MA(41–55), MA(47–59) and MA(71–85) showed anti-HIV or dimerization inhibitory activity as discussed above.[16–18] These peptides have no R_8 sequence and thus cannot penetrate cell membranes. They exhibit inhibitory activity on the surface of cells, not intracellularly.

The structures of MA peptides 8L and 9L, dissolved in PBS buffer (2.7 mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 9.59 mM Na_2HPO_4) at pH 7.4, were determined by CD spectroscopy (Fig. 3). When peptides form α-helical structures, minima can be observed at approximately 207 and 222 nm in their CD spectra. The amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in an α-helical region (helix 4) of the parent MA protein (Fig. 4), and peptides 8L and 9L were presumed to have an α-helical conformation.[26–28] However, the CD spectra shown in Figure 3, suggest that these peptides lack any characteristic secondary structure. This is because the 15-mer peptide derived from MA is not sufficiently long to form a secondary structure even though Gly, Cys and octa-Arg are attached to their C-terminus. Analysis of the CD spectra suggests MA fragment peptides need a longer sequence in order to form a secondary structure. The CD spectra of the control MA peptides 8C and 9C were not determined because the aqueous solubility of these peptides is inadequate.

Fluorescent imaging of live cells was used to evaluate the cell membrane permeability of the MA peptides 8L and 14L, which showed high and zero significant anti-HIV activity, respectively. The MA fragment 14 is a hybrid of the fragments 2 and 3, and the MA peptides 14L and 14C, which are based on the conjugation of the N-terminal chloroacetyl group of an R_8 peptide and iodoacetamide to the thiol group of the Cys residue, respectively (Supplementary data), are control peptides lacking significant anti-HIV activity (Tables 1 and 2). These peptides were labeled with 5(6)-carboxyfluorescein via a GABA linker at the N-terminus to produce 8F–L and 14F–L (Fig. 5A). The fluorophore-labeled peptides 8F–L and 14F–L were incubated with live cells of HeLa, A549 and CHO-K1, and the imaging was analyzed by a fluorescence microscope (Fig. 5B and C). A549 cells are human lung adenocarcinomic human alveolar basal epithelial cells.[29] Similar penetration of both peptides 8F–L and 14F–L into these cells was observed. Even peptides without significant anti-HIV activity can penetrate cell membranes. The penetration efficiency of both peptides into A549 was relatively high and into HeLa was low. In CHO-K1 the penetration efficiency of 8F–L is relatively low, but that of 14F–L is high. These imaging data confirm that the MA peptides with the R_8 sequence can penetrate cell membranes and suggest that MA peptides such as 8L and 9L should be able to inhibit HIV replication inside cells.

4. Conclusions

Several HIV-1 inhibitory fragment peptides were identified through the screening of an overlapping peptide library derived from the MA protein. Judging by the imaging experiments, peptides possessing the R_8 group can penetrate cell membranes and might exhibit their function intracellularly thus inhibiting HIV replication.

Two possible explanations for the inhibitory activity of these MA fragment peptides can be envisaged: (1) The fragment peptides might attack an MA protein and inhibit the assembly of MA proteins. (2) These peptides might attack a cellular protein and inhibit its interaction with MA. Further studies to elucidate detailed action mechanisms and identify the targets of these peptides will be performed in future. The technique of addition of the R_8 group to peptides enabled us to screen library peptides that function within cells. Thus, the design of an overlapping peptide library of fragment peptides derived from a parent protein with a cell membrane permeable signal is a useful and efficient strategy for finding potent cell-penetrating lead compounds.

In the present study, the MA peptides 8L and 9L were shown to inhibit HIV-1 replication with submicromolar to micromolar EC_{50} values in cells using the MT-4 assay (NL4-3 and NL(AD8) strains) and the p24 ELISA assay (JR-CSF strain). Our findings suggest that these peptides could serve as lead compounds for the discovery of novel anti-HIV agents. Amino acid residues covering fragments 8 and 9 corresponding to 8L and 9L are located in the exterior surface of MA, and in particular in the interface between two MA trimers (Fig. 4C).[26–28] The interaction of two MA trimers leads to the formation of an MA hexamer, which is the MA assembly with physiological significance. Thus, the region covering fragments 8 and 9 is critical to oligomerization of MA proteins. This suggests that MA peptides 8L and 9L might inhibit the MA oligomerization through competitive binding to the parent MA, and that more potent peptides or peptidomimetic HIV inhibitors could result from studies on the mechanism of action of these MA peptides and identification of the interaction sites. Taken together, some seeds for anti-HIV agents are inherent in MA proteins, including inhibitors of the interaction with PM such as the MA peptide 2C.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.055.

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—Review—

ケミカルバイオロジーを基盤とした抗HIV剤の創製

玉村啓和

Development of Anti-HIV Agents Based on Chemical Biology

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Recently, highly active anti-retroviral therapy (HAART), which involves a combinational use of reverse transcriptase inhibitors and HIV protease inhibitors, has brought us a great success in the clinical treatment of AIDS patients. However, HAART has several serious clinical problems. These drawbacks encouraged us to find novel drugs and increase repertoires of anti-HIV agents with various action mechanisms. The recent disclosing of the dynamic supramolecular mechanism in HIV-entry has provided potentials to find a new type of drugs. To date, we have synthesized HIV-entry inhibitors, especially coreceptor CXCR4 antagonists. In addition, CD4 mimics in consideration of synergic effects with other entry inhibitors or neutralizing antibodies have been developed. The development of the above anti-HIV agents is based on the concept of reverse chemical genomics, in which target molecules are fixed. On the other hand, based on the concept of forward chemical genomics, in which active compounds are searched according to the screening of random libraries, effective peptide leads such as integrase inhibitors derived from fragment peptides of HIV-1 Vpr have been discovered. As such, from a point of view on chemical biology, anti-HIV leads have been found utilizing reverse and forward chemical genomics. Furthermore, antibody-based therapy or AIDS vaccine is still thought to be a promising treatment. Thus, peptidic antigen molecules based on artificial remodeling of the dynamic structures of a surface protein gp41 in HIV fusion have been developed. The present chemical biology approaches would be essential for discovery of anti-HIV agents in consideration of cocktail therapy of AIDS.

Key words——anti-human immunodeficiency virus (HIV) agent; AIDS vaccine; chemical biology; CXCR4 antagonist; CD4 mimic; integrase inhibitor

1. はじめに

1980年頃サンフランシスコやニューヨークにおいて，これまで知られていなかった進行性の呼吸器障害により死亡する例が相次いで報告された．患者らは体内のCD4陽性リンパ球が急速に減少又は消失し，免疫不全状態を呈しており，この疾患は後天性免疫不全症候群（acquired immunodeficiency syndrome, AIDS）と名付けられた．この疾患はすぐに全世界に広がり，原因ウイルスであるヒト免疫不全ウイルス（human immunodeficiency virus, HIV）が単離された．HIVは空気感染ではなく主に性的感染，血液感染，母子感染の3つの経路により感染し，現在世界で4000万人以上のHIV感染者がおり，多くの発展途上国においては今もなお感染者数が増加している．さらに，多くの先進国では感染者数が減少しているのに対し，日本では増加傾向にある．[1,2]

大きく分けてHIVには，HIV-1及びHIV-2が存在し，HIV-1は西半球，ヨーロッパ，アジア，アフリカ中央部・南部・東部で多くみられ，HIV-2はアフリカ西部で多くみられる．HIV-2に比べHIV-1は感染例が多く，感染力も強いため，抗HIV薬やエイズワクチン開発は主にHIV-1を標的として行われている．

HIV-1は，直径約110 nmのRNA型エンベロープウイルス（レトロウイルス）で，約9500塩基からなる2コピーのRNAゲノムや逆転写酵素，インテグラーゼなどを含む核（キャプシド，capsid）と，それを取り囲む球状エンベロープによって構成され

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本総説は，日本薬学会第131年会シンポジウムS05で発表したものを中心に記述したものである．

る．ウイルス粒子の外側を構成するエンベロープには，外に突き出している糖タンパク質 gp120 とその内側に存在する脂質二重膜を貫通する糖タンパク質 gp41 からなるスパイクがある．この gp120 と gp41はヘテロダイマーを形成し，さらにヘテロダイマーを形成したタンパク質がホモ三量体を形成して HIV 膜上に十数個存在している［Fig. 1(A)］．これまでの研究により HIV-1 の宿主細胞への侵入から新ウイルスの出芽までの一連の複製サイクル（ライフサイクル）が詳細に解明されてきた．このサイクルは数ステップに分けることができ，ウイルスの宿主細胞への吸着・膜融合，RNA ゲノムの逆転写，ウイルス DNA の宿主 DNA への組込み（インテグレーション）によるプロウイルス DNA の形成と複製，ウイルス構成タンパク質のプロセッシング，ウイルス粒子の構築，出芽，ウイルスの成熟化の過程を経て増殖していく［Fig. 1(B)］．[3,4]

これまで，上述のような HIV のライフサイクルを各ステップで阻害するような薬剤の研究・開発が進められてきた．主に HIV 固有の酵素をターゲットとして，ウイルスのライフサイクルの異なる作用点に働くものがある．代表的な抗 HIV 薬として核酸系若しくは非核酸系逆転写酵素阻害剤，プロテアーゼ阻害剤，インテグラーゼ阻害剤が開発され，臨床において用いられている．また，侵入過程を阻害する膜融合阻害剤，CCR5 阻害剤も FDA で認可されている．[5] しかし，HIV-1 は変異を起こし易いため，単剤療法では薬剤耐性があらわれ薬剤の効果がなくなってしまう．そのため HIV 感染症の治療には，薬剤の耐性変異が重ならないように数種の薬剤を組み合わせた多剤併用療法を行っている．これは，highly active anti-retroviral therapy（HAART）と呼ばれ，主に逆転写酵素阻害剤，プロテアーゼ阻害剤，インテグラーゼ阻害剤の中から 2，3 剤の組み合わせで用いられる．[6] HAART は有効な治療法として成果を挙げているが，長期投与による高額な治療費，重篤な副作用，耐性ウイルスの出現などの問題点もある．そのため，これまでの薬剤とは異なる作用機序を持つ新たな薬剤の開発が望まれており，創薬化学者には実際に使用できる薬剤のレパートリーを増やすことが求められている．われわれは以前からコレセプター CXCR4 阻害剤を中心に抗 HIV 剤を創製しており，最近ではケミカルバイオ

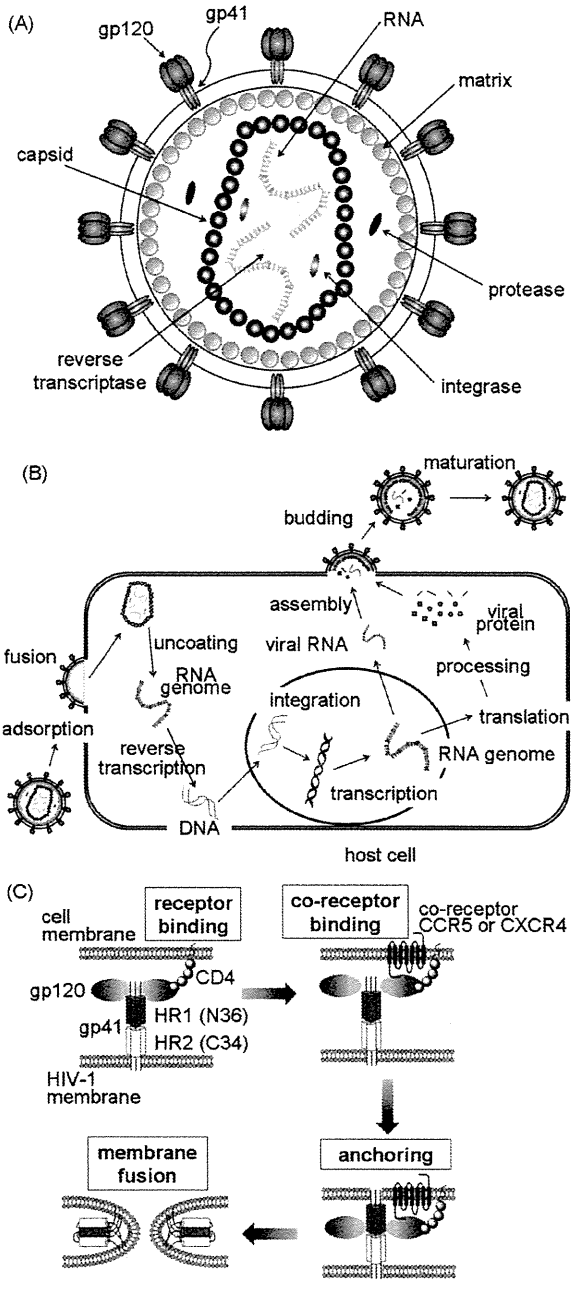

Fig. 1. (A) Structure of HIV Virion, (B) Replication Cycle of HIV, (C) Mechanism of HIV-1 Entry and Fusion

玉村啓和

1964 年生まれ．京都大学大学院薬学研究科修士課程在学中に助手に着任，博士（薬学）．京都大学大学院薬学研究科助教授を経て，2005 年より東京医科歯科大学生体材料工学研究所教授．1999–2000 年 米国国立衛生研究所/米国国立がん研究所へ留学．専門はペプチド・天然物を基盤とした創薬化学，ケミカルバイオロジー．

ロジー的手法を駆使して，吸着阻害剤 CD4 ミミック，膜融合阻害剤，インテグラーゼ阻害剤，HIV の侵入の膜融合機構をターゲットとした人工設計型抗原分子を創製している．本論文では，その研究内容について報告する．

2. コレセプター CXCR4 阻害剤

HIV の宿主細胞への吸着・膜融合に関して，まず，HIV エンベロープタンパク質 gp120 が細胞表面上の第一受容体である糖タンパク質 CD4 に結合し，gp120 のコンフォメーション変化の後に，gp120 は第二受容体（コレセプター）である CCR5 あるいは CXCR4 に結合する．[7,8] CCR5 と CXCR4 は 7 回膜貫通 G タンパク質共役型受容体（7TM-GPCR）に属するケモカイン受容体である．CCR5 は HIV 感染の初期に主流になるウイルス株マクロファージ指向性 HIV-1（R5-HIV-1）が主に使用するコレセプターであり，CXCR4 は HIV 感染の後期に主流になるウイルス株 T 細胞指向性 HIV-1（X4-HIV-1）が主に使用するコレセプターである．gp120 の CD4，CCR5 あるいは CXCR4 に対する結合より，gp120 と非共有結合的にヘテロダイマーを形成している gp41 の N 末端側が露出され，gp41 に存在する膜挿入ペプチドが標的細胞の細胞膜にアンカリングする．アンカリングの後，三量体の gp41 の N 末端側の helix 領域である HR1（NHR, N-region）領域（N36 配列を含む領域）と C 末端側 helix 領域である HR2（CHR, C-region）領域（C34 配列を含む領域）が逆平行に結合し，六量体を形成することで HIV の膜と標的細胞の膜が近づき膜融合を引き起こす［Fig. 1(C)］．[9]

まず，玉村の以前の所属機関である京都大学大学院薬学研究科の藤井信孝教授の下で，コレセプター CXCR4 阻害剤の創製に取り組んだ．コレセプター CXCR4 を抗エイズ薬のターゲットとすることは，もう 1 つの主要なコレプターである CCR5 の阻害剤 Maraviroc（Pfizer Inc.）[10]が臨床使用されたことより妥当であると考えられ，CXCR4 阻害剤も早急な開発と安全性の確認が期待される．HIV のコレセプター指向性を考えると，HIV 感染直後の前期からエイズを発症する後期に移行するに従って，CCR5 指向性の株から CXCR4 指向性の株が主流になっていく．このことから，CCR5 阻害剤だけでなく，片手落ちにならないように CXCR4 阻害剤も必要と思われる．1989 年頃から，カブトガニの血球由来の防御ペプチド polyphemusin の構造活性相関研究を精力的に行い，T22 という 18 残基からなる侵入阻害ペプチドを見い出した．[11]CXCR4 がコレセプターとして同定された後，T22 は CXCR4 アンタゴニストであることが証明され，[12] 構造最適化により 14 残基からなる強力な CXCR4 アンタゴニスト活性を示す T140 を見い出した．[13] その生体内安定性を向上させた T140 誘導体は現在，臨床試験（phase II）中である．[14,15] また，T140 のファルマコフォアのアミノ酸残基を基にした環状ペンタペプチドライブラリーを構築し，この中から T140 と同等のアンタゴニスト活性を有する誘導体 FC131 の創出に成功した［Fig. 2(A)］．[16]

これらのリード化合物を基にさらに低分子量のペプチドミミックも見い出しており，[17] さらに，非ペプチド性の CXCR4 アンタゴニストである二核亜鉛錯体[18]やその誘導体[19]を創出している［Fig. 2(B)］．CXCR4 が HIV のコレセプターであることが報告されてから数年後に，CXCR4 とその内因性リガンド CXCL12 の相互作用が，種々の固形がんの転移や血液がんの進行，関節リウマチの炎症等に大きく係わっていることが明らかにされた．[20] それに伴い，T140 誘導体等ががん転移阻害活性，白血病の進行の阻害活性，抗関節リウマチ作用を有することを明らかにした．[20-22] また，他の研究者からも AMD3100（Genzyme Corp.）や KRH-1636（Kureha Chemical & Daiichi Sankyo Co., Ltd.）等種々の CXCR4 アンタゴニストが報告されているが，誌面の関係上，総説を参照されたい．[23] 生理的には，CXCR4 は CXCL12 との相互作用により，胎生時の血管形成や心形成，造血，神経形成において progenitor cell の遊走や活性化等の重要な作用を示すことから，CXCR4 アンタゴニストの副作用を十分検討する必要がある．以上より CXCL12/CXCR4 情報伝達系の制御は，HIV 感染症，がん転移，白血病，関節リウマチ等の多くの疾病に対する創薬戦略として有望であり，言い方を変えれば，CXCR4 は multiple 創薬ターゲットとして魅力的である．現在，立体配座固定化テンプレートを活用し，より有用な創薬リードへ導くよう進めている．

3. 吸着阻害剤 CD4 ミミック

HIV は細胞侵入時に，エンベロープタンパク質

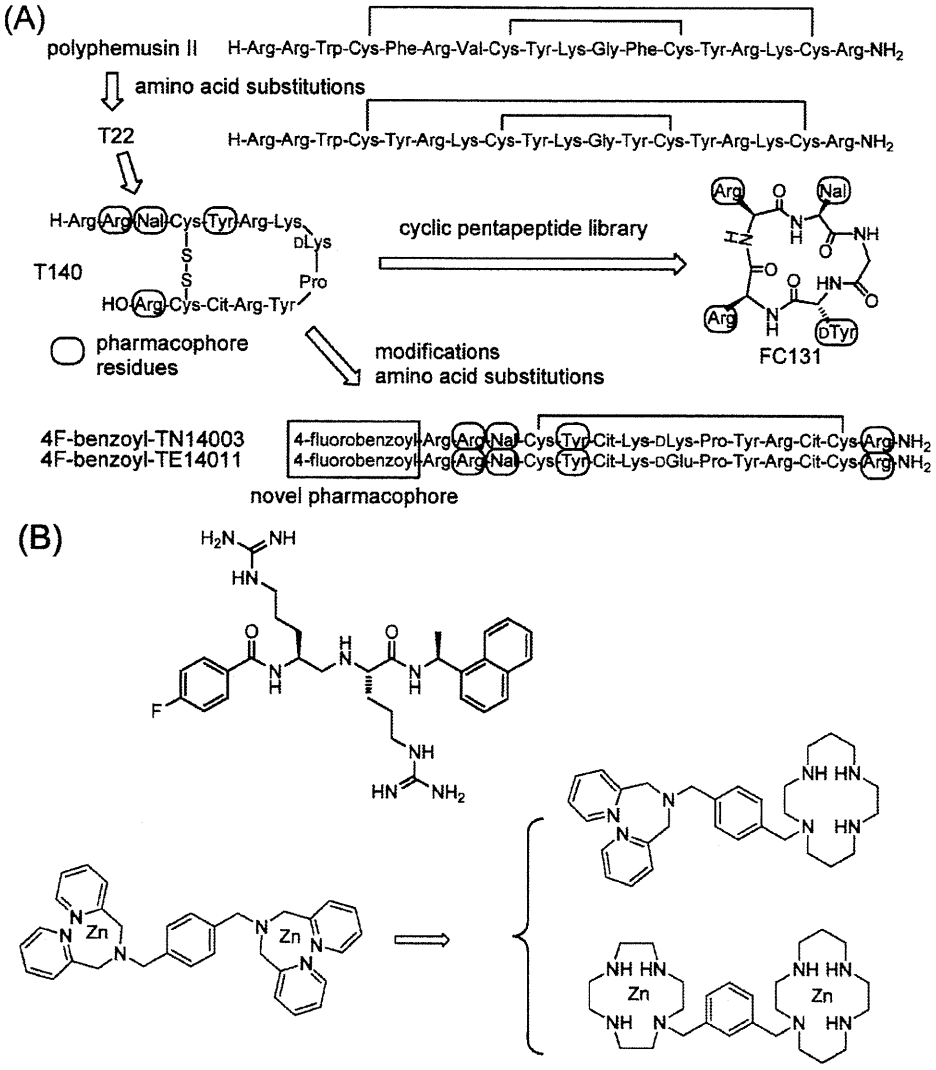

Fig. 2. (A) Development of CXCR4 Antagonists Based on Horseshoe Crab Peptides, Nal=L-3-(2-naphthyl) alanine, Cit=L-citrulline, (B) Structures of a Peptide Mimic CXCR4 Antagonist with Low Molecular Weight (upper), a Dipicolylamine (Dpa)-Zinc(II) Complex (lower left) and Its Derivatives (lower right)

gp120 が細胞表面上の第一受容体 CD4 に結合し，gp120 のコンフォメーション変化が生じ，gp120-CD4-コレセプター（非共有結合複合体）の形成，gp41 と宿主細胞膜の相互作用を経て膜融合する (Fig. 1)．NBD-556 は HIV-1 の複合体形成阻害スクリーニングにより見い出された HIV 侵入阻害剤である．[24] また，NBD-556 (Fig. 3) は可溶性 CD4 と同様に gp120 と相互作用することにより gp120 の構造変化を促すことから，低分子型 CD4 ミミックとして注目されている．これまでに可溶性 CD4 と gp120 の共結晶構造が明らかにされ，CD4 の Phe^{43} の側鎖が gp120 の特徴的な空洞 (Phe-43 cavity) に入り込む形で相互作用することが明らかにされている．[25] われわれは，可溶性 CD4 と gp120 の共結晶構造を基にした分子モデリング解析 (Flex-SIS module of SYBYL 7.1) を行い，Phe^{43} だけでなく Arg^{59} も gp120 と相互作用することを示し，[26] NBD-556 のアニリン部位が Phe^{43} の側鎖と，テトラメチルピペリジン環部位が Arg^{59} の側鎖と対応するように gp120 と相互作用することが示唆された (Fig. 3)．さらに，NBD-556 のクロロアニリン部位は CD4 に比べ，Phe-43 cavity に 6.5 Å 程度深く入り，gp120 の芳香族アミノ酸 (Trp^{447}, Phe^{382}, Trp^{112}) と，リンカーであるオキサミド構造は水素

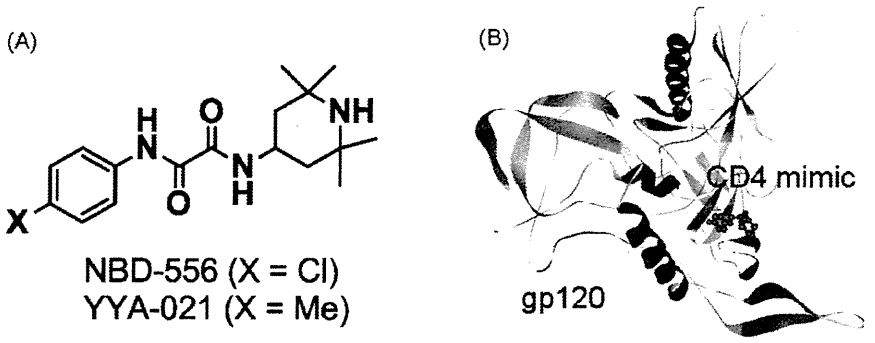

Fig. 3. (A) Structures of CD4 Mimics NBD-556 and YYA-021, (B) Docking Model of gp120 and NBD-556

結合供与体としてgp120と相互作用していることも示唆されている.[25] すなわち，このCD4ミミックは適切なリンカーを用いることにより，CD4のペプチド二本鎖上の2つの部位を含む広範囲な領域をミミックしている．われわれはPhe-43 cavity周辺の構造的及び静電的要求を明らかにする目的で，NBD-556の芳香環パラ位に関する構造活性相関研究を行った.[26] これにより，芳香環パラ位にある程度嵩高くかつそれほど電子供与性の強くない官能基を有する誘導体（YYA-021等）が顕著な抗HIV活性及びgp120の構造変化能を有することを明らかにした（Fig. 3）．また，この構造変化によりCCR5/CXCR4などのコレセプター結合領域やCD4-induced siteを認識する中和抗体の結合能が上昇することも併せて明らかにしている.[27] さらに，これらCD4ミミックは前章で述べたCXCR4アンタゴニストT140と併用すると，顕著な相乗効果を示し,[26] CD4ミミック-T140誘導体のハイブリッド化合物の創製にも成功している.[28] このようにCDミミックは中和抗体やコレセプター阻害剤との併用により，さらに有用性が上がると思われる．

一般にタンパク質-タンパク質間の相互作用では，相互作用領域が連続した一次構造（アミノ酸配列）で存在するのではなく，高次構造の中で不連続に存在することが少なくない．この場合，相互作用領域に係わる官能基を適当なリンカーで架橋した低分子化合物により，分子量が数百倍という元の親タンパク質の機能を模倣することも可能である．このHIV侵入阻害剤CD4ミミックはその一例であろう．

4. インテグラーゼ阻害剤

これまで臨床で使用された抗エイズ薬や上述のわれわれが創製した抗HIV剤はすべて標的分子設定型のリバースケミカルジェネティクス的手法により創出されたものである．例えば，逆転写酵素阻害剤やプロテアーゼ阻害剤を開発する場合は，それぞれの酵素（標的分子）に結合するように分子設計されている．CXCR4アンタゴニストやCD4ミミックを創製した際は，CXCR4やgp120（標的分子）に結合するようにデザインした．次に，創薬候補品等有用なリード化合物を創出するケミカルバイオロジー的手法として，このようなリバースケミカルジェネティクス的手法とは方向性が正反対になるフォワードケミカルジェネティクス的手法に着目した．すなわち，ランダムライブラリーから抗HIV活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，有用な抗HIV剤のリード化合物を見い出そうとした．

まず，HIV構成タンパク質の中にHIV自身の複製を阻害するものが存在するだろうという仮説の下に，上述のランダムライブラリーのソースをHIV-1の遺伝子産物であるタンパク質（Gag・Pol・Env・Vpu・Vpr・Rev・Tat）由来のアミノ酸配列を基にしたオーバーラッピングペプチドライブラリー（アミノ酸10–17残基）とした．このライブラリーを用いて，簡便に評価できるインテグラーゼ（IN）阻害活性（細胞内で阻害活性を評価する系ではない）のスクリーニングを行った結果，アクセサリータンパク質であるVpr由来の3個の部分ペプチドに抗IN活性が見い出された．これらの部分ペプチドには共通してLQQLLF配列が含まれていた（Fig. 4）.[29] LQQLLF配列が阻害活性の発現に重要なモチーフであると考えられ，また，細胞内で活性を発現させるため，LQQLLFモチーフを中心に数種類のペプチドを合成し，細胞膜透過性モチーフで

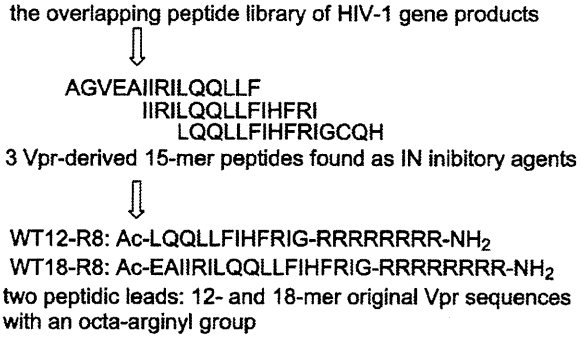

Fig. 4. Development of Vpr-derived IN Inhibitors

あるocta-Arg（RRRRRRRR）をC末端側に付与した．その結果，抗IN活性だけでなく細胞レベルでの抗HIV-1活性ともに強力なペプチドWT18-R8（Ac-EAIIRILQQLLFIHFRIG-RRRRRRRR-NH_2）及びWT12-R8 (Ac-LQQLLFIHFRIG-RRRRRRRR-NH_2）を同定した（Fig. 4）．LQQLLFモチーフはこのVpr中のαヘリックス構造部分に存在していることから，αヘリックス構造が阻害活性の発現に重要であると考えられる．[30]感染の前期過程において，VprもINも酵素や鋳型RNA及び様々な細胞内宿主因子を含むプレインテグレーション複合体（Pre-Integration Complex, PIC）と呼ばれる複合体の中に存在し，VprはINと相互作用することでHIV自身のオートインテグレーションを制御している（BAFのような作用）かもしれない．いずれにしても，HIV構成タンパク質の中にHIV自身の複製を阻害するものが存在する可能性がある．また，PIC中でVprは溶液中とは異なるコンフォメーションをとり，LQQLLFモチーフを含むαヘリックス構造部分が外側に提示され，INと相互作用して，INの作用をマスクしているのかもしれない．これは，大きなタンパク質の中に秘められた神秘的な活性が部分ペプチドに存在する，すなわち，親タンパク質の作用とは異なる作用が部分ペプチドに存在する，といういわゆるクリプタイド（＝cryptic peptide）の概念にも関係すると思われる．[31]このIN阻害ペプチドはさらに構造を最適化することにより，Raltegravir（Merck Sharp & Dohme Corp.）[32]とは違うアロステリック機構を持つ新たな抗IN剤の創出として期待できる．

現在同様に，ランダムライブラリーから抗HIV活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，HIV-1 matrix protein（MA）由来の部分ペプチドが抗HIV活性を示すことを見い出しており，本法を用いることにより，有用な抗HIV剤のリード化合物を見い出すことができる可能性がある．

5. 膜融合機構をターゲットとしたワクチンと膜融合阻害剤

これまで臨床で使用できる多くの抗HIV薬が開発され，多剤併用療法（HAART）は大きな効果を上げてきた．さらに，広範囲のウイルス株に対して中和活性を持つワクチンを開発することによって，特に最近感染者が増加しているアフリカ，アジア等の発展途上国でAIDS及びHIV感染症で苦しむ患者のQOLの改善や治療における選択肢の拡大に貢献できると考えられる．HIVワクチンの開発においてはこれまでの通常の感染症に対して有効である弱毒化ワクチン，生ワクチンといった方法はHIVの易変異性ゆえに危険視されたこともあり使うことができない．さらに，通常抗体誘導を行う際には，ウイルス粒子表面のタンパク質における部分配列を合成しその配列特異的な抗体を誘導しているが，誘導された抗体はそのアミノ酸配列に特異的に結合するものの，中和標的の立体構造に対しての特異性や結合活性は概して低い．変異の激しいHIVを標的とする場合，アミノ酸の一次配列だけではなく，立体構造を認識して結合する中和抗体を誘導することが望ましいと考えられ，これまでとは違ったワクチン開発が必要となっている．そこで，立体構造を保持した状態の抗原分子を用いることによって，立体構造に対しても特異的な抗体を誘導することができれば，HIVに対してもより高い中和活性を有する抗体が誘導できるのではないかと考えた．

HIVは先に述べたように，gp120-CD4-コレセプター（非共有結合複合体）の形成後，ホモ三量体のgp41が宿主細胞膜にアンカリングし，HR1領域とHR2領域の会合により6ヘリックスバンドル（六量体）を形成して宿主細胞膜と膜融合する（Fig. 1）．これまでにこの膜融合過程を標的とした創薬研究が広く行われ，Enfurvirtide（fuzeon / T20）（Roche/Trimeris）が膜融合阻害剤としてFDAから認可されている．[33] EnfurvirtideはHR2（CHR, C-region）領域の部分ペプチドであり，HR2ミメティックとしてHR1領域に相互作用することで，

6 ヘリックスバンドル形成を阻害する．また，HR1 及び HR2 ヘリックス領域は HIV 株間で高く保存されていることから，gp41 のアミノ酸配列を認識する中和抗体（2F5 及び 4E10）が見い出されている．[34] さらに，近年では gp41 に対する特異性向上を目的として，その立体構造を模倣した gp41 ミメティックの創製研究が行われ，HR1 又は HR2 ヘリックス領域を 3 価型テンプレートで束ねた gp41 ミメティックが複数合成され，阻害剤や人工抗原分子として応用されている．[35-37] しかし，これら gp41 ミメティックは非等価なテンプレートの構造上，ヘリックス領域の位相にずれが生じ，天然の三量体構造を再構築しているとは言いがたい．われわれは天然型の gp41 が有する等価な三量体構造を模倣するために，ヘリックス領域を等価に配置可能な C3 対称性テンプレートを新たにデザインし，3 本の HR1（NHR, N-region）領域由来ペプチド（N36）を N 端側から集積させた gp41 ミメティック（N36 三量体）を創製した（Fig. 5）．[38] 合成した N36 三量体の CD スペクトル測定により，三量体は単量体に比べ高い α ヘリックス性を有していることが明らかになり，C3 対称性テンプレート上での N36 三量体構造の形成が示唆された．また，HR2（CHR, C-region）領域由来ペプチド（C34）と混合させると α ヘリックス性が上昇したことから，この gp41 ミメティックは天然の構造を高く反映していると考えられる．合成した N36 三量体をマウスに免疫した結果，得られた血清は N36 単量体よりも N36 三量体に対する抗体価が約 30 倍高く，三量体構造を特異的に認識する抗体が誘導されていることが示唆された．さらに，N36 三量体の免疫によって得られた血清は，単量体の免疫に比べ，強い中和活性がみられたことから，三量体構造を特異的に認識する抗体の有用性が示唆された．われわれが合成した N36 三量体ミミックは C3 対称性テンプレートを用いることにより，天然の三量体構造を再構築することができ，これを抗原分子として使用することにより天然の高次構造を特異的に認識する抗体が誘導されたと考えられる．なお，この N36 三量体ミミックは，阻害剤としても N36 の量論比に相当する抗 HIV 活性を有していた．また，HR1 領域ペプチドの対になっている HR2 領域ペプチド（C34）の三量体ミミックに関しても，別の C3 対称性テンプレートを合成し，3 本の C34 を C 端側から集積させた gp41 ミメティック（C34 三量体）を創製した．同様に，C34 三量体ミミックは単量体よりも三量体

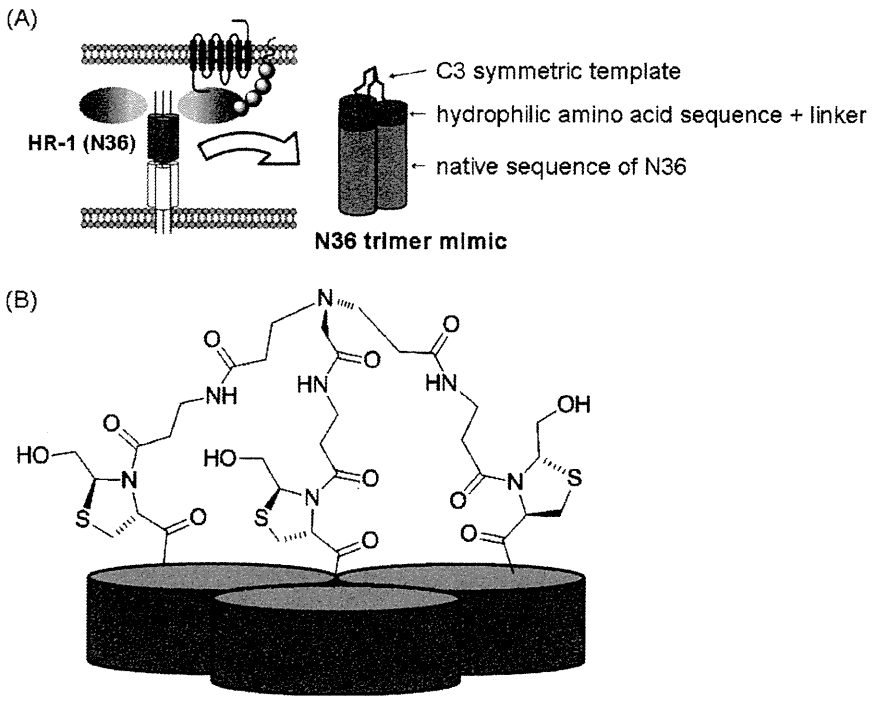

Fig. 5. (A) Development of an N36 Trimer Mimic Using a C3-Symmetric Template, (B) Structure of the C3-Symmetric Template

構造を特異的に認識する抗体の誘導，及び，中和抗体の誘導において優れていることが示唆された．なお，このC34三量体ミミックは，阻害剤としては単量体よりも100倍強い抗HIV活性を有しており，現在，この理由を解明中である．

第3章のCD4ミミックは，タンパク質-タンパク質間（gp120-CD4間）の相互作用の領域に係わる官能基を適当なリンカーで架橋した低分子化合物であり，元の親タンパク質の機能を模倣したものである．本章のgp41ミメティックは，タンパク質-タンパク質間の相互作用に係わる部分ペプチドを適当なテンプレート上に配置することで，タンパク質の高次構造そのものを再構築したものである．このケミカルバイオロジー的手法は規則的な二次構造の組み合わせで構成されるタンパク質を模倣する場合に有効である．

これらのgp41三量体ミメティックは今後実験動物レベルを上げていくことにより，立体構造を保持した抗原分子の有効性を示すことができると考えられる．ワクチンに関しては，gp41以外にも宿主細胞側のタンパク質CXCR4の細胞外領域を標的とした人工抗原分子を創製し，免疫により抗体誘導を確認している．宿主側のタンパク質を抗原分子とすることは，常識的ではないが，広範囲のウイルス株に対して有効性が期待でき，耐性出現の危険性も少ないという利点もある．

6. おわりに

毎年種々の抗HIV薬が開発され，薬を飲み続ければAIDSは死なない病のようになってきた．しかし，一生投薬を続ける必要があり，いずれの抗HIV薬も根治に至るものではない．そのため，臨床で使用できる薬剤のレパートリーを増やすことが求められている．われわれは以前からHIV感染のコレセプターであるCXCR4の阻害剤を中心に，標的分子設定型のリバースケミカルジェネティクス的手法により抗HIV剤を創製してきた．最近さらに抗HIV剤のターゲットを増やし，HIV侵入の動的超分子機構をターゲットとしたCD4ミミックを創製した．このCD4ミミックはCXCR4阻害剤や中和抗体等との併用において，相乗効果を示した．さらに，ランダムライブラリーから抗HIV活性を指標にスクリーニングするというフォワードケミカルジェネティクス的手法を用い，リード化合物を見い出した．すなわち，インテグラーゼ阻害活性を有するVprの部分ペプチドを見い出した．このようにケミカルバイオロジー的方法も取り入れ，いろいろな観点からリード化合物を探索し，種々の抗HIV剤の創製を行っている．また，最近再度注目されてきたエイズワクチンに関しても，HIV侵入の動的超分子機構をターゲットとしてテンプレート等ケミカルバイオロジーの概念を用い，人工設計型抗原分子を創製している．阻害剤及びワクチンの両方に力を入れており，カクテル療法を視野に入れた抗AIDS薬の創製を考えている．

謝辞　以上で述べた研究は，CXCR4に関しては京都大学大学院薬学研究科，藤井信孝教授の下で始めたものであり，また，共同研究としてシンガポール国立大学医学科，山本直樹教授，大庭賢二博士，国立感染症研究所エイズ研究センター，村上努博士，駒野　淳博士，熊本大学エイズ学研究センター，松下修三教授，吉村和久准教授，原田恵嘉博士，及び上述の研究室のメンバーにお世話になりました．また，実際に実験を担当して頂いた玉村研の野村　渉助教，鳴海哲夫助教，相川春夫助教，田中智博博士，橋本知恵修士，山田裕子学士，落合千裕修士，中原　徹修士，大矢亜紀修士に感謝いたします．

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Small molecular CD4 mimics as HIV entry inhibitors

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ABSTRACT

Derivatives of CD4 mimics were designed and synthesized to interact with the conserved residues of the Phe43 cavity in gp120 to investigate their anti-HIV activity, cytotoxicity, and CD4 mimicry effects on conformational changes of gp120. Significant potency gains were made by installation of bulky hydrophobic groups into the piperidine moiety, resulting in discovery of a potent compound with a higher selective index and CD4 mimicry. The current study identified a novel lead compound **11** with significant anti-HIV activity and lower cytotoxicity than those of known CD4 mimics.

1. Introduction

The dynamic supramolecular mechanism of HIV cellular invasion has emerged as a key target for blocking HIV entry into host cells.[1] HIV entry begins with the interaction of a viral envelope glycoprotein gp120 and a cell surface protein CD4.[2] This triggers extensive conformational changes in gp120 exposing co-receptor binding domains and allowing the subsequent binding of gp120 to a co-receptor, CCR5[3]/CXCR4.[4] Following the viral attachment and co-receptor binding, gp41, another viral envelope glycoprotein mediates the fusion of the viral and cell membranes, thus completing the infection. Molecules interacting with each of these steps are potential candidates for anti-HIV-1 drugs. In particular, discovery and development of novel drugs that inhibit the viral attachment are required for blocking the HIV infection at an early stage.[5]

In 2005, small molecular CD4 mimics targeting the viral attachment were identified by an HIV syncytium formation assay and shown to bind within the Phe43 cavity, a highly conserved pocket on gp120,[6] which is a hydrophobic cavity occupied by the aromatic ring of Phe43 of CD4.[7] These molecules are comprised of three essential moieties: an aromatic ring, an oxalamide linker, and a piperidine ring (Fig. 1) and show micromolar order potency against diverse HIV-1 strains including laboratory and primary isolates. Furthermore, they possess the unique ability to induce the conformational changes in gp120 required for binding with soluble CD4.[8] Such CD4 mimicry can be an advantage for rendering the envelope more sensitive to neutralizing antibodies.[9] While such properties are promising for the development of HIV entry inhibitors and the use combinatorially with neutralizing antibodies, cytotoxicity is one of the drawbacks of CD4 mimics.

To date, we and others have performed structure–activity relationship (SAR) studies of CD4 mimics based on modifications of the aromatic ring, the oxalamide linker, and the piperidine moiety of CD4 mimics. In an initial survey of SAR studies of NBD-556 and NBD-557, Madani et al. revealed that potency (i.e., CD4 binding and mimicry) was highly sensitive to modifications of the aromatic ring, which is thought to bind in the Phe43 cavity of gp120 (Fig. 1). The CD4 mimic analogs (JRC-II-191) with a *para*-chloro-*meta*-fluorophenyl ring had significantly increased affinity for gp120.[10] Our SAR studies also revealed that a certain size and electron-withdrawing ability of the *para*-substituents are indispensable for potent anti-HIV activity.[11] Furthermore, the replacement of the chlorine group at the *para* position with a methyl group which is almost as bulky as a bromine atom leads to improvement of solubility of the compounds in buffer to provide the reproducibility in the biological studies with comparable biological activities.

Further SAR studies were focused on the piperidine moiety of CD4 mimics to investigate its contribution to biological activities, and we found that the piperidine ring is critical for the CD4 mimicry on the conformational changes in gp120 and that substituents on the nitrogen of the piperidine moiety can contribute significantly to both anti-HIV activity and cytotoxicity.[12] Based on these SARs and our modeling study, we speculate that interactions of the piperidine moiety with several amino acids in the vicinity of the Phe43 cavity in gp120, specifically an electrostatic interaction with

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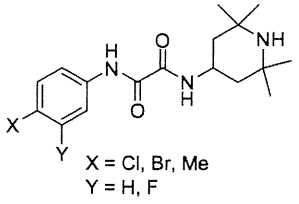


Figure 1. CD4 mimics.

Asp368 and a hydrophobic interaction with Val430, are critical for biological activity. LaLonde et al. focused on modifications of the piperidine moiety using computational approaches, adducing evidence for the importance of these interactions to the binding affinity against gp120.[13] Based on these results, we envisioned that an enhancement of the interaction of CD4 mimics with residues associated with the Phe43 cavity in gp120 would lead to the increase of their potency and CD4 mimicry inducing the conformational changes of gp120, and the decrease of their cytotoxicity. Thus, in this study a series of CD4 mimics, which were designed to interact with the conserved residues in the Phe43 cavity, were synthesized to increase binding affinity for gp120, and the appropriate SAR studies were performed.

2. Results and discussion

Two types of CD4 mimic analogs were designed: (1) CD4 mimics with the ability to interact electrostatically with Asp368, and (2) CD4 mimics with the ability to interact hydrophobically with Val430 (Fig. 2). The X-ray structure of gp120 bound to soluble CD4 (PDB: 1RZJ) revealed that the guanidino group of Arg59 of CD4 is involved in a hydrogen bond with Asp368 of gp120. In order to mimic this interaction, a guanidino and related groups such as thiourea and urea were introduced to the piperidine moiety of the CD4 mimic derivative COC-021, which was developed in order to modify the nitrogen of the piperidine moiety and which showed biological activity, including anti-HIV activity and CD4 mimicry, similar to that of the parent compound NBD-556.[12] Furthermore, to interact with Val430 by hydrophobic interaction, the methyl groups on the piperidine ring were replaced with cyclohexyl groups to prepare a novel CD4 mimic analog with enhanced hydrophobicity.

2.1. Chemistry

The syntheses of CD4 mimics are outlined in Scheme 1. CD4 mimics with guanidine, thiourea, and urea groups on the piperidine moiety were prepared using our previously reported method.[12] Coupling of *p*-chloroaniline with ethyl chloroglyoxylate followed by aminolysis of the ethyl ester with 4-amino-*N*-benzylpiperidine under microwave conditions (150 °C, 3 h) gave the corresponding amide. Removal of the benzyl group with 1-chloroethyl chloroformate[14] gave the free piperidine moiety, which was modified to produce the desired compounds **4–8** (Scheme 1).

For synthesis of a CD4 mimic derivative with two cyclohexyl groups, treatment of 2,2,6,6-tetramethylpiperidin-4-one **9** with cyclohexanone in the presence of ammonium chloride furnished a 2,6-substituted piperidin-4-one derivative,[15] and reductive amination with benzylamine and subsequent removal of benzyl group provided a primary amine **10**. Microwave-assisted aminolysis of ester **2** with amine **10** yielded the desired dicyclohexyl-substituted analog **11** (Scheme 2). The synthesis of the other compounds is described in Supplementary data.

2.2. Biological studies

The anti-HIV activity of synthetic CD4 mimics was evaluated in a single-round viral infective assay. Inhibition of HIV-1 infection was measured as reduction in β-galactosidase gene expression after a single-round of virus infection of TZM-bl cells as described previously.[9] IC_{50} was defined as the concentration that caused a 50% reduction in the β-galactosidase activity (relative light units [RLU]) compared to virus control wells. Cytotoxicity

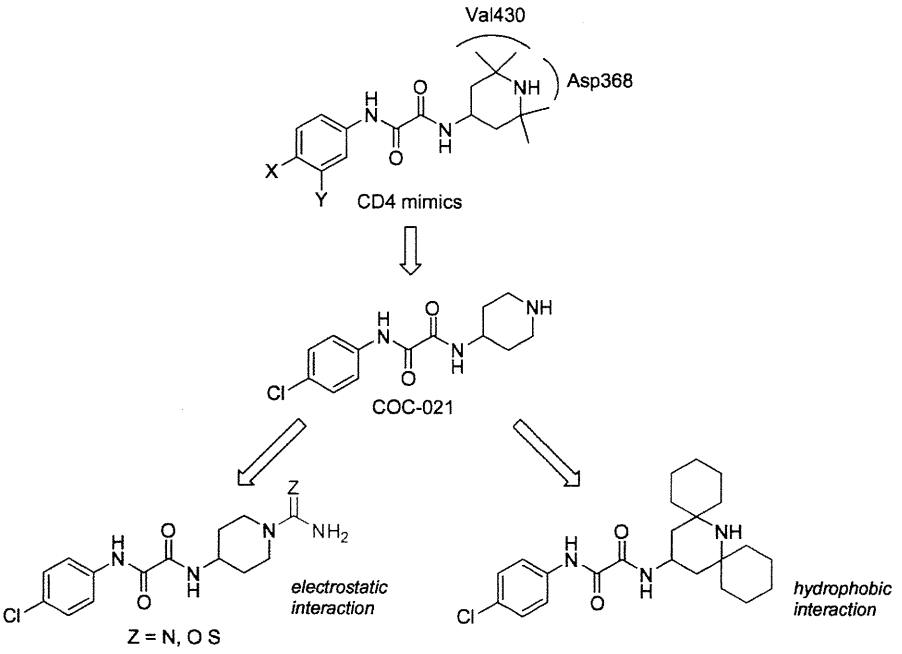
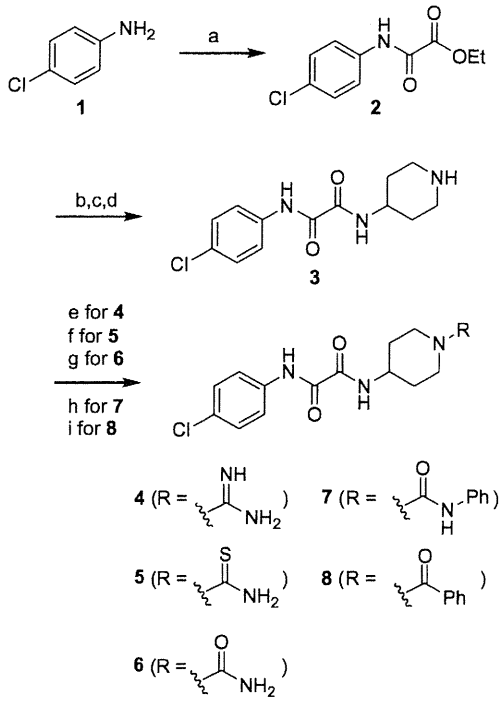
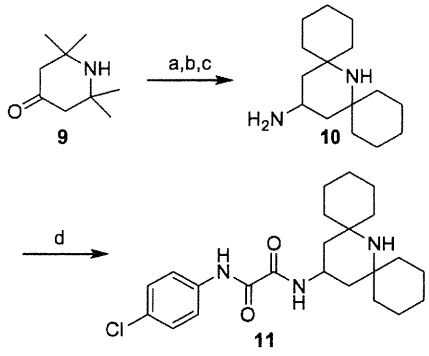


Figure 2. Design strategy for novel CD4 mimics with enhanced electrostatic/hydrophobic interaction.



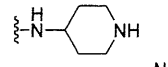
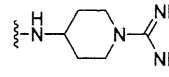
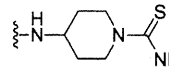
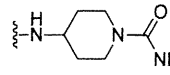
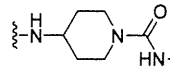
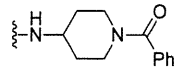
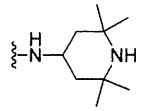
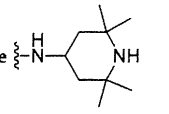
Scheme 1. Synthesis of N-modified piperidine derivatives **4–8**. Reagents and conditions: (a) Ethyl chloroglyoxylate, Et_3N, THF, quant.; (b) 1-benzyl-4-aminopiperidine, Et_3N, EtOH, 150 °C, microwave, 78%; (c) 1-chloroethyl chloroformate, CH_2Cl_2; (d) MeOH, reflux, 64% in two steps; (e) 1*H*-pyrazole-1-carboxamidine hydrochloride, Et_3N, DMF, 61%; (f) (trimethylsilyl)isothiocyanate, $CHCl_3$, 36%; (g) (trimethylsilyl)isocyanate, $CHCl_3$, 30%; (h) phenyl isocyanate, $CHCl_3$, 32%, (i) benzoyl chloride, Et_3N, CH_2Cl_2, 68%.



Scheme 2. Synthesis of dicyclohexyl derivative **11**. Reagents and conditions: (a) Cyclohexanone, NH_4Cl, DMSO, 60 °C; (b) benzylamine, $NaBH_4$, MeOH; (c) 10% Pd/C, H_2, MeOH, 7% from **9**; (d) **2**, Et_3N, EtOH, 150 °C, microwave, 17%.

of the compounds based on the viability of mock-infected PM1/CCR5 cells was evaluated using WST-8 method. The assay results for the CD4 mimics **3–8** are shown in Table 1. Compound **12** (NBD-556) showed potent anti-HIV activity; its IC_{50} value was 0.61 μM, and it is thus 13–20-fold more potent than the reported values.[11,12] Although previous studies found that compound **13**, with a methyl group at the *p*-position of the phenyl ring, and compound **3**, with no dimethyl groups on the piperidine ring, showed potent anti-HIV activity, only moderate activities were observed in the current study; this is about 12–14-fold less potency than reported for compound **12** and is probably due to

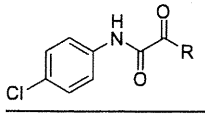
Table 1
Effects of the nitrogen-substituents on anti-HIV activity and cytotoxicity of CD4 mimic analogs[a]

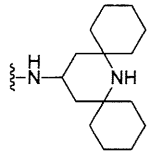
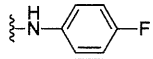
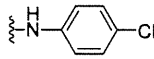
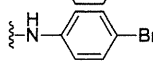
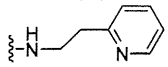
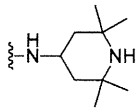
Compd	X	R	IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI (CC_{50}/IC_{50})
			YTA (R5)		
3[d]	Cl		7.0	51	7.3
4[e]	Cl		6.1	72	12
5	Cl		5.5	42	7.6
6	Cl		8.3	310	37
7	Cl		11	6.2	0.56
8	Cl		5.1	ND	–
12 (NBD-556)	Cl		0.61	35	57
13	Me		8.4	260	31

[a] All data with standard deviation are the mean values for at least three independent experiments (ND = not determined)
[b] IC_{50} values are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.
[d] Desalted by satd $NaHCO_3$ aq.
[e] TFA salts.

the different assay system. All of the synthesized novel derivatives of compound **12** showed moderate to potent anti-HIV activity. A guanidine derivative **4** and thiourea derivative **5** showed potent anti-HIV activities (IC_{50} of **4** = 6.1 μM and IC_{50} of **5** = 5.5 μM) but their potency was approximately 10-fold lower than that of the parent compound **12**. A urea derivative **6** also showed potent anti-HIV activity (IC_{50} = 8.3 μM) and exhibited lower cytotoxicity (CC_{50} = 310 μM). On the other hand, introduction of a phenyl group in the urea derivative **6**, led to an *N*-phenylurea derivative **7**, with an increase of cytotoxicity (CC_{50} = 6.2 μM). To examine the influence of the N–H group on anti-HIV activity, an *N*-benzoyl derivative **8** was also tested. The IC_{50} value of **8** was 5.1 μM, which is equipotent with the thioamide derivative **5**. The *N*-benzoyl derivative **8** was essentially equipotent with **3** and this result suggests the presence of the hydrogen atom of the N–H group does not contribute to an increase in anti-HIV activity. The thiourea derivative **5** and the *N*-phenylurea derivative **7**, which have more acidic protons (pK_a of thiourea and *N*-phenylurea; 21.0 and 19.5,[16] respectively) than the urea derivative **6** (pK_a of urea; 26.9[16]), were found to exhibit relatively strong cytotoxicity. This observation indicates that

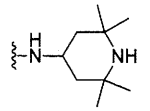
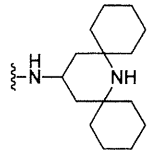
Table 2
Anti-HIV activity and cytotoxicity of CD4 mimic analogs **11**, **12**, and **14–17**[a]



Compd	R	IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI (CC_{50}/IC_{50})
		YTA (R5)		
11		0.68	120	176
14		3.1	>500	>160
15		>100	>500	–
16		>100	>500	–
17		19.8	480	24
12 (NBD-556)		0.61	35	57

[a] All data with standard deviation are the mean values for at least three independent experiments
[b] IC_{50} values are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

Table 3
Anti-HIV activity of CD4 mimic **12** and dicyclohexyl derivative **11**[a]

Compd	R	IC_{50}[b] (μM) Single-round assay	IC_{50}[c] (μM) Multi-round assay
12 (NBD-556)		0.61	0.90
11		0.68	0.56

[a] All data with standard deviation are the mean values for at least three independent experiments.
[b] IC_{50} values of the single-round assay are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.

substitution on the piperidine moiety of acidic functional groups was unfavorable.

The assay results for CD4 mimics that target hydrophobic interactions are shown in Table 2. Compound **11** showed significant anti-HIV activity (IC_{50} = 0.68 μM) comparable to that of the lead compound **12**, but exhibited lower cytotoxicity. Compound **11** showed approximately four-fold less cytotoxicity than **12**. The SI of **11** is 176, 3 times higher than that of **12** (SI = 57). This result suggests that substitution of bulky hydrophobic groups into the piperidine moiety may be consistent with lower cytotoxicity of CD4 mimics. It is noteworthy that compound **14**, which has a *p*-fluoroanilino group in place of the piperidine ring, exhibits potent anti-HIV activity (IC_{50} = 3.1 μM) without significant cytotoxicity (CC_{50} >500 μM). The SI of compound **14** is >160, which is comparable to that of **11**. However, replacement of the piperidine moiety with a *p*-bromo- or *p*-chloroanilino group resulted in the loss of anti-HIV activity. These results suggest that the introduction of a fluorine atom to the piperidine moiety might be consistent with improvement of the anti-HIV activity. Extension of the alkyl chain by two carbons, as in **17** resulted in a 30-fold loss of anti-HIV activity, indicating that relatively rigid structures are preferable for anti-HIV activity.

The anti-HIV activities of **12** and compound **11**, which has a higher SI than the parent compound **12** were evaluated in a multi-round viral infective assay and the results are shown in Table 3. In this assay, the IC_{50} value of **12** was 0.90 μM, which was slightly larger value than measured in a single-round assay (IC_{50} = 0.61 μM). Compound **11** showed higher anti-HIV activity (IC_{50} = 0.56 μM) than compound **12**, indicating that the introduction of hydrophobic cyclohexyl groups into the piperdine moiety has a positive effect on not only the cytotoxicity but also the anti-HIV activity. This is possibly due to the stability in the assay condition derived from the hydrophobicity of cyclohexyl group(s). These results are consistent with a previous study of the analog with one hydrophobic *gem*-dimethyl group on the piperidine moiety, a compound with potent anti-HIV activity and efficient binding affinity for gp120.[13]

To gain insight into the interactions involved in the binding, molecular modeling of compound **11** docked into gp120 (1RZJ) was carried with Sybyl 7.1 (Fig. 3). The binding mode of compound **11** in the Phe43 cavity suggested that the orientation of the piperidine moiety of **11** is different from that in compound **12**, and that the cyclohexyl group can be positioned near Val430 with whose isopropyl group it can interact hydrophobically.

Fluorescence activated cell sorting (FACS) analysis was performed as previously reported,[11,12] to evaluate the CD4 mimicry effects on conformational changes of gp120 and the results are shown in Figure 4. Comparison of the binding of an anti-envelope CD4-induced monoclonal antibody (4C11) to the cell surface pretreated with the above CD4 mimics was measured in terms of the mean fluorescence intensity (MFI). Our previous studies revealed that the profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **12** was entirely similar to that of pretreatment of soluble CD4. In this FACS analysis, the MFI of pretreatment with compound **12** is 23.13. The profiles of the binding of 4C11 to the cell surface pretreated with compounds **3**, **4** and **5** were comparable to that of compound **12** [MFI (**3**) = 20.54, MFI (**4**) = 20.85, MFI (**5**) = 20.24, respectively], suggesting that these derivatives offer a significant enhancement of binding affinity for 4C11. On the other hand, pretreatment with **6** and **8** did not cause significant enhancement of the binding affinity for 4C11, indicating that introduction of a carbonyl group on the piperidine nitrogen is not conducive to CD4 mimicry. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **11**, which had significant anti-HIV activity and lower cytotoxicity than compound **12**, (MFI (**11**) = 22.17) was similar to that of compound **12**, suggesting that compound **11** offers significant enhancement of binding affinity for 4C11. This result indicates that compound **11** retains the CD4 mimicry on the conformational changes of gp120. Although compound **14** and compound **17** showed potent anti-HIV activity and no significant cytotoxicity, the profiles pretreated with (MFI (**14** and **17**) = 15.20 and 15.38) were similar to that of the control (MFI = 14.94), suggesting that these compounds **14** and **17** failed to produce a significant increase in binding affinity for 4C11. These

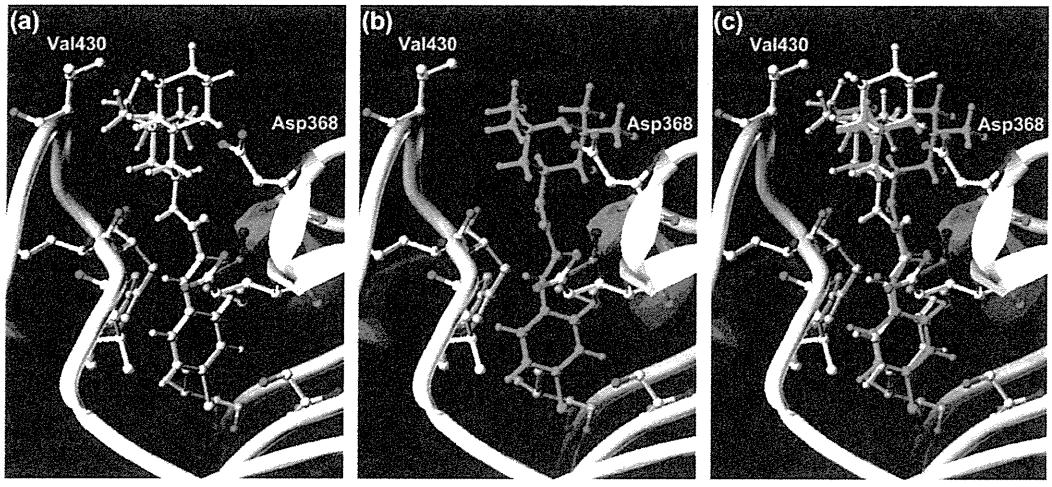


Figure 3. Docking structures of (a) compound **11** and (b) compound **12** bound in the Phe43 cavity of gp120 (1RZJ); (c) merge image of compounds **11** and **12**. Compounds **11** and **12** are represented in yellow and green sticks, respectively. Key residues in the cavity forming interactions with compounds are represented in gray sticks.

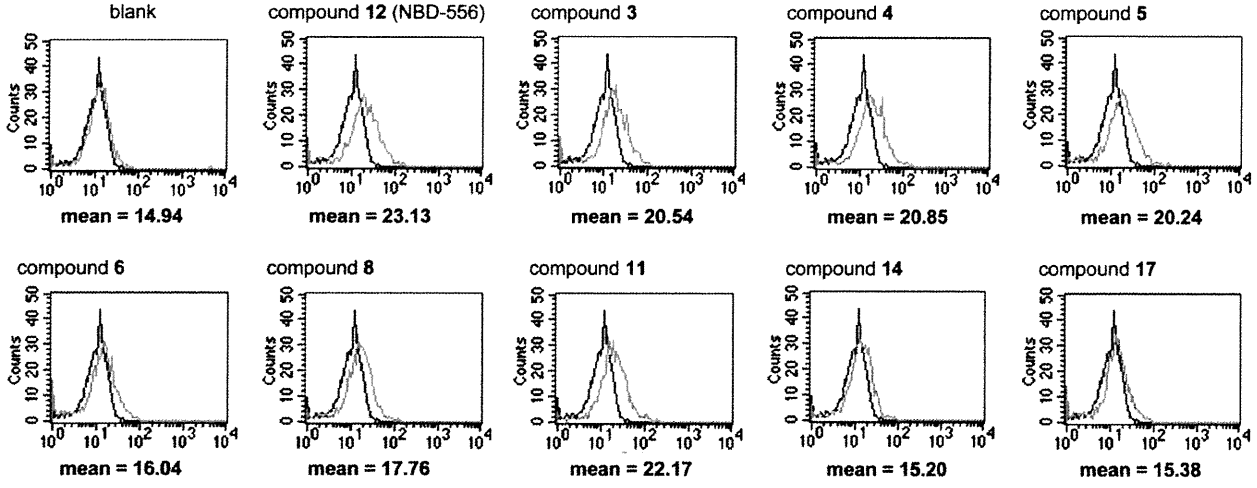


Figure 4. FACS analysis of compounds **12**, **3–6**, **8** (Table 1), **11**, **14**, and **17** (Table 2).

results are consistent with our previous finding that the piperidine ring is critical to the CD4 mimicry of the conformational changes in gp120.

3. Conclusion

A series of CD4 mimics were designed and synthesized to interact with the conserved residues in the Phe43 cavity of gp120 to investigate their anti-HIV activity, cytotoxicity, and CD4 mimicry as a function of conformational change of gp120. The biological activities of the synthetic compounds indicate that (1) the hydrogen atom of the piperidine moieties contributes significantly to cytotoxicity, and (2) installation of bulky hydrophobic groups into the piperidine moiety can increase anti-HIV activity and decrease cytotoxicity thus providing a novel compound with higher selective index than those of the original CD4 mimics. Furthermore, this modification has no great influence on the CD4 mimicry on the conformational change of gp120. Thus, compound **11** is promising for further studies. More detailed SAR investigations with respect to the substitution on the piperidine moiety have been ongoing studies.

4. Experimentals

^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Avance III spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as internal standard. Low- and high-resolution mass spectra were recorded on a Bruker Daltonics microTOF focus in the positive and negative detection mode. For flash chromatography, Wakogel C-200 (Wako Pure Chemical Industries, Ltd) and silica gel 60 N (Kanto Chemical Co., Inc.) were employed. For analytical HPLC, a Cosmosil 5C_{18}-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 cm^3 min^{-1} on a JASCO PU-2089 plus (JASCO Corporation, Ltd., Tokyo, Japan), and eluting products were detected by UV at 220 nm. Preparative HPLC was performed using a Cosmosil 5C_{18}-ARII column (20 × 250 mm, Nacalai Tesque, Inc.) on a JASCO PU-2087 plus (JASCO Corporation, Ltd, Tokyo, Japan) in a suitable

gradient mode of CH_3CN solution containing 0.1% (v/v) TFA at a flow rate of 7 cm^3 min^{-1}. Microwave reactions were performed in Biotage Microwave Reaction Kit (sealed vials) in an Initiator™ (Biotage). The wattage was automatically adjusted to maintain the desired temperature for the desired period of time.

4.1. Chemistry

4.1.1. N^1-(4-Chlorophenyl)-N^2-(piperidin-4-yl)oxalamide (3)

To a stirred solution of *p*-choroaniline (**1**) (14.0 g, 110 mmol) in THF (146 mL) were added ethyl chloroglyoxylate (8.13 mL, 73.2 mmol) and triethylamine (Et_3N) (15.2 mL, 110 mmol) at 0 °C. The mixture was stirred for 6 h at room temperature. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolve in EtOAc, and washed with 1 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure gave the crude ethyl oxalamate, which was used without further purification. To a solution of the above ethyl oxalamate (1.27 g, 5.25 mmol) in EtOH (13.0 mL) were added Et_3N (1.46 mL, 10.5 mmol) and 4-amino-1-benzylpiperidine (2.97 mL, 15.8 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and *n*-hexane, and dried under reduced pressure to provide the corresponding amide (1.58 g, 81% yield) as colorless crystals. To a stirred solution of **S1** (1.46 g, 3.90 mmol) in CH_2Cl_2 (39.0 mL) was added dropwise 1-chloroethyl chloroformate (0.860 mL, 7.80 mmol) at 0 °C. After being stirred at room temperature for 30 min, the mixture was refluxed for 1 h. After concentration under reduced pressure, the residue was dissolved in MeOH and then refluxed for 1 h. After concentration under reduced pressure, the residue was diluted with EtOAc and washed with saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. After concentration under reduced pressure, the residue was washed with cold EtOAc, and dried under reduced pressure to provide the title compound **3** (778 mg, 71% yield) as white powder.

^1H NMR (400 MHz, $CDCl_3$) δ 1.39–1.52 (m, 2H), 1.92–2.01 (m, 2H), 2.67–2.79 (m, 2H), 3.06–3.19 (m, 2H), 3.83–3.95 (m, 1H), 7.34 (d, J = 8.80 Hz, 2H), 7.44 (d, J = 7.64 Hz, 1H), 7.59 (d, J = 8.80 Hz, 2H), 9.28 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 33.0 (2C), 45.2 (2C), 47.9, 21.0 (2C), 129.3 (2C), 130.5, 135.0, 157.6, 158.8; HRMS (ESI), *m/z* calcd for $C_{13}H_{17}ClN_3O_2$ (MH^+) 282.1004, found 282.1002.

4.1.2. N^1-(1-Carbamimidoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (4)

To a stirred solution of **3** (50.0 mg, 0.178 mmol) in DMF (20.0 mL) was added 1-aminopyrazole hydrochlride (312 mg, 2.13 mmol) and Et_3N (0.390 mL, 28.1 mmol). The reaction mixture was stirred at room temperature for 24 h. After concentration under reduced pressure, purification by preparative HPLC gave the trifluoroacetate of the title compound **4** as white powder (36.0 mg, 61% yield).

^1H NMR (500 MHz, DMSO) δ 1.41–1.55 (m, 2H), 1.59–1.71 (m, 2H), 2.70–2.74 (m, 2H), 3.74–3.87 (m, 1H), 3.88–4.03 (m, 2H), 5.93 (s, 2H), 7.42 (d, J = 9.00 Hz, 2H), 7.85 (d, J = 9.00 Hz, 2H), 8.95 (d, J = 9.00 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 31.3 (2C), 43.0 (2C), 47.6, 122.4 (2C), 128.6, 129.1 (2C), 137.1, 158.2, 159.3, 159.5; HRMS (ESI), *m/z* calcd for $C_{14}H_{19}ClN_5O_2$ (MH^+) 324.1222, found 324.1213.

4.1.3. N^1-(1-Carbamothioylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (5)

To a stirred solution of **3** (140 mg, 0.498 mmol) in $CHCl_3$ (5.00 mL) was added trimethylsilyl isothiocyanate (141 mL, 1.00 mmol) and stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **5** as white powder. (62.0 mg, 36% yield).

^1H NMR (400 MHz, DMSO) δ 1.45–1.69 (m, 2H), 1.69–1.81 (m, 2H), 2.67–2.81 (m, 2H), 3.02–3.16 (m, 2H), 3.75–3.89 (m, 1H), 7.41 (d, J = 9.00 Hz, 2H), 7.85 (d, J = 9.00 Hz, 2H), 9.00 (d, J = 8.50 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 27.8 (2C), 42.3 (2C), 44.4, 122.0 (2C), 128.2, 128.6 (2C), 129.5, 136.6, 158.6, 159.4; Anal. calcd for $C_{14}H_{18}ClN_4O_2S$: C, 49.34; H, 5.03; N, 16.44. Found: C, 49.32; H, 4.76; N, 16.11.

4.1.4. N^1-(1-Carbamoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (6)

To a stirred solution of **3** (60.0 mg, 0.213 mmol) in $CHCl_3$ (1.10 mL) was added trimethylsilyl isocyanate (56.0 μL, 0.421 mmol), and the mixture was stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **6** (20.1 mg, 30% yield) as white powder.

^1H NMR (500 MHz, DMSO) δ 1.44–1.55 (m, 2H), 1.58–1.71 (m, 2H), 2.65–2.78 (m, 2H), 3.76–3.87 (m, 1H), 3.87–4.01 (m, 2H), 5.94 (s, 1H), 7.42 (d, J = 9.00 Hz, 2H), 7.86 (d, J = 9.00 Hz, 2H), 8.95 (d, J = 9.00 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 30.8 (2C), 42.6 (2C), 47.1, 122.0 (2C), 128.1, 128.6 (2C), 136.7, 157.8, 158.8, 159.0; HRMS (ESI), *m/z* calcd for $C_{14}H_{18}ClN_4O_3$ (MH^+) 325.1062, found 325.1060.

4.1.5. N^1-(4-Chlorophenyl)-N^2-(1-(phenylcarbamoyl)piperidin-4-yl)oxalamide (7)

To a stirred solution of **3** (140 mg, 0.498 mmol) in $CHCl_3$ (5.00 mL) was added phenyl isocyanate (54.0 μL, 0.500 mmol) and stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **7** as white powder. (64.1 mg, 32% yield).

^1H NMR (500 MHz, DMSO) δ 1.52–1.66 (m, 2H), 1.68–1.80 (m, 2H), 2.81–2.95 (m, 2H), 3.84–3.96 (m, 1H), 4.08–4.20 (m, 2H), 6.91–6.94 (m, 2H), 7.21–7.24 (m, 2H), 7.36–7.52 (m, 4H), 7.86 (d, J = 9.00 Hz, 2H), 8.53 (s, 1H), 8.99 (d, J = 8.50 Hz, 2H), 10.81 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 31.3 (2C), 43.4 (2C), 47.5, 120.0 (2C), 122.0, 122.4 (2C), 128.6, 128.7 (2C), 129.1 (2C), 137.1, 141.1, 155.2, 159.2, 159.5; HRMS (ESI), *m/z* calcd for $C_{20}H_{22}ClN_4O_3$ (MH^+) 401.1375, found 401.1372.

4.1.6. N^1-(1-Benzoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (8)

To a stirred solution of **3** (500 mg, 1.78 mmol) in $CHCl_3$ (17.8 mL) was added benzoyl chloride (307 μL, 2.67 mmol) and the mixture was stirred at room temperature for 1 h. The precipitate was collected and washed with cold EtOAc, and dried under reduced pressure to provide the title compound **8** (232 mg, 34% yield).

^1H NMR (500 MHz, $CDCl_3$) δ 1.21–1.68 (br, 4H), 1.96–2.08 (br, 2H), 3.02–3.16 (br, 2H), 4.04–4.07 (m, 1H), 7.35 (d, J = 9.00 Hz, 2H), 7.41–7.43 (m, 5H), 7.52 (d, J = 8.00 Hz, 1H), 7.59 (d, J = 9.00 Hz, 2H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 31.4 (2C), 41.0 (2C), 47.6, 121.0 (2C), 126.9 (2C), 128.6 (2C), 129.3 (2C), 129.9, 130.6, 134.8, 135.6, 157.2, 159.0, 170.5; HRMS (ESI), *m/z* calcd for $C_{20}H_{21}ClN_3O_3$ (MH^+) 386.1266, found 386.1276.

4.1.7. Amine (10)

To a stirred solution of 2,2,6,6-tetramethylpiperidin-4-one (7.75 g, 50.0 mmol) and cyclohexanone (15.5 mL, 150 mmol) in DMSO (71.0 mL) was added NH_4Cl (16.1 g, 300 mmol) and stirred at 60 °C for 5 h. The reaction mixture was diluted with H_2O

(150 mL), acidified with 7% aq HCl, and extracted with Et_2O (200 mL × 3). The water layer was adjusted to pH 9 using 10% aq K_2CO_3 and then back-extracted with EtOAc. The extract was washed with brine and dried over Na_2SO_4. After concentration under reduced pressure, the residue was dissolve in MeOH (60.0 mL) and benzylamine (10.9 mL, 100 mmol) was added. After being stirred at room temperature for 1 h, sodium cyanoborohydride was added and stirred at room temperature for 6 h. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with EtOAc, then dried over $MgSO_4$. After concentration under reduced pressure, the residue was dissolve in MeOH (150 mL) and 10% Pd/C (5.32 g, 5.00 mmol) was added and stirred at room temperature for 24 h under hydrogen atmosphere. After the reaction mixture was filtered through celite, the filtrate solution was concentrated under reduced pressure followed by flash chromatography over silica gel with EtOAc–EtOH (4:1) to gave the title compound **10** (820 mg, 7 % yield) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.730 (t, J = 12.0 Hz, 2H), 1.15–1.85 (m, 23H), 2.01–3.7 (m, 2H), 2.95–3.05 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.2 (2C), 22.8 (2C), 26.2 (2C), 37.3 (2C), 42.3 (2C), 43.6 (2C), 47.0, 53.2 (2C); HRMS (ESI), m/z calcd for $C_{15}H_{29}N_2$ (MH^+) 237.2325, found 237.2321.

4.1.8. N^1-(4-Chlorophenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl) oxalamide (11)

To a solution of **10** (722 mg, 3.05 mmol) in EtOH (15.0 mL) was added ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (363 mg, 1.50 mmol) and triethylamine (0.415 mL, 3.00 mmol) and stirred for 3 h at 150 °C under microwave irradiation. The mixture was filtered and the precipitate was collected and washed with cold EtOH, and dried under reduced pressure to provide the compound **11** (108 mg, 17% yield) as white powder.

^{1}H NMR (500 MHz, DMSO) δ 1.12–1.91 (br, 24H), 4.02–4.07 (m, 1H), 7.42 (d, J = 9.00 Hz, 2H), 7.84 (d, J = 9.00 Hz, 2H), 8.76 (br, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.1 (2C), 22.7 (2C), 26.0 (2C), 37.2 (2C), 42.5 (2C), 42.9 (2C), 43.6, 52.7 (2C), 120.9 (2C), 129.3 (2C), 130.4, 135.0, 157.6, 158.8; HRMS (ESI), m/z calcd for $C_{23}H_{33}ClN_3O_2$ (MH^+) 418.2256, found 418.2261.

4.1.9. N^1-(4-Chlorophenyl)-N^2-(4-fluorophenyl)oxalamide (14)

To a solution of the ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (1.21 g, 5.00 mmol) in EtOH (25.0 mL) were added Et_3N (1.38 mL, 10.0 mmol) and 4-fluoroaniline **12** (1.44 mL, 15.0 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and n-hexane, and dried under reduced pressure to provide the compound **14** (601 mg, 41% yield) as colorless crystals. Compounds **15** and **16** were similarly synthesized.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07–7.14 (m, 2H), 7.35–7.40 (m, 2H), 7.59–7.63 (m, 4H), 9.29 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 115.8 (d, J = 22.5 Hz, 2C), 122.5 (2C), 122.8 (d, J = 7.5 Hz, 2C), 128.8, 129.1 (2C), 134.4, 137.1, 158.3, 158.9 (d, J = 42.5 Hz), 160.2; HRMS (ESI), m/z calcd for $C_{14}H_{11}ClFN_2O_2$ (MH^+) 293.0488, found 293.0485.

4.1.10. N^1-(4-Chlorophenyl)-N^2-(2-(pyridin-2-yl)ethyl) oxalamide (17)

To a solution of the ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (726.3 mg, 3.00 mmol) in EtOH (10.0 mL) were added Et_3N (0.831 mL, 6.00 mmol) and 2-(pyridin-2-yl)ethanamine 14 (1.07 mL, 9.00 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and n-hexane, and dried under reduced pressure to provide the title compound **17** (336 mg, 37% yield) as colorless crystals.

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.08 (t, J = 6.50 Hz, 2H), 3.82 (q, J = 6.50 Hz, 2H), 7.12–7.21 (m, 2H), 7.30–7.37 (m, 2H),7.54–7.66 (m 3H), 8.40 (s, 1H), 8.60 (d, J = 5.00 Hz, 1H), 9.26 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 36.5, 39.0, 121.0 (2C), 121.8, 123.4, 129.2 (2C), 130.3, 135.1, 136.7, 149.5, 157.5, 158.6, 159.6; HRMS (ESI), m/z calcd for $C_{15}H_{15}ClN_3O_2$ (MH^+) 304.0847, found 304.0850.

4.2. Molecular modeling

The structures of compounds **11** and **12** were built in Sybyl and minimized with the MMFF94 force field and partial charges.[17] Dockings were then performed using FlexSIS through its SYBYL module, into the crystal structure of gp120 (PDB: 1RZJ).

4.3. FACS analysis

JR-FL (R5, Sub B) chronically infected PM1 cells were pre-incubated with 100 μM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines, Fig. 4) to the Env-expressing cell surface in the presence of a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines, Fig. 4). Data are representative of the results from a minimum of two independent experiments. The number at the bottom of each graph in Figure 4 shows the mean fluorescence intensity (MFI) of the antibody 4C11.

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Supplementary data

Supplementary data (NMR charts of compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.045.

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