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G．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総合研究報告書
NBD誘導体の活性測定、NBD誘導体の最適化の研究

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研究要旨：
平成22年度は、HIV-1 エンベロープの立体構造を変化させて、中和抗体の感受性を増強させるCD4類似低分子化合物 (NBD-556)が、実際にどのような抗体の中和感受性を増強するのかを検討した。その結果、治療用坑 HIV-1 中和抗体 KD-247 のみならず、CD4i 抗体の中和活性もNBD-556 の添加により著しく増強することがわかった。また、感染者から分離したウイルスに対する同時期の血清 IgG による中和も、NBD-556 存在下では中和できるようになることが確認でき、これらを報告した。また、NBD-556 より毒性の低い YYA-021(NBD-559)を選び、NBD-556との比較検討を行った。平成 23 年度は、引き続き NBD-556 およびその誘導体に関する研究を行った。候補となる新規化合物を用いて in vitro 耐性誘導を行い、誘導された NBD 耐性ウイルスの Env gp120 領域のシークエンスを行い、化合物の構造と結合部位及び活性の相関を調べた。その結果、耐性変異は大きく３つに分類できることが分かった。すなわち、i)V255M 単独変異を持つグループ、ii)V255M+T375I を中心とした複数の変異を持つグループ、そして、iii) CD4 cavity の入り口付近に変異を持つグループであった。平成 24 年度は、NBD 誘導体の芳香環部位とオキサミド部位をインドール骨格に置換することで平面構造を付与しつつ、分子全体としてのエントロピーの減少を目指したインドール骨格含有化合物群を中心に約 40 種類の化合物に対してウイルス学的解析を行った。その中で弱いながらも抗 HIV 活性を示す化合物はいずれも 5位に塩素、臭素、またはフッ素原子を有するものだった。一方、新たに構築したクローンウイルスを用いて in vitro 耐性誘導を行い、de novo 変異をはじめとする耐性変異機序の更なる検討を行った。昨年度のバルクウイルス同様に変異は 3 通りに大別され、これらの変異が de novo 変異として獲得されることが明らかになった。

A. 研究目的
我々は、中和抗体の感受性増強能はNBD-556 並み、もしくはそれ以上に維持しつつ、細胞毒性の低い低分子化合物の検索を目的として研究を行っている。また、候補化合物による in vitro 耐性誘導をバルクの臨床分離ウイルスとクローンウイルスで行う事により、予想される結合部位と立体構造変化誘導活性の相関を調べ、より効果的な Env 構造変化誘導薬剤の開発を目指した。

B. 研究方法
全期間を通じ、東京医科歯科大学生体材料工学研究所機能分子部門分子認識分野の玉村啓和教授に HIV-1 のエンベロープに立体構造変化を起こさせる低分子化合物(NBD-556 およびその誘導体)の合成を行っていただき、抗ウイルス効果と抗体の反応性の変化を WST-8 assay と FACS で解析した。また、候補となる新規化合物をバルクの臨床分離ウイルスとクローンウイルスを用いて in vitro 耐性誘導を行い、誘導された NBD 耐性ウイルスの Env gp120 領域のシークエンスから、化合物の構造と結合部位及び活性の相関を検討した。
（倫理面での配慮）
研究の倫理的妥当性は熊本大学医学部先進医療審査会、倫理委員会にて承認されている。また、遺伝子組み換え生物等を用いる実験については、必要に応じた国立感染症研究所の機関承認を取得済みである。

C. 研究結果
NBD-556がgp120のどの部位と結合しているかを詳細に知るために、in vitro耐性誘導システムを用いて、この化合物に耐性のウイルスをとり、sCD4の耐性ウイルスとシークエンスを比較検討した。その結果、NBD-556の変異部位はsCD4のそれと非常に似通った部位に集中していることがわかった。
一般的に、HIV感染症例においては、血中

のウイルスに対する同時期の抗体には、ほとんど中和活性が見られない。ところが、NBD-556が1μMでも存在すると、自己血清IgGによる明らかなウイルス感染阻害効果が認められるようになった。同じウイルスを使って、中和単クローン抗体KD-247(抗V3抗体)や4E9C(CD4i抗体)を用いて、抗体の中和感受性のNBD-556による増強効果を調べた結果も、同様にNBD-556低濃度存在下で、著明な中和感受性の増強が認められた。これらのことは、CD4i抗体や抗V3抗体が血中に存在している場合、エンベロープの立体構造変化を誘発するような化合物（NBD誘導体）を加えるだけで、それまで中和抵抗性であったウイルスを感受性に変えることが可能となることが示された。

ただ、NBD-556の最大の問題点として細胞毒性が強いことがあげられる。そこで新しい低毒性のNBD誘導体の開発を主たる目的として、NBD-556類似の低分子化合物の探索を進めた。その結果、エンベロープの三量体構造変化はNBD-556と遜色なく維持され、かつ細胞毒性の低い誘導体YYA-021の開発に成功した。この誘導体は、NBD-556と同様に臨床分離株に対する中和抗体の感受性を増加させることが確認された。そこで、引き続き玉村啓和教授にお願いして合成していただいた数十個のNBD誘導体から、NBD-556以外に立体構造変化を惹起するYYA-021を含む11個の誘導体を選んで、sCD4とともに、in vitro耐性誘導実験を行った。よりin vivo に近いウイルスを使用する目的で、臨床分離R5ウイルス株（Y1）を用いて行った。

すべての化合物を5－9回パッセージし、最終濃度が20-100μM(CD4は5μg/ml)に到達するまで継代した。得られたそれぞれの耐性ウイルスは、すべてのNBD誘導体に対して交差耐性を示した。最終パッセージのウイルスのgp120のシークエンスを比較したところ、大きく３つに分類できることが分かった。それらは、i)V255M単独変異を持つグループ、ii)V255M+T375Iを中心とした複数の変異を持つグループ、そして、iii) CD4 cavityの入り口付近に変異を持つグループであった。

最終年度は、NBD誘導体の芳香環部位とオキサミド部位をインドール骨格に置換することで平面構造を付与しつつ、分子全体としてのエントロピーの減少を目指したインドール骨格含有化合物群を中心に約40種類の化合物の検討を行った。インドール骨格含有化合物群の中で弱いながらも抗HIV活性を示す化合物はいずれも5位に塩素、臭素、またはフッ素原子を有するものであり、昨年度までに得られた芳香環部位誘導体の構造活性相関と一致したが、顕著な抗HIV活性およびgp120構造変化誘起能を示さなかった。

一方、臨床分離R5ウイルス株（Y1）を用いたin vitro耐性誘導実験で、NBD誘導体の耐性獲得機序には、やはり(i)V255M変異、(ii)T375I変異、または（iii)M426I変異、の3通りに大別されることが明らかになった。

次に、NBD-556以外に立体構造変化を惹起する7個の誘導体を選んで、新たにin vitro耐性誘導実験を臨床分離R5ウイルス株（Y1）の全env領域をNL4-3 backboneに組換えたクローンウイルス(Y1c)を用いて行った。得られたそれぞれの耐性ウイルスは、昨年度のバルクウイルス同様にすべてのNBD誘導体に対して交差耐性を示した。最終パッセージのウイルスのgp120のシークエンスを比較したところ、de novo変異として獲得される主要変異が、(i)V255M変異、(ii)T375I変異、または（iii)M426I変異、の3通りに大別されることが明らかになった。これらの事は、NBD骨格を有する立体構造変化誘導剤はCD4 cavity内もしくは近位部位との結合によりCD4様効果を示す事が示された。

D.考察

一連の研究結果から、NBD-556は、実際の感染症例でも血中に存在する中和抗体の中和感受性を増強する可能性が期待できることが示唆された。このことは、このようなエンベロープに立体構造変化を誘導する化合物が侵入阻害剤だけでなく、既存の血中抗体の中和感受性を増強するのみならず、治療用の抗HIV抗体の増強をも可能性とすることを意味している。また新たに毒性の低いYYA-021を見いだすことに成功し、この化合物を用いての動物実験への移行へとつなげる事ができた。継続して新しい化合物の発見に務めた結果、基本骨格のregion Iのフェニル環のメタ位とパラ位の両方にClとFを持つものや、NBD-556のregion IIIをシ

クロヘキシル化した化合物の効果が大きい事が分かった。また、インドール骨格含有化合物群の中で、弱いながらも抗HIV活性を示す化合物はいずれも5位に塩素、臭素、またはフッ素原子を有するものであり、それまでに得られた芳香環部位誘導体の構造活性相関と一致した。

これまで行ったin vitro耐性誘導実験により、主要変異が、(i)V255M変異、(ii)T375I変異、または(iii)M426I変異、の3通りに大別されることが明らかになった。今後、芳香環部位のパラ・メタ位（5・6位）およびgp120のV255, T375, およびM426残基が、どのように感染阻害能や立体構造変化誘導能に関与しているかを詳細に検討することにより、より効果的な低分子化合物の開発が可能となると考えている。また、より毒性が低くPKの良い化合物の探索も引き続き行っていく予定である。

E. 結論

NBD-556様の中和抗体増強物質の存在下では血清IgGで同時期のウイルスを中和できるようになり、これらの化合物は新しいコンセプトの抗HIV治療の道を開くものとして、今後期待できる。また、より毒性の低いYYA-の発見により、抗体と併用した場合の効果をin vivoで確認するためのサルを用いた実験が可能となった。一貫して行った、iv vitro耐性誘導によって、NBD誘導体の結合様式および耐性機序を推定し、構造と結合様式の大まかな推定ができた。これらの知見をもとに、より立体構造変化誘導能に優れた化合物の開発に努めていきたい。

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H. 知的財産権の出願・登録状況

特になし

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

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別紙4

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Enhanced Exposure of Human Immunodeficiency Virus Type 1 Primary Isolate Neutralization Epitopes through Binding of CD4 Mimetic Compounds▽

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N-(4-Chlorophenyl)-N′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide (NBD-556) is a low-molecular-weight compound that reportedly blocks the interaction between human immunodeficiency virus type 1 (HIV-1) gp120 and its receptor CD4. We investigated whether the enhancement of binding of anti-gp120 monoclonal antibodies (MAbs) toward envelope (Env) protein with NBD-556 are similar to those of soluble CD4 (sCD4) by comparing the binding profiles of the individual MAbs to Env-expressing cell surfaces. In flow cytometric analyses, the binding profiles of anti-CD4-induced epitope (CD4i) MAbs toward NBD-556-pretreated Env-expressing cell surfaces were similar to the binding profiles toward sCD4-pretreated cell surfaces. To investigate the binding position of NBD-556 on gp120, we induced HIV-1 variants that were resistant to NBD-556 and sCD4 *in vitro*. At passage 21 in the presence of 50 μM NBD-556, two amino acid substitutions (S375N in C3 and A433T in C4) were identified. On the other hand, in the selection with sCD4, seven mutations (E211G, P212L, V255E, N280K, S375N, G380R, and G431E) appeared during the passages. The profiles of the mutations after the selections with NBD-556 and sCD4 were very similar in their three-dimensional positions. Moreover, combinations of NBD-556 with anti-gp120 MAbs showed highly synergistic interactions against HIV-1. We further found that after enhancing the neutralizing activity by adding NBD-556, the contemporaneous virus became highly sensitive to antibodies in the patient's plasma. These findings suggest that small compounds such as NBDs may enhance the neutralizing activities of CD4i and anti-V3 antibodies *in vivo*.

Human immunodeficiency virus type 1 (HIV-1) replicates continuously in the face of a strong antibody (Ab) response, although Abs effectively control many viral infections (3). Neutralizing Abs (NAbs) are directed against the HIV-1 envelope (Env) protein, which is a heterodimer comprising an extensively glycosylated CD4-binding subunit (gp120) and an associated transmembrane protein (gp41). Env proteins are present on the virion surface as "spikes" composed of trimers of three gp120-gp41 complexes (20, 21, 29). These spikes resist neutralization through epitope occlusion within the oligomer, extensive glycosylation, extension of variable loops from the surface of the complex, and steric and conformational blocking of receptor binding sites (16, 18, 20).

Ab access to conserved regions is further limited because viral entry is a stepwise process involving conformational changes that lead to only transient exposure of conserved domains such as the coreceptor binding site (4, 5). However, some early strains of HIV-1 appear to be highly susceptible to neutralization by Abs (1, 10). For instance, subtype A HIV-1 envelopes from the early stage of infection exhibit a broad range of neutralization sensitivities to both autologous and heterologous plasma (1), suggesting that at least a subset of the envelopes have some preserved and/or exposed neutralization epitopes. It is well known that the potential for neutralizing properties of particular Abs is enhanced after binding of soluble CD4 (sCD4), especially NAbs against CD4-induced epitopes (CD4i Abs) (27) and some anti-V3 Abs (22). CD4i Abs are detected in plasma samples from many patients at an early stage of HIV-1 infection (9). Consequently, we hypothesize that small compounds such as sCD4 can enhance the neutralizing activities of CD4i Abs and some anti-V3 Abs not only *in vitro* but also *in vivo*.

In a previous report, two low-molecular-weight compounds that presumably interfere with viral entry of HIV-1 into cells were described (35). These two N-phenyl-N′-(2,2,6,6-tetramethyl-piperidin-4-yl)-oxalamide analogs, NBD-556 and NBD-557, comprise a novel class of HIV-1 entry inhibitors that block the interaction between gp120 and CD4. These compounds were found to be equally potent inhibitors of both X4 and R5 viruses in CXCR4- and CCR5-expressing cell lines, respectively (35). Schön et al. (25) also reported that NBD-556 binds to gp120 in a process characterized by a large favorable change in enthalpy that is partially compensated for by a large unfavorable entropy change, representing a thermodynamic signature similar to that observed for binding of sCD4 to gp120. In a recent study, Madani et al. (23) reported the following findings: (i) NBD-556 binds within the Phe43 cavity, a highly conserved and functionally important pocket formed as gp120 assumes the CD4-bound conformation; (ii) the NBD-556 phenyl ring projects into the Phe43 cavity; (iii) the enhancement of CD4-independent infection by NBD-556 requires the induction of conformational changes in gp120; and (iv) increased

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affinities of NBD-556 analogs toward gp120 improve the antiviral potency during infection of CD4-expressing cells. The latter two studies demonstrated that low-molecular-weight compounds such as NBDs can induce conformational changes in the HIV-1 gp120 glycoprotein similar to those observed upon sCD4 binding (23, 25). The authors of these studies concluded that their data supported the importance of gp120 residues near the Phe43 cavity in binding to NBD-556 and lent credence to the docked binding mode.

In the present study, we investigated the binding position of NBD-556 on gp120 by inducing HIV-1 variants that were resistant to NBD-556 by exposing HIV-1_{IIIB} to increasing concentrations of the compound *in vitro*. We also induced sCD4-resistant HIV-1_{IIIB} variants and compared the profile of the sCD4-resistant mutations to that of the NBD-556-resistant mutations. We subsequently examined the virological properties of pseudotyped HIV-1 clones carrying the NBD-556 and sCD4 resistance-associated *env* gene mutations. Our findings provide a foundation for understanding the interaction of NBD-556 with the CD4-binding site of HIV-1 gp120. We also evaluated the anti-HIV-1 interactions between plasma NAbs and NBD-556 *in vitro* and considered the possibility of using the data as a key to opening the shield covering the conserved epitopes targeted by NAbs.

(This study was presented in part at the 15th Conference on Retroviruses and Opportunistic Infection, Boston, MA, 3 to 6 February 2008 [Abstract 736].)

MATERIALS AND METHODS

Cells, culture conditions, and reagents. The CD4-positive T-cell line PM1 was maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone Laboratories, Logan, UT), 50 U of penicillin/ml, and 50 μg of streptomycin/ml. PM1/CCR5 cells were generated by standard retrovirus-mediated transduction of PM1 cells with pBABE-CCR5 provided by the National Institutes of Health AIDS Research and Preference Reagent Program (NIH ARRRP) (24, 34). PM1/CCR5 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 U of penicillin/ml, 50 μg of streptomycin/ml, and 0.1 mg of G418 (Invitrogen, Carlsbad, CA)/ml. The TZM-bl cell line was obtained from the NIH ARRRP and maintained in Dulbecco modified Eagle medium (Sigma) supplemented with 10% FCS.

NBD-556 (molecular weight, 337.84) and YYA-004 (molecular weight, 303.4), which has the same structure as JRC-I-300 (23), were synthesized as previously described (23, 25, 30). KD-247 (12), 3E4, and 0.5γ (unpublished) are anti-gp120-V3 monoclonal Abs (MAbs). 17b (27), 4C11, and 4E9C (unpublished) are MAbs against CD4-induced epitopes (CD4i Abs). 17b, 2G12 (a MAb against the gp120 glycan), and b12 (a MAb against the CD4-binding site [CD4bs] epitope) were provided by the NIH ARRRP. The 0.5δ antibody established in our laboratory is an anti-CD4bs MAb (unpublished results). RPA-T4 (an anti-CD4 MAb) was purchased from BD Biosciences Pharmingen (San Jose, CA). Recombinant human sCD4 was purchased from R&D Systems, Inc. (Minneapolis, MN).

MAbs 3E4, 0.5γ, 0.5δ, 4C11, and 4E9C were human MAbs established from a patient with long-term nonprogressive illness. B cells from the patient's peripheral blood mononuclear cells (PBMC) were transformed by Epstein-Barr virus, followed by cloning. Culture supernatant from an individual clone was screened for reactivity to $gp120_{SF2}$ by enzyme-linked immunosorbent assay (ELISA). The specificity of the antibodies was determined by gp120 capture ELISA and fluorescence-activated cell sorting analysis of HIV-1_{JR-FL}-infected PM1 cells in the presence or absence of sCD4. The binding specificity was further assessed by an ELISA using peptides corresponding to the V3 sequence of various isolates. Based on these binding data, we classified them as follows: V3 MAbs, 3E4 and 0.5γ; CD4bs MAb, 0.5δ; and CD4i MAbs, 4C11 and 4E9C.

The laboratory-adapted HIV-1 strains HIV-$1_{89.6}$, HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} were propagated in phytohemagglutinin-activated PBMC. These viruses were then passaged in PM1/CCR5 cells, and the culture supernatants were stored at −150°C prior to use. R5 primary HIV-1 isolates (HIV-$1_{Pt.1}$, HIV-$1_{Pt.2}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$) were isolated from four Japanese patients in our laboratory. All patients were at a stage of chronic infection. HIV-$1_{Pt.1}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$ were isolated from drug-naive patients, and HIV-$1_{Pt.2}$ was isolated from a drug-experienced patient and passaged in phytohemagglutinin-activated PBMC. Infected PBMC were cocultured with PM1/CCR5 cells for 4 to 5 days, and the culture supernatants were stored at −150°C until used. Nucleotide sequences of the gp120 from the four primary isolates were deposited in the DNA Data Bank of Japan under accession numbers AB553911 to AB553914.

Susceptibility assay. The sensitivities of six laboratory-adapted viruses, four primary isolates, and HIV-1_{IIIB} viruses passaged in the presence of sCD4 or NBD-556 were determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as previously described with minor modifications (31). Briefly, PM1/CCR5 cells (2×10^3 cells/well) were exposed to 100 times the 50% tissue culture infective dose ($TCID_{50}$) of the viruses in the presence of various concentrations of sCD4 or NBD-556 in 96-well round-bottom microculture plates, followed by incubation at 37°C for 7 days. After removal of 100 μl of the medium, 10 μl of MTT solution (7.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well. The plate was then incubated at 37°C for 3 h. Subsequently, the produced formazan crystals were dissolved by adding 100 μl of acidified isopropanol containing 4% (vol/vol) Triton X-100 to each well. The optical densities at a wavelength of 570 nm were measured in a microplate reader. All assays were performed in duplicate or triplicate. We also determined the concentration for 50% cytotoxicity (CC_{50}) by using the MTT assay.

The sensitivities of the HIV-$1_{Pt.3}$ primary isolate to KD-247 (anti-V3 MAb), 4E9C (CD4i MAb), and autologous plasma IgG in the presence or absence of NBD-556 were also determined by using the MTT assay. To exclude any influence of plasma factors, such as antiviral drugs, cytokines, and chemokines, on the neutralization activities, we used IgG from the patient's plasma, which was purified using protein A-Sepharose (Affi-gel Protein A; Bio-Rad, Hercules, CA) (19).

Flow cytometric analysis. HIV-1_{JR-FL} chronically infected PM1 cells were preincubated with or without sCD4 (0.5 μg/ml) and NBD-556 (1, 3, 10, 30, 90, and 100 μM) for 15 min and then incubated with various anti-HIV-1 MAbs (17b, 4C11, KD-247, 3E4, and 0.5γ) at 4°C for 30 min. The cells were washed with PBS, and a fluorescein isothiocyanate-conjugated goat anti-human IgG Ab was used for Ab detection. Flow cytometry was performed with a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed by using the BD CellQuest version 3.1 software (BD Biosciences).

Data analysis and evaluation of synergy. Analyses of the synergistic, additive, and antagonistic effects of the antiviral agents were initially performed according to the median effect principle using the CalcuSyn version 2 computer program (6) to provide estimates of the 50% inhibitory concentration (IC_{50}) values of the antiviral agents in combination. Combination indices (CIs) were estimated from the data and reflected the nature of the interactions between KD-247 and sCD4 or NBD-556 and between NBD-556 and CD4i MAb 4C11 or anti-CD4bs MAb 0.5δ against HIV-1_{JR-FL} or HIV-1_{IIIB} on PM1/CCR5 cells as determined by the MTT assay. A CI of <0.9 indicated synergy, a CI between 0.9 and 1.1 indicated additivity, and a CI of >1.1 indicated antagonism. The CI value was directly proportional to the amount of synergy for the combination regimen. For example, values of <0.5 represented a high degree of synergy, while values of >1.5 represented significant antagonism. This approach has been widely used in analyses of antiviral interactions and was chosen to allow comparability with published literature.

Docking simulation. The structure for NBD-556 was built in SYBYL 7.1 (Tripos, St. Louis, MO) and minimized with the MMFF94 force field and partial charges (15). Using FlexSIS through its SYBYL module, docking of NBD-556 was performed into the crystal structure of gp120 obtained from the Protein Data Bank (PDB; entry 1RZJ). The binding site was defined as residues Val255, Asp368, Glu370, Ser375, Ile424, Trp427, Val430, and Val475, including residues located within a radius of 4.4 Å. The structure of the ligand was treated flexibly, and all other options were set to their default values. Figures were generated using SwissPdb Viewer version 3.9 (SPdbViewer) (13) and ViewerLite version 5.0 (Accelrys, Inc., San Diego, CA). We also generated a simian immunodeficiency virus (SIV) gp120 figure (PDB entry 2BF1) to compare the sites of the mutations in HIV-1 gp120 using the same software programs.

Isolation of NBD-556- and sCD4-resistant mutants from HIV-1_{IIIB} *in vitro*. To select NBD-556 and sCD4 escape viruses, HIV-1_{IIIB} was treated with various concentrations of NBD-556 or sCD4 and then infected into PM1/CCR5 cells as previously described with minor modifications (32). Viral replication was monitored by observation of any cytopathic effects in PM1/CCR5 cells. The culture supernatants were harvested on day 7 and used to infect fresh PM1/

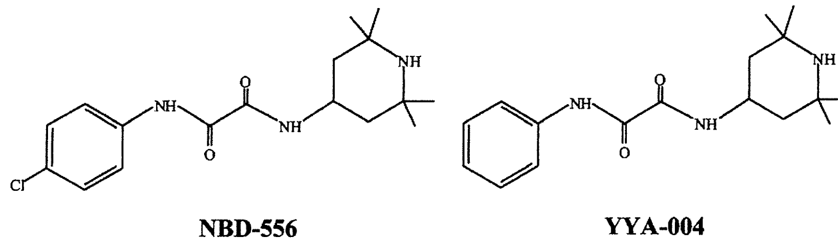

FIG. 1. Structures of NBD-556 and YYA-004.

CCR5 cells for the next round of culture in the presence of increasing concentrations of NBD-556 or sCD4. When the viruses began to propagate in the presence of NBD-556 or sCD4, the concentration was further increased. After the viruses were passaged using up to 50 μM NBD-556 or 20 μg of sCD4/ml in PM1/CCR5 cells, the resulting viruses, designated NBD-556(20)14p, NBD-556(50)17p, and sCD4(20)5p, were recovered from the passaged cell culture supernatants.

Proviral DNA extracts from cells cultured with several concentrations of NBD-556 and sCD4 were subjected to PCR amplification using *Taq* polymerase (Takara, Shiga, Japan). The amplified products were cloned into pCR2.1 (Invitrogen), and the *env* regions in both the passaged and selected viruses were sequenced by using an ABI Prism 3110 automated DNA sequencer (ABI, Foster City, CA).

Construction of mutant Env expression vectors. Proviral DNA was extracted from the passaged HIV-1_{IIIB}-infected PM1/CCR5 cells by using a QIAamp DNA blood minikit (Qiagen, Valencia, CA). For the construction of Env expression vectors, we used pCXN2, which contains a chicken actin promoter. Briefly, we amplified the passaged HIV-1_{IIIB} gp160 regions using LA *Taq* (Takara) with the primers ENVA (5′-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3′) and ENVN (5′-CTGCCAATCAGGGAAGTAGCCTTGTGT-3′). The PCR products were inserted into pCR-XL-TOPO (Invitrogen). The EcoRI fragment of pCR-XL-IIIB containing the entire *env* region was ligated into pCXN2 to give pCXN-IIIBwt. pCXN-IIIB(S375N), pCXN-IIIB(V255E), and pCXN-IIIB(A433T) were generated by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in accordance with the manufacturer's instructions with the primer pairs S375Nfw (5′-AAATTGTAACGCACAATTTTAATTGTGGAGG-3′) and S375Nrv (5′-CCTCCACAATTAAAATTGTGCGTTACAATTT-3′), V255Efw (5′-GAATTAGGCCAGTAGAATCAACTCAACTGCT-3′) and V255Erv (5′-AGCAGTTGAGTTGATTCTACTGGCCTAATTC-3′), and A433Tfw (5′-CAGGAAGTAGGAAAAACAATGTATGCCCCTC-3′) and A433Trv (5′-GAGGGGCATACATTGTTTTTCCTACTTCCTG-3′), respectively.

Pseudovirus preparation. Portions, 5 μg of pSG3ΔEnv and 0.5 μg of pRSV-Rev (17), supplied by the NIH ARRRP, and a 4.5-μg portion of HIV-1_{IIIB} Env-expressing pCXN2 were cotransfected into 293T cells using the Effectene transfection reagent (Qiagen). At 48 h after transfection, the pseudovirus-containing supernatants were harvested, filtered through a 0.2-μm-pore-size filter, and stored at −80°C. The pseudovirus activities were measured with a luminescence assay using TZM-bl cells as previously described (28).

Single-round virus infection assay. A single-cycle infectivity assay was used to measure the neutralization of HIV-1_{IIIB} pseudoviruses as described previously (26, 28). Briefly, NBD-556, YYA-004, sCD4, 2G12, b12, RPA-T4, or 4C11 at various concentrations and a pseudovirus suspension corresponding to 100 $TCID_{50}$ were preincubated in the absence or presence of 1 μM NBD-556 for 15 min on ice. The virus-compound mixtures were added to TZM-bl cells, which had been seeded in a 96-well plate (1.5×10^4 cells/well) on the previous day. The cultures were incubated for 2 days at 37°C, washed with PBS, and lysed with lysis solution (Galacto-Star mammalian reporter gene assay system; ABI). After transfer of the cell lysates to luminometer plates, the β-galactosidase activity (in relative light units) in each well was measured by using 50-fold-diluted Galacto-Star substrate in a reaction buffer diluent (100 μl/well; ABI) in a TR717 microplate luminometer (ABI). The reduction in infectivity was determined by comparing the relative light units in the presence or absence of each compound and expressed as the percentage of neutralization. Each assay was repeated two to three times.

RESULTS

Anti-HIV-1 activities of sCD4, NBD-556, and YYA-004 for laboratory strains and primary HIV-1 isolates. Initially, we determined the inhibitory activities of sCD4, NBD-556, and YYA-004, which has a phenyl group instead of the *p*-chlorophenyl group of NBD-556 (Fig. 1), on the infection of PM1/CCR5 cells by different laboratory-adapted HIV-1 strains and different HIV-1 primary isolates of subtype B, including both X4 and R5 viruses, by using a previously reported method (33). sCD4 inhibited the laboratory-adapted HIV-1 strains HIV-1_{IIIB}, HIV-$1_{89.6}$, HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} with IC_{50}s ranging from 0.26 to 6.1 μg/ml (Table 1). NBD-556 inhibited the X4 virus HIV-1_{IIIB} and dualtropic virus HIV-$1_{89.6}$ with IC_{50}s of 7.8 and 11.4 μM, respectively, but did not inhibit the R5 viruses HIV-1_{BaL}, HIV-1_{SF162}, HIV-1_{JR-FL}, and HIV-1_{YU2} with IC_{50}s of >30 μM. We also tested sCD4 and NBD-556 against the R5 primary isolates HIV-$1_{Pt.1}$, HIV-$1_{Pt.2}$, HIV-$1_{Pt.3}$, and HIV-$1_{Pt.4}$. sCD4 effectively inhibited all of the primary isolates at concentrations of 0.2 to 7.4 μg/ml. On the other hand, NBD-556 inhibited two of the four primary

TABLE 1. Inhibitory activities of sCD4 and NBD-556 toward infection by laboratory and primary strains of HIV-1

Virus	Subtype	Cell	Mean IC_{50}[a] ± SD		
			sCD4 (μg/ml)	NBD-556 (μM)	YYA-004 (μM)
Laboratory-adapted viruses					
X4					
HIV-1_{IIIB}	B	PM1/CCR5	0.26 ± 0.17	7.8 ± 2.6	>100
Dual					
HIV-$1_{89.6}$	B	PM1/CCR5	0.87 ± 0.09	11.4 ± 2.4	>100
R5					
HIV-1_{BaL}	B	PM1/CCR5	1.7 ± 0.28	>30	>100
HIV-1_{SF162}	B	PM1/CCR5	3.6 ± 0.64	>30	>100
HIV-1_{JR-FL}	B	PM1/CCR5	3.6 ± 0.71	>30	>100
HIV-1_{YU2}	B	PM1/CCR5	6.1 ± 2.00	>30	>100
Primary isolates					
R5					
HIV-$1_{Pt.1}$	B	PM1/CCR5	0.2 ± 0.04	3.6 ± 0.67	>100
HIV-$1_{Pt.2}$	B	PM1/CCR5	1.6 ± 0.21	>30	>100
HIV-$1_{Pt.3}$	B	PM1/CCR5	3.7 ± 0.42	11.8 ± 1.6	>100
HIV-$1_{Pt.4}$	B	PM1/CCR5	7.4 ± 1.30	>30	>100

[a] PM1/CCR5 cells (2×10^3) were exposed to 100 $TCID_{50}$ of each virus and then cultured in the presence of various concentrations of sCD4, NBD-556, or YYA-004 as indicated. The IC_{50}s were determined by using the MTT assay on day 7 of culture. All assays were conducted in duplicate, and the data shown represent the means derived from the results of two to three independent experiments. For NBD-556, CC_{50} = 140 μM; for YYA-004, CC_{50} = 350 μM. (The CC_{50} is the concentration for 50% cytotoxicity.)