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H. 知的財産権の出願・登録状況（予定を含む。）

1. 特許
   1) 出願予定
   発明者:玉村啓和、廣田雄樹、苛原 優、野村渉、鳴海哲夫、松下修三、吉村和久、原田恵嘉
   発明の名称:抗 HIV 活性を有する低分子 CD4 mimic の開発

様式A（8）

別紙3

厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

霊長類を用いた新規治療法POC試験

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研究要旨

昨年度本研究課題の前身であるH22-政策創薬一般-007で、単クローン抗体KD-247及び小分子CD4ミミックYYA-021の複合投与により観察されたSHIV-KS661曝露後ウイルス複製抑制効果が複合投与による相乗効果か明らかにするため、KD-247単独投与によるウイルス複製への影響を検索した。

単独投与によってもウイルス複製及びCD4陽性T細胞枯渇阻止効果が見られ、これらの効果は複合投与により得られたものと差がなかった。また、ウイルス遺伝子の変異解析及び変異ウイルスの表現型の検索から、KD-247はウイルスに変異を起こさせる選択圧となった事が示唆された一方で、YYA-021は選択圧となった証拠が得られなかった。

以上より、複合投与に用いたYYA-021の用量、投与頻度及び投与経路では試験管内で観察された複合投与によるウイルス複製抑制相乗効果は個体レベル感染で再現出来ない事を明らかにした。

今後はより薬理学的特性（長い血中半減期及び低い毒性）が良好な小分子CD4ミミックの開発が本研究のコンセプトを個体レベルで証明するために必要と考えられる。

A．研究目的

HIV 感染症は多剤併用療法の確立により不治の病から制御可能な慢性感染症に変容した。しかし、既存薬剤の長期投与により派生する種々の解決すべき問題が顕在化している事からも、服薬せずウイルスを生涯制御する、「機能的治癒」が治療における目標となっている。

研究代表者は、中和抗体の臨床応用に向けた研究の過程でHIV-1 gp120 のCD4 結合部位に作用してEnv 三量体の立体構造を変化させ、中和抗体の反応性及び中和活性を飛躍的に増強する低分子化合物、NBD-556 を同定した。感染者が誘導する中和抗体の多くは感染ウイルスのエピトープが保存されているにもかかわらず、中和活性が見られない。これは、Env がウイルス粒子上で三量体を形成し、その立体構造によりエピトープを遮蔽しているためと考えられている。この立体遮蔽を解除し中和抗体が中和エピトープに到達可能となれば、感染者体内で誘導された抗体によるウイルス中和が期待される。

中和抗体はウイルス膜上の機能的Env に結合するばかりでなく、Env を発現する感染細胞の表面に結合し、ADCC などの機構でこれを攻撃する。これらの効果がin vivo で実証できれば、体内の感染細胞を減少させることが可能となり、現在の抗ウイルス療法との併用により機能的治癒への道が

開かれる。本分担研究では、このような新しい治療戦略の in vivo における有効性を動物モデルで検証することを目的とする。

本研究課題の前身である、「HIV エンベロープ蛋白（Env）の立体構造変化誘導剤(NBD 誘導体)の臨床応用に向けた基礎研究」（H22-政策創薬-一般-007）において、我々は SIV-HIV-1 キメラウイルス（SHIV）/アカゲザル感染モデルへの抗 V3 ループ抗体 KD-247 および NBD 誘導体 YYA-021 複合投与介入が、非投与時と比較して血中ウイルス量を有意に抑制可能な事を見いだした。しかし、観察された抑制効果が複合投与によるかの検討はなされていなかった。

そこで、本年度は KD-247 単独投与による複製抑制効果を検索し、複合投与時の抑制効果と比較を行った。

B．研究方法

1. SHIV KS661/アカゲザル感染系

H22-政策創薬-一般-007 で用いたのと同一の系を用いた。即ち、SHIV KS661 株 10,000 $TCID_{50}$ をインド産アカゲザルに静脈内接種した。本モデル系ではウイルス感染 10-12 日後に血漿中 RNA ウイルス量が 10^8-10^9 copies/ml のピーク値を示し、その後一端減少するものの、10^6-10^7 copies/ml の値を維持する。末梢血 CD4 陽性 T 細胞数はウイルス RNA がピークに達するのとほぼ同時に非可逆的に枯渇する。

2. 抗体投与

16 mg/kg の KD-247 をウイルス接種 24 時間、8 および 15 日後に麻酔下の感染アカゲザルに静脈内投与した。投与量及び投与頻度・回数は H22-政策創薬-一般-007 で行ったと同様とした。

3. ウイルス学的及び免疫学的解析

ウイルス接種前より経時的に採血し、血漿より RNA を抽出、逆転写反応後、ウイルス *gag* 遺伝子領域を増幅するリアルタイム PCR 法により、血中ウイルス量を評価した。また、実験終了時に動物を安楽殺し、末梢血リンパ球、脾臓及び腸間膜リンパ節から単核細胞を調製後、同様に RNA 抽出、PCR を行い、組織におけるウイルス量を評価した。末梢血を蛍光色素標識単クローン抗体で染色し、リンパ球サブセットの推移を追跡した。

4. 投与抗体の動態追跡

H22-政策創薬-一般-007 で検索した個体及び今年度本研究計画で検索した個体の経時血漿中の KD-247 を、抗原に Env 蛋白を用いた ELISA 法により検索した。

5. 抗 KD-247 抗体応答の検索

血漿中の KD-247 結合性蛋白の検索に surface plasmon resonance 法（Biacore 2000）を用いた。アビジン化されたセンサーチップにビオチン化 KD-247 をコーティングし、希釈経時血漿を導入して結合性蛋白を検出した。

6. ウイルス遺伝子変異及び中和感受性の解析

H22-政策創薬-一般-007 で検索した個体及び今年度本研究計画で検索した個体の経時血漿のうち、抗体投与中または投与後にウイルス量がリバウンドを示したものから RNA を抽出、逆転写後、env 遺伝子全長を single genome amplification 法により増幅、クローン化し、塩基配列を決定した。それら env クローンを用いて pseudotype ウイルスを作製し、KD-247 に対する中和感受性を元株 SHIV KS661 と比較した。

（倫理面への配慮）

動物実験に当たっては、「研究機関等における動物実験等の実施に関する基本指針」に基づいた「京都大学における動物実験の実施に関する規定」を遵守した。京都大学ウイルス研究所附属動物実験施設におけるアカゲザルの飼養については、「特定外来生物による生態系等に係わる被害の防止に関する法律」の規定に基づき、環境大臣より許可を受けている。また、「感染症の予防及び感染症の患者に対する医療に関する法律」の輸入禁止地域等を定める省令に基づき輸入サル飼育施設の指定を受けている。また、「動物の愛護及び管理に関する法律」にも遵守し、実験を行った。

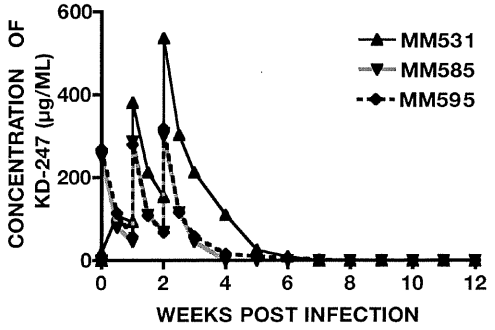
組換え SHIV 感染実験については第二種使用等をする間に執る拡散防止措置について大臣確認されている。

C．研究結果

1. KD-247 血中濃度の推移

KD-247 をウイルス曝露 24 時間、8 及び 15 日後

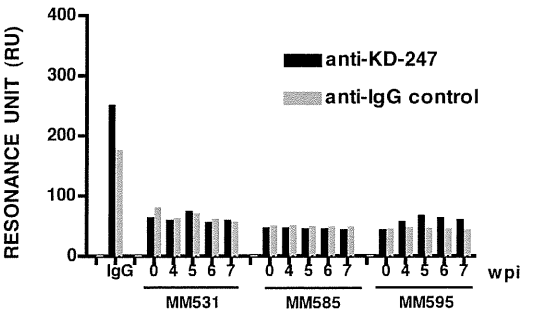
に投与した３頭のアカゲザルの血中抗体濃度を下図に示す（解析は熊本大学エイズ学研究センターの丸田秦広氏により行われた）。



３頭のうち、MM531 のみ血中濃度が高くなったものの、全ての個体で同様の血中抗体動態を示した。即ち、投与直後に抗体濃度は急激に上昇、その後減衰して行くが次回投与時には検出可能な抗体が残存し、次の投与で前回よりも高い血中濃度に達し、再び前回投与時と同様に減衰した。これらの結果は当該抗体及び小分子 CD4 ミミック YYA-021 を複合投与した（H22-政策創薬一般-007）際に観察された抗体の動態と同様であり、2 投与群間で有意な違いはなかった。

2. KD-247 投与による抗イディオタイプ抗体応答

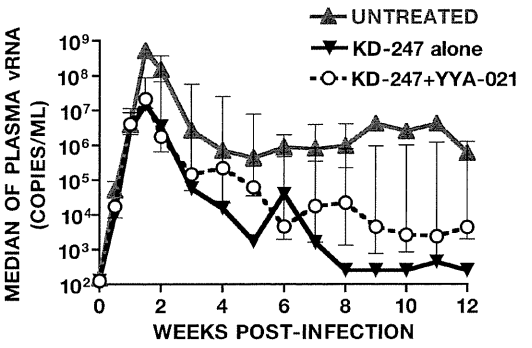
抗体投与後の経時血漿中の KD-247 反応性物質の検索結果を下図に示す解析は熊本大学エイズ学研究センターの丸田秦広氏により行われた）。。



抗体の血中濃度が最も高く且つ長期間保たれた MM595 において対照 IgG と比べて高値の KD-247 反応性物質の産生が見られたが、他の個体では観察されなかった。また、MM595 においても反応性物質の産生により当該抗体の消失が加速された様子はなかった。KD-247 および YYA-021 複合投与群でも６頭中１頭でのみKD-247反応性物質の産生が見られた個体があったが、この個体においても抗体のクリアランスが他の個体と比べて加速する事はなかった。

3. KD-247 単独投与によるウイルス複製抑制

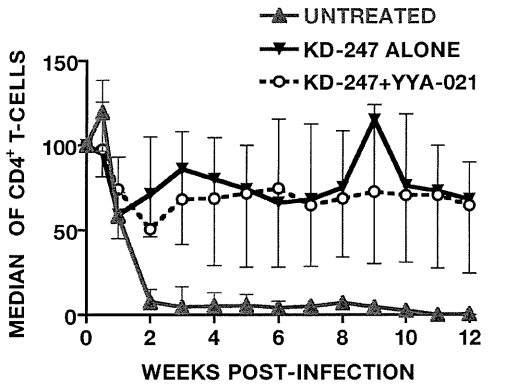
ウイルス曝露後の血中ウイルス RNA 量の推移を、非投与群及びYYA-021 およびKD-247 複合投与群の結果（H22-政策創薬一般-007 で実施）と共に下図に示す。



単独投与（黒実線）群においてもウイルス感染阻止は達成しなかったが、非投与群（灰色実線）と比較してウイルス複製は抑制され、特にウイルス曝露 1.5 及び８週後では有意な差が見られた。複合投与群（黒破線）と単独投与群では観察期間中ウイルス複製抑制に有意な差が見られる事はなかった。

4. CD4 陽性 T 細胞の動態

ウイルス曝露後の血中 CD４T 陽性細胞数の推移を、非投与群及び YYA-021 および KD-247 複合投与群の結果（H22-政策創薬一般-007 で実施）と共に下図に示す。



抗体投与群は単独、複合を問わず、SHIV-KS661 が

引き起こす非可逆的CD4陽性T細胞枯渇を阻止した。枯渇阻止効果において両群に有意な差はなかった。

5. ウイルスの逃避変異解析

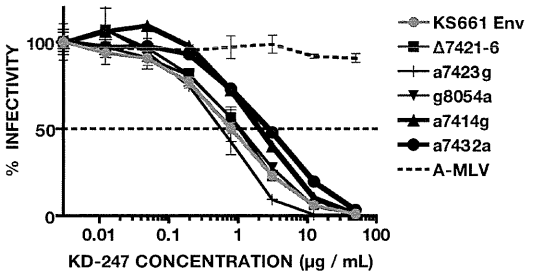
今年度検索した結果を H22-政策創薬-一般-007で実施した結果と比較すると、小分子CD4ミミックを加えた事による抑制効果の増強があった事を支持する証拠は血中ウイルス量および末梢血リンパ球の推移からは示唆されていない。そこで投与したKD-247およびYYA-021がSHIV-KS661に対して逃避変異を誘導する様な選択圧となったかをウイルス遺伝子の解析から検討した。

YYA-021が相互作用するHIV-1 Envのアミノ酸に就いては既に報告されている(Yoshimura et al. J. Virol. 2010)。得られたenvのsingle genome ampliconクローンの遺伝子解析の結果、これらアミノ酸に変異を持つものは見られなかった。

KD-247はEnvの可変領域の一つであるV3ループ上の一次配列を抗原決定基として認識するが、ここに変異のあったクローンも存在しなかった。しかし、他の可変ループ（V1/V2およびV4）に潜在的糖鎖付加部位の消失、移動または糖鎖付加部位の近傍で電位の異なるアミノ酸への置換が見られた（下表参照)。

V1/V2 LOOP			
CLONE ID	ANIMAL ID, WPI	DEDUCED AMINO ACID SEQUENCE	FREQUENCY
	SHIV KS661	CSFYITTSIRNKVKKEYALFNRLDVVPVKNTSNTKYRLISC	
	MM542, 6wpi	.....K.....	1/8
a7414g	MM585, 6wpi	.....E.....	5/6
Δ7421-6	MM566, 7wpi	.....---.....	2/10
a7423g	MM542, 6wpi	.....I.....	8/8
g7424a	MM566, 7wpi	.....N.....	3/10
	MM538, 6wpi		12/12
a7432g	MM566, 7wpi	.....E.....	5/10
V4 LOOP			
CLONE ID	ANIMAL ID, WPI	DEDUCED AMINO ACID SEQUENCE	FREQUENCY
	SHIV KS661	CNTAQLFNSTWNVAGGTNGTEGNDIITLQC	
g8036a	MM538 6wpi	.....N.....	12/12
g8054a	MM566 7wpi	.....E.....	4/10

観察された変異を持つ env クローンを用いてpseudotypeウイルスを作製し、中和試験を行ったところ、2クローンが元株と比べて中和抵抗性を示した（a7414g及びa7432g、下図参照)。即ち、各ウイルスクローンの中和曲線が50%感染性（点線）と交わる箇所を比較すると、元株SHIV-KS661（灰色）よりもa7414g(黒線三角)およびa7432g(黒線丸)は数倍量のKD-247を要する事が示された。



D. 考察

以前に達成したKD-247およびYYA-021複合投与による曝露後 SHIV-KS661 複製抑制への YYA-021の貢献を検索したが、その貢献は見られなかった。試験管内では20μMのYYA-021を用いてKD-247とのウイルス抑制相乗効果を観察したが、個体レベルではこの濃度を達成出来なかった。理由は、

1. 6.25 mg/kg以上投与すると左心不全を疑う急性毒性が観察された、

2. 6.25 mg/kgでは血中濃度が投与完了15分後にのみ検出可能で、以後は速やかに検出限界以下になったためである。

試験管内では相乗効果が見られたため、急性毒性を抑える事が出来ればより高用量のYYA-021を投与出来、結果としてKD-247との相乗効果が観察された可能性がある。また、本CD4ミミックが速やかに血中から検出出来なくなった理由に就いては明らかではないが、試験管内で擬似的な状況を再現する目的で行った実験（抗凝固血液にYYA-021を混合、30分間静置した後、血漿及び血球成分に分けて分析を行った）では、約半分のYYA-021は血球に吸着、または取り込まれていた。更に血漿を交換後、静置するとYYA-021は再び血漿中に検出された事から、本薬剤は血漿と血球の間にある平衡状態を以て分布する事が示唆された。このような性質が血漿中または組織間液に存在するウイルスへの作用を考えた時に長時間体内に留まる事が期待出来るので有利である一方、高用量を適用し、血漿中に薬効濃度を保つ必要がある。より薬理学的特性（長い血中濃度の維持及び低毒性）の良好な誘導体の開発が必要である。

E. 結論

本研究計画の前身である H22-政策創薬-一般

-007 において観察された KD-247 および YYA-021 の複合投与により観察されたウイルス複製抑制効果に、YYA-021 は貢献しなかったと結論された。当該試験に用いた YYA-021 の用量、投与頻度及び投与経路では試験管内で観察された複合投与によるウイルス複製抑制相乗効果は個体レベル感染では再現出来ない。今後はより薬理学的特性（長い血中半減期及び低い毒性）が良好な小分子 CD4 ミミックの開発が本研究のコンセプトを個体レベルで証明するために必要と考えられる

F．研究発表

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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

別紙４

研究成果の刊行に関する一覧表レイアウト

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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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A CD4 mimic as an HIV entry inhibitor: Pharmacokinetics

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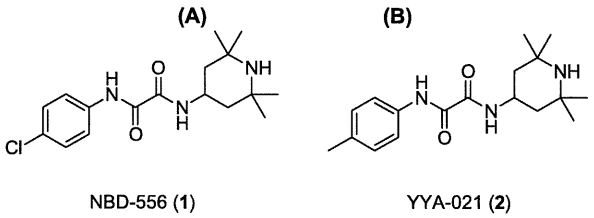
ABSTRACT

To date, several small molecules of CD4 mimics, which can suppress competitively the interaction between an HIV-1 envelope glycoprotein gp120 and a cellular surface protein CD4, have been reported as viral entry inhibitors. A lead compound **2** (YYA-021) with relatively high potency and low cytotoxicity has been identified previously by SAR studies. In the present study, the pharmacokinetics of the intravenous administration of compound **2** in rats and rhesus macaques is reported. The half-lives of compound **2** in blood in rats and rhesus macaques suggest that compound **2** shows wide tissue distribution and relatively high distribution volumes. A few hours after the injection, both plasma concentrations of compound **2** maintained micromolar levels, indicating it might have promise for intravenous administration when used combinatorially with anti-gp120 monoclonal antibodies.

1. Introduction

Several anti-HIV-1 drugs, including protease inhibitors and integrase inhibitors have been developed and have contributed to the highly active anti-retroviral therapy (HAART) used to treat AIDS.[1] Prevention of the HIV-1 infection of its target cells[1,2] is however a legitimate goal and the viral attachment process is an important target for the development of the drugs which could forestall such infection. The dynamic and supramolecular entry of human immunodeficiency virus type 1 (HIV-1) into target cells is initiated by the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.[3] Sequential binding of CD4 and a co-receptor (CCR5 or CXCR4) to gp120 can trigger a series of conformational rearrangements of gp41, a viral transmembrane glycoprotein mediating fusion between the viral and cellular membranes.[3–6] Control, especially of dynamic conformational changes of the envelope glycoproteins is a very attractive option.[7] To date, several small molecules that mimic CD4 have been developed as HIV-1 entry inhibitors, which competitively block the binding of gp120 to CD4[8] and the potential of these CD4 mimics has been explored (Fig. 1).[9–11] Furthermore, the interaction of CD4 mimics with a highly conserved pocket on gp120, designated as the 'Phe43 cavity', induces conformational changes in gp120,[12] a process which occurs with unfavorable binding entropy, and leads to a favorable enthalpy change similar to that caused by binding of the soluble CD4 binding to gp120. Thus, these unique properties render CD4 mimics valuable not only as entry inhibitors but also as 'envelope protein openers' and putatively, stimulants when combined with neutralizing antibodies.[13]



NBD-556 (**1**) YYA-021 (**2**)

Figure 1. Structures of NBD-556 (**1**) and YYA-021 (**2**).

Through our SAR studies a lead compound YYA-021 (**2**) with relatively high potency and low cytotoxicity has been found,[11] although the original compound NBD-556 (**1**) had relatively high cytotoxicity. Pharmacokinetic (PK) studies were performed to assess the potential of compound **2** for clinical application and in addition, the possibility and the effectiveness of its use in combination with neutralization antibodies are discussed.

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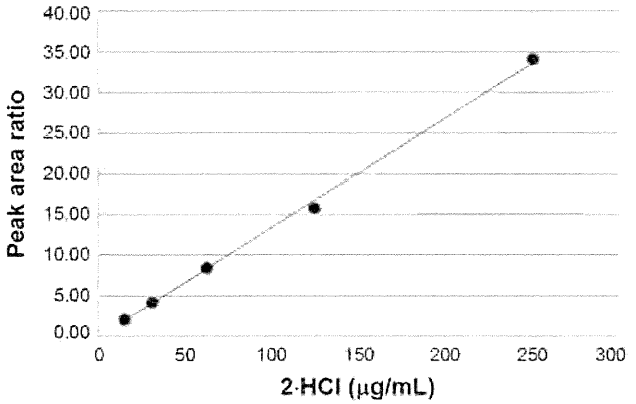

Figure 2. A calibration curve of compound **2·HCl** for calculating PK in rats.

2. Results and discussion

2.1. Calibration curve

Compound **2** was converted to its hydrochloride salt **2·HCl**, by treatment with 4 M HCl in dioxane. Standard solutions in saline of this HCl salt at concentrations over a range of 0–250 μg/mL were prepared and a calibration curve was constructed using the ratio of observed HPLC peak areas and concentrations of **2·HCl** to demonstrate the linearity shown in Figure 2.[14] The corresponding linear regression equation is $Y = 0.1345X$ ($R^2 = 0.9981$). Plasma concentrations of **2·HCl** were measured using this equation. The calibration curve shown in SD Figure S5C for the concentrations of compound **2** in plasma of a rhesus macaque was constructed using standard solutions of **2·HCl** in saline at concentrations over a range of 0–312.5 μg/mL. The linear regression equation in this case is $Y = 0.034X$ ($R^2 = 0.9927$). For adsorption of compound **2** by blood cells of a rhesus macaque, a calibration curve was constructed using standard solutions of **2·HCl** in saline at concentrations over a range of 0–950 μg/mL. The linear regression equation is $Y = 0.0553X$ ($R^2 = 0.9263$) and the curve is shown in SD Figure S7C.

2.2. Pharmacokinetics in rats

The primary stock solution of **2·HCl** was prepared in saline (2.5 mg/mL). Initially, the acute toxicity of compound **2** in rats was investigated to determine its maximum tolerated dose. The HCl salt, compound **2·HCl**, (0, 0.5, 1.0, 2.5 and 5.0 mg) was administered by tail vein injection into Jcl:SD rats (3 or 4 rats for each dose). Rats with the 5.0 mg administration showed a relatively low increase in the body weight although the intake amount of bait and water increased remarkably two weeks after administration, compared to the other rats (Supplementary data, SD Figs. S1 and S2). This observation suggested abnormalities such as renal and hepatic dysfunction and as a result, the maximum dose in rats of **2·HCl** was determined to be 2.5 mg.

Compound **2·HCl** (2.5 mg) in saline (1 mL) was administered at a level of 14 mg/kg by tail vein injection into Jcl:SD rats. Blood was collected from the tail vein into centrifugal blood collection tubes 15, 30, 45, 60, 120 and 240 min after administration of **2·HCl**. Plasma (50 μL) obtained from the blood sample was mixed with phenol in saline (final volume 60 μL) and centrifuged at 2000*g* for 3 min to remove any protein. The resultant supernatant was used in the analysis. A 50 μL aliquot of each filtrate was injected into HPLC by an autosampler. Compound **2** was detected in the HPLC analysis and was characterized by ESI-TOF-MS. The HPLC chart of the filtrate collected 30 min after administration of compound **2** is shown in Figure 3. Concentrations in blood of compound **2** after administration (15, 30, 45, 60, 120 and 240 min) were calculated using the calibration curve (SD Fig. S4) and plotted as shown in Figure 4.[15]

Using the data measured at 15, 30, 45, 60, 120 and 240 min, the half-life was calculated as 8.4 min by curve fitting with a one-compartment model based on GraphPad Prism Version 5.04 (GraphPad Software, CA, U.S.A). The initial concentration was calculated as 17.3 μg/mL. Compound **2** has a low molecular weight (317.4) and some level of hydrophobicity based on its structure, suggesting that the renal excretion is unlikely. At a dose of 2500 (μg)/initial concentration 17.3 (μg/mL), the volume of distribution was calculated as 145 mL, suggesting widespread tissue distribution of **2** as a result of its hydrophobicity.

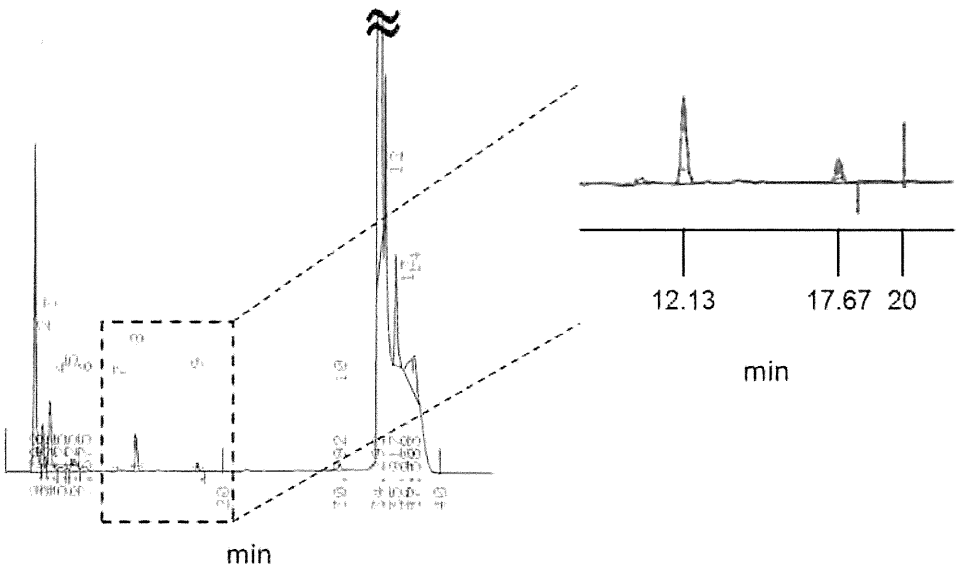

Figure 3. HPLC chart of rat plasma 30 min after intravenous administration of compound **2**. The plasma sample was eluted with a linear gradient of 20–35% CH_3CN (0.1% TFA, in 30 min). Peak 8 (retention time: 12.13 min) corresponds to the internal standard (phenol) and peak 9 (retention time: 17.67 min) corresponds to compound **2**.

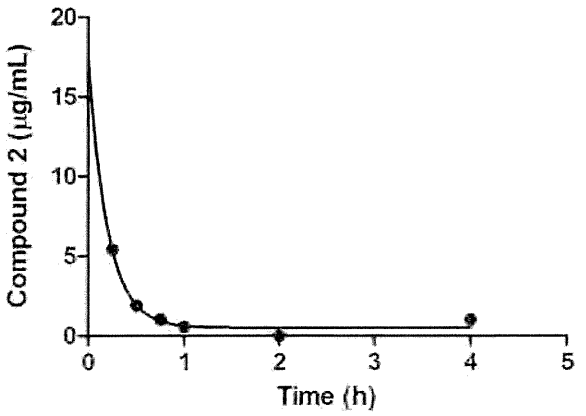

Figure 4. Plots of concentrations in rat blood of compound **2** after administration (15, 30, 45, 60, 120 and 240 min). Half-life is calculated as 0.141 h (8.4 min). The plasma of a representative rat was used for the analyses.

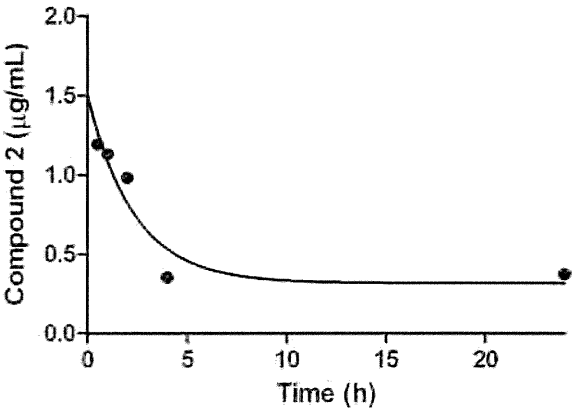

Figure 6. Plots of macaque blood concentrations of compound **2** after administration of **2·HCl** (0.5, 1, 2, 4 and 24 h). Half-life is calculated as 1.64 h (98.4 min). The plasma of a rhesus macaque was used for the analyses.

2.3. Pharmacokinetics in a rhesus macaque

The primary stock solution of the **2·HCl** was prepared in potassium-free phosphate-buffered saline ($Na_2HPO_4 + NaH_2PO_4 + NaCl$, pH 7.4) (2.4 mg/mL) to avoid hyperpotassemia and acidosis. Initially, the acute toxicity observed by treatment with **2·HCl** in a rhesus macaque was investigated to determine the maximum tolerated dose. **2·HCl** (14.1, 35.3 and 70.6 mg) was administered by cephalic vein injection into a rhesus macaque (one macaque for each dose). A macaque administered 70.6 mg, showed abnormalities such as mydriasis, prolonged PR interval in the electrocardiogram and bradycardia, while acute toxicity was not observed following the administration of **2·HCl** at levels up to 35.3 mg[16] and the maximum tolerated dose in a rhesus macaque was determined to be 35.3 mg (6.7 mg/kg). The macaque which had been administered 70.6 mg of **2·HCl** was treated by an emergency intervention with dobutamine (iv).

Compound **2·HCl** (70.6 mg) was intravenously administered at 13.4 mg/kg into a rhesus macaque. Blood (3.0 mL) was collected from cephalic vein 0, 0.5, 1, 2, 4 and 24 h after administration of the hydrochloride using winged needles. Plasma (60 μL) obtained from the blood sample was mixed with MeOH (200 μL) to remove plasma proteins and then centrifuged at 2000*g* for 3 min at room temperature in a microcentrifuge (MCF-2360, LMS Co., Ltd., Tokyo, Japan). A 228 μL aliquot of each supernatant was mixed with 12 μL of phenol in saline (stock solution: 0.3 mg/mL) to give a final concentration of phenol of 150 μg/mL, then filtered. A 200 μL aliquot of each filtrate was analyzed by HPLC using phenol as an internal standard. Compound **2** was detected in the HPLC analysis of each filtrate and its peak was characterized by ESI-TOF-MS. The HPLC of the filtrate prepared 30 min after administration of **2·HCl** is shown in Figure 5. Blood concentrations of compound **2** after 0.5, 1, 2, 4 or 24 h administration were calculated using the calibration curve (SD Fig. S6) and plotted as shown in Figure 6.

Using the time-course data, the half-life was calculated as 98.4 min by curve fitting based on GraphPad Prism Version 5.04 (GraphPad Software, CA, U.S.A.). The initial concentration in blood was also calculated as 1.50 μg/mL. In a macaque, this suggests that the renal excretion is not possible and this was by the absence of compound **2** in the HPLC analysis of the urine (SD). The distribution volume was calculated as 47.1 L; the dose 70.6 (mg)/initial concentration 1.50 (μg/mL) ratio suggests tissue distribution.

2.4. Adsorption of compound 2 to blood cells of a rhesus macaque

The question of whether compound **2** can be attached to and adsorbed into blood cells was investigated. Initially, **2·HCl** was added to blood (1 mL) of a rhesus macaque reaching a final concentration of 1 mg/mL. This mixture was incubated at 37 °C for 0, 1 and 2 h, and then centrifuged at 3600 rpm for 5 min at room temperature in a microcentrifuge to separate plasma (730 μL) from

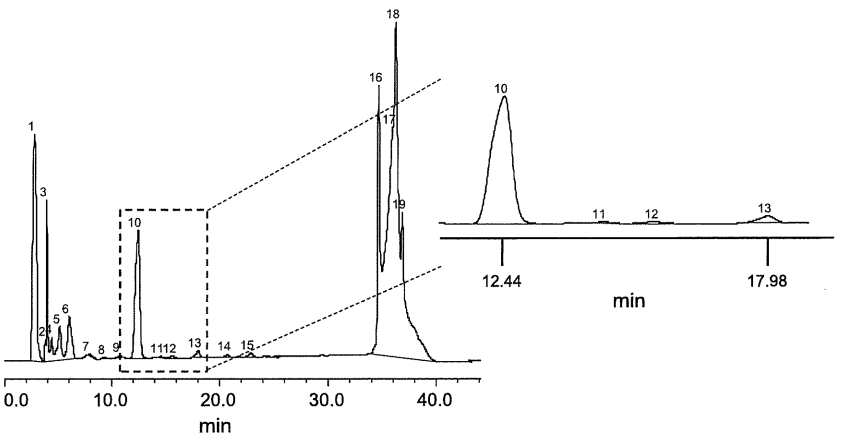

Figure 5. HPLC chart of macaque plasma 30 min after intravenous administration of **2·HCl**. The plasma sample was eluted with a linear gradient of 20–35% CH_3CN (0.1% TFA, in 30 min). Peak 10 (retention time: 12.44 min) corresponds to the internal standard (phenol) and peak 13 (retention time: 17.98 min) corresponds to compound **2**.

blood cells. In addition, plasma (0.5 mL) was added to the blood cells incubated for 1 h, then the mixture was incubated again for 1 h to separate plasma (730 μL) from the blood cells. Blood cell samples were prepared by mixing the blood cells with 480 μL of PBS and diluting the mixture to 1 mL with PBS. Blood cell samples were sonicated to disrupt cell membranes then both plasma and blood cell samples (60 μL each) were vortex-mixed with 200 μL of MeOH, then centrifuged at 2000*g* for 3 min at room temperature. A 228 μL aliquot of each supernatant was mixed with 12 μL of phenol in saline (final concentration: 600 μg/mL) and filtered. A 200 μL aliquot of each filtrate was injected to HPLC by an autosampler (SD Fig. S8).

The fractions of plasma and blood cells were analyzed by HPLC to quantify compound **2** (Fig. 7). The results showed that incubation enhanced the attachment or adsorption of compound **2** to blood cells; incubation for 1 or 2 h led to percentages of 39.7% and 39.3%, respectively, in distribution in cells, prior to incubation there was a distribution percentage of 34.8% in cells. Furthermore, compound **2**, which was adsorbed into blood cells, was significantly redistributed in plasma when fresh plasma was added; the addition of plasma to blood cells incubated for 1 h and a further incubation for 1 h caused redistribution of compound **2** to plasma and a reduction of distribution percentages to cells from 39.7% to 11.5%. This suggests that compound **2**, which was attached to blood cells, might be transferred from the cells to plasma and subsequently distributed to tissues.

These results indicate that the concentration in blood of compound **2** might be reduced, ultimately to ~1 mg/mL (3 μM) with 2–4 h treatments in rats (14 mg/kg) and rhesus macaques (13.4 mg/kg). The EC_{50} value of compound **2** has been determined as 10–50 μM in laboratory and primary HIV-1 strains.[11] Thus, in several hours after intravenous administration of compound **2** the efficacy might be diminished because the concentration in blood of compound **2** is being maintained below EC_{50} levels. However, the amount administered cannot be increased because of the acute toxicity which is described in the previous section, although the concentration in blood of compound **2** does not reach its CC_{50} level of 210 μM.[11] Recently, we reported that CD4 mimics such as compound **1** enhance the binding potency of anti-gp120 monoclonal antibodies such as KD-247[16] toward an envelope protein gp120, showing synergistic neutralization.[13] Compound **2** also enhances the neutralizing activity of KD-247 against simian–human immunodeficiency virus (SHIV)-KS661 strain via highly synergistic interactions. When compound **2** is used in combination with anti-gp120 monoclonal antibodies such as KD-247, the level of compound **2** of 3 μM might be sufficient for neutralization in vivo and thus it might be possible to reduce the dose of compound **2**. In addition, we also found that compound **1** and CXCR4 antagonists such as T140[2] showed synergistic anti-HIV-1 activity,[11] Thus, a combinatorial use with co-receptor antagonists would be also effective for reduction of the dose of compound **2**.

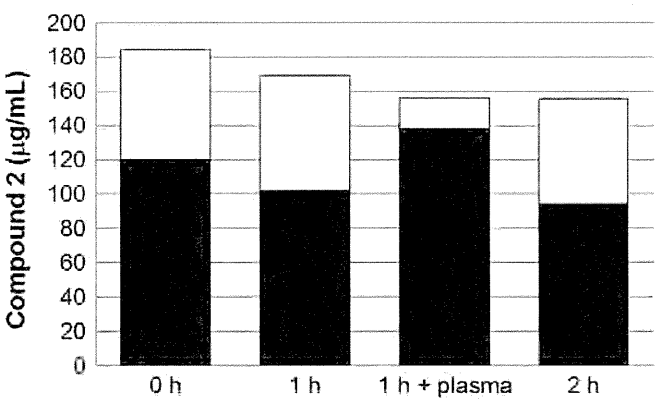

Figure 7. Quantitative analysis of compound **2** contained in macaque blood cells and plasma after incubation for 0, 1, or 2 h. 1 h + plasma means the addition of plasma (0.5 mL) to 1 h incubated blood cells followed by further incubation for 1 h. Concentrations of compound **2** in plasma are shown in black bars, and those in blood cells are shown in white bars.

3. Conclusion

CD4 mimics are attractive not only as HIV entry inhibitors but also possibly as cooperating agents for neutralizing antibodies. Binding of CD4 mimicking compounds to gp120 causes a conformational change in gp120. In this way, CD4 mimics function as 'envelope openers' and enhance the binding ability of anti-gp120 neutralizing antibodies. We discovered lead compound **2** with relatively high potency and low cytotoxicity in our previous study. In the current study, the pharmacokinetics of compound **2** in rats and rhesus macaques in the intravenous administration were investigated. Plots of plasma concentrations of compound **2** fitted with a one-compartment model provided calculation of half-lives of compound **2** in blood in rats and rhesus macaques: 8.4 and 98.4 min, respectively, suggesting that compound **2** is broadly distributed in tissues. A few hours post-injection, plasma concentrations of compound **2** in both species stabilized at micromolar levels. Consequently, compound **2** might have promise as a lead compound for the intravenous administration in a cocktail therapy with anti-gp120 monoclonal antibodies such as KD-247 and with co-receptor antagonists such as T140.[2]

4. Experimental

4.1. General information

A Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was used for analytical HPLC, 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 eluents were detected by UV at 220 nm on a JASCO UV-2075 plus (JASCO Corporation, Ltd, Tokyo, Japan). Samples were injected by an autosampler on a JASCO AS-2075 plus (JASCO Corporation, Ltd, Tokyo, Japan). ESI-TOF-MS were recorded on a micrOTOF-2focus (Bruker Daltonics) mass spectrometer.

4.2. Calibration curve

To compound **2** (1.0 g) in MeOH (5 mL) was added 4 M HCl/dioxane (10 mL) and the mixture was stirred for 30 min at room temperature. The mixture was concentrated under reduced pressure and the **2**·**HCl** was precipitated in cooled Et_2O (yield: 1.2 g, quantitative).

To construct a pharmacokinetics calibration curve in a rat, standard stock solutions in saline of **2**·**HCl**; 263, 131.5, 65.8, 32.9 and 16.4 μg/mL and an internal standard stock solution; 1.25 mg/mL phenol in saline were prepared. Each standard stock solution (22.8 μL) was mixed with 1.2 μL of the internal stock solution to give a total volume of 24 μL, then filtered. A 20 μL aliquot of each filtrate was injected to HPLC. The final concentration of **2**·**HCl** was 250, 125, 62.5, 31.3 or 15.6 μg/mL and each sample contains 62.5 μg/mL phenol. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min (SD Fig. S3). A calibration curve was constructed using the ratio of HPLC peak areas and concentrations of **2**·**HCl** to demonstrate the linearity as shown in Figure 2. The linear regression equation is $Y = 0.1345X$ ($R^2 = 0.9981$).

To construct a pharmacokinetics calibration curve in a rhesus macaque, standard stock solutions in saline of **2**·**HCl** (329, 164.5,

82.3, 41.1, 20.6 and 10.3 μg/mL) and an internal standard stock solution of phenol in saline (3 mg/mL) were prepared. Each standard stock solution (22.8 μL) was mixed with 1.2 μL of the internal stock solution and filtered. A 20 μL aliquot of each filtrate was injected to HPLC. The final concentration of **2·HCl** was 312.5, 156.3, 78.1, 39.1, 19.5 and 9.77 μg/mL, each contained 150 μg/mL phenol. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min. A calibration curve was constructed using the ratio of HPLC peak areas and concentrations of **2·HCl** to demonstrate the linearity as shown in SD Figure S5. The linear regression equation is $Y = 0.034X$ ($R^2 = 0.9927$).

To construct a calibration curve for the adsorption experiments, standard stock solutions in saline of **2·HCl**; 1000, 500, 250, 100, 50 μg/mL and an internal standard stock solution of 12 mg/mL phenol in saline were prepared. Each standard stock solution (228 μL) was mixed with 12 μL of the internal stock solution and filtered. A 200 μL aliquot of each filtrate was injected to HPLC by an autosampler. The final concentration of **2·HCl** was 950, 475, 238, 95.0 and 47.5 μg/mL and contained 600 μg/mL phenol. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min. A calibration curve was constructed using the ratio of HPLC peak areas and concentrations of **2·HCl** to demonstrate the linearity shown in SD Figure S7. The linear regression equation is $Y = 0.0553X$ ($R^2 = 0.9263$).

4.3. Pharmacokinetics in rats

Experiments using rats were conducted in an animal facility under specific pathogen-free conditions, in compliance with institutional regulations approved by the Committee of Tokyo Medical and Dental University (Tokyo, Japan). Five-week-old Jcl:SD rats purchased from CLEA Japan, Inc. (Tokyo, Japan) were maintained for one week before experiments. Compound **2·HCl** (2.5 mg) in 1.0 mL of saline was administered by tail vein injection into 6 week-old Jcl:SD rats. Blood was collected from the tail vein into centrifugal blood collection tubes (E-DS11, Eiken Chemical Co., Ltd., Tokyo, Japan) 15, 30, 45, 60, 120 and 240 min after administration. Blood was centrifuged at 2000*g* for 3 min at room temperature to separate plasma and stored at −80 °C before use. A freeze-thawed plasma sample (50 μL) was mixed with 10 μL of phenol in saline (stock solution: 0.375 mg/mL, giving a final concentration of 62.5 μg/mL) and filtered at 2000*g* for 3 min in a microcentrifuge (MCF-2360, LMS Co., Ltd., Tokyo, Japan). A 50 μL aliquot of each filtrate was injected into an HPLC. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min shown in SD Figure S4. Compound **2** was detected in the HPLC analysis and its peak was characterized by ESI-TOF-MS (*m/z* calcd for $C_{18}H_{28}N_3O_2$ $[M+H]^+$ 318.22, found 318.19).

4.4. Pharmacokinetics in a rhesus macaque

Experiments using an adult female rhesus macaque, seven years old, were conducted in a biosafety level 3 animal facility, in compliance with institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University (Kyoto, Japan). Compound **2·HCl** (70.6 mg) in 30 mL of 0.1 M sodium phosphate buffer containing NaCl (pH 7.4) was administered by cephalic vein injection. Blood (3.0 mL) was collected from the cephalic vein 0, 0.5, 1, 2, 4 and 24 h after administration using winged needles. Blood was centrifuged at 3600 rpm for 5 min at room temperature to separate plasma then stored at −80 °C before use. A freeze-thawed plasma sample (60 μL) was vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. A 228 μL aliquot of each supernatant was mixed with 12 μL of phenol in saline (stock solution: 0.3 mg/mL, final concentration: 150 μg/mL) and filtered. A 200 μL aliquot of each filtrate was injected into HPLC by an autosampler. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min shown in SD Figure S6. Compound **2** was detected in the HPLC analysis of each filtrate and its peak was characterized by ESI-TOF-MS (*m/z* calcd for $C_{18}H_{28}N_3O_2$ $[M+H]^+$ 318.22, found 318.19).

4.5. Adsorption experiments of compound 2 to blood cells of a rhesus macaque

Compound **2·HCl** in saline (5 mg/mL, 0.25 mL) was added to blood (1.0 mL) collected from a macaque, and incubated at 37 °C for 0, 1 and 2 h. After incubation, plasma samples (730 μL) were separated by centrifugation at 3600 rpm for 5 min at room temperature, and PBS (480 μL) was added to the resulting precipitates, increasing their total volume to 1.0 mL, and producing blood cell samples. In addition, plasma (0.5 mL) was added to the blood cells after 1 h incubation and the mixture was incubated again for 1 h to separate plasma (730 μL) and blood cells. The separated plasma and blood cell samples were stored at −80 °C before use. Freeze-thawed plasma samples (60 μL) were vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. Freeze-thawed blood cell samples (200 μL) were sonicated to disrupt cell membranes (Sonifier 250, Branson Ultrasonics, Emerson Japan Ltd., Kanagawa, Japan), and 60 μL of the sonicated products was vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. A 228 μL aliquot of each supernatant obtained from both plasma and blood cell samples was mixed with 12 μL of phenol in saline (stock solution: 12 mg/mL, final concentration: 600 μg/mL) and filtered. After filtration, a 200 μL aliquot of each filtrate was injected into HPLC by an autosampler. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min and is shown in SD Figure S8.

Acknowledgments

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Supplementary data

Supplementary data (figures of changes on body weight and on intake amounts of bait and water in rats, tables of HPLC peak areas and HPLC charts) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.005.

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Short Communication

Generation of a replication-competent simian–human immunodeficiency virus, the neutralization sensitivity of which can be enhanced in the presence of a small-molecule CD4 mimic

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Simian–human immunodeficiency virus (SHIV) carrying the envelope from the clade B clinical human immunodeficiency virus type 1 (HIV-1) isolate MNA, designated SHIV MNA, was generated through intracellular homologous recombination. SHIV MNA inherited biological properties from the parental HIV-1, including CCR5 co-receptor preference, resistance to neutralization by the anti-V3 loop mAb KD-247 and loss of resistance in the presence of the CD4-mimic small-molecule YYA-021. SHIV MNA showed productive replication in rhesus macaque PBMCs. Experimental infection of a rhesus macaque with SHIV MNA caused a transient but high titre of plasma viral RNA and a moderate antibody response. Immunoglobulin in the plasma at 24 weeks post-infection was capable of neutralizing SHIV MNA in the presence but not in the absence of YYA-021. SHIV MNA could serve a model for development of novel therapeutic interventions based on CD4-mimic-mediated conversion of envelope protein susceptible to antibody neutralization.

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Control of primate lentiviral infection by antibodies directed against viral envelope protein is theoretically feasible. This was confirmed by the successful protection of macaque monkeys from challenge inoculation with simian–human immunodeficiency virus (SHIV) carrying an envelope protein (Env). Env was derived from a laboratory strain of human immunodeficiency virus type 1 (HIV-1) through the passive immunization of neutralizing mAbs directed against HIV-1 (Mascola *et al.*, 2000; Nishimura *et al.*, 2003). This neutralization is consistent with the results normally seen in cell culture systems.

In contrast, clinical isolates of HIV-1 that have not been subjected to extensive passage in T-cell lines are generally resistant to antibody-mediated neutralization (Moore *et al.*, 1995). It has been shown that virus in infected individuals is under selective pressure to develop a variety of means to evade attack by neutralizing antibodies, including sequence variation, glycosylation, tertiary structural shielding formed by the Env trimer and the rapid kinetics of conformational changes of Env, which affect fusion between the viral envelope and the plasma membrane of target cells (Kong & Sattentau, 2012). Although four major neutralizing epitopes have been identified in HIV-1 Env (i.e. the V1/V2 loop, the glycan-V3 site and CD4-binding site of gp120, and the membrane-proximal external region of gp41), for reasons that are as yet unclear few reports of antibodies directed against these epitopes capable of neutralizing a broad range of isolates have been published (Kwong & Mascola, 2012). High titres of antibodies directed against the V3 loop are elicited in individuals during the early phase of HIV-1 infection, but these are incapable of neutralizing the virus because the epitope in functional Env trimer is probably shielded from the antibody (Davis *et al.*, 2009b). Therefore, it is necessary to develop a means of rendering these epitopes accessible to

One supplementary table and five supplementary figures are available with the online version of this paper.

the antibodies, to make antibody-mediated suppression of HIV-1 a valid therapeutic option.

It has been reported that neutralization mediated by antibodies directed against the V3 loop (Lusso *et al.*, 2005) or CD4-induced epitope (CD4i) (Thali *et al.*, 1993) can be enhanced in the presence of soluble CD4 (sCD4). It is known that the interaction of Env with sCD4 drives a conformational change of the viral protein and makes the cryptic/occult epitopes accessible to these antibodies (Wyatt *et al.*, 1998). Small molecules that emulate sCD4 for its interaction and subsequent induction of conformational change of Env may be employed to intensify antibody-mediated interventions against HIV-1 infection. Compounds with the above-mentioned properties (i.e. NBD-556 and NBD-557) have been reported previously (Zhao *et al.*, 2005). NBD-556 has been shown in cell culture to interact with the CD4-binding pocket to induce a conformational change in gp120 (Madani *et al.*, 2008) and enhance exposure of the Env of primary HIV-1 isolates to neutralizing epitopes (Yoshimura *et al.*, 2010).

The present study was performed to evaluate small-molecule CD4-mimic-based enhancement of antibody-mediated virus neutralization, in the context of virus infection *in vivo*. The SHIV/macaque monkey model of AIDS is particularly suitable for such studies, as SHIV carries the HIV-1 Env and the neutralization sensitivity of SHIV is comparable to that of the parental HIV-1 (Shibata & Adachi, 1992).

As NBD-556, unlike sCD4, inhibits infection with select HIV-1 isolates (Yoshimura *et al.*, 2010), we generated a new SHIV strain carrying Env, the sensitivity of which to antibody-mediated neutralization is enhanced in the presence of a CD4 mimic. An HIV-1 isolate (MNA), previously designated primary isolate HIV-1 Pt.3, was used as the source of Env, as the viral protein has been reported to interact with NBD-556 (Yoshimura *et al.*, 2010). While the virus belongs to a distinct subset of HIV-1 isolates, as mentioned above, it has also been reported to utilize the CCR5 molecule to gain entry into target cells, a property that is shared by the majority of HIV-1 strains (Yoshimura *et al.*, 2010). A mAb directed against the tip of the V3 loop (GPGR motif), KD-247 (Eda *et al.*, 2006), was employed to assess this concept, as HIV-1 MNA is resistant to KD-247-mediated neutralization, despite carrying the exact epitope sequence in the tip of the V3 loop, and is converted to being sensitive to the antibody by NBD-556 in a dose-dependent manner (Yoshimura *et al.*, 2010).

First, we reproduced the results of Yoshimura *et al.* (2010) using a neutralization assay employing TZM-bl cells (Platt *et al.*, 1998), obtained from the National Institutes of Health (NIH) AIDS Reagent Program (Fig. S1, available in JGV Online). The virus was resistant to KD-247, as described previously, and required almost 50 μg ml^{-1} of the antibody to achieve 50 % neutralization in our assay. However, the observed resistance was eliminated in the presence of 2 μM NBD-556; 50 % neutralization was achieved in the presence of ~0.1 μg KD-247 ml^{-1}, corresponding to 1/500 of the amount of the antibody to achieve the same degree of neutralization in the absence of the CD4 mimic.

With reproduction of the properties of HIV-1 MNA Env, we generated an SHIV strain carrying Env through intracellular homologous recombination, as described previously (Fujita *et al.*, 2013) with minor modifications (Fig. S2). DNA fragments representing the 5′ and 3′ ends of the SHIV genome (fragments I and II, respectively) were amplified by PCR from the proviral DNA plasmid SHIV KS661. A DNA fragment containing *env* (fragment III) was amplified from cDNA of the HIV-1 MNA genome, which was prepared from virus particles (virion-associated RNA) in the culture supernatant of PM1/CCR5 cells (Yusa *et al.*, 2005) infected with the virus. The PCR primers used are listed in Table S1. Using a FuGENE HD transfection reagent, lipofection was performed on the C8166-CCR5 cells (Shimizu *et al.*, 2006) to co-transfect them with 0.2 μg DNA. A cytopathic effect, presumably caused by the emerged recombinant virus, was observed on day 13 post-transfection. The emerged virus, designated SHIV MNA, carried the entire gp120 and three-quarters of gp41 from HIV-1 MNA Env (Fig. 1a). The rest of Env was from SHIV KS661, the Env of which was derived from HIV-1 89.6 (Shinohara *et al.*, 1999). The CD4 binding site, and the regions and elements that reportedly interact with NBD-556 (Madani *et al.*, 2008; Yoshimura *et al.*, 2010), are preserved in SHIV MNA Env (Fig. S3). The virus was replication competent in PM1/CCR5 cells (data not shown).

As HIV-1 MNA has been suggested to be a CCR5-utilizing virus, we were intrigued as to whether SHIV MNA inherited the trait from the parental virus. We subjected SHIV MNA and the parental HIV-1 MNA to a co-receptor usage assay as described previously (Nishimura *et al.*, 2010), with minor modifications (Fig. S4). As expected, SHIV MNA was shown to utilize CCR5 as an entry co-receptor.

We next assessed the neutralization profiles of SHIV MNA in comparison with the parental HIV-1 MNA, as described previously (Li *et al.*, 2005; Wei *et al.*, 2002). Both SHIV MNA and HIV-1 MNA showed essentially no neutralization by KD-247 up to 25 μg ml^{-1}, and 50 % neutralization was achieved at 50 μg ml^{-1} (Fig. 1b). As the CD4 mimic, we employed YYA-021, a compound generated and characterized through studies concerning the structure–activity relationships of small molecules (Narumi *et al.*, 2010, 2011, 2013; Yamada *et al.*, 2010). The compound was shown to be slightly less potent but to exhibit substantially lower toxicity than NBD-556, and was therefore a suitable choice for our purposes in future studies in animal models. SHIV MNA was resistant to neutralization by YYA-021 at all concentrations examined, except 25 and 50 μM, and showed a neutralization profile almost identical to that of HIV-1 MNA (Fig. 1c). To further characterize the

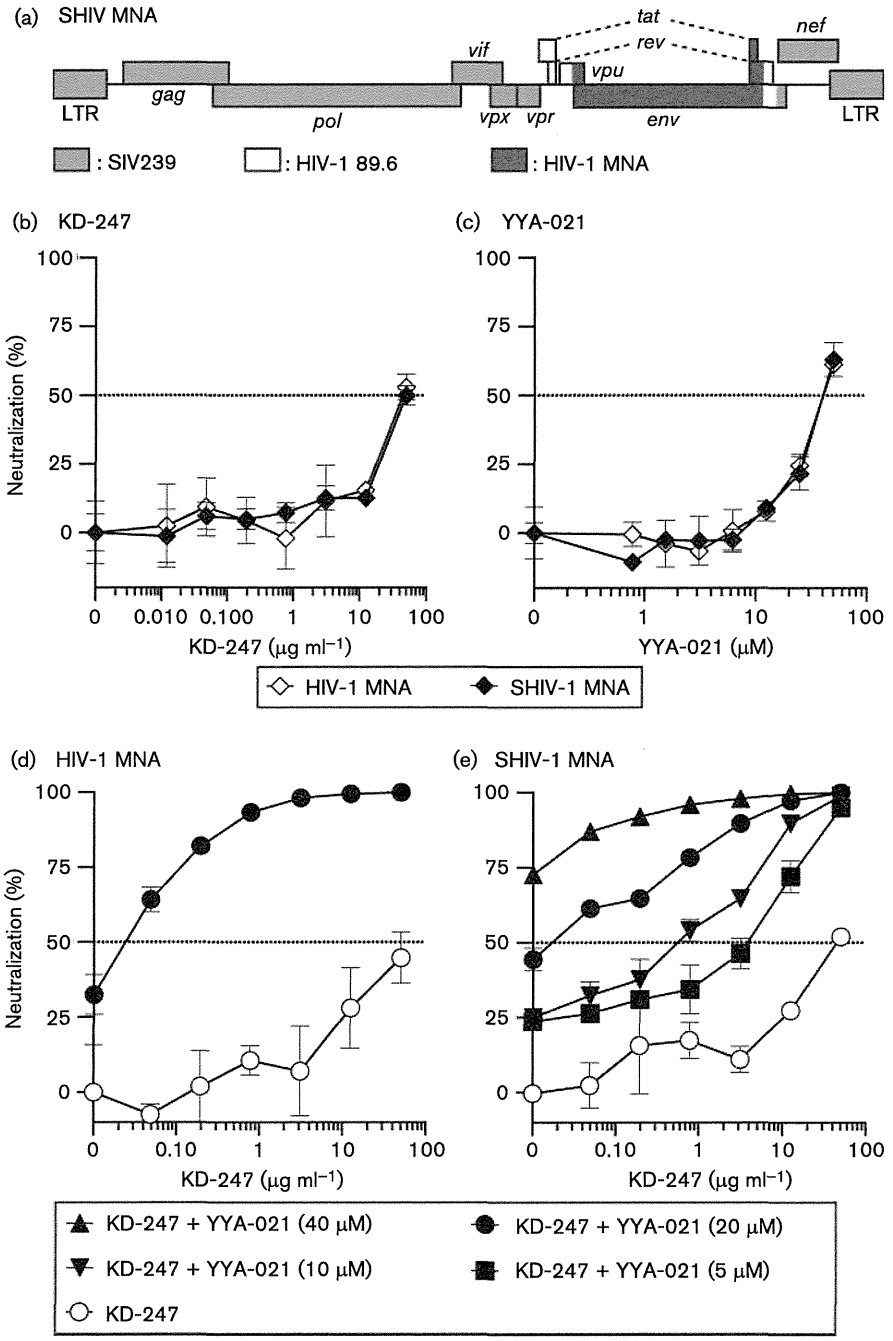

Fig. 1. Genomic organization (a) and neutralization sensitivity (b–e) of SHIV MNA. (a) Grey shaded boxes represent genes derived from SIV239, open boxes those from HIV-1 89.6 and dark grey shaded boxes those from HIV-1 MNA. LTR, long terminal repeat. (b–e) Percentage neutralization was calculated as 100×[1−(RLU.N−RLU.B)/(RLU.V−RLU.B)], where RLU is relative luciferase units; RLU.N is RLU in wells with cells, virus and KD-247 and/or YYA-021; RLU.V is RLU in wells with cells and virus; and RLU.B is RLU in wells with cells.

biological properties of SHIV MNA Env, a set of entry assays was conducted (Fig. S5). The *env* genes cloned from SHIV MNA and HIV-1 MNA were utilized to generate pseudotyped viruses. These pseudotypes were inoculated into TZM-bl cells in the presence of increasing amounts of NBD-556, YYA-021 or sCD4. A control group was derived from another virus preparation pseudotyped with amphotropic murine leukemia virus (A-MLV) Env (Landau *et al.*, 1991). When the efficiency of entry was defined by intracellular luciferase activities, virtually no difference was observed between Envs of SHIV MNA and the parental HIV-1. Thus, SHIV MNA Env replicated in