られることを明らかとした。

D．考察

本研究期間では、初年度にスクリーニング法の確立、次年度以降で融合阻害剤の開発、最終年度、ウイルスレセプター結合阻害剤の開発を行った。初年度確立したスクリーニングに用いたNP2細胞はHIV感染に必要となるレセプターを発現していないため、目的とするレセプターcDNAを遺伝子導入することにより正確なアンタゴニズムを検討可能である。HIV感染に中心的な役割を果たすCD4、CXCR4、CCR5を今回導入しが、今後必要に応じてCCR3等を導入した細胞の確立も行っていきたいと考えている。検出方法もこれまでのMAGI法はX-Galによる染色・計測しかないが、我々が作製した方法では、X-Galによる染色だけでなく、SEAP活性によってもその効果を検討可能である。また時間がかかるもののMTT色素法によるオートメーション化も可能であることがわかっている。この有用な方法を本研究で見出した薬剤の測定や作用機序の解明に利用した。

T-20はgp41の六量体の形成を阻害することで膜融合を抑制するため、逆転写酵素阻害剤やプロテアーゼ阻害剤に耐性を持つHIV-1の複製も強く抑制する。しかしながら、gp41の38番目のアミノ酸がバリンからアラニンに変異したV38A変異や43番目のアスパラギンからアスパラギン酸に変異したN43D変異が導入されるとT-20の効果を減弱させてしまう(Table 3)。したがってT-20耐性ウイルスの複製を抑制できる新規の融合阻害剤が望まれている。一方でC-HR由来のペプチドの一つであるC34はin vitroにおいてT-20より効果的に融合を阻害することから我々はC34を母核として高活性の誘導体を作製した。これらの誘導体はN-HRとの相互作用にとって必要ではない溶媒接触面のアミノ酸残基をグルタミン酸 (E) とリジン (K) に置換させている。これらの置換はC34のアミノ酸配列のiとi+4の位置にあり (Figure 2)、それによりXEEXXKK(Xは置換されていないアミノ酸)モチーフの繰り返しとなっている。SC34EKは2個の完全なXEEXXKKモチーフと3個の不完全なモチーフを持ち、元の配列であるC34と比べて抗HIV-1活性が増強されていた。興味深いことに、5個の完全なモチーフを持つSC35EKの抗HIV-1活性はSC34EKのそれと同等であることから、我々は4個のモチーフを持つSC29EKが十分な活性をもつことを示したが、3個になるとその活性は急激に低下した。一方で、T-20EKではアミノ酸数を減らすと活性が減弱し、36アミノ酸よりも小分子化が困難であった。我々が作製したEKシリーズのペプチドは配列の約30%を変更したものであるが、米国Trimeris/Roche社が開発を進めているT-20由来ペプチドは約50%を改変し、またアミノ酸数を減らすことはできていない。おそらくT-20対応配列の中、特に両端に活性維持部位が含まれていると考えられる。この点に関してC末側のトリプトファンに富む部位がこれまで詳細に調べられて来たが、N末側に関しての報告はいまだ少ないのが現状である。今後N末側の解析が必要と考えられた。

N36/SC29EK複合体のTm値はN36/C29複合体のそれより高い値を示したことから、EK置換はα-ヘリックス性を増強させることにより六量体の安定性を高めたと考えられた。EKの導入による安定した -ヘリックス構造はペプチド内での塩橋形成によるものだと考えられていたが、近年EKによる静電気的な相互作用がα-ヘリックス性を強めていることを明らかとした。従って -ヘリックス性の増強は少なくてもC34の低分子量化にも役立つことが考えられる。ペプチド製剤は、その製造に高いコストがかかり低分子化が必要であるが、単純にT-20を短くしただけでは抗ウイルス活性が失われてしなう。本研究で用いたEK置換はα-ヘリックス性を増強させることにより抗ウイルス効果を増強させる事が可能であり、低分子化への可能を提示した。

これらのペプチドは少なくとも30%以上のアミノ酸を変更しており、感染者に投与す

るに当たり吸収性、安定性、抗原性、有効性、毒性などほぼすべての臨床試験を新規薬剤と同様にやり直す必要がある。そのため、開発には莫大な費用と時間が必要であり、早期臨床応用は困難である。しかしながら我々が見出した耐性変異を導入した T-20 等は実際に T-20 投与によって出現してきた自然界に存在しうる HIV-1 の配列であり、これら薬理学的問題は比較的少なくなり、有効な薬剤の早期臨床応用を加速させると考えられる。この1アミノ酸変異導入による活性増強はT-20に限らず C34 でも認められた。このことは我々が提唱した新しい耐性克服型のペプチド創製がある程度普遍的に応用することができることを示唆する。第2世代の融合阻害剤が開発されても HIV-1 はおそらくそれに対しても耐性を獲得すると予想されるが、その場合においても導入された耐性変異を導入することにより、耐性ウイルスの複製を抑制しうると考えられ、ペプチド配列の設計をより簡便化することが可能となる。耐性変異の導入パターンがある程度症例をかさねることで明らかになればあらかじめその配列をもつペプチドを作製しておくことも可能であろう。

最終年度は、融合阻害剤にこだわらず、広くウイルスの細胞進入を阻害する薬剤のスクリーニングを行い、ポルフィリン誘導体にその効果があることを明らかとした。これまでにもポルフィリン誘導体が抗 HIV 効果を示すことは知られていたが（*J Med Chem* 37:1099-108, 1994）、主に gp120 との in vitro 結合性で議論されており、その作用機序は明確にはなっていなかった。後の研究(*Antimicrob Agents Chemother*, 46:3917-25 2002)においても、ウイルス粒子の不活化や融合阻害剤とも考えられていた。メチレンブルー等でも知られている UV による photodamage が、ポルフィリン誘導体でも起こることが示されているが(*Antiviral Res*, 61:37-47, 2004)、実験は gp120 由来ペプチドとそれに対する抗体の結合性を指標に検討しており、直接ウイルスを用いていないため、実際のウイルス感染において主たる機序であるとは結論できないと考えられる。

このようなことを踏まえ、分担研究者は感染性 HIV を用いてウイルス学的にその作用機序を検討した結果、ポルフィリン誘導体は gp120 の V3 loop に結合し、R5 指向性、X4 指向性を問わずHIVの entryを阻害、この際、UV 照射はなくとも効果を示すことを明らかとした。また time of addition の結果より、CD4分子との結合からコレセプター結合に関与するステップで効果を示すことからも確認し、これまで融合阻害剤とも考えられてきたポルフィリン誘導体が、融合ではなく、V3とレセプター結合の阻害が主な作用点であることを明らかにした。

E．結論

本研究期間において、多剤耐性 HIV 感染症を克服するために HIV の細胞侵入過程を標的とした薬剤の開発を 3 年間にわたり行った。はじめに新規アッセイ系を確立し、その後耐性 HIV に効果を示す融合阻害ペプチドとレセプター吸着阻害剤の開発研究を行った。耐性変異をペプチドデザインに応用し、融合阻害剤耐性 HIV に効果を示すペプチドの作製に成功した。また、ポルフィリン誘導体が HIV のレセプター結合を阻害することによって抗 HIV 効果を示すことを明らかにした。一部の化合物は経口吸収性もあることから今後誘導体のさらなる合成・検討が望まれる。

F．健康危険情報

基礎的研究であり該当しない

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H．知的財産権の出願・登録状況

なし

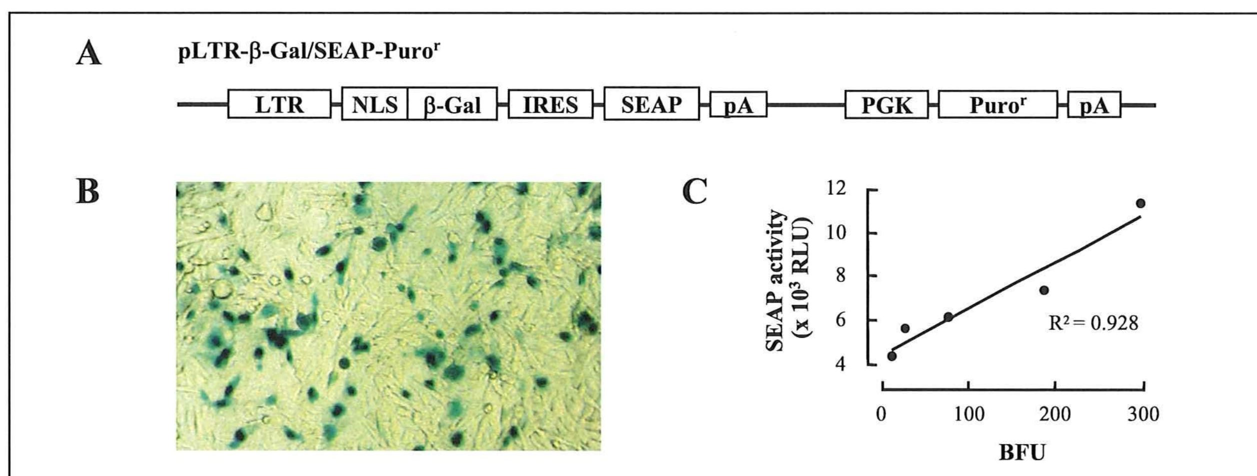

Figure. 1 Establishment of a cell line with β-galactosidase (β-Gal) and secretary alkaline phosphatase (SEAP) genes driven by a long terminal repeat (LTR). (A) Schematic diagram of the vector used in the present study, which simultaneously expresses genes for β-Gal and SEAP under the control of the HIV-1 LTR promoter (pLTR-β-Gal/SEAP-Puro^r). The enhancer region (positions -138 to +89) of the LTR, nuclear localization signal (NLS) derived from the T-antigen of SV40, internal ribosome entry site (IRES), phosphoglycerate kinase promoter (PGK) and polyadenylation signal (pA) are also shown. (B) Microscopic image of X-gal-stained NCK45-β-Gal/SEAP cells at 48 h after virus inoculation. (C) Correlation between β-Gal and SEAP activities in culture supernatants. NCK45-β-Gal/SEAP cells were infected with HIV at various infectious doses and incubated for 48 h. Culture supernatants were examined for their SEAP activities and expressed as relative right units (RLU). BFU: blue-cell-forming units.

Table 1. 抗HIV 活性の比較

		EC$_{50}$ (μM) [a]						CC$_{50}$ (μM) [c]
		HIV-1$_{IIIB}$ [b]			HIV-1$_{Ba-L}$ [b]			
		MAGI	NCK45-β-Gal/SEAP		MAGI	NCK45-β-Gal/SEAP		NCK45-β-Gal/SEAP
	Target		β-Gal	SEAP		β-Gal	SEAP	
DS5000	gp120	0.14	0.076	0.07	0.36	0.49	0.42	>100
AZT	RT	0.031	0.0043	0.0035	0.05	0.0035	0.0094	>100
ddC	RT	0.4	0.53	0.42	0.48	0.72	0.67	>100
T-140	CXCR4	0.006	0.006	0.0025	>100	>100	>100	>100
TAK-779	CCR5	>100	>100	>100	0.003	0.035	0.027	>100

[a] EC$_{50}$; 50% effective concentration.
Data represent mean values of at least three independent experiments (mean value ± standard deviation).
[b] HIV-1$_{IIIB}$ and HIV-1$_{Ba-L}$ utilize CXCR4 (X4) and CCR5 (R5) as a co-receptor, respectively.
[c] CC$_{50}$; 50% cytotoxic concentration. CC$_{50}$ was determined by MTT method.

Table 2. SC29EKの抗ウイルス活性

Substitution	EC$_{50}$ [a] (nM)					
	T-20	SC22EK	C29	SC29EK	C34	SC34EK
HIV-1$_{WT}$ [b]	15 ± 1.3 [c] (6.3) [d]	77 ± 14	245 ± 42 (4.7)	2.4 ± 0.1 (1.3)	7.3 ± 0.7 (1.1)	3.0 ± 0.3 (1.1)
HIV-1$_{D36G}$	2.4 ± 0.6	N.D. [e]	52 ± 18	1.9 ± 0.0	6.5 ± 1.8	2.7 ± 0.7
HIV-1$_{D36G/V38A}$	23 ± 8.2 (9.6)	>1000	504 ± 193 (9.7)	3.4 ± 0.9 (1.8)	9.2 ± 2.0 (1.4)	1.5 ± 0.3 (0.6)
HIV-1$_{D36G/N43D}$	49 ± 10 (20)	>1000	>1000 (>19)	3.0 ± 0.6 (1.6)	9.3 ± 3.5 (1.4)	2.2 ± 0.4 (0.8)
HIV-1$_{D36G/N43D/S138A}$	84 ± 16 (35)	N.D.	>1000 (>19)	4.1 ± 0.6 (2.2)	15.7 ± 5.5 (2.4)	1.6 ± 0.4 (0.6)
HIV-1$_{\Delta V4/D36G/I37K/N126K/L204I}$	390 ± 155 (163)	252 ± 71	>1000 (>19)	50 ± 11 (26)	171 ± 15 (26)	3.0 ± 0.2 (1.1)

[a] EC$_{50}$: 50% Effective Concentration
[b] Wild type (WT) indicates HIV-1$_{NL4-3}$
[c] Antiviral activity was determined by the MAGI assay.
[d] Parenthesis indicates fold of decreased EC$_{50}$ value compared to EC$_{50}$ value in D36G.
[e] Not Determined
[f] ΔV4 indicates five amino acids deletion in V4 region of gp120

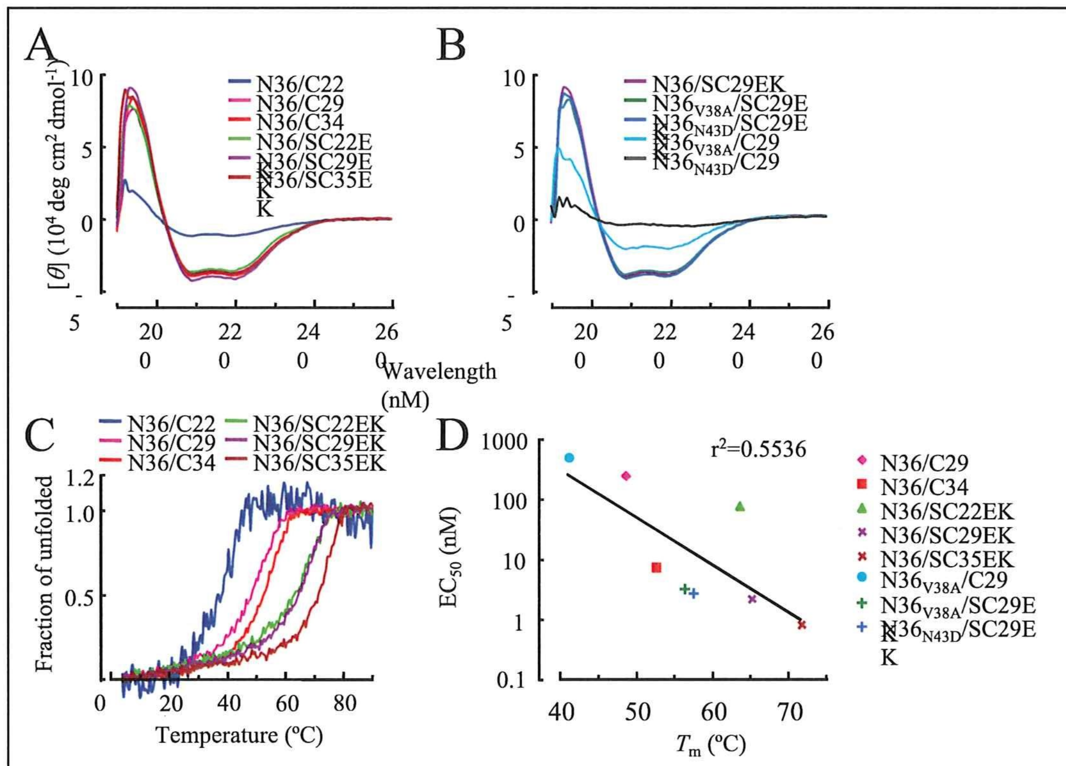

Figure 2. Circular dichroism (CD) spectra of various N-HR derived peptides and C-HR derived peptides complexes.

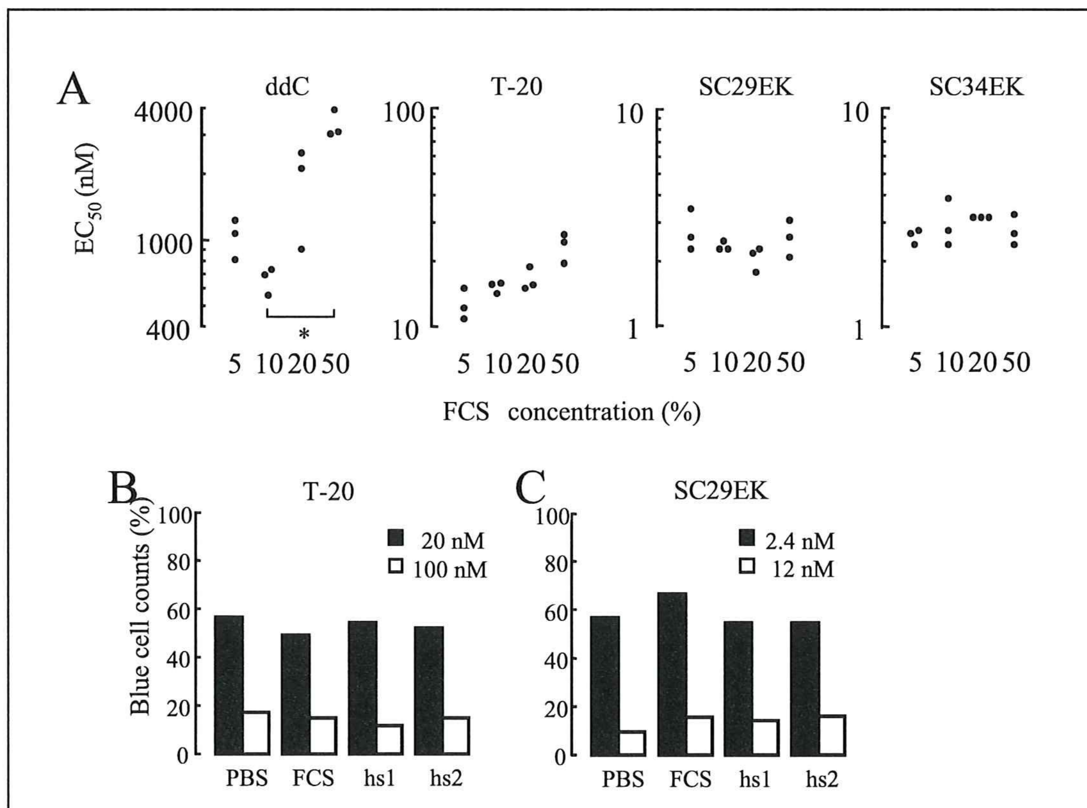

Figure 3. Effect of serum components on antiviral activity. Antiviral activities in various serum concentrations, 5, 10, 20 and 50%, were determined by the MAGI assay. (A) Black dots indicate EC_{50} (nM) values of each determinations that were carried out three times independently (*p=0.01 by student's *t* test). Antiviral activities of T-20 (B) and SC29EK (C) in human HIV-seronegative sera were assessed by counting the number of blue cells of HeLa-CD4/CCR5/LTR- β -galactosidase. Bars indicate percentage of the number of blue cells in PBS, FCS and human sera against that of control which added only virus not antiviral agents to cells.

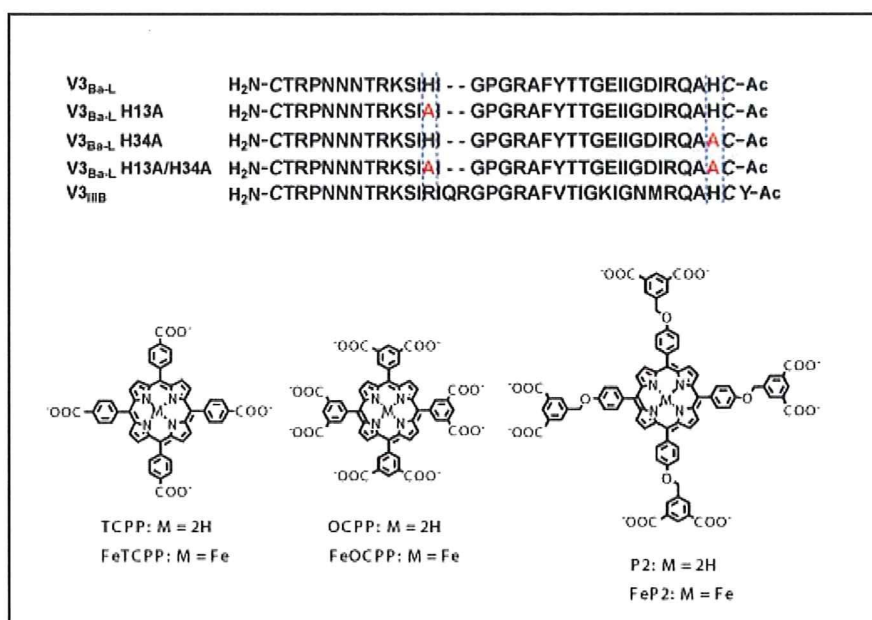

Figure 4. Amino acid sequences of the V3Ba-L, V3Ba-LH13A, V3Ba-LH34A, V3Ba-LH13A/H34A, and V3IIIB peptides (A) and multivalent anionic porphyrins and their iron complexes used in this work (B).

Table 4. Anti-HIV activity of porphyrin derivatives

Inhibitor	EC_{50} [a]		CC_{50} [b]
	HIV-$1_{Ba\text{-}L}$	HIV-1_{IIIB}	
		μM	
AZT	0.034 ± 0.009	0.023 ± 0.010	>10
DS5000 [(c)]	0.27 ± 0.021	0.36 ± 0.021	>21
AMD3100	>0.2	0.0034 ± 0.0002	>0.2
TCPP	4.7 ± 0.15	2.6 ± 0.23	40 ± 5
FeTCPP	0.89 ± 0.21	1.4 ± 0.30	>100
OCPP	1941 ± 196	1699 ± 562	36 ± 5
FeOCPP	353 ± 25	488 ± 187	>100
P2	187 ± 29	205 ± 46	35 ± 5
FeP2	116 ± 16	59 ± 14	>100

[a]The EC_{50} values were determined by the MAGI assay.
[b]The CC_{50} values were determined by the MTT method.

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分担研究報告書

HIV 吸着・膜融合過程を標的とする多剤耐性克服型耐性克服型 HIV 化学療法剤の開発

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研究要旨: 本分担研究の目的は、多剤耐性克服型 HIV 化学療法剤の候補となりうる経口吸収性を示す CXCR4 阻害剤 KRH-3955 についてその抗 HIV-1 活性（培養細胞レベルと動物モデルの両方）や CXCR4 への作用点について詳細な解析を行うとともに、これらの CXCR4 阻害剤の試験管内での耐性誘導を試み、その解析を通してより耐性の出にくい薬剤をデザインや耐性変異パターンの予測を行うことである。最終年度では、サルを用いたエイズの動物モデルにおいて KRH-3955 が事前内服による感染予防効果の検討において CD4 陽性細胞数の維持に効果を示すなど HAART 療法のバックアップとして期待できる薬剤候補であることが示された。

A. 研究目的

本研究班の課題である HIV 吸着・膜融合過程を標的とする多剤耐性克服型 HIV 化学療法剤の開発に関して、共同研究者（株）クレハが開発した KRH-1636 の誘導体で、経口吸収性を示す CXCR4 阻害剤 KRH-3955 に関して行った研究成果を報告する。本研究の目的は、新しい作用機序を有する HIV 阻害剤として期待される CXCR4 阻害剤に対する耐性 HIV-1 を誘導し、その耐性変異のパターンや耐性機構を解析することによって、より耐性の出にくい薬剤をデザインしたり、耐性変異パターンを予測することである。 材料としては、共同研究者（株）クレハが開発した経口吸収性を示す 2 種類の高活性 CXCR4 阻害剤 KRH-3955、 KRH-3148 を使用した。また、KRH-3955 と KRH-3148 を用いた PM1/CCR5-NL4-3 の感染系による薬剤耐性誘導実験は、AMD3100、AMD070 を対照薬剤として使用して行い、現在も継続中である。

B. 研究方法

（1）抗 HIV-1 活性測定：活性化 PBMC を標的細胞として X4、R5、R5X4 の各種 HIV-1 を感染させ培養上清中の p24 抗原量を市販の ELISA を用いて測定後算出した。

（2）hu-PBL-SCID mice を用いた HIV-1 感染モデルにおける抗ウイルス活性測定：感染 2 週間前に KRH-3955 を 10 mg/kg 単回経口投与した。分離したヒト PBMC をマウス腹腔に導入し、1 日後に HIV-1 NL4-3 を感染させた。感染 7 日後にマウス腹腔から PBMC を回収し、IL-2 存在下で 4 日間培養した。抗 HIV-1 活性は培養上清中の p24 抗原量を市販の ELISA を用いて測定後算出した。

（3）SDF-1α結合阻害活性の測定：CXCR4 を強制発現させた CHO 細胞を用いて薬剤存在、非存在下で細胞に結合した ^{125}I-SDF-1α の放射活性を測定した。

（4）各種抗 CXCR4 抗体結合阻害活性の測定：Molt-4 細胞に結合した CXCR4 抗体量を FACS にて定量した。

（5）CXCR4 阻害剤との相互作用に影響を与える CXCR4 中のアミノ酸の同定：CXCR4 点変異体を安定発現させた 293 細胞を使用し、CXCR4 阻害剤が変異 CXCR4 と抗 CXCR4 抗体 12G5 との結合阻害活性に与える影響を測定することによって、 阻害剤と相互作用する CXCR4 中のアミノ酸を推定した。

（6）CXCR4 阻害剤耐性 HIV-1 誘導実験
PM1/CCR5 細胞を標的細胞として、NL4-3 をウイルスとして用い、KRH-3955、

KRH-3148、AMD3100、AMD070 の4薬剤について平成19年10月に開始した。

（7）事前内服による感染予防効果の検討(共同研究者：エイズ研究センター仲宗根主任研究官)：カニクイサルに SHIV-KS661$_C$ を感染させる AIDS モデルにおいて KRH-3955を100 mg/kgで感染24時間前に投与（対照薬剤：TDF/FTC は、30/20 mg/kg を感染 24 時間前と感染直前の2回投与）後、10 AID50 の SHIV-KS661$_C$を経直腸感染させ、12週にわたって観察する。主なモニター項目は、CBC、CD4/CD8 細胞数、ウイルス量、などである。

（倫理面での配慮）

本研究で使用を予定する複製可能な組換えウイルスは大臣確認を得ている（大19-6 および大 17-23）ほか、組換え DNA 実験の申請は本研究所に機関承認されている（機 19-63 ほか)。

C. 研究結果及び考察

（1）KRH-3955 の抗 HIV-1 活性：KRH-3955は、用いたX4, R5X4 HIV-1 の活性化PBMCにおける複製をEC_{50}: 1-4 nMで抑制した。また、活性化 PBMC や MT-4 細胞に対して25 mMまで顕著な細胞毒性を示さなかった。

（2）hu-PBL-SCID mice を用いた HIV-1 感染モデルにおける抗ウイルス活性：感染前に単回経口投与した KRH-3955 は、hu-PBL-SCID mice 腹腔内における NL4-3 の感染・複製をほぼ完全に抑制した。

（3）SDF-1α結合阻害活性： KRH-3955、AMD3100、の SDF-1α結合に対する IC_{50} (nM)はそれぞれ、0.8、281.1 であった。

（4）各種抗 CXCR4 モノクローナル抗体結合阻害活性： KRH-3955はN末端を認識する抗体以外の抗体の CXCR4 発現細胞への結合を強く阻害した。一方、コントロールとして用いたCXCR4 阻害剤 AMD3100は、ECL 1と2を認識する抗体である 12G5 の結合は抑制したが、それ以外の抗体結合の阻害は弱いかほとんど認められなかった。

（5）CXCR4 阻害剤との相互作用に影響を与えるCXCR4 アミノ酸の同定： KRH-3955はHis^{281}と相互作用すると推定された。一方、AMD3100 は過去の論文で報告されているとおりその相互作用するアミノ酸はAsp^{171}、Asp^{262} 、Glu^{288}であり KRH-3955 の作用するアミノ酸との重なりは認められなかった。

（6）CXCR4 阻害剤耐性 HIV-1 誘導実験：PM1/CCR5 細胞を標的細胞として、NL4-3 を親株とした薬剤耐性株誘導実験を 2007. 10.11 に開始した。実験開始時の薬剤濃度は EC_{50}よりやや低い濃度に設定し、ほぼ4日おきに 1:5 に培養物を継代した。ウイルス感染による CPE が培養全体に観察されるようになった時点で薬剤濃度を 1.5 倍上昇させた。なお、コントロールとして薬剤無添加での感染細胞の継代培養（この場合は、CPE でほぼ完全に細胞が死滅するので培養上清のみを継代した）も併行して行った。2009年 12 月下旬の時点での薬剤濃度は、KRH-3955 、 KRH-3148 、 AMD3100 、AMD070 でそれぞれ 15、750、3000、4500 nM である。現在、それぞれの薬剤に対する耐性ウイルスについてそれらの Env 領域に蓄積した変異を解析中である。

（7）事前内服による感染予防効果の検討: KRH-3955を100 mg/kgで感染24時間前に投与した3頭の末梢血 CD4 数は、感染後3ヶ月経ってもウイルス感染時のレベルを維持していた。一方、対照薬剤の TDF/FTC では、感染後3ヶ月において3頭中2頭の末梢血 CD4 数はウイルス感染時のレベルを維持していたが、1頭は感染後1ヶ月で急激な末梢血 CD4 数の減少を起こした。

D. 結論

経口投与可能な CXCR4 阻害剤 KRH-3955 が抗 HIV-1 剤として有望であることを示すことができた。薬剤としての有効性や CXCR4 への作用様式をさらに明らかにするためまた臨床応用への可能性を評価するため耐性誘導実験を継続中である。

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F. 知的財産権の出願・登録状況（予定を含む）

該当事項なし。

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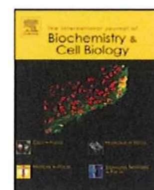

Electrostatically constrained α-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide

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ABSTRACT

α-Helical peptides, such as T-20 (enfuvirtide) and C34, derived from the gp41 carboxyl-terminal heptad repeat (C-HR) of HIV-1, inhibit membrane fusion of HIV-1 and the target cells. Although T-20 effectively suppresses the replication of multi-drug resistant HIV variants both in vitro and in vivo, prolonged therapy with T-20 induces emergence of T-20 resistant variants. In order to suppress the emergence of such resistant variants, we introduced charged and hydrophilic amino acids, glutamic acid (E) and lysine (K), at the solvent accessible site of C34. In particular, the modified peptide, SC34EK, demonstrates remarkably potent inhibition of membrane fusion by the resistant HIV-1 variants as well as wild-type viruses. The activity was specific to HIV-1 and little influenced by serum components. We found a strong correlation between the anti-HIV-1 activities of these peptides and the thermostabilities of the 6-helix bundles that are formed with these peptides. We also obtained the crystal structure of SC34EK in complex with a 36 amino acid sequence (N36) comprising the amino-terminal heptad repeat of HIV-1. The EK substitutions in the sequence of SC34EK were directed toward the solvent and generated an electrostatic potential, which may result in enhanced α-helicity of the peptide inhibitor. The 6-helix bundle complex of SC34EK with N36 appears to be structurally similar to that of C34 and N36. Our approach to enhancing α-helicity of the peptide inhibitor may enable future design of highly effective and specific HIV-1 inhibitors.

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1. Introduction

Enfuvirtide (T-20), which has been clinically approved as the first fusion inhibitor of HIV-1, is derived from a 36 amino acid region of the carboxyl-terminal heptad repeat (C-HR) of gp41, an HIV-1 transmembrane envelope glycoprotein, which plays central role in the fusion of HIV-1 with host cells. T-20 prevents the formation of a 6-helix bundle, which is comprised of a trimer of dimers formed from the amino-terminal heptad repeat (N-HR) and the carboxyl-terminal heptad repeat (C-HR) in an antiparallel orientation. Six-helix formation by physiological gp41 enables host cell and virus membranes to contact and fuse, enabling the virus entry into the cells. Therefore, inhibition of the formation of this 6-helix bundle prevents fusion of HIV-1 and targeted host cell membranes (Derdeyn et al., 2000; Wild et al., 1992). Notably, T-20 effectively suppresses the replication of HIV-1 variants, which are resistant to multiple reverse transcriptase and protease inhibitors, and has been used in the optimized regimens for HIV-1-infected patients harboring multi-drug resistant HIV-1 variants (Lalezari et al., 2003; Lazzarin et al., 2003).

Emergence of T-20-resistant HIV-1 was reported not only in patients receiving T-20 monotherapy in a phase I clinical trial (Wei et al., 2002), but also in patients treated with a combination of T-20

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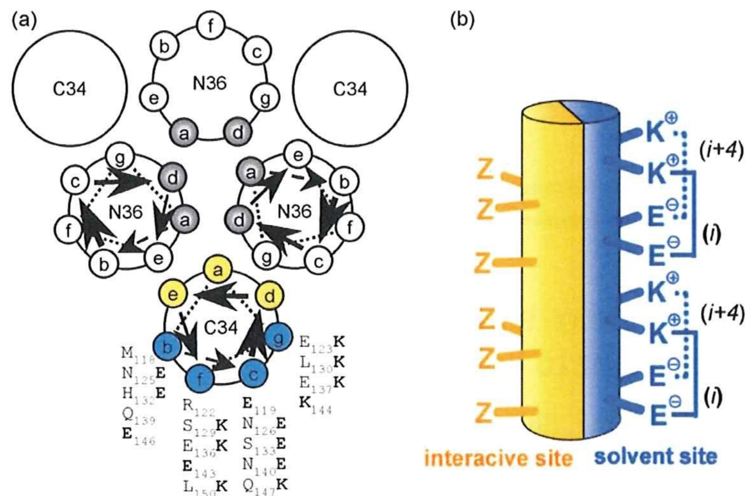

Fig. 1. Helical wheel representation of the 6-helix bundle structure and the design of SC34EK. (a) Amino acid residues at positions a, d, e of C34 are interactive sites that form the 6-helix complex with N36, while the remaining amino acid residues at positions b, c, f, g are solvent accessible sites, which are substituted with Glu (E) or Lys (K) in SC34EK. (b) The design concept of introducing the EK motif to the solvent accessible site. The α-helical C-HR peptide could be divided into interactive (red) and solvent (blue) sites. Z indicates the original amino acids of C34. Only amino acid residues at solvent sites were replaced by E at the i position and K at the $i+4$ position.

and other inhibitors in subsequent phases II and III trials (Matthews et al., 2004; Poveda et al., 2002). These resistant variants frequently acquired mutations in gp41, especially in amino acids 36–45 of the N-HR region (Aquaro et al., 2006; Cabrera et al., 2006; Mink et al., 2005; Poveda et al., 2002; Rimsky et al., 1998; Wei et al., 2002) (Fig. 1). Additionally, complementary mutations in the C-HR region, such as S138A mutation, were found in some T-20 resistant variants (Cabrera et al., 2006; Poveda et al., 2004; Xu et al., 2005). Introduction of these complementary mutations compensates for impaired HIV-1 replication stemming from the primary mutations that give rise to resistance. The N43D mutation in the N-HR region that confers resistance to T-20 is a well documented example (Xu et al., 2005).

Although T-20 inhibits gp41-mediated fusion (Derdeyn et al., 2000; Wild et al., 1992), it has additional effects on HIV-1 replication. For instance, baseline sensitivity of HIV-1 to T-20 is influenced not only by the amino acid sequence of gp41, but also by the co-receptor specificity (CCR5/CXCR4) defined by the structure of the V3 loop of gp120, a glycoprotein capping gp41, which binds to the CD4 cells (Derdeyn et al., 2000; Derdeyn et al., 2001). Moreover, substitutions within the CD4 binding domain of gp120 also contribute to the resistance of the virus to T-20 (Baldwin and Berkhout, 2006). Thus, the mode of action and the mechanism of resistance to T-20 seem to be complicated. In contrast, another fusion inhibitor known as C34, has been clearly shown to bind to the N-HR in vitro and act as a decoy of gp41 C-HR and prevent the formation of the 6-helix bundle (Chan et al., 1997; Liu et al., 2005; Xu et al., 2007). Its inhibitory effect is over 10-fold greater than that of T-20 (Armand-Ugon et al., 2003; Nameki et al., 2005). Thus, C34 appears to be a suitable peptide to employ in the rational design of an improved HIV fusion inhibitor, based on the interaction between the peptide and the target.

It has been reported that α-helicity of the C-HR and N-HR peptide complexes correlates with the anti-HIV-1 activity of the peptide inhibitor (Chan et al., 1998), suggesting that enhancement of α-helicity of C34 may provide higher affinity to the N-HR region, thus resulting in more potent anti-HIV-1 activity. To design potent fusion inhibitors using the enhancement of α-helicity approach, we divided the α-helical peptide C34 into two characteristic interactive (a, d, e) and solvent accessible (b, c, f, g) sites according to the reported N36/C34 structure (Fig. 1) (Chan et al., 1997). When HIV-1 gp41 is folded, a tryptophan-rich domain (WRD) in the N-terminus of C-HR plays an important role in tight and specific binding, through the interaction of the hydrophobic aromatic ring with a deep groove formed by the N-HR coiled coil (Chan and Kim, 1998; Salzwedel et al., 1999). In fact, C34 contains the N-terminal WRD, which binds to a hydrophobic pocket formed by the amino acid residues L57, W60 and K63 on the N-HR trimer surface (Chan et al., 1998; Ferrer et al., 1999), resulting in higher anti-HIV-1 activity of C34 compared to T-20, which lacks the N-terminal WRD. On the other hand, the solvent accessible site appears to contribute little to the formation of the 6-helix bundles, as demonstrated by the crystal structure of C34 bound to N36 (Chan et al., 1997). Therefore, amino acids in the interactive site are indispensable for binding, whereas those in the solvent accessible site may be replaceable (Fig. 1). To enhance the α-helicity of C34, we introduced a series of systematic replacements of amino acid residues in the solvent accessible site, where the original amino acid residues were substituted with charged and hydrophilic glutamic acid (E) or lysine (K) with the intention of forming possible intrahelical salt-bridges (Marqusee and Baldwin, 1987) (Fig. 1b). We obtained two peptides, SC34 and SC34EK (Fig. 2a), both of which gratifyingly demonstrated increased anti-HIV-1 activity (Otaka et al., 2002).

In this study, we demonstrate that SC34EK maintains highly potent activity against T-20 resistant clones of HIV-1, as well as several clinical isolates, and we reveal that the enhanced α-helicity of SC34EK is indeed involved in the improvement of activity. The activities are specific to HIV-1 and are not influenced by serum components. Structural analysis indicates that electrostatic interactions introduced by EK substitutions enhance the conformational stability of the 6-helix bundle, thus preventing HIV-1 fusion with the host cell. The information from our investigations involving the enhanced α-helicity of SC34EK should enable further design of highly effective and specific HIV-1 inhibitors.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI1640- and Dulbecco's modified Eagle medium (DMEM)-based culture medium, respec-

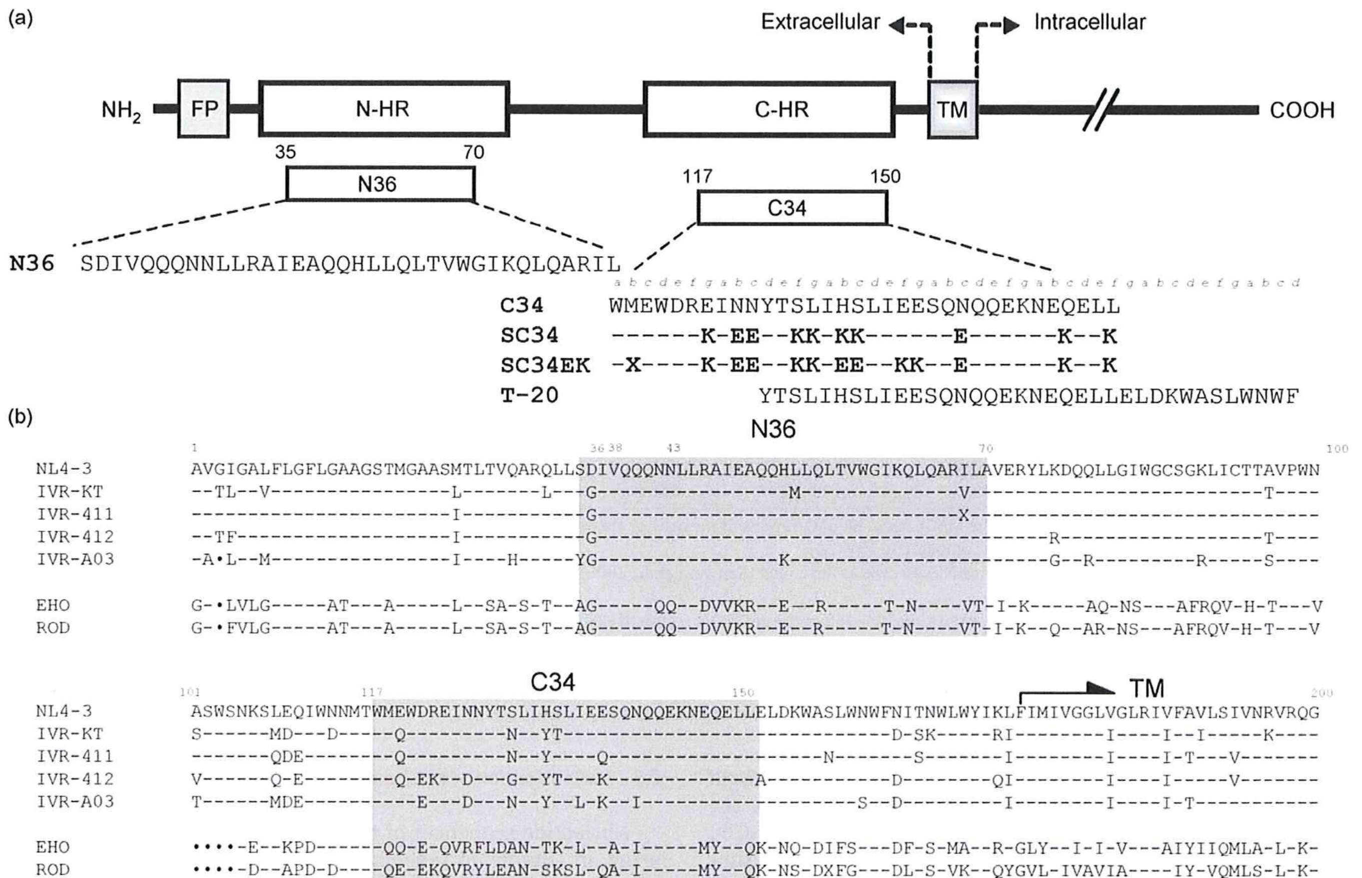

Fig. 2. Schematic view of gp41 and C34 derivatives and amino acid alignment of gp41. (a) The locations of the fusion peptide (FP), the amino-terminal heptad repeat region (N-HR), the carboxyl-terminal heptad repeat region (C-HR), and the transmembrane domain (TM) and the amino acid sequences of N36, T-20, C34 and its derivatives are shown. The residue numbers of each peptide correspond to their positions in gp41 of the NL4-3 strain. The X in SC34EK indicates norleucine, introduced to avoid oxidation of the methionine residues. No differences between the original methionine- and norleucine-containing peptide were observed (Otaka et al., 2002). (b) Alignment of amino acid sequence of clinical isolates (KT, IVR411, IVR412 and IVR-A03; GenBank accession number; AB222704, AB222705, AB222706 and AB222703, respectively) and HIV-2 strains (EHO and ROD) are shown. Corresponding regions of N36 and C34 are indicated in gray. Identical or deleted amino acids from the sequence of NL4-3 are indicated with a bar or a dot, respectively. The X in amino acid sequences of IVR411 and ROD indicates the mixture of I and V for IVR411, and mixture of I and M for ROD.

tively. HeLa-CD4-LTR-β-gal cells were kindly provided by Dr. M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (NIAID) (Bethesda, MD) and were used for the drug susceptibility assay (MAGI assay) as described previously (Kimpton and Emerman, 1992; Kodama et al., 2001; Maeda et al., 1998). The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (EC_{50}).

Laboratory HIV-1 (III_B) and HIV-2 (EHO and ROD) strains were used. An HIV-1 infectious clone pNL4-3 was used for constructions and for the production of HIV-1 variants as described (Nameki et al., 2005). A wild-type HIV-1, HIV-1_{WT}, was generated by transfection of pNL4-3 into 293T cells. Clinical isolates obtained from drug-naïve and heavily drug-experienced patients, were kindly provided by Dr. S. Oka (AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan). Their co-receptor tropisms were determined using NCK45 cells as described previously (Kajiwara et al., 2006).

2.2. Antiviral agent

The peptide-based fusion inhibitors used in this study were synthesized as described previously (Otaka et al., 2002), and the sequences can be identified in Fig. 2a. 3′-Azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxycytidine (ddC) were purchased from Sigma (St. Louis, MO, USA). MKC-442 was provided by Dr. S. Shigeta (Fukushima Medical University, Fukushima, Japan).

2.3. Determination of drug susceptibility of HIV-1

The peptide sensitivity of infectious clones was determined using the MAGI assay with as described previously (Kodama et al., 2001; Maeda et al., 1998). The activity of test compounds was determined as the concentration that blocks HIV-1 replication by 50% (EC_{50}). For clinical isolates, PHA-stimulated peripheral blood mononuclear cells (PBMCs) were used as described previously (Kodama et al., 2001). PBMCs (10^6 cells/ml) were exposed to test compounds and HIV-1, and were cultured in the presence of interleukin 2 for 7 days. Amounts of p24 protein in the supernatants of the cultures were then determined using the commercially available p24 antigen enzyme linked solvent assay kit.

2.4. Construction of recombinant HIV-1 clone

Recombinant infectious HIV-1 clones with substituted V3 regions, pNL-$V3_{ADA}$ and pNL-$V3_{SF162}$ were generated using pNL4-3. The V3 region, corresponding to n.t. 7029–7249 of pNL4-3, was amplified using primers containing appropriate BglII and NheI restriction enzyme cleavage sites for directional cloning into pBS-

Table 1
Antiviral activity of gp41-derived peptides against gp41 and gp120 V3 recombinant virus[a]

Clone	Tropism[b]	EC_{50} (nM)					
		ddC	N36	T-20	C34	SC34	SC34EK
gp41 recombinant virus							
WT[c]		404 ± 196	180 ± 70	35 ± 17	3.2 ± 0.9	1.4 ± 0.7	0.7 ± 0.3
L33S		289 ± 24	39 ± 11	**>1000**	2.9 ± 0.9	1.3 ± 0.1	0.9 ± 0.3
V38A		714 ± 109	407 ± 76	**402 ± 68**	**96 ± 29**	2.0 ± 0.5	1.1 ± 0.6
V38E		291 ± 57	41 ± 14	**>1000**	**492 ± 85**	**37 ± 12**	**4.3 ± 1.3**
N43K		321 ± 8.5	234 ± 63	114 ± 19	**50 ± 9.5**	2.5 ± 0.3	2.7 ± 0.3
N43D		430 ± 42	461 ± 266	**>1000**	**>100**	**9.0 ± 6.6**	1.0 ± 0.8
D36S/V38M		296 ± 88	178 ± 31	42 ± 6.4	7.2 ± 4.0	1.9 ± 0.1	0.8 ± 0.3
V38E/N42S		273 ± 105	227 ± 20	**>1000**	**322 ± 7.5**	**32 ± 3.1**	3.2 ± 1.0
ΔFNSTW/L33S/N43K[d]		276 ± 39	152 ± 31	**>1000**	**248 ± 56**	2.7 ± 0.3	**4.4 ± 0.5**
ΔFNSTW/D36G/I37K/N126K/L204I[d]		246 ± 67	547 ± 7.8	**754 ± 174**	**67 ± 21**	4.6 ± 0.9	2.9 ± 0.8
gp120 V3 recombinant virus							
V3-ADA	R5	362 ± 102	360 ± 91	**289 ± 19**	6.8 ± 3.3	0.7 ± 0.4	2.0 ± 0.2
V3-SF162[e]	R5	995 ± 219	383 ± 9.9	19 ± 2.8	7.8 ± 3.5	0.5 ± 0.2	0.5 ± 0.2
V3-CH1[f]	R5X4	649 ± 4.5	**2207 ± 42**	16 ± 1	5.6 ± 0.1	1.3 ± 0.1	0.7 ± 0.1
V3-CH2[g]	R5	1515 ± 177	192 ± 13	35 ± 32	3.8 ± 0.1	0.4 ± 0	0.9 ± 0.8

[a] Anti-HIV-1 activity was determined using the MAGI assay. All data represent means ± standard deviation obtained from the results of three independent experiments. Bold indicates over 5-fold increase in EC_{50} value compared to HIV-1_{WT}.
[b] The co-receptor tropism was determined using NCK45 cells as described (Kajiwara et al., 2006).
[c] HIV-1_{NL4-3} served as a wild-type virus.
[d] ΔFNSTW is the deletion of five amino acids at position 364–368 in the gp120 V4 region of HIV-1_{NL4-3} (Nameki et al., 2005). Fusion inhibitor resistant variants used have been previously reported (Armand-Ugon et al., 2003; Nameki et al., 2005).
[e] The V3 region of NL4-3 gp120 was replaced with the corresponding region of HIV-1_{SF162}.
[f] HIV-1_{V3-CH1} has mutations in the gp120 V3 region of primary isolate HIV-1_{KMT}, where GKI is substituted by GEI.
[g] HIV-1_{V3-CH2} has mutation in the gp120 V3 region of the primary isolate HIV-1_{KMT}, where GKI is substituted by GQI.

$gp120_{WT}$. The resulting amplified V3 region was subjected to BglII and NheI digestion, subcloned into pBS-$gp120_{WT}$ containing the corresponding region in the DNA fragment of EcoRI–NheI (1510 bp containing gp120 V1, V2 and V3, n.t. 5740–7249 of pNL4-3) and subsequently ligated into pNL4-3. pNL-$V3_{CH1}$ and $V3_{CH2}$, CCR5 and dual (CXCR4 and CCR5) tropic molecular clones, were kindly donated by Dr. Y. Maeda, Kumamoto University (Kumamoto, Japan) (Foda et al., 2001; Maeda et al., 2000).

Recombinant infectious HIV-1 clones carrying various mutations in gp120 and/or gp41 were also generated using pNL4-3. Briefly, the desired mutations were introduced using site directed mutagenesis into the region of pSL-$gp41_{WT}$ flanked by the NheI–BamHI restriction enzyme sites (1220 bp containing gp120 V4, V5 and gp41 ectodomain n.t. 7250–8469 of pNL4-3) (Weiner et al., 1994). After restriction enzyme digestion and purification the NheI–BamHI fragments were ligated into pNL4-3, generating a series of molecular clones with the desired mutations.

Each molecular clone was transfected into 293T cells (10^5 cells/6-well culture plate). After 48 h, MT-2 cells (10^6 cells/well) were added and co-cultured with the 293T cells for an additional 24 h. When an extensive cytopathic effect was observed, the supernatants were harvested and stored at −80 °C for further use.

Table 2
Antiviral activity of gp41-derived peptides against clinical isolates[a]

Strain	EC_{50} (nM)				
	AZT	T-20	C34	SC34	SC34EK
NL4-3 (WT)[b]	2.0	36	3.2	0.36	0.4
KT (WT)[b]	2.0	11	0.2	0.1	0.03
IVR411	**7600**	4.1	0.2	3.1	0.04
IVR412	**9060**	23	7.2	**4.8**	0.1
IVR-A03	**1200**	7.0	**17**	**4.1**	0.7

[a] Anti-HIV-1 activity was determined using the amounts of p24 protein in the supernatants of the PHA-stimulated PBMC cultures using commercially available ELISA kit (Kodama et al., 2001). Bold indicates over 5-fold increase in EC_{50} value compared to HIV-1_{WT}.
[b] HIV-1_{NL4-3} and HIV-1_{KT} served as controls.

2.5. Determination of gp41 amino acid sequence

Nucleotide sequences of the clinical isolates were determined using an automated sequencer. Briefly, DNA was extracted from PBMCs infected with the clinical isolates, subjected to nested PCR for the gp41 coding region, and then directly sequenced as described previously (Nameki et al., 2005).

2.6. Measurement of circular dichroism (CD) spectra

N-HR peptides (N36, $N36_{V38A}$ or $N36_{N43D}$) and C-HR peptides (C34 or SC34EK) were incubated at 37 °C for 30 min (the final concentration of both the N-HR peptide and the C-HR peptide were 10 μM in pH 7.4, 12 mM phosphate-buffered solution containing 50 mM NaCl). The wavelength-dependence of molar ellipticity [θ] was monitored at 25 °C as the average of eight scans, and the thermal stability was estimated by monitoring the change in the CD signal at 222 nm in a spectropolarimeter (Model J-710; Jasco, Tokyo, Japan) equipped with a thermoelectric temperature controller. The midpoint of thermal unfolding transition (melting temperature [T_m]) of each complex was determined as described previously (Otaka et al., 2002). The percentages of a-helicity in 6-helix complexes were calculated by comparing the CD signal at 222 nm of N36/C34 or N36/SC34EK complexes in a spectropolarimeter.

2.7. Crystallization, data collection and refinement

Samples for crystallization were prepared by mixing solutions of N36 and SC34EK dissolved in 10 mM sodium acetate buffer at a concentration of 10 mg/mL. The mixture was incubated for 30 min at 37 °C, then was passed through a 22 μm filter. Crystallization was performed by the hanging drop vapor diffusion method at 4 °C. Droplets were prepared of equal amounts (2 μL) of reservoir solution and the peptide solution. Hexagonal prism crystals were obtained under the following conditions: 100 mM sodium acetate buffer (pH 4.0), 200 mM ammonium sulphate, 14% polyethylene glycol monomethyl ether 2000. After screening of