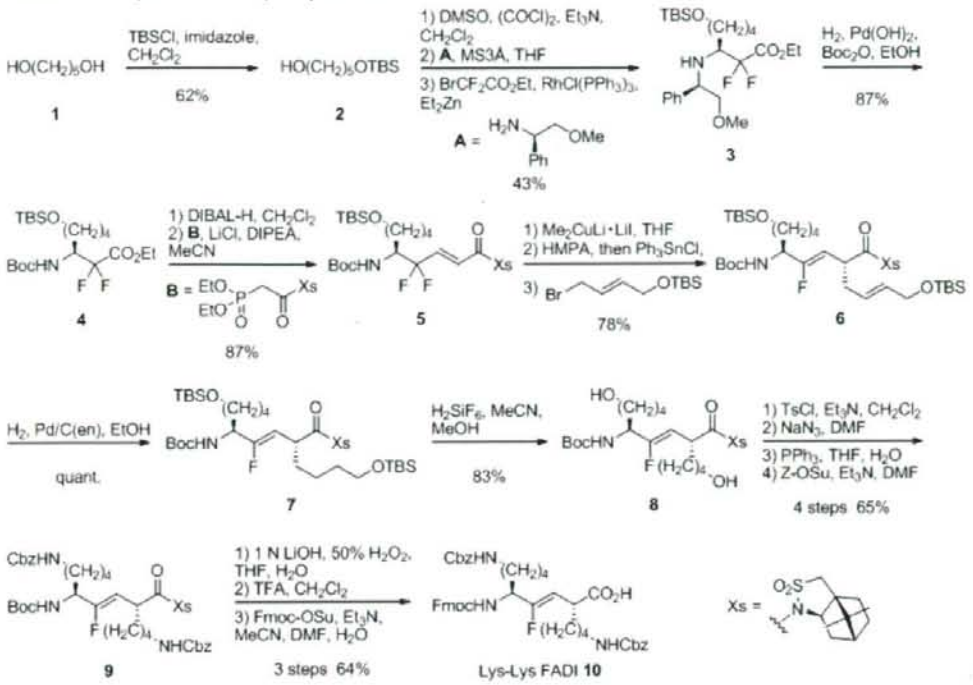
Scheme 1. Synthesis of Lys-Lys FADI



する影響などの機能評価もほとんどなされていない。SC29EK はαヘリックス構造を安定化させる X-EE-XX-KK モチーフを有する。そこで、Lys-Lys に相当する FADI を合成し、ペプチド二次構造に対する影響を EADI との比較も含めて検討した。

B．研究方法

X-EE-XX-KK モチーフの Lys-Lys に相当する FADI や EADI を化学合成し、Fmoc 固相合成法で SC29EK に導入した。そして、α ヘリックス構造形成を CD スペクトル測定により、抗 HIV 活性発現に重要な 6-helix bundle 構造形成を T_m 値測定により、抗 HIV 活性を MAGI アッセイにより、それぞれ評価した。

（倫理面への配慮）

該当事項なし

C．研究結果

Lys-Lys 型 FADI **10** の合成を Scheme 1 に示す。まず、市販の 1,5-pentanediol **1** を出発物質として、一方の水酸基を TBS 基で保護したアルコール **2** に対し、Swern 酸化、不斉補助基 **A** を用いた Reformatsky-Honda 型の反応によりアミン **3** を中程度の収率で得た。続いて Boc_2O 存在下、接触還元によりキラル補助基を除去して Boc 体 **4** とし、DIBAL 還元、Oppolzer 型不斉補助基を有するホスホネート **B** との Horner-Wadsworth-Emmons 反応によってエノン **5** を得た。エノン **5** を、有機銅試薬を用いたジアステレオ選択的一電子還元－アルキル化に付すことにより、単一の異性体としてα-アルキルサルタム **6** を得た。Pd/C-エチレンジアミン複合体により側鎖アルケンを選択的に還元した後、TBS 基の脱保護、水酸基のアジドを経由した Cbz 保護アミン **9** への変換、次いで不斉補助基の除去、Boc 基の Fmoc 基への変換により目的の FADI **10** を合成した。

続いて Lys-Lys 型 EADI **19** の合成を Scheme 2 に示す。Boc-Lys(Cl-Z)-OH **11** から誘導したアリルアルコール **13** の分子内光延反応によりビニルアジリジン **14** を得た。続いてオゾン酸化、ホスホネート **C** との Horner-Wadsworth-Emmons 反応によってアジリジンエノエート **15** へと誘導した。アジリジンエノエート **15** に対する *anti*-S_N2' 反応によって側鎖を導入し、β,γ-不飽和エステル **16** を得た。続いて TBS 基の脱保護、光延反応による水酸基の保護アミンへの変換で **17** へと誘導した後、Ns 基の脱保護、

Scheme 2. Synthesis of Lys-Lys EADI

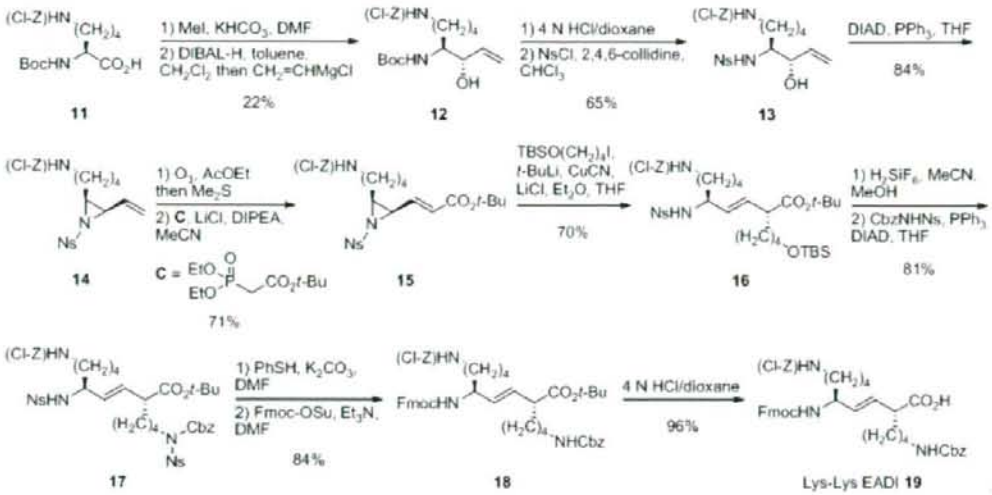


Table 3. Sequences of SC29EK and its derivatives

Peptide		Sequence[a]
SC29EK		WEEWDKKIEEYTKKLEELIKKSEEQQKKN
SC29EK-F1	SC29EK-E1	WEEWDKKIEEYTKKLEELIKKSEEQQ<u>KK</u>N
SC29EK-F2	SC29EK-E2	WEEWDKKIEEYTKKLEELI<u>KK</u>SEEQQKKN
SC29EK-F3	SC29EK-E3	WEEWDKKIEEYT<u>KK</u>LEELIKKSEEQQKKN
SC29EK-F4	SC29EK-E4	WEEWD<u>KK</u>IEEYTKKLEELIKKSEEQQKKN

[a] Underlined residues indicate Lys-Lys FADI or EADI.

Fmoc 化、*t*-Bu 基の除去により、目的の EADI **19** を合成した。

SC29EK の 4 か所の Lys-Lys 部位に FADI、EADI を導入した誘導体、FADI 含有 SC29EK-F1～F4、EADI 含有 SC29EK-E1～E4 は、通常の Fmoc 固相合成法により合成した（Table 3）。

合成した一連の SC29EK 誘導体に対する CD スペクトル測定の結果、SC29EK の Lys-Lys 部位への FADI や EADI の導入はαヘリックス性の低下をもたらした（Fig. 6）。

SC29EK 誘導体の 6-helix bundle 構造形成能を調べるため、N-領域部分ペプチド N36 との複合体の T_m 値を測定した結果（Table 4）、FADI および EADI の導入部位が N-末端側に近づくにつれ N36 との結合親和性が低下することが明らかとなった。FADI と EADI との比較においては SC29EK 配列中央部に導入した場合（SC29EK-F3 および -E3）において顕著な差が認められた。

また、一連の誘導体の抗 HIV 活性を MAGI アッセイにより評価した（Table 5）。FADI、EADI 共に置換部位が N 末端に近づくにつれて活性が減弱し、N36 との混合 CD スペクトル及び T_m 値との相関が認められた。全ての置換部位において、FADI 含有誘導体は対応する EADI 含有誘導体より強力な抗 HIV 活性を示す傾向が認められた。特に、6-helical bundle の安定性に差が認められた SC29EK-F3 と SC29EK-E3 の間に最も顕著な活性の差が認められ、SC29EK-F3 が弱いながらも活性を示したのに対し、SC29EK-E3 では活性が失われた。

Table 4. Thermal stability of 6-helix bundle structures consisting of N36 and SC29EK derivatives

Peptide		T_m (°C) FADI	EADI
SC29EK		67.4	
SC29EK-F1	SC29EK-E1	64.8	64.1
SC29EK-F2	SC29EK-E2	60.9	62.2
SC29EK-F3	SC29EK-E3	49.5	40.1
SC29EK-F4	SC29EK-E4	44.1	43.9

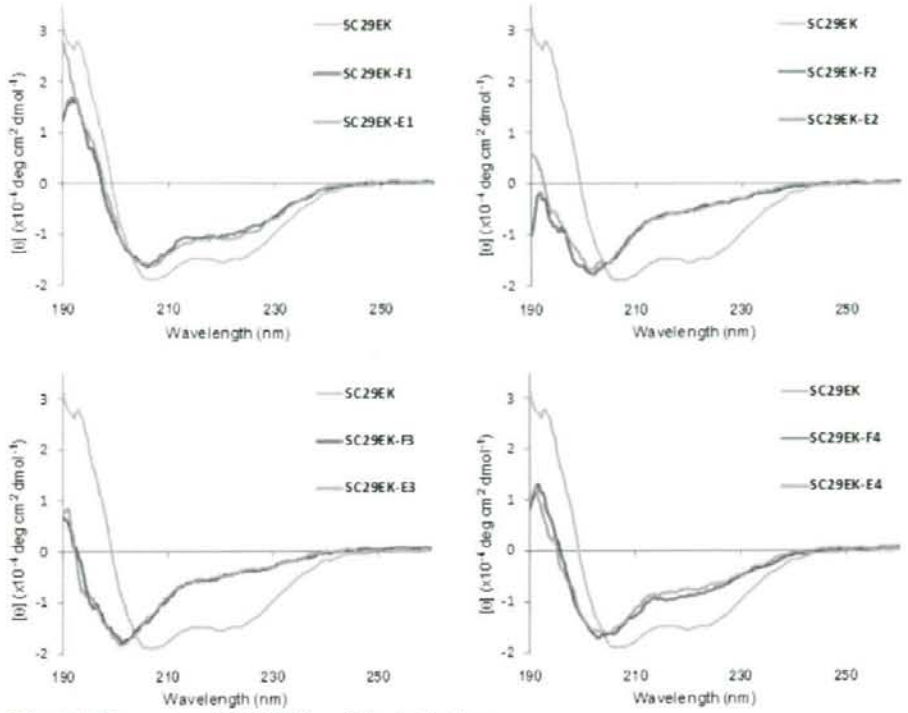


Fig. 6. CD spectra of SC29EK and its derivatives.

Table 5. Anti-HIV activity of SC29EK and its derivatives against HIV-1 NL4-3 strain

Peptide	EC_{50} (nM)[a] FADI	EADI
SC29EK	2.2 ± 0.2	
SC29EK-F1, -E1	37 ± 6	43 ± 7
SC29EK-F2, -E2	663 ± 242	865 ± 317
SC29EK-F3, -E3	599 ± 96	> 10000
SC29EK-F4, -E4	5216 ± 202	> 10000

[a] EC_{50} was determined as the concentration that blocked HIV-1 replication by 50%.

D．考察

SC29EK の Lys-Lys 部位への FADI および EADI の導入により α ヘリックス性が低下したことから、FADI および EADI は α ヘリックス構造形成に関与するペプチド主鎖の水素結合を十分に模倣できていないと考えられる。また、FADI および EADI の導入部位が N-末端側に近づくにつれ N36 との結合親和性を低下させたことから、その部位のペプチド結合が N36 との相互作用に重要な役割を果たしていることが示唆された。

FADI および EADI の導入部位が N-末端側に近づくにつれ抗 HIV 活性が減弱していたが、全ての導入部位において FADI 含有 SC29EK が EADI 含有 SC29EK よりも高い抗 HIV 活性を示した。このことから、FADI の導入は EADI の導入と比較して生理活性（α ヘリックス）構造をとるのに有利であり、FADI は EADI よりも優れたペプチドイソスターであることが実証された。

E．結論

Lys-Lys 型 FADI および EADI をジアステレオ選択的に化学合成し、高活性 HIV-1 膜融合阻害剤 SC29EK の X-EE-XX-KK α ヘリックス誘起モチーフに導入した。SC29EK の Lys-Lys 部位への FADI 導入は EADI の導入と同様に α ヘリックス性の低下をもたらし、フルオロアルケンでは α ヘリックス構造に必要な水素結合を十分に模倣できない可能性が示唆された。しかしながら、FADI 置換 SC29EK は EADI 置換 SC29EK と比較して SC29EK の抗 HIV 活性を維持したことから、ペプチド結合のカルボニル酸素（水素結合アクセプター）を模倣することの重要性が示された。

F．健康危険情報

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H．知的財産権の出願・登録状況

1．特許取得

1. **PCT/JP2006/326069：新規ＣＸＣＲ４拮抗剤及びその用途**

   日本：特許証発行（特許第4122441号：2008年5月16日）

   欧州：移行手続き完了（06 843 451.3：2008年6月26日）

   米国：移行手続き完了（No. 12/087, 160：2008年6月27日）

２．実用新案登録

該当事項なし

３．その他

該当事項なし

厚生労働科学研究費補助金(政策創薬総合研究事業)

分担研究報告書

経口投与可能なCXCR4阻害剤に関する研究

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研究要旨:本年度は、まず抗 X4HIV-1 活性を有する CXCR4 阻害剤 KRH-3955 についてその抗 HIV-1 活性や CXCR4 への作用点について詳細な解析を加えた。平成19年秋に開始した 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 KRH-3148 とコントロールの一つ AMD070 で中程度(20~30 倍)の耐性ウイルスが得られた。また、KRH-3955 がカニクイサル PBMC への SHIV 感染も顕著に阻害することが明らかになった。

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。今年度はまず、抗X4HIV-1活性を有するCXCR4阻害剤KRH-3955についてその抗HIV-1活性や CXCR4 への作用点について詳細な解析を加えた。また、昨年度に開始した 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を継続している。

B. 研究方法

(1)抗 HIV-1 活性測定:固定化抗 CD3 抗体で刺激し、IL-2 存在下で増殖させた PBMC を標的細胞として X4、R5、R5X4 の各種 HIV-1 を MOI=0.001 で感染させ、種々の濃度の薬剤存在下で7日から 10 日培養した。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。

(2)薬剤耐性 HIV-1 に対する抗ウイルス活性測定:CD4 と CXCR4 を導入した U87 細胞を標的細胞として、HIV-1(HXB2 または NL4-3) Env 発現ベクターと Env 領域に Luciferase 遺伝子を導入した HIV ゲノムベクター(薬剤耐性遺伝子搭載)から作製したシュードタイプウイルスを種々の濃度の薬剤存在下で感染させ、感染させた標的細胞抽出液の Luciferase 活性を測定して抗ウイルス活性を算出した。

(3)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。

(4)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。

(5)各種抗 CXCR4 抗体結合阻害活性の測定:Molt-4 細胞を on ice で薬剤処理した後細胞を洗浄し、抗体反応後結合した CXCR4 抗体量を FACS にて定量した。

(6)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:主に酸性アミノ酸をアラニンに置換した 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。

(7)CXCR4 阻害剤耐性 HIV-1 誘導実験

PM1/CCR5 細胞を標的細胞として、X4 株である 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。

(倫理面での配慮)

該当事項なし

C. 研究結果

(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。一方、JR-CSF など R5 HIV-1 に対しては 200 uM においても顕著な抗ウイルス活性を示さなかった。また、活性化 PBMC や MT-4 細胞に対して 25 uM まで顕著な細胞毒性を示さなかった。なお、PBMC における R5X4 HIV-1 の複製阻害は、今回使用したウイルスが PBMC において CXCR4 を優先的に使用することによると考えられる。

(2)KRH-3955 の薬剤耐性 HIV-1 に対する抗ウイルス 活性(single-round 感染系): KRH-3955 は、NRTI、NNRTI、PI 耐性、MDR (NRTI、NNRTI、PI 耐性)、T-20 耐性株のいずれに対しても親株である HXB2、NL4-3 とほぼ同程度の抗ウイルス活性を示した。

(3)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。

(4)SDF-1α結合阻害活性:コントロールとして使用した CXCR4 阻害剤 AMD3100、とともに検討した。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。 KRH-3955 の IC_{50} 値は、MT-4 細胞に HIV-1IIIB を感染させる系における化合物の EC_{50} 値によく対応していた。一方、AMD3100 の SDF-1α結合阻害活性は HIV-1 複製阻害活性より顕著に弱かった。

(5)各種抗 CXCR4 モノクローナル抗体結合阻害活性: KRH-3955 の CXCR4 に対する作用点を明らかにする実験の一つとして、これらの化合物が、各種抗 CXCR4 モノクローナル抗体の CXCR4 への結合を阻害するかを検討した。CXCR4 の N 末端を認識する抗体(A145)、レセプターの細胞外領域(ECL)1と2を認識する抗体(12G5)、ECL2 を認識する抗体(44717)、ECL3 を認識する抗体(A80)の4種類の抗体を用いた。KRH-3955 は N 末端を認識する抗体以外の抗体の CXCR4 発現細胞(Molt-4)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、ECL1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。なお、KRH-3955 で CXCR4 発現細胞を処理して 37℃でインキュベートしても A145 の結合量が変化しないことから、これらの CXCR4 阻害剤には CXCR4 をダウンモジュレートする活性はないことも明らかになった。

(6)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: CXCR4 の細胞外領域、膜貫通領域と推定される中で細胞外領域に近接する領域に存在する主に酸性アミノ酸をアラニンに置換した点変異体を作製し 293 細胞に導入して安定発現株を樹立した。CXCR4 阻害剤が変異 CXCR4 と抗 CXCR4 抗体 12G5 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。その結果、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。

(7)カニクイサル PBMC への SHIV 感染阻害実験:2頭のカニクイサルから調製した活性化 PBMC への SHIV-KS661c と SHIV-89.6P の in vitro における感染を KRH-3955 は顕著に抑制した。

(8)CXCR4 阻害剤耐性 HIV-1 誘導実験:

PM1/CCR5 細胞(共同研究者 熊本大・前田先生分与)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平成19年 10 月に開始した。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)も併行して行っている。平成2年1月下旬の時点での薬剤濃度は、KRH-3955、KRH-3148、AMD3100、AMD070でそれぞれ 12.5、500、900、1350 nM である。中程度の耐性が得られた KRH-3148 と AMD070 の耐性ウイルスについてそれらの Env 領域に蓄積しているよ予想される変異の解析を予定している。

D. 考察

KRH-3955 が経口吸収性を有する強力な CXCR4 阻害剤であり、X4 HIV-1 の複製阻害剤として有望であると考えられる。抗 CXCR4 抗体の結合阻害パターンや CXCR4 変異体を用いた 12G5 結合阻害実験から示された相互作用する CXCR4 中のアミノ酸において AMD3100 とは明らかな差異が認められた。

E. 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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G. 知的財産権の出願・登録状況(予定を含む)
該当事項なし。

研究成果の刊行に関する一覧表

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雑誌

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Novel screening systems for HIV-1 fusion mediated by two extra-virion heptad repeats of gp41

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ABSTRACT

Entry of human immunodeficiency virus type 1 (HIV-1) into target cells is mediated by its envelope protein gp41 through membrane fusion. Interaction of two extra-virion heptad repeats (HRs) in the gp41 plays a pivotal role in the fusion, and its inhibitor, enfuvirtide (T-20), blocks HIV-1 entry. To identify agents that block HIV-1 fusion, two screening methods based on detection and quantification by the enzyme-linked immunosorbent assay (ELISA) principle have been established. One method uses an alkaline phosphatase (ALP)-conjugated antibody (Ab-ELISA) and the other uses an ALP-fused HR (F-ELISA) to detect and quantify the interaction of the two HRs. The F-ELISA was more simple and rapid, since no ALP-conjugated antibody reaction was required. Both ELISAs detected all the fusion inhibitors tested except for T-20. Interaction of the two HRs was observed in both ELISAs, even in the presence of 10% dimethyl sulfoxide. Ab-ELISA performed best in a pH ranging from 6 to 8, while F-ELISA performed best at a pH ranging from 7 to 8. These results indicate that both established ELISAs are suitable for the identification of HIV-1 fusion inhibitors.

1. Introduction

Combination chemotherapy has been widely used and reduces the mortality caused by HIV-1 infection. During prolonged therapy, however, in some patients, such efficacy is attenuated by the emergence of drug-resistant variants (Calmy et al., 2004). Moreover, combination chemotherapy occasionally induces various adverse effects and may also increase the costs of the therapy. Therefore, development of novel anti-HIV-1 drugs that suppress replication of resistant variants, and are less toxic and less cost is urgently needed.

There are at least two approaches to controlling replication of resistant variants and/or to reducing unfavorable adverse effects induced by the therapy. One approach is the development of anti-HIV-1 drugs which inhibit new targets such as viral integrase (Hazuda et al., 2004) or cellular receptors such as CCR5 (Tagat et al., 2004). Actually, an integrase inhibitor, raltegravir (Grinsztejn et al., 2007), and a CCR5 antagonist, maraviroc (Fätkenheuer et al., 2005) have been approved for clinical application. The other is the development or modification of current drugs that inhibit well-established targets, to make them effective against resistant variants while reducing adverse side-effects. In this study, we focus on the recently established and promising target of virus–cell membrane fusion.

The mechanism of virus–cell membrane fusion has already been disclosed (Eckert and Kim, 2001). Briefly, one of the HIV-1 envelope glycoproteins, gp120, binds to the host cell receptor CD4 and CXCR4 or CCR5, and then, another membrane-spanning protein gp41 in trimer anchors itself to the host cell membrane. After anchoring, heptad repeats 1 and 2 (HR1 and HR2), which are two extra-virion α-helical regions in the gp41, form an anti-parallel 6-helical bundle and lead to fusion of HIV-1 with the host cell membrane. On the basis of this molecular mechanism, compounds which prevent 6-helical bundle formation will be potential HIV-1 fusion inhibitors. Enfuvirtide (T-20) is the first peptide approved and used against HIV-1 variants that are refractory to the effect of reverse transcriptase and protease inhibitors (Lalezari et al., 2003; Lazzarin et al., 2003). Previously, we and others have developed novel potent fusion inhibitors, in the form of gp41 HR2-derived peptides (Bewley et al., 2002; Otaka et al., 2002; Root et al., 2001) (Fig. 1) and small molecules (Cai and Gochin, 2007; Frey et al., 2006). However, no fusion inhibitors, except for T-20, have been approved for clinical use. To screen further potential fusion inhibitors, we have established two simple, rapid and reproducible

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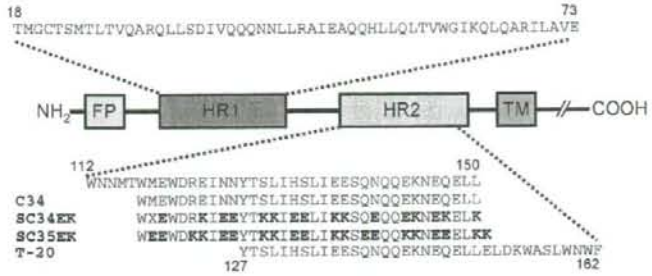

Fig. 1. Schematic view of gp41. The locations of the fusion peptide (FP), N-terminal heptad repeat region (HR1), C-terminal heptad repeat region (HR2), transmembrane domain (TM) and amino acid sequence of HR1, HR2, T-20, C34 and its derivatives (Otaka et al., 2002) are shown. The residue numbers of each peptide correspond to their positions in the envelope protein gp41 of HIV-1 NL4-3 clone. Representative regions of HR1 and HR2 used in this study are defined by the amino acids 18–73 and 112–150, respectively, and designated as MBP-HR1- and GST-HR2- or TRX-ALP-HR2-fused protein as described in Section 2. The X in SC34EK indicates an artificial amino acid norleucine instead of methionine, to avoid oxidation of the methionine residue.

in vitro screening systems using the enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Antiviral agents

The peptide-based fusion inhibitors were synthesized as described previously (Otaka et al., 2002), and their sequences are shown in Fig. 1. CCR5 antagonist TAK-779 (Baba et al., 1999) was provided by Takeda Pharmaceutical Company Ltd. (Osaka, Japan) through an AIDS research and reference reagent program. CXCR4 antagonist AMD-3100 (De Clercq et al., 1994) was provided by S. Shigeta (Fukushima Medical University, Fukushima, Japan). Adsorption inhibitor dextran sulfate MW 5000, DS-5000 (Baba et al., 1988) was purchased from Sigma (St. Louis, MO).

2.2. Protein expression and purification

A DNA fragment of the alkaline phosphatase (ALP) coding region without its secretory signal sequence, corresponding to amino acids 22–471 (Dodt et al., 1986; Kikuchi et al., 1981), was amplified by PCR from the *E. coli* JM109 genome (K12 strain; GenBank accession number: U00096). The amplified ALP region was ligated into the pET32a vector (Novagen, Madison, WI) to create pET32-ALP, a thioredoxin (TRX)-ALP fusion construct. A DNA fragment coding the HR1 region of HIV-1 gp41, amino acid positions 18–73, was amplified by PCR from an HIV-1 molecular clone pNL4-3 (GenBank accession number: AF324493). The amplified HR1 region was ligated into the pMAL-C2 vector (New England Biolabs, Ipswich, MA) to express HR1 with maltose-binding protein (MBP) as a tag, designated pMAL-HR1. The HR2 region, gp41 amino acid positions 112–150, was also amplified and ligated into both the pGEX-5X vector (GE Healthcare, Buckinghamshire, UK) and the pET32-ALP construct to express HR2 fusion protein with glutathione *S*-transferase (GST) and TRX-ALP, designated pGEX-HR2 and pET32-ALP-HR2, respectively. All vectors were verified by DNA sequencing and transformed into *E. coli* BL21-CodonPlus (DE3)-RIL strain (Stratagene, La Jolla, CA) for bacterial expression. The expressed MBP-HR1, GST-HR2 and TRX-ALP-HR2 proteins were purified by Amylose Resin (New England Biolabs), Glutathione Sepharose 4B (GE Healthcare) and Ni-NTA Agarose (Qiagen, Valencia, CA), respectively, according to the Manufacturers' recommended protocols. Purity was determined by SDS–PAGE and concentration by the Bradford protein assay (Bio-Rad, Hercules, CA).

2.3. Indirect detection of interaction of HR1 and HR2 (Ab-ELISA) (Fig. 2A)

Fifty nanomolar MBP-HR1 dissolved in 50 mM sodium carbonate buffer (pH 9.4) was coated on a 96-well ELISA plate (Costar, Cambridge, MA) by incubation at 4 °C for 8 h. After washing three times with PBS containing 0.025% Tween 20 (T-PBS) (pH 7.4), the plate was blocked using bovine serum albumin (BSA) at a concentration of 1 mg/ml in T-PBS at 4 °C for 2.5 h, and then washed again as described above. The MBP-HR1 on the plate was allowed to bind GST-HR2 (50 nM) by incubation at 37 °C for 1.5 h in the presence or absence of various concentrations of compounds for testing. After washing, binding of GST-HR2 was detected by using alkaline phosphatase (ALP)-conjugated anti-GST antibody (Sigma) in 1:2000 dilution at 4 °C for 1 h, then washed as before, prior to the addition of phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (BluePhos Microwell Phosphatase Substrate; KPL, Gaithersburg, MD). After incubating at room temperature for 30 min, absorbance at 595 nm was measured by a plate reader (model 3550, Bio-Rad).

2.4. Direct detection of interaction of HR1 and HR2 (F-ELISA) (Fig. 2D)

All procedures were performed as described above, except that TRX-ALP-HR2 (50 nM) was used in place of GST-HR2, with binding directly detected by BluePhos Microwell Phosphatase Substrate without the interaction of ALP-conjugated anti-GST antibody.

2.5. Anti-HIV activity

Anti-HIV-1 activity was determined by the multinuclear activation of a galactosidase indicator (MAGI) assay as described previously (Kimpton and Emerman, 1992; Kodama et al., 2001). Briefly, the MAGI cells (10^4 cells/well) were seeded in flat bottom 96-well microtitre plates. The following day, the cells were inoculated with HIV-1 and cultured in the presence of various concentrations of inhibitors in fresh medium. After 48 h incubation, all the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in each well were counted.

3. Results

3.1. Establishment of ELISA

To establish a novel assay system representing the specific interaction of HR1 and HR2 regions of the HIV-1 gp41 protein, a

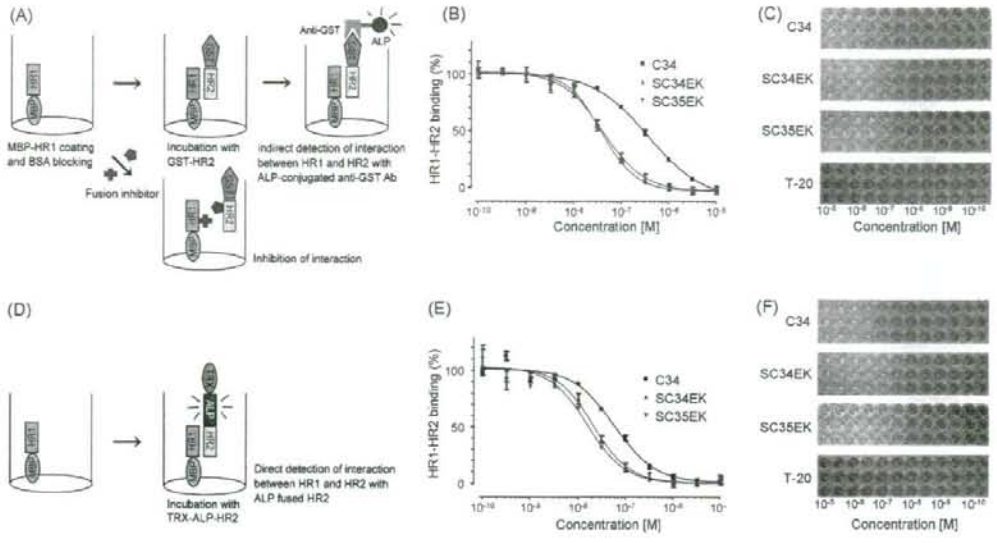

Fig. 2. Flow chart of the established ELISA systems (A and D) and the inhibitory effects of peptide-based fusion inhibitors determined by these systems (B, C, E and F). The schemes of Ab-ELISA and F-ELISA are shown. In Ab-ELISA (A), GST-HR2 interacts with MBP-HR1 on the ELISA plate, and the amounts of GST-HR2 are quantified by using ALP-conjugated anti-GST antibody and ALP substrate. In the presence of fusion inhibitors, GST-HR2 cannot interact with MBP-HR1, resulting in no ALP activity. In F-ELISA (D), ALP-fused HR2 protein enables the detection of the interaction of HR2 directly without ALP-conjugated anti-GST antibody. Inhibition curves of binding by Ab-ELISA (B) and F-ELISA (E) at peptide concentrations 10^{-10} to 10^{-5} M are illustrated. The actual appearance of ELISA plates observed in Ab-ELISA (C) and F-ELISA (F) is shown.

simple ELISA was first established with ALP-conjugated antibody (Ab-ELISA) as shown in Fig. 2A. MBP-HR1 was coated onto a 96-well ELISA plate. After blocking with BSA, GST-HR2 solution was added to the MBP-HR1 coated well. Using ALP-conjugated anti-GST antibody, the interaction of HR1 and HR2 was colorimetrically measured by a plate reader. Agents that block the interaction of HR2 with HR1 can reduce optical density at 595 nm (OD_{595}). The period for efficient coating of MBP-HR1 to the plate was measured by detection of ALP-conjugated anti-MBP antibody. After 8 h and up to 24 h little increase in efficiency of MBP-HR1 coating was observed (data not shown). When coating and blocking were performed prior to the assay, total time of the procedure, excluding washing, was only 3 h.

Prior to evaluation of fusion inhibitors, we examined interaction of GST-HR2 with the MBP-HR1 coating. We first coated MBPs with or without HR1 at a concentration of 50 nM, incubated them with various concentrations of GST-HR2, and then detected bound GST-HR2 with anti-GST antibody. GST-HR2 interacted with MBP-HR1 in a dose-dependent manner, at least up to 100 μM and provided sufficient OD_{595} values, over 1.0 (Fig. 3). Thus, 50 nM of GST-HR2 was used for further experiments.

Next, we modified the Ab-ELISA by using ALP-fused HR2 instead of GST-HR2 in the reaction with coated MBP-HR1, as shown in Fig. 2D (F-ELISA). The ALP-fused HR2 enabled us to directly detect the HR1 and HR2 interaction without the antibody reaction step, thus providing an even more rapid and simple procedure than the Ab-ELISA which uses ALP-conjugated antibody for detection. The total time required for the F-ELISA, excluding coating and blocking, was approximately 2 h. These results demonstrate that the ELISA systems detect the interaction of HR1 and HR2 interaction, enables the screening of potential fusion inhibitors without the need for infectious HIV-1 material, and is both simple and rapid.

3.2. Inhibitory effect of HR2-derived peptides and other entry inhibitors

The efficacy of the fusion inhibitory peptides C34, SC34EK and SC35EK and other compounds was determined by both Ab-ELISA (Fig. 2A) and F-ELISA (Fig. 2D). Both ELISAs only detected the activities of these three fusion inhibitory peptides, but not of other entry inhibitors (Table 1). The inhibitory effects of these peptide fusion inhibitors were reproducible and displayed a sigmoidal dose-dependent curve (Fig. 2B and E). These results suggested that our established ELISAs were specific for the interaction between HR1 and HR2 in the fusion process. Higher sensitivities for peptides tested were obtained by F-ELISA compared with those by Ab-ELISA (Table 1). However, compared with the MAGI assay, sensitivities of both ELISAs were between 14- and 50-fold lower. Neither ELISA technique was able to detect the inhibitory effect of T-20, which

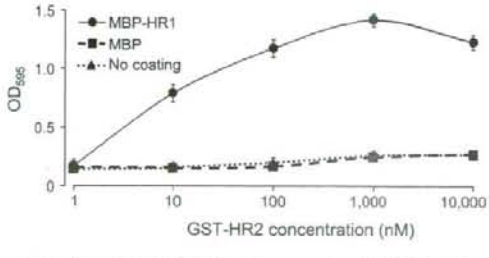

Fig. 3. The binding efficacy of GST-HR2. Fifty nanomolars of MBP-HR1 (circle), MBP (square) and mock (triangle with broken line) were coated on the plate. Various concentrations of GST-HR2 were added and incubated at 37 °C for 1.5 h. Bound GST-HR2 was detected with ALP-conjugated anti-GST antibody by measuring the optical density at 595 nm (OD_{595}).

Table 1
The efficacy of HR2-derived peptides and other entry inhibitors as determined by Ab-ELISA or F-ELISA and the cell-based MAGI assay

Compounds	EC_{50} (nM)[a]			
	Ab-ELISA[b]	F-ELISA[c]	MAGI[d]	
			NL4-3[e]	Bal.[f]
C34[g]	365 ± 43	59 ± 7.7	4.0 ± 0.86	N.D.[h]
SC34EK[g]	41 ± 5.0	21 ± 3.2	1.6 ± 0.61	N.D.
SC35EK[g]	38 ± 3.0	16 ± 2.8	0.35 ± 0.030	N.D.
T-20[g]	>10,000	>10,000	35 ± 17	N.D.
TAK-779	>100,000	>100,000	>100,000	1.85 ± 0.19
AMD-3100	>100,000	>100,000	0.39 ± 0.030	>100,000
DS-5000	>100,000	>100,000	19 ± 6.0	348 ± 46

[a] EC_{50} refers to the concentration of peptides which show 50% inhibition relative to the control.
[b] The amount of binding GST-HR2 measured by ALP-conjugated anti-GST antibody.
[c] Direct detection of HR1 and HR2 interaction without antibody reaction by using ALP-fused HR2 protein.
[d] Multinuclear activation of a galactosidase indicator assay using HeLa CD4-LTR/β-galactosidase indicator cells (Kimpton and Emerman, 1992).
[e] CXCR4 (X4) tropic HIV-1 strain.
[f] CCR5 (R5) tropic HIV-1 strain.
[g] Peptide sequences are shown in Fig. 1.
[h] Not determined.

has anti-fusion activity in vitro and in vivo, even though the gp41 amino acid region 23–58, which is a predictive site for T-20 interaction, is included in the MBP-HR1 fusion protein (Figs. 1 and 2C and F; Table 1). We further examined the effect on T-20 susceptibility of changing the coating and interaction. In this experiment, first GST-HR2 was coated, then exposed to MBP-HR1, and finally detected by anti-MBP antibody. Again C34 and its derivatives were effective, but T-20 was not (data not shown).

3.3. Effect of DMSO concentration and pH

For screening, compounds are frequently dissolved in dimethyl sulfoxide (DMSO). However, high concentrations of DMSO (over 1%) reduced cell viability in the cell-based assay, e.g., MAGI assay. The ELISA systems described here do not require cells, thus should be less influenced by DMSO concentration compared to the MAGI assay. To verify this, we determined the concentration of DMSO that affects the interaction of HR1 and HR2 in our ELISAs. In both the Ab-ELISA and F-ELISA, DMSO concentrations up to 10% did not influence the optical densities to any significant extent (Fig. 4A). At these concentrations, optical densities recorded were less than 20% lower compared to those recorded in the absence of DMSO, indicating that the sensitivities of these tests would be sufficient to screen compounds that are dissolved in reagents containing up to 10% DMSO.

Next, we investigated the effect of pH on detection by ELISA. High concentrations of some compounds that are highly acidic or basic may decrease viability of the cells in cell-based assays. The pH of the reaction buffer was modified by addition of HCl and NaOH as control acidic or basic compounds, respectively. In the F-ELISA, binding of HR1 and HR2 was 2–2.5-fold greater at pH less than 7 than at pH 7.4, while in the Ab-ELISA, the binding was relatively stable at pH 6 (Fig. 4B) and reduction of HR1 and HR2 binding was less than 20%. On the other hand, at basic pH, binding of HR1 and HR2 were relatively stable up to pH 9 in both ELISAs. These results indicate that both systems are less influenced by DMSO concentrations up to 10% and in basic reaction conditions compared to cell-based assays. However, in acidic reaction conditions, interaction of HR1 and HR2 is likely to be overestimated in the F-ELISA.

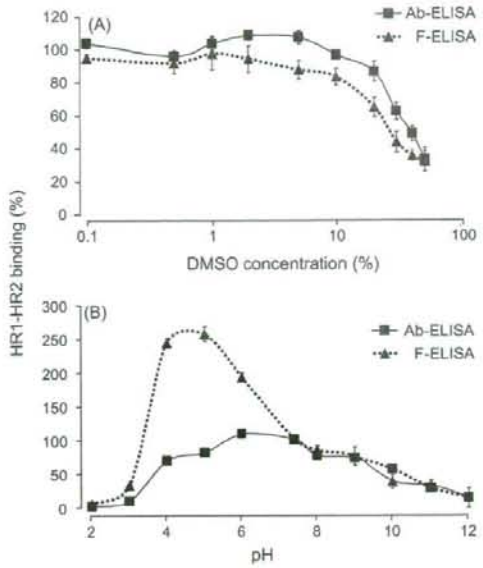

Fig. 4. Effects of DMSO concentration and pH. The effect of DMSO from 0.1 to 50% added to the reaction of HR1 and HR2 is shown (A). Binding is expressed as a percentage of that in the absence of DMSO. Alteration of the pH from 2 to 12 at the HR1 and HR2 reaction was performed by using HCl or NaOH (B). Binding is expressed as a percentage of that at pH 7.4.

4. Discussion

Our newly established ELISA systems successfully detected the HIV-fusion inhibitory activities of C34, a peptide-based fusion inhibitor (Fig. 1), and its derivatives in a dose-dependent manner. However, T-20 lacking the N-terminal 10 amino acids of C34 but containing an additional 12 amino acids in the C-terminal region did not show activity in either of the ELISA systems (Fig. 2; Table 1). T-20 is believed to inhibit 6-helical bundle formation through competition with the physiological HR2 region of gp41. This hypothesis is strongly supported by the introduction of a site of mutations for T-20 resistance in vivo. Variants isolated from T-20 treated patients frequently display mutations in the HR1 region, especially at amino acids 36–45, including D36G/V/S, V38A/E and N43D (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). Interestingly, amino acid positions 36–45 are also crucial for C34 binding, and some C34 resistant variants also show cross-resistance to T-20 (Nameki et al., 2005). Moreover, our preliminary data in the time course of addition experiments showed that the profile of inhibition is identical between C34 and T-20 (data not shown).

Our designed MBP-HR1 contains the presumed interaction site of T-20 (amino acid positions 23–58), as determined by crystal structure analysis of the N36–C34 complex (Chan et al., 1997) (Fig. 1). However, we failed to detect T-20 inhibitory activity in our ELISA systems (Fig. 2C and F). To the best of our knowledge, there are no reports that describe the potent activity of T-20 in protein- or peptide-based assays (Cai and Gochin, 2007; Huang et al., 2006, 2007; Jiang et al., 1999; Liu et al., 2007; Ryu et al., 1998; Xu et al., 2007).

In this regard, two groups have tried to reveal the mechanism of action of T-20 mainly through physicochemical experiments, with both groups proposing that T-20 may act through the lipid mem-

brane. Jiang et al. has proposed that HR2 peptides have two different functional domains, an HR1-binding domain, and a lipid-binding domain (Liu et al., 2007). C34 contains an HR1-binding sequence but not a lipid-binding domain, while T-20 has only a lipid-binding domain, suggesting that T-20 might be functional only in the presence of lipid membrane. Wexler-Cohen and Shai (2007). also found that the C-terminal region of T-20 which was not included in C34 could be replaced with fatty acid, indicating that T-20 acts through the lipid membrane.

It is possible that MBP hampers the proper conformation of HR1. However, in the 6-helix bundle crystal structure of human T cell leukemia virus type 1 gp21, MBP remained fused to the N-terminal of HR1 (Kobe et al., 1999). Thus, it is unlikely that the inability of HR1 to bind T-20 is due to improper conformation of HR1. Moreover, even synthetic peptides of HR1 and T-20 do not bind each other (Liu et al., 2005).

To date, several peptide-based detection systems have been reported, although they failed to demonstrate T-20 activity. Most of them utilize the NC-1 monoclonal antibody which recognizes discontinuous epitopes presented on the 6-helix complex between N36 and C34 to detect 6-helical conformations (Huang et al., 2006, 2007; Jiang et al., 1999; Liu et al., 2007). It is predicted that this system may not detect the peptide-based fusion inhibitor C34 itself or may not detect C34 derivatives, since the antibody NC-1 was derived from the 6-helix conformation of N36 and C34 peptides. Ryu et al. (1998) also reported similar ELISA systems, but showed an inhibitory effect only for C51 with an EC_{50} value of 1.0 μg/ml (approximately 200 nM). Other groups have reported the development of assay systems using fluorescence resonance energy transfer (FRET) (Cai and Gochin, 2007; Xu et al., 2007). Although FRET requires no coating and washing steps, it seems to be less sensitive compared to our ELISA systems. In fact, EC_{50} values of C34 in the FRET system were described as approximately 5 μM (Xu et al., 2007), while those in our Ab-ELISA and F-ELISA were 365 and 59 nM, respectively (Table 1).

The sensitivities of our ELISA systems were lower than those of the cell-based MAGI assay (Table 1). However, the ELISA systems could detect the interaction between HR1 and HR2 even at a high concentration of DMSO, and in a relatively wide pH range (Fig. 4), indicating their capacity for screening of highly concentrated compounds. Decreased concentrations of MBP-HR1 and GST-HR2 or ALP-HR2 increased the antiviral sensitivity, although this also reduced detection sensitivity of ALP activity. Detection sensitivity could be increased by using a highly sensitive chemiluminescent probe as an alternative to the BCIP substrate we used.

At pH greater than 8, both ELISAs showed decreased optical density, while at pH less than 7, enhanced ALP activity was observed in F-ELISA compared with the neutral pH 7.4 (Fig. 4B). Although we could not elucidate the detailed mechanism at present, even in Ab-ELISA, the optical density was also enhanced by using an acidic buffer in the incubation of GST-HR2 with anti-GST antibody (data not shown). Thus, low pH enhances ALP activity rather than enhancing the interaction of HR1 and HR2. These results indicate that we should take note of this artificial enhancement when acidic compounds are screened by F-ELISA.

Major difference between class I and class II fusion is based upon the structure of the glycoproteins involved in the fusion process. For instance, HIV and FluV utilize alpha-helix structure domains located in gp41 and HA2, respectively. In contrast, Flaviviruses, which fuse through class II, utilize beta-sheet structure domains in E protein. Although both glycoproteins complete fusion with trimer of hairpins (alpha-helix and beta-sheet, respectively), in the pre-fusion state, they form trimers and dimers, for class I and class II, respectively. Moreover, the fusion peptide domain which is directly inserted into target cell membrane, is located at N-terminus and internal loop of the env-protein, for class I and class II, respectively.

At the virus–cell membrane fusion step, the interaction between viral envelope proteins HR1 and HR2 is a common mechanism of class I fusion (Jahn et al., 2003; Schibli and Weissenhorn, 2004). It is expected that establishment of a similar ELISA screening system for other viruses using class I fusion for cell entry, such as influenza virus (Eckert and Kim, 2001), feline immunodeficiency virus (FIV) (Medinas et al., 2002), severe acute respiratory syndrome coronavirus (SARS-CoV) (Bosch et al., 2004) and Ebola virus (Watanabe et al., 2000) is possible. For some highly virulent agents, such as SARS-CoV and Ebola virus, our system will be an extremely useful tool since it does not require infectious material.

In this study, we have developed two novel in vitro assay systems for fusion inhibitors by focusing on the interaction of envelope proteins HR1 and HR2. Hydrophobic pocket in HR1 and tryptophan rich domain in HR2 acting as "pocket" and "knob", respectively, play a key role in the virus–cell membrane fusion process, indicating that these interactions are an attractive target for small molecule fusion inhibitors (Ferrer et al., 1999). C34, GST-HR2 and ALP-TRX-HR2 used in this study contain "knob" region but T-20 does not. The developed systems are also ideal for initial screenings because of low variability and good reproducibility even at high compound concentration, and since they allow for a non-infectious rapid and simple procedure. These assays will be useful for the discovery of novel fusion inhibitors not only of HIV-1, but also of other viruses which utilize the class I fusion mechanism.

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