FR901724, a Novel Anti-human Immunodeficiency Virus (HIV) Peptide Produced by *Streptomyces*, Shows Synergistic Antiviral Activities with HIV Protease Inhibitor and 2',3'-Dideoxynucleosides

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A novel tricyclic 21-amino-acid peptide, FR901724, was isolated from the cultured broth of *Streptomyces* sp. No. 73264. This peptide appears to possess potent anti-human immunodeficiency virus (HIV) activity *in vitro* and might represent a lead to a new class of anti-HIV agents; it qualifies as an HIV-cell fusion inhibitor because of its weak inhibition of virus-cell binding and strong inhibition of syncytium formation. From the time-of-addition experiments, the mode of action of FR901724 was found to definitely differ from that of KNI-272, a peptide mimetic allophenylnorstatine-derivative HIV protease inhibitor. FR901724 appears to interact with a stage of the virus replicative cycle that may well correspond to virus-cell fusion. We also found that FR901724 was synergistic or had a strong tendency toward synergism when combined with other antiviral drugs, such as KNI-272, AZT, ddI and dextran sulfate.

Key words AIDS; anti-HIV compound; protease inhibitor; dideoxynucleoside; drug combination

The relative complexity of the human immunodeficiency virus (HIV) replicative cycle suggests that several stages of the replication might represent potential targets for anti-HIV therapy. Most drugs available at present act to inhibit the two HIV-specific enzymes, reverse transcriptase and HIV protease. However, developing strategies aimed at perturbing other steps of the virus replicative cycle, such as gp120-CD4 interaction, gp120-gp41 interaction, membrane fusion, and so forth, could provide an additional means of treating the viral infection. Early stages of viral replication are being studied intensively because of their high specificity and sensitivity to antiviral drugs. Although the exact nature of the events leading up to membrane fusion after the binding of gp120 to CD4 is unknown, the interactions between the infected cell or virus particle and its cellular components have been considered.^{1–3)} Specific binding of gp120 to the CD4 receptor initiates spatial transformation of the viral envelope spikes, resulting in exposure of the transmembrane glycoprotein, gp41.^{4–6)} Among the gp41 regions implicated in fusion activity, a discrete stretch of hydrophobic amino acids, referred to as the fusion peptide, is located at the N-terminus of the glycoprotein.⁷⁾ Synthetic peptides with amino acid sequences corresponding to the N-terminus of gp41 (fusion peptides) were shown to lyse and fuse liposomes, and to inhibit syncytium formation in HIV-infected cell cultures.^{8–11)} In addition, we have reported that a synthetic peptide, T22, showed potent syncytium formation, but did not inhibit direct virus-cell binding. Using various peptide derivatives of T22, we demonstrated that the anti-HIV activities are dependent not only upon the amino acid sequence of the peptides, but also on changes in the high-order structure of their surroundings, as well as on their spatial orientation.^{12,13)}

In the course of our anti-HIV screening program, we discovered a novel peptide, FR901724, in the culture broth

of *Streptomyces* sp. No. 73264. In this paper, we report on the *in vitro* anti-HIV activity of FR901724 and its mechanism of action. The efficacy of this compound in combination with other antiviral drugs, such as an HIV-1 protease inhibitor, dideoxynucleosides or sulfated polysaccharides is also addressed.

MATERIALS AND METHODS

Compounds *Streptomyces* sp. No. 73264, the strain that produces FR901724, was isolated from a soil sample collected in Nagano prefecture (Japan). The cultured broth (90 l) of this strain was filtered with an aid of diatomaceous earth (10 kg). The mycelium cake was extracted with acetone (30 l), yielding 37 l of the extract. The acetone extract was concentrated to remove acetone under reduced pressure. The concentrate (20 l) was passed through a column of a high porous polymer resin Diaion HP-20 (Mitsubishi Chemical Industries, Ltd., Tokyo, Japan) (6 l). The column was washed with water (20 l) and 50% aqueous methanol (20 l), and then eluted with 80% aqueous methanol (20 l). The eluate was diluted with water to 50% aqueous methanol solution (30 l), and applied to a column chromatography on a highly porous polymer resin Diaion HP-20ss (Mitsubishi Chemical Industries, Ltd.) (4 l) pre-packed with 50% aqueous methanol. The column was developed with 60% (7 l) and 80% (20 l) aqueous methanol containing 0.2% sodium dihydrogen phosphate. Active fractions (80% aqueous methanol containing 0.2% sodium dihydrogen phosphate) were combined and diluted with water to a 50% aqueous methanol solution (32 l) again, and then applied on a column of reversed phase silica gel YMC ODS-AM 120-S-50 (YMC Co., Kyoto, Japan) (4 l) equilibrated with 50% aqueous methanol before use. After washing the column with 60% aqueous methanol containing 0.2%

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Fig. 1. Chemical Structure of FR901724

sodium dihydrogen phosphate (10 l), the active compound was eluted with 73% aqueous methanol containing 0.2% sodium dihydrogen phosphate (8 l). The eluate was diluted in the same way as above and adjusted to pH 3.0 with 6 N HCl. The resultant solution (12 l) was passed through a column of Diaion HP-20 (2 l), and then washed with water (6 l) and 5% aqueous methanol (6 l). The objective compound was eluted with methanol (5 l). The active fractions were combined and evaporated *in vacuo* to give a white powder of FR901724 (3.8 g).

FR901724 has a melting point of 265 to 271 °C and a molecular weight of 2164. (FAB-MAS: $M+H^+$). The chemical structure of FR901724 is shown in Fig. 1. We found that FR901724 was identical with Siamycin I, which was recently reported by Tsunakawa *et al.*¹⁴⁾

KNI-272, a peptide-based HIV-1 protease inhibitor, was prepared as described elsewhere.^{15,16)} Dextran sulfate was purchased from Kowa Co., Ltd. (Tokyo, Japan), and 3'-azido-2',3'-dideoxythymidine (AZT) and 2',3'-dideoxyinosine (ddI) were obtained from Yamasa Shoyu Co., Ltd. (Chiba, Japan). Recombinant N-terminal peptides of HIV-1 gp41 (517-685) produced in *E. coli* were purchased from ABT (Cambridge, MA).

Cells Human T-lymphotropic virus type I (HTLV-I) positive human T cell line, MT-4, and the HTLV-I non-infected T cell line, MOLT-4, were grown and maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 100 IU of penicillin per ml and 100 µg of streptomycin per ml (complete medium). Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors, stimulated with phytohemagglutinin (5 µg/ml) for 3 d, then cultured in RPMI 1640 medium containing 20% FCS, antibiotics, and 5 ng of recombinant interleukin 2 (IL-2) (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan) per ml. Human peripheral macrophage cells were also used as target cells of HIV infection.

Viruses Two strains of HIV-1 (HIV-1_{III}B and HIV-1_{JR-FL}) and one strain of HIV-2 (HIV-2_{ROD}) and simian immunodeficiency virus (SIV_{MAC}) were used for the anti-HIV assay in a cell-free virus infection system. HIV-1_{JR-FL} is a monocyte tropic HIV-1 strain.¹⁷⁾ These viruses were obtained from the culture supernatant of MOLT-4, MT-4 cells or human peripheral macrophages which were infected with the virus and stored in a small volume (1 ml) at -80 °C until use. The titer of the virus stocks was determined as 50% tissue culture infectious doses (TCID₅₀).

Anti-HIV Assay The anti-HIV activity of the test compounds was determined from the protection they provided from HIV-induced cytopathic effects (CPE). MT-4 cells were infected with HIV-1_{III}B at a multiplicity of infection (MOI) of 0.01. HIV- or mock-infected MT-4 cells (1.5×10^5 cells/ml, 200 µl) were placed into 96-well microtiter plates and incubated in the presence of various concentrations of the test compounds. All experiments were carried out in triplicate. After 5 d of culture at 37 °C in a CO₂ incubator, the number of viable cells was determined by the 3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.^{12,18)} The antiviral activity against HIV-1_{III}B was also determined as the inhibitory effect on virus-specific antigen expression. Briefly, HIV-1-infected MT-4 cells (MOI=0.01) were cultured with various concentrations of a compound, then viral antigen expression was examined by indirect immunofluorescence (IF), with polyclonal anti-HIV-1 antibody-positive human serum obtained from an HIV-1-infected patient as a probe, and monitored by laser flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

The assay procedure for measuring the anti-HIV-1 activity of the test compounds in PBMC was based on the quantitative detection of an HIV-1 core protein, p24, in the cell culture supernatant with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Abbott, Chicago, IL). The antiviral activities of the tested compounds in human peripheral macrophages were also investigated as described by Perno *et al.*¹⁹⁾ Briefly, PBMC were isolated by the Ficoll-Hypaque technique and washed twice in phosphate-buffered saline (PBS). The adherent cells were obtained by incubating 10^6 PBMC/well in 24-well plates in 1 ml of complete medium for 5 d. Non-adherent cells were removed by washing the well 3 times with RPMI 1640 medium; they were then infected with HIV-1_{JR-FL}. After a 2-h adsorption period at 37 °C, the cells were washed 5 times with RPMI 1640 medium. Finally, the complete medium with varying concentrations of test compounds was added. Neither IL-2 nor mitogens were added to any of the macrophage cultures. After infection, the cells were cultured at 37 °C in a CO₂ incubator and half of the culture medium was changed every 4 d. Fifteen days after infection, the p24 core antigen in the culture supernatant was determined by ELISA. The cytotoxicity of the compounds was evaluated on the basis of the viability of mock-infected MT-4 cells and PBMC, as determined by the MTT method.

IF Virus Binding Assay The procedure used for the detection of the binding of HIV-1 particles to the cell surface has been described previously.²⁰⁾ Briefly, MT-4 cells were exposed to an HIV-1 preparation (which had been concentrated 100 times from the supernatant of MOLT-4/HIV-1_{III}B cultures) in the absence or presence of the test compounds. After incubation at 37 °C for 60 min, the cells were washed twice in PBS to remove unbound virus particles. The cells were then processed for indirect IF measurement as described above.

Syncytium Formation Inhibition Assay MOLT-4 cells and MOLT-4/HIV-1_{III}B cells were mixed in a ratio of 1 : 1 (final cell concentration: 10^6 /ml). The mixed cell suspen-

sion was then co-cultured with various concentrations of the test compound at 37 °C in a CO₂ incubator. Twenty hours after the co-culture, the number of viable cells was determined by the trypan blue dye exclusion method, and the fusion index (*FI*) was calculated as follows: $FI = 1 - [\text{cell number in test well (MOLT-4 mixed with MOLT-4/HIV-1}_{\text{IIB}} \text{ cells})] / [\text{cell number in control well (MOLT-4 cells)}]$. The *FI* values obtained for each concentration of the compound can be expressed as a fraction of the control value, leading to the following definition of the percentage of fusion inhibition: % fusion inhibition = $[1 - (FI_T / FI_C)] \times 100$, where *FI_T* is the *FI* of the test sample and *FI_C* is that of the control sample.²¹⁾

Time-of-Addition Experiment To determine the stage of the HIV replication cycle with which FR901724 interferes, a time-of-addition experiment was carried out. MT-4 cells were exposed to HIV-1_{IIB} at a high multiplicity (MOI > 1.0) to ensure that the virus replicative steps were synchronized in the whole-cell population, then incubated for 1 h at 0 °C. After adsorption, the unadsorbed virus was removed by washing 3 times, shifting the temperature to 37 °C, then adding the compounds (*e.g.*, FR901724; 40 µg/ml, dextran sulfate; 20 µg/ml, AZT; 0.5 µM, and KNI-272; 5 µM) to parallel cultures in microtiter plates at different times after adsorption or immediately after HIV-1_{IIB} exposure to MT-4 cells without adsorption.

To determine whether or not HIV infection was suppressed, HIV-1-antigen positive cells were determined at 25 h post-infection (p.i.) by the IF method as described in the anti-HIV assay section. In addition, the production and processing of HIV-1 protein was analyzed by SDS-PAGE and Western immunoblots.²²⁾ Samples were separated on 12% acrylamide and 0.32% bis-acrylamide gels at 150 V for 90 min, then transferred to clear blot membrane-p filters (Atto Co., Tokyo, Japan) for 60 min at 2.0 mA/cm². After blocking the reaction with 10% nonfat dry milk overnight, the filters were rinsed briefly and washed 3 times for 5 min in PBS with 0.1% Tween 20. Membranes were incubated with 500-fold diluted anti-HIV-1 antibody positive human serum for 60 min at 37 °C, washed as described above, and incubated for 60 min at 37 °C with 1500-fold diluted horse radish peroxidase-conjugated sheep antibody to human immunoglobulin (Amersham, Buckinghamshire, UK). After washing as previously described, proteins were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and exposure to X-ray film (Konica Co., Tokyo, Japan).

Analysis of Compound Combination Effect The inhibition of HIV replication by FR901724 and KNI-272, dextran sulfate, AZT, or ddI was examined by a checkerboard method. The antiviral activity in combination-treated HIV-infected MT-4 cells was determined for the protection against HIV-induced CPE provided by the MTT method, as described above. For an experiment involving multiple ratios, five to six concentrations of each compound were obtained by checkerboard combinations of various concentrations of the test compounds. The combination effect was evaluated by the method of Prichard and Shipman²³⁾ employing a computer program using a Macintosh™ Excel spreadsheet (Microsoft Corp.,

Redmond, WA) and Deltasoft™ graphics (Deltapoint, Inc., Monterey, CA). The amount of synergy observed with combinations of the two compounds is represented by the height of the bars in the graph when the percentage of drug interaction is plotted *versus* drug concentrations. Additive levels were subtracted out by the program and are represented by the floor of the graph. The extent of synergistic interaction can be quantitated by computing the volume of synergy from a surface area plot of the same data.

RESULTS

In Vitro Anti-HIV Activities of FR901724 The anti-HIV-1 activities of FR901724 were assessed by its protection of HIV-1-induced CPE and its inhibition of virus-specific antigen expression in MT-4 cells *in vitro* (Fig. 2). Although HIV-1-infected MT-4 cells were not able to survive under viral infection, FR901724 showed cell-protective activity against virus-induced CPE with an apparent dose dependency; the 50% effective concentration (EC₅₀) value of FR901724 was 1.60 µg/ml. The 50% cytotoxic concentration (CC₅₀) of FR901724 was 67.8 µg/ml and its selectivity index (ratio of CC₅₀ to EC₅₀) was 42. We also investigated the antiviral activity of FR901724 against HIV-2_{ROD} and SIV_{MAC} in MT-4 cells and against HIV-1_{IIB} in normal human peripheral blood mononuclear cells (PBMC) and macrophages (Table 1). Irrespective of the criteria used to assess anti-HIV activity, *i.e.*, inhibition of viral induced CPE or p24 production, FR901724 invariably inhibited not only HIV-1, but also HIV-2 and SIV strains.

Inhibition of Virus Binding To elucidate the mechanism of action of FR901724, we investigated whether it inhibited the binding of HIV-1 particles to MT-4 cells, as assessed by laser flow cytometry using an IF assay. As shown in

Fig. 2. Inhibitory Effect of FR901724 on HIV-1-Induced Cytopathic Effects (CPE) and Viral Antigen Expression in MT-4 Cells

The viability of HIV-infected (hatched columns) and mock-infected MT-4 cells (open columns) was measured by the MTT method 5 d after infection. The number of viable cells was expressed as the percentage of mock-infected drug-free control cells. HIV-1 antigen-positive cells were detected by indirect IF and laser flow cytometry, using a polyclonal antibody as a probe. The number of viral antigen-positive cells was expressed as a percentage of the HIV-1-infected drug-free control cells. Each experiment was performed at least three times and the results are the means of three experiments.

Table 1. Antiviral Activity Spectra of FR901724, KNI-272, Dextran Sulfate and Dideoxynucleoside Analogues

Virus strain	Cell	Assay	Day of analysis	Antiviral activity ^{a)} (EC ₅₀)				
				FR901724 (μg/ml)	KNI-272 (μM)	Dextran sulfate (μg/ml)	AZT (μM)	ddI (μM)
HIV-1 _{IIIB}	MT-4	MTT	5	1.60	0.24	0.78	0.024	14.03
	PBMC ^{b)}	p24	12	3.71	1.23	2.68	0.011	10.73
HIV-1 _{JR-FL}	hu-Mφ ^{c)}	p24	15	0.76	1.03	0.33	0.164	0.25
HIV-2 _{ROD}	MT-4	MTT	5	3.04	0.66	0.86	0.014	19.89
SIV _{MAC}	MT-4	MTT	5	0.70	0.87	4.41	0.011	3.01
Cytotoxicity ^{d)} (CC ₅₀)	MT-4	MTT	5	67.8	> 60	> 1000	28.5	> 500

a) Anti-HIV-1 activity (EC₅₀), concentration required to achieve 50% protection of the MT-4 cells against HIV-1-induced cytopathic effects is determined by the MTT method. b) EC₅₀ in PBMC is calculated based on the 50% reduction of p24 expression in the culture supernatant. c) EC₅₀ in human peripheral macrophages is calculated based on the 50% reduction of p24 expression in the culture supernatant. d) Cytotoxic concentration of compounds which was required to achieve 50% toxicity of the MT-4 cells (CC₅₀) is determined by the MTT method.

Fig. 3. Effects of FR901724 and Dextran Sulfate on HIV-1 Binding to MT-4 Cells

The white histograms represent cellular fluorescence resulting from nonspecific binding of anti-HIV-1 antibody to MT-4 cells (which had not been exposed to HIV-1). The shaded histograms represent the cellular fluorescence resulting from specific binding of anti-HIV-1 antibody to virus-exposed MT-4 cells in the absence of a test compound (A), or in the presence of dextran sulfate (25 μg/ml) (B) or FR901724 (25 μg/ml) (C).

Fig. 3, FR901724 was not able to inhibit HIV-1 binding at 25 μg/ml. In contrast, dextran sulfate (25 μg/ml) almost completely inhibited HIV-1 binding. Therefore, FR901724 does not have direct inhibitory action on the binding of HIV to CD4-positive cells.

Inhibition of Syncytium Formation When we investigated inhibitory activity against multinuclear giant cell (syncytium) formation in co-cultures of persistently HIV-1-infected MOLT-4 cells (MOLT-4/HIV-1_{IIIB}) and uninfected MOLT-4 cells, FR901724 inhibited giant cell formation more efficiently than dextran sulfate; their 50% inhibitory concentrations were 0.7 and 1.8 μg/ml, respectively (Fig. 4). The inhibitory effect of HIV-1 gp41 on syncytium formation in the co-culture system was also investigated. It was found that 25 μg/ml of recombinant N-terminal gp41 peptides inhibited 20% syncytium formation; however, no significant inhibition was observed when levels of less than 10 μg/ml of gp41 were added to the co-culture (data not shown). We then investigated whether or not the anti-HIV-1 activity of FR901724 was affected by interaction with HIV-1 gp41. Antiviral activity was assessed by fusion inhibitory activity in the co-culture of MOLT-4 and MOLT-4/HIV-1 cells. The result indicated that the anti-HIV-1 activity of FR901724

Fig. 4. Inhibitory Effects of Dextran Sulfate (open columns) and FR901724 (hatched columns) on Syncytium Formation in a Co-culture of MOLT-4 and MOLT-4/HIV-1_{IIIB} Cells

The number of viable cells was determined 20h after co-culture in the presence of various concentrations of the compounds, and percent fusion inhibition was calculated as described under Materials and Methods.

was not significantly affected by the additional recombinant N-terminal gp41 peptides (data not shown).

Time-of-Addition Experiment To delineate the stage

Fig. 5. Time-of-Addition Experiment

MT-4 cells were infected at a high multiplicity of infection with HIV-1_{IIIb}. After 60-min adsorption with HIV-1 at 0°C, unadsorbed virus was removed by 3 washing steps, then the temperature was shifted to 37°C. After the temperature was raised, FR901724 (40 µg/ml) (○), dextran sulfate (20 µg/ml) (●), AZT (0.5 µM) (▲), and KNI-272 (5 µM) (□) were added to parallel cultures in microtiter plates at different times post-infection or immediately after HIV-1 exposure to MT-4 cells without adsorption (at the point “-1”). HIV-1-antigen-positive cells were determined 25 h post-infection by an IF method.

Fig. 6. Western Immunoblotting Analysis of HIV-1-Infected MT-4 Cells

MT-4 cells were exposed and adsorbed with HIV-1 for 60 min at 0°C, unadsorbed virus was removed by washing, and the temperature was shifted to 37°C. After the temperature had been raised, FR901724 (40 µg/ml)(A) or KNI-272 (5 µM)(B) was added to HIV-1-infected MT-4 cells at the indicated time. Cell culture supernatant was collected at 25 h after infection and the viral lysate was analyzed with 12% SDS-PAGE followed by the immunoblotting reaction.

of the HIV-1 replicative cycle at which FR901724 may interact, the compound was added at different times after the adsorption of HIV-1 to the MT-4 cells. Dextran sulfate, AZT and KNI-272 were run in parallel as reference compounds. The compounds were added at concentrations that were approximately 20 to 25-fold higher than their EC₅₀ values. Dextran sulfate, which interferes with virus binding, was no longer able to inhibit HIV-1 replication if added 0 min (or later) after the adsorption. In contrast, AZT completely inhibited virus antigen expression from 0 to 4 h after adsorption; more specifically, it began to lose its inhibitory effect if the treatment was delayed for 6 h. In the treatment with FR901724, the compound inhibited HIV infection if it was added until 60 min after adsorption. However, the inhibitory potency decreased if it was added 1.5 h after

adsorption. Almost all cells were HIV-1-antigen-positive if KNI-272 was added at any period after adsorption (Fig. 5).

For the immunoblot analysis, the HIV-1 core protein, p24, was not detected when FR901724 was added to MT-4 cells at 0 and 30 min after adsorption. However, FR901724 failed to inhibit the viral protein expression when it was added 60 min after adsorption (Fig. 6A). HIV proteins did not appear when AZT was added to HIV-infected MT-4 cells at times from 0 to 4 h after adsorption (data not shown), because AZT inhibited the reverse transcriptional process of HIV replication. When KNI-272 was added to HIV-infected MT-4 cells at different times after adsorption, processing was also analyzed by immunoblots. Studies of virus proteins by means of immunoblots demonstrated the effective inhibition of the

Fig. 7. Combined Drug Interaction When HIV-1-Infected MT-4 Cells Were Treated with FR901724 and KNI-272

Panel A represents the experimental dose response surface. Panel B represents the surface of response corresponding to the null hypothesis that drug interactions are additive. The calculated additivity surface was subtracted from the experimental surface and plotted in 3-D form (Panel C). The amount of synergy is represented by the height of the bars.

processing of Pr55 to p24 by KNI-272. The p24-band was not detected when KNI-272 was added to HIV-infected MT-4 cells 10 h after adsorption, whereas it was detectable if KNI-272 was added 16 h after adsorption (Fig. 6B). This result indicates that the Pr55 Gag precursor processing was inhibited if KNI-272 was added to HIV-infected cells before protease started to act.

Combinations of FR901724 and KNI-272, Dextran Sulfate, AZT, or ddI in MT-4 Cells Next, we investigated whether FR901724 showed synergistic or additive effects when it was used concomitantly with other anti-viral drugs. We first investigated the antiviral effect of FR901724 com-

Fig. 8. Drug Interaction Analysis of FR901724 and AZT, ddI or Dextran Sulfate in HIV-1-Infected MT-4 Cells

bined with KNI-272 in acutely HIV-infected MT-4 cells, which was monitored by the protection against HIV-induced CPE. Figure 7A demonstrates the experimental dose-response surface of the combination treatment of these two compounds. Figure 7B represents the calculated additivity of the drug combination. The calculated additivity surface was subtracted from the experimental surface and plotted in 3-D form (Fig. 7C). These plots represent deviations from expected interactions and would reveal a horizontal plane at 0% if the interactions were purely additive. As shown in Fig. 7C, combinations of FR901724 and KNI-272 in the dose range tested were consistently more effective than the use of either compound alone, because the plots appeared above the plane with a height corresponding to the percent above the

calculated additivity. When we tested the combination effect of FR901724 and AZT, ddI or dextran sulfate, a synergistic antiviral effect was observed (Fig. 8). In particular, the combinations of compounds at concentrations near to their EC_{50} values (e.g., FR901724 in test ranges from 1.1 to 3.3 $\mu\text{g/ml}$ and AZT at 0.011 to 0.033 μM , ddI at 3.7 to 33.3 μM or dextran sulfate at 1.1 to 3.3 $\mu\text{g/ml}$, respectively) showed strong synergistic interactions.

DISCUSSION

Of the anti-HIV treatment strategies tested to date, none is curative and only certain inhibitors of viral reverse transcriptase have demonstrated clinical benefit. As a result, the search for new, more effective compounds and other sites on the virus at which to target antiviral therapy continues at an urgent pace. In the present study, we demonstrated that a novel peptide produced by the *Streptomyces* strain, FR901724, appeared to possess potent anti-HIV activity *in vitro* and might represent a lead to a new class of anti-HIV agents. This oligopeptide qualifies as an HIV-cell fusion or HIV-infected cell-to-cell fusion inhibitor; it is only weakly inhibitory to virus-cell binding, yet it is strongly inhibitory to syncytium formation.

Several peptide-based anti-HIV compounds have been reported. Protease is responsible for proteolytic processing of the Gag/Pol precursor into mature proteins and leads to the formation of infectious virus particles. Identification of the HIV protease cleavage site has proved useful in designing appropriate inhibitors. From the time-of-addition experiments, the mode of action of FR901724 was found to definitely differ from that of KNI-272, a peptide mimetic allophenylnorstatine-derivative HIV protease inhibitor.^{15,16} The chemical structure of FR901724 is fairly unusual. Given the presence of an internal amide bond and two disulfide bridges, it is very rigid (Fig. 1). Recently, a 21-mer tricyclic polypeptide, RP71955, which was isolated from a *Streptomyces* strain, was reported by Helynck *et al.*²⁴ and Frechet *et al.*²⁵ The amino acid sequence of RP71955 is the same as that of FR901724 except in two amino acid positions; RP71955 has Ile-4 and Val-17 in its amino acid sequence, whereas FR901724 has Val-4 and Ile-17, respectively. Although the precise molecular target of RP71955 has not yet been determined, they indicated that the hydrophobic character of the peptide makes it a likely candidate for interaction with membranes. Moreover, a 33% sequence similarity was observed between RP71955 and residues 608-628 of gp41.^{24,26}

	608
gp41	L L G I W G C S G K L I C T T A V P W N A
RP71955	C L G I G S C N D F A G C G Y A V V C F W
FR901724	C L G V G S C N D F A G C G Y A I V C F W

This sequence is part of the ectodomain of gp41 (residues 527-655), and both FR901724 and RP71955 may interfere with the molecular function of gp41. Indeed, these compounds have been found to inhibit the formation of HIV-induced syncytia, but not the interaction of direct

binding of gp120 to CD4. The formation of syncytia is considered to involve a mechanism analogous to cell fusion.²⁷ It is generally agreed that the N-terminal hydrophobic domain of gp41 plays a critical role in membrane fusion²⁸⁻³¹; it contains an N-terminal fusion peptide (residues 512-527) and a disulfide loop near the middle of the extracellular domain (residues 598-604). Some changes affecting amino acids in the extracellular domain of HIV-1 gp41 resulted in envelope glycoproteins that were partially or completely defective in membrane fusion.³² Syncytium formation was more sensitive than virus entry to these changes.

In the present study, a recombinant N-terminal HIV-1 gp41 (residues 517-685) did not affect syncytium formation strongly, and this recombinant peptide did not affect the anti-HIV activities of FR901724 in the co-culture of MOLT-4 and MOLT-4/HIV-1 cell systems. However, this does not conclusively negate the interaction of FR901724 and gp41. More precisely, it overlaps with several antigenic epitopes, which have been shown to be crucial for the activity of gp41.^{27,33} Other studies have shown that monoclonal antibodies with epitopes corresponding to residues 581-614 have increased binding to gp41 upon the complexing of soluble-CD4 with gp120 at 4°C, *i.e.*, without dissociation of the gp120-soluble-CD4 complex from gp41.²⁷ It has been proposed that CD4 binding induces conformational changes in gp41, increasing the exposure of this antigenic region, and that furthermore, this event is an essential step in inducing fusion. It could, for example, involve binding to a membrane receptor other than CD4.³ The importance of the N-terminal peptides of gp41 in the pathobiology of HIV-1 infection needs further study.

FR901724 appears to interact with a stage of the virus replicative cycle that may correspond well to virus-cell fusion, and the mode of action of this compound closely resembles that of the synthetic peptide T22 ([Tyr-5,12, Lys-7]polyphemusin II), which is a derivative of polyphemusin that is highly abundant in hemocyte debris of the horseshoe crab *Limulus polyphemus*.¹² However, T22, which has two disulfide linkages and four basic amino acid side chains on the plane of the β -sheet, has distinct anti-HIV activity, and this activity might be associated with these disulfide linkages and/or basic amino acid side chains. On the other hand, FR901724 contains no basic amino acids. Therefore, FR901724 and T22 appear to use different mechanisms in exerting anti-HIV activity. Studies of the interaction between FR901724 and virus and/or cellular components, especially triptase TL2² and the V3 domain of the HIV envelope protein, are under way.

In this study, we also investigated whether FR901724 had synergistic antiviral activities when combined with other anti-HIV compounds. Combination chemotherapeutic approaches have been used extensively to reduce drug toxicity, to prevent or delay the development of resistant virus mutation, and to obtain a greater effect than is achieved with one active drug alone. Using a 3-D analysis method, we found that FR901724 was synergistic or had a strong tendency toward synergism when combined with KNI-272, AZT, ddI and dextran sulfate. If we added 10 $\mu\text{g/ml}$ of FR901724 to HIV-infected MT-4 cells,

the additive effect was observed with any of the other antiviral drugs, because almost 100% of the anti-HIV activity was obtained with this concentration of FR901724 alone. This was also true for 15 μM of KNI-272, 0.1 μM of AZT, 100 μM of ddI and 10 $\mu\text{g/ml}$ of dextran sulfate. Therefore, the antiviral activities of combined treatment should be discussed at the dose ranges of each compound that achieved less than 100% efficacy with the use of one compound alone. In the present study, combined treatment with concentrations near the EC_{50} values of the compounds (e.g., FR901724 in test ranges from 1.1 to 3.3 $\mu\text{g/ml}$ and KNI-272 at 0.56 to 5 μM , AZT at 0.011 to 0.033 μM , ddI at 3.7 to 33.3 μM or dextran sulfate at 1.1 to 3.3 $\mu\text{g/ml}$, respectively) showed strong synergistic interactions. This result indicates that higher anti-HIV activities can be expected if FR901724 is used with a protease inhibitor or with 2',3'-dideoxynucleosides. More in-depth studies must be conducted to further investigate this finding.

Very recently, Mitsuya *et al.* reported a novel anti-HIV polypeptide, NP-06, produced by a *Streptomyces* sp.³⁴⁾ The chemical structure of NP-06 is exactly the same as that of FR901724. However, they isolated NP-06 from a different species strain of soil collections than what we found. They also concluded that NP-06 blocked the early stage of HIV infection, most likely at the stage of virus-cell fusion. Taken together, in view of its potent anti-HIV activities and synergistic interactions *in vitro* with other anti-HIV compounds, FR901724 appears to have considerable potential as a candidate antiviral drug.

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