Chloropeptins, New Anti-HIV Antibiotics Inhibiting gp120-CD4 Binding from *Streptomyces* sp.

I. Taxonomy, Fermentation, Isolation, and Physico-chemical Properties and Biological Activities

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Chloropeptins I and II, which are gp120-CD4 binding inhibitors, were isolated as pale yellow-brown powders from the mycelia of a soil actinomycete, *Streptomyces* sp. WK-3419. Their physico-chemical properties showed that they are chlorinated peptides. Chloropeptin I ($C_{61}H_{45}N_7O_{15}Cl_6$) is a novel compound, but chloropeptin II was identified as complestatin. Both compounds inhibited gp120-CD4 binding (IC_{50}: 1.3 and 2.0 μM, respectively), the cytopathic effect of HIV in MT-4 cells (EC_{50}: 1.6 and 1.7 μM, respectively) and syncytium formation in co-cultured HIV-1-infected and uninfected MOLT-4 cells (IC_{50}: 0.5 and 1.1 μM, respectively). Chloropeptin I was synergistic in the inhibition of the cytopathic effect when combined with other anti-HIV drugs such as zidovudine (AZT), didanosine (ddI), zalcitabine (ddC) and nevirapine.

Blocking human immunodeficiency virus (HIV) entry, which begins with highly specific binding of the HIV envelope glycoprotein gp120 to a CD4 molecule on the surface of most susceptible cells[1~3], is one of the most important targets of HIV therapy[4].

While screening for activities against gp120-CD4 binding from microorganisms, we discovered the novel inhibitors, isochromophilones I and II, from the fungus, *Penicillium multicolor*[5~7]. Here, we report additional two gp120-CD4 binding inhibitors, chloropeptins I and II (Fig. 1), produced by *Streptomyces* sp. isolated from a soil sample. This paper describes the taxonomy, fermentation, isolation, physico-chemical properties and biological activities of these compounds, a preliminary account of which was published in this journal[8]. The structure elucidation will be separately reported[9].

Materials and Methods

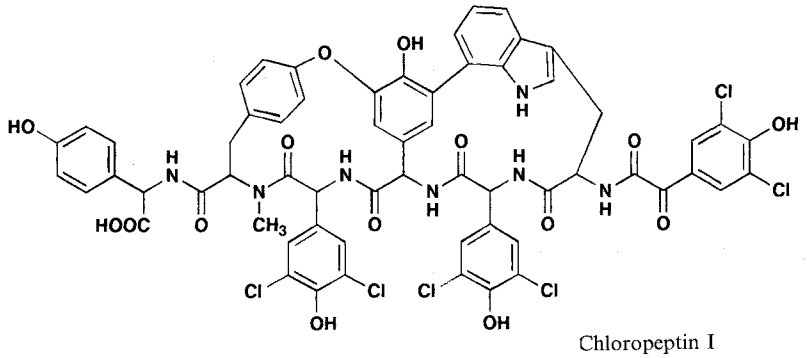
Producing Organism and Taxonomy

To examine the morphological and cultural properties, strain WK-3419 was cultured as described by International Streptomyces Project[10,11]. Morphology was observed under a light microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430). The culture was examined after an incubation at 27°C for 14 days. The color was judged by the Color Harmony Manual[12]. The physiological properties including carbon utilization were examined as described by PRIDHAM and GOTLIEB[13]. Diaminopimelic acid was analyzed as described by BECKER *et al.*[14].

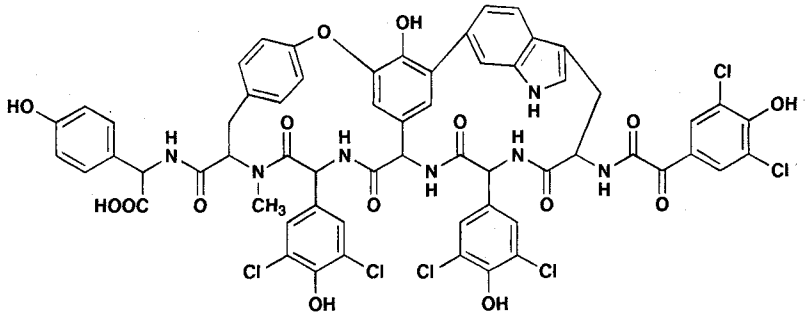
Fermentation

A loopful of mycelia from a dense slant culture of strain WK-3419 was transferred into a large test tube (2 × 20 cm) containing 10 ml of a medium consisting of starch 2.4%, glucose 1.0%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.4% (adjusted to pH 7.0 before sterilization). The tube was incubated at 27°C on a reciprocal shaker at 300 rpm for 3 days to give a first seed culture. This culture (2 ml) was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the above medium, and was incubated at 27°C on a rotary shaker (200 rpm) for 4 days to give a second seed culture. This culture (600 ml) was transferred into a 50-liter jar fermenter containing 30 liters of the above medium supplemented with allophane 0.5% (non-crystalline aluminosilicic clay from Asahi Chemical Co., Ltd.) and 4 ml/liter of trace metal solution, containing 1.0 g each of $FeSO_2 \cdot H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 2H_2O$ per liter. Chloropeptin production was monitored by HPLC using a CAPCELL PAK C18 column and acetonitrile - THF - 0.1% phosphoric acid (37 : 8 : 55) as the developing solvent. Fermentation proceeded at 27°C for 7 days under aerobic conditions.

Fig. 1. Structures of chloropeptins I and II (complestatin).



Chloropeptin I



Complestatin
(Chloropeptin II)

Spectral Analysis

UV and visible spectra were recorded on a Shimadzu UV-160 A spectrophotometer and IR spectra were recorded on a Jasco A-102 spectrophotometer. Mass and NMR spectra were obtained on JMS-SX102A and Varian XL-400 spectrometers, respectively.

Gp120-CD4 Binding Assay

Binding of recombinant sCD4 (rsCD4) to recombinant gp120 (rgp120) adsorbed on a 96-well plate was assayed by ELISA as described by GILBERT *et al.*[15]. To 96-well plates coated with rgp120 (2.0 μg/ml), rsCD4 (1.0 μg/ml), bovine serum albumin (BSA, 5.0 mg/ml) and sample solution were added, then incubated for 1 hour at room temperature. The plates were washed, then anti-CD4-mAB (8E7.H8) was added and incubated for 1 hour at room temperature. The plates were treated with mouse anti-IgG-HRP, then bound rsCD4 was assayed colorimetrically.

Anti-HIV Assay

The activity of a sample against HIV-1_{IIIB} was determined on the basis of virus-induced cytopathogenicity in MT-4 cells as described by NAKASHIMA *et al.*[16]. The cytotoxicity of the sample was evaluated in parallel with the antiviral activity and compared with the viability of mock-infected cells by the MTT method. In the assay, after HIV-1-infected cells were incubated with a test sample at 37°C for 5 days, the viable cells were counted by the MTT method.

Syncytium Formation Inhibition Assay

This assay preceeded as described by NAKASHIMA *et al.*[16]. In the assay, MOLT-4 cells and HIV-1-infected MOLT-4 cells (MOLT-4/HIV-1_{IIIB}) were mixed and incubated at 37°C for 20 hours. Thereafter, the cells were counted and the percent fusion inhibition was calculated.

Cytotoxicity

HeLa-S3 and CEM cells were anchorage-dependent and -independent, respectively. Cells were incubated in EAGLE's minimum essential medium containing 10% fetal calf serum at 37°C in a 5% CO_2 - 95% atmosphere. To determine cytotoxicity, cells suspended in 200 μl of the medium (3×10^4 cells/ml) were plated in 96-well culture plates and incubated for 24 hours at 37°C. To each well was added a sample solution dissolved in dimethylsulfoxide (DMSO; final concentration, 1.25%). After 72-hour incubation, the cell growth was measured colorimetrically by the tetrazolium salt (MTT) method.

Antimicrobial Activity

Antimicrobial activity was assayed by the conventional paper disc method using the following media and 8 mm paper discs (50 μl of sample was added to each disc). Bacteria were incubated in medium containing peptone

0.5%, meat extract 0.5% and agar 1.0% (pH 7.0) at 37°C, fungi in medium containing glucose 1.0%, yeast extract 0.5% and agar 1.0% (pH 7.0) at 27°C, and mycoplasma in that containing PPLO Broth (Difco) 2.1%, glucose 0.1%, horse serum 15%, phenol red 10 μg/ml and agar 1.2% at 37°C.

Analysis of Compound Combination Effect

The inhibition of HIV replication by chloropeptin I and AZT, ddI, ddC or nevirapine 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. Five or six concentrations of each compound were examined by checkerboard combinations with various concentrations of the test compounds. The combination effect was evaluated by the method of PRICHARD and SHIPMAN[17] 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. Zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) were obtained from Yamasa Shoyu Co., Ltd. (Chiba, Japan). Nevirapine was synthesized by Dr. T. KINO, Fujisawa Pharmaceutical Co., Ltd. (Ibaraki, Japan).

Results

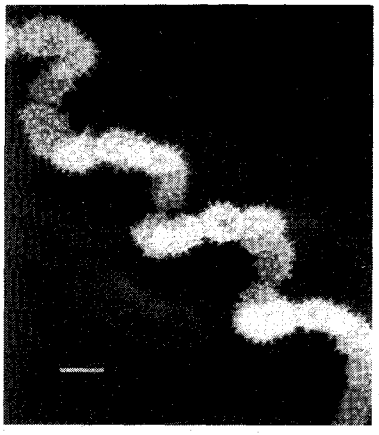
Taxonomy of the Chloropeptin-producing Strain WK-3419

Vegetative mycelia grew abundantly on yeast extract-malt extract, oatmeal and other agar media, but did not fragment into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract-malt extract, inorganic salts-starch and other agar media. The spore chains were of the spirales type and each had over 20 spores per chain. The spores were ovoid in shape, 1.4 × 0.9 mm in size and had a spiny surface (Fig. 2). Whirls, sclerotic granules, sporangia and flagellate spores were not observed.

The cultural characteristics and the physiological properties are shown in Tables 1 and 2, respectively. The vegetative mycelia were yellow. The aerial mass was bluish gray. Melanoid pigment was produced in tyrosine-yeast extract, broth and on tyrosine agar and the soluble pigment was yellow. The strain utilized all of the carbon sources tested (D-glucose, L-arabinose, D-xylose, raffinose, melibiose, D-mannose, D-fructose, L-rhamnose, inositol and sucrose) when cultured on PRIDHAM and GOTTLIEB's medium[13] supplemented with a 1.0% carbon source at 27°C.

Fig. 2. Scanning electron micrograph of spore chains of *Streptomyces* sp. WK-3419 grown on glucose-asparagine agar for 14 days.

Bar represents 1 μm.



The taxonomic properties described above indicated that strain WK-3419 belongs to the genus *Streptomyces*[18]. The strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. WK-3419 and the accession No. FERM P-13583.

Fermentation

Fig. 3 shows typical time course of chloropeptin production by *Streptomyces* sp. WK-3419 using a 50-liter jar fermenter. The production of chloropeptins I and II began after day-1, became active after day-2 and reached a maximum 5 or 6 days later. More chloropeptin I than II (complestatin) was produced.

Isolation

The isolation procedures for chloropeptins I and II are summarized in Fig. 4. The mycelial cake was extracted with 70% aqueous acetone. The extract was evaporated to give a crude material. The methanol-soluble fraction from the material was subjected to Sephadex LH-20 column chromatography to give a pale yellow-brown powder (1.1 g) of chloropeptin I. Another pale yellow-brown powder (chloropeptin II, 720 mg) was obtained from the residue by preparative HPLC.

Table 1. Properties of strain WK-3419 in culture.

Medium	Culture characteristics	Medium	Culture characteristics
Yeast extract-malt extract agar [a]	G: Good, mustard gold (2ne) R: Mustard gold (2pg) AM: Abundant, no name (17ig) SP: Antique gold ($1^1/_2$pe)	Tyrosine agar [a]	G: Moderate, mustard (2le) R: Adobe brown (3lg) AM: Moderate, pearl (2ba) SP: None
Oatmeal agar [a]	G: Good, bamboo (2gc) R: Light ivory (2ca) AM: Moderate, teal gray (19ih) SP: None	Sucrose-nitrate agar [b]	G: Good, mustard (2le) R: Yellow maple (3ng) AM: Abundant, alabaster tint (13ba) SP: Maple (4le)
Inorganic salts-starch agar [a]	G: Good, light antique gold ($1^1/_2$ic) R: Gold (2lc) AM: Abundant, slate (15ih) SP: None	Glucose-nitrate agar [b]	G: Good, light ivory (2ca) R: Light ivory (2ca) AM: Moderate, alabaster tint (13ba) SP: None
Glycerol-asparagine agar [a]	G: Good, ivory (2db) R: Bamboo (2gc) AM: Abundant, teal gray (19ih) SP: Chartreuse yellow (1ic)	Glycerol-calcium malate agar [b]	G: Moderate, light ivory (2ca) R: Pearl (2ba) AM: Abundant, teal gray (19ih) SP: None
Glucose-asparagine agar	G: Good, bamboo (2gc) R: Light mustard tan (2ie) AM: Abundant, fog blue (16ig) SP: Chartreuse yellow (1ic)	Glucose-peptone agar [b]	G: Good, bamboo (2gc) R: Bamboo (2gc) AM: Abundant, fog blue (15ig) SP: Gold ($1^1/_2$lc)
Peptone-yeast extract-iron agar [a]	G: Moderate, biscuit ecru (2ec) R: Biscuit ecru (2ec) AM: Trace SP: None	Nutrient agar [b]	G: Good, light ivory (2ca) R: Light wheat (2ea) AM: Moderate, no name (17ig) SP: None

[a] Medium recommended by ISP.[10]
[b] Medium recommended by Waksman S. A.[11]
Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

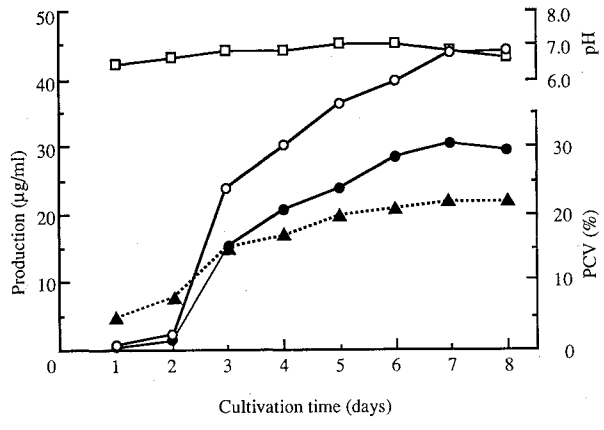
Table 2. Physiological properties of strain WK-3419.

Melanin formation	
Tyrosine agar	+
Peptone-yeast extract-iron agar	±
Tryptone-yeast extract broth	+
Reduction of nitrate	+
Liquefaction of gelatin (21~23°C)	-
Hydrolysis of starch	+
Coagulation of milk (37°C)	-
Peptonization of milk (37°C)	+
Decomposition of cellulose	-
Temperature range for growth	12 ~ 38°C

+, positive; —, negative ; ±, doubtful

Fig. 3. Typical time course of chloropeptin production by *Streptomyces* sp. WK-3419 in a 50-liter jar fermenter.

○ Chloropeptin I, ● chrolopeptin II (complestatin), ▲ PCV, □ pH.



Physico-chemical Properties

Physico-chemical properties of chloropeptins I and II are summarized in Table 3. The properties of chloropeptin II coincided with those of complestatin[19,20]. Chloropeptin II was identified as complestatin by its direct comparison with the authentic sample provided by Dr. S. TAKAHASHI of Sankyo Co., Ltd., (Tokyo, Japan). However, chloropeptin I differed from complestatin in terms of optical rotation, UV spectrum and solubility in methanol, although their molecular formulae ($C_{61}H_{45}N_7O_{16}Cl_6$) were identical. Both compounds were soluble in dimethyl sulfoxide, pyridine and alkaline water, but not in hexane, chloroform and ethyl acetate. However, their solubility in lower alcohols differed. Complestatin (chloropeptin II) is essentially in-soluble in methanol whereas chloropeptin I is soluble. The UV spectra of chloropeptin I and complestatin both under-

went a bathochromic shift. We considered from the data shown in Table 3 that chloropeptin I is also a peptide containing chlorinated phenol groups. The structure elucidation will be separately reported[9].

Biological Activities

Chloropeptin I and complestatin potently inhibited gp120-CD4 binding (IC_{50}: 1.3 and 2.0 μM, respectively) even in the presence of 5.0 mg/ml BSA as shown in Fig. 5, but vancomycin (100 μM) containing a similar chlorinated heptapeptide did not. Without BSA, they exhibited more potent activities (IC_{50}: 32 and 53 nM, respectively). Chloropeptin I inhibited the cytopathic effect of HIV and syncytium formation as shown in Table 4. Complestatin also inhibited them at somewhat higher concentrations than those of chloropeptin I. The cytotoxicity of chloropeptin I against mock-infected MT-4 cells was very low (CC_{50}: >600 μM).

The cytotoxicity of chloropeptin I and complestatin against HeLa-S3 cells as anchorage-dependent cells and CEM cells as anchorage-independent cells was also examined. The CC_{50} values for chloropeptin I and complestatin against HeLa-S3 cells were 160 and 90 μM, respectively, and those against CEM cells were 330 and 150 μM, respectively, indicating that chloropeptin I is more selectively toxic than complestatin, because the former had more potent anti-HIV activity than complestatin.

A shown in Table 5, chloropeptin I and complestatin exhibited relatively low antimicrobial activities against some Gram-positive bacteria and a mycoplasma, and none against the other microorganisms tested, (*Mycobacterium smegmatis* ATCC607, *Escherichia coli*: NIHJ and NIHJ JC-2 (IFO-12734), *Pseudomonas aeruginosa* P-3, *Xanthomonas orizae* KB88, *Bacteroides fragilis* KB169, *Candida albicans* KF1, *Mucor racemosus* KF223

Fig. 4. Isolation procedures for chloropeptins I and II.

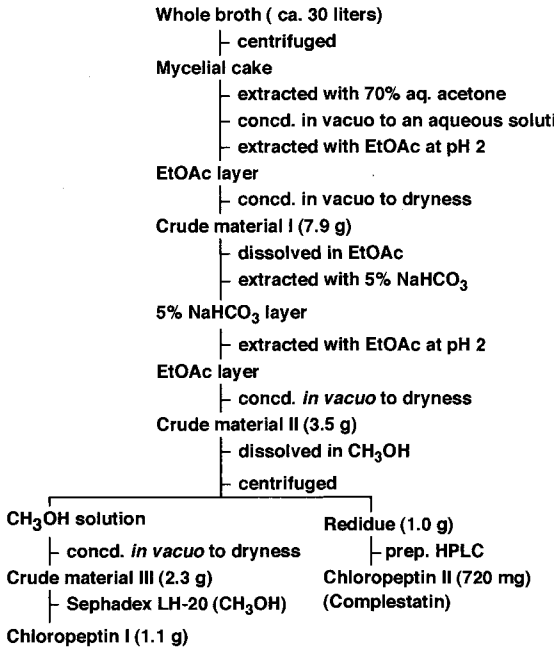
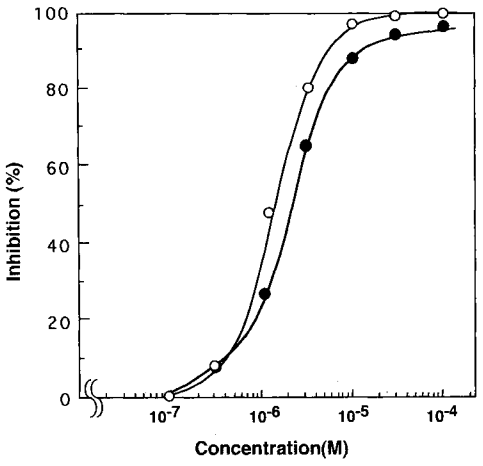


Table 3. Physico-chemical properties of chloropeptins I and II.

	Chloropeptin I	Chloropeptin II (complestatin)
Appearance	Pale yellow brown powder	Yellow powder
Molecular formula	$C_{61}H_{45}N_7O_{15}Cl_6$	$C_{61}H_{45}N_7O_{15}Cl_6$
HRFAB-MS*m/z* (M^+)	Found 1325.1093 Calcd. 1325.1105	Found 1325.1122 Calcd. 1325.1105
MP	>300°C	>300°C
$[\alpha]_D^{26}$ (DMSO)	-18.8°(*c*=1.6)	+16.3°(*c*=1.6)
UV λ_{max}nm(ε) (in CH_3OH)	214(64,600), 239(sh), 285(sh), 291(14,600), 304(sh)	213(62,800), 239(sh), 283(14,400), 288(14,300)
UV λ_{max}nm(ε) (in CH_3OH-0.01N NaOH)	242(72,500), 283(sh), 292(16,100), 307(16,200), 357(21,900)	210(66,800), 241(sh),291(sh), 307(sh), 357(11,400)
IR $\nu_{max}cm^{-1}$ (KBr)	3280, 3060, 1640, 1500, 1490, 1410, 1210	3390, 3280, 1650, 1500, 1490, 1410, 1210
Color reaction positive to negative to	 Ehrlich Ninhydrin, Dragendorff	 Ehrlich Ninhydrin, Dragendorff
Solubility soluble in insoluble in	 DMSO, pyridine, alkaline water, CH_3OH hexane, $CHCl_3$, EtOAc, water acetone, benzene	 DMSO, pyridine, alkaline water, hexane, $CHCl_3$, EtOAc, water acetone, benzene

Fig. 5. Inhibitory activities of chloropeptin I and complestatin against gp120-sCD4 binding.



Chloropeptin I (○) or complestatin (●) and rsCD4 (1.0 μg/ml) at the indicated concentration were added to rgp120-coated plates and then incubated for 60 minutes. at room temperature in the presence of 5.0 mg/ml BSA. The rgp120-bound rsCD4 was determined by ELISA as described in Materials and Methods. Results are expressed as inhibition (%) against control gp120-CD4 binding in the absence of a sample.

(IFO-4581) and *Aspergillus niger* KF103 (ATCC-6275)).

Chloropeptin I administered intravenously to mice at a dose of 100 mg/kg did not cause acute toxicity.

Combination of Chloropeptin I and AZT, ddI, ddC or Nevirapine in *In Vitro* Anti-HIV Assay

Next, we investigated whether chloropeptin I showed synergistic or additive effects when it was used concomitantly with reverse transcriptase inhibitors. We first investigated the antiviral effect of chloropeptin I combined with AZT in acutely HIV-infected MT-4 cells, which was monitored by the protection against HIV-induced CPE. The calculated additivity surface was subtracted from the experimental surface and plotted in 3-D form (Fig. 6A). These plots would reveal a horizontal plane at 0% if the interactions were purely additive. The combinations of chloropeptin I and AZT 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 present above the calculated additivity. When we tested

Table 4. Anti-HIV-1 activities of chloropeptin I, complestatin, dextran sulfate and AZT.

Compound	CPE [a]			Fusion [b]
	EC_{50} (μM)	CC_{50} (μM)	SI	IC_{50} (μM)
Chloropeptin I	1.6	>600	>380	0.5
Complestatin	1.7	530	320	1.1
Dextran sulfate	2.9 (μg/ml)	>1000 (μg/ml)	>350	2.1 (μg/ml)
AZT	0.011	260	25000	—

a) Inhibition effects of chloropeptin I, complestatin, dextran sulfate and AZT on HIV-1-induced cytopathic effect (CPE) in MT4 cells. The viability of virus- and mock-infected cells was assessed by the MTT method as described in Materials and Methods. Anti-CPE effects are expressed as the EC_{50} value compared with the CC_{50} value against mock-infected control cells. Selectivity index (SI) was the ratio of IC_{50} for CPE to CC_{50}.

b) Inhibition effects of chloropeptin I, complestatin and dextran sulfate on HIV-1-induced syncytia formation in coculture of virus-infected and uninfected Molt-4 cells. The extent of cell fusion was assayed as described in Materials and Methods. Anti-cell fusion effects are expressed as IC_{50} value against fusion of control cells in the absence of a sample.

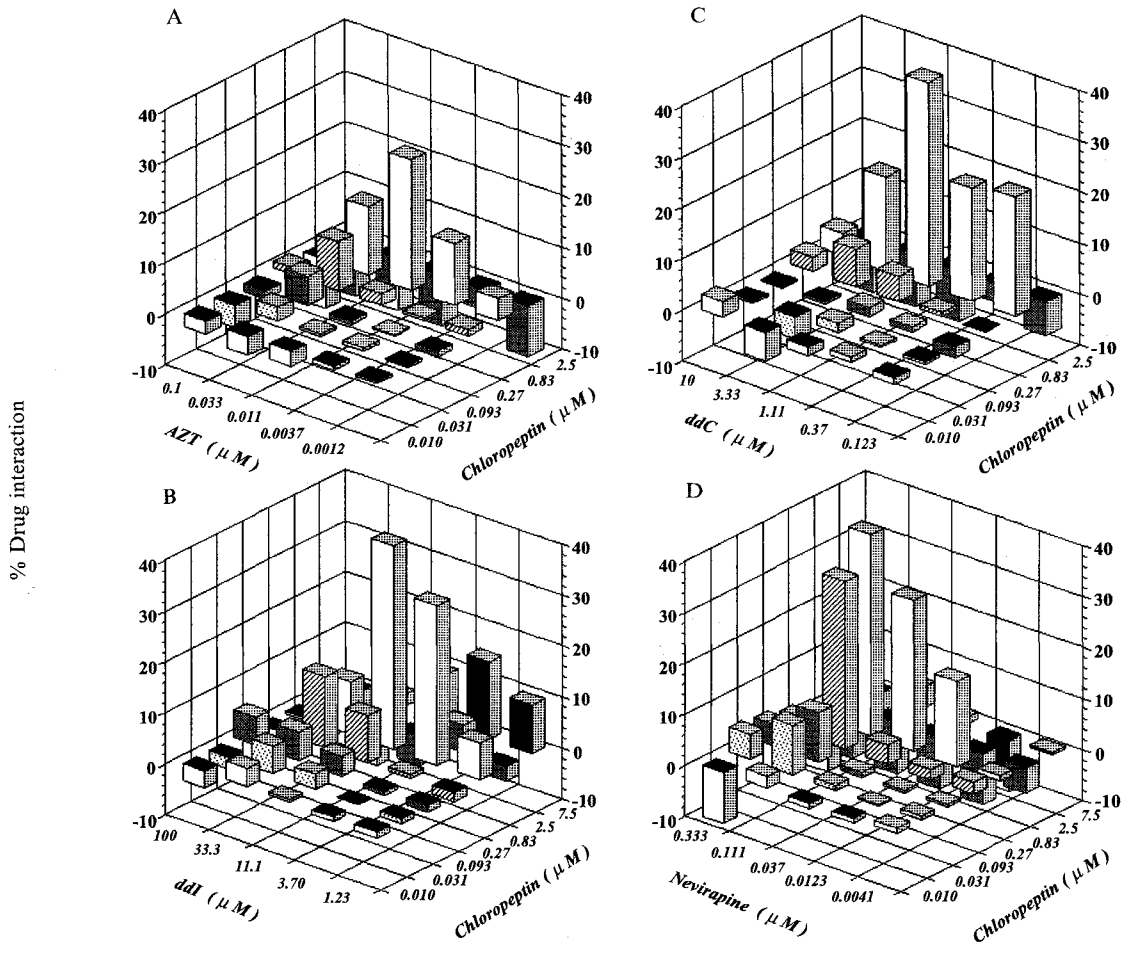
Table 5. Antimicrobial activities of chloropeptin I and complestatin assayed using paper discs.

Microorganism	Inhibitory zone (Φ mm)			
	Chloropeptin I		Complestatin	
	1.0	0.25	1.0	0.25 (mg/ml)
Staphylococcus aureus FDA 209P	11.3	9.8	11.0	9.7
Micrococcus luteus PCI 1001	13.8	11.0	13.1	10.8
Bacillus subtilis PCI 219	11.1	9.6	11.0	9.4
Acholeplasma laidlawii PG-8 KB 174	12.4*	-	11.0*	-

* hazy zone

50 μl/8 Φ mm disc

Fig. 6. Combined drug interaction when HIV-1-infected MT-4 cells were treated with chloropeptin I and AZT (A), ddI (B), ddC (C) or nevirapine (D).



The calculated additivity surface was subtracted from the experimental surface and plotted in 3-D form. The amount of synergy is represented by the height of the bars.

the combination effect of chloropeptin I and ddI, ddC and nevirapine, a synergistic antiviral effect was also observed (Figs. 6B, 6C and 6D). In particular, the combinations of compounds at concentrations near to their EC_{50} values (*e.g.*, chloropeptin I in test ranges from 0.37 to 3.3 μM and AZT at 0.0037~0.033 μM, ddI at 3.7~33.3 μM, ddC at 0.1~3.3 μM or nevirapine at 0.037~0.32 μM, respectively) showed significant synergistic interactions.

Discussion

None of the drugs tested to date for anti-HIV treatment is curative. Only several inhibitors against viral reverse transcriptase and protease have demonstrated any clinical benefit. The search of more effective drugs and other targets for antiviral therapy is thus extremely important. In the present study, we found the novel hexapeptide chloropeptin I as the first gp120-CD4 binding inhibitor from *Streptomyces* with potent inhibitory activities against HIV-1 induced cytopathic effects and syncytium formation. Another gp120-CD4 binding inhibitor (chloropeptin II) isolated from the same strain was identified as complestatin[19,20]. Complestatin also exhibited anti-HIV activity *in vitro* and inhibited syncytium formation as reported by MOMOTA *et al.*[21]. However, it was less soluble than chloropeptin I and a little inferior in terms of selective toxicity.

Chloropeptin I inhibited not only gp120-CD4 binding [1.3 μM with BSA (5.0 mg/ml)], but also HIV-1-induced cytopathic affect assayed in MT-4 cells (EC_{50}: 1.6 μM) and syncytium formation in MOLT-4 and MOLT-4/HIV-1_{IIIB} cells (IC_{50}: 0.5 μM), suggesting that the inhibition of cytopathic effect and syncytium formation would be due to the inhibition of gp120-CD4 binding.

Some biological activities of chloropeptin I, a novel chlorinated hexapeptide, were compared with those of vancomycin, a glycopeptide antibiotic that contains a chlorinated heptapeptide. Vancomycin did not inhibit gp120-CD4 binding even at a concentration of 100 μM,

while chloropeptin I exhibited only very low antimicrobial activity as described above. The structure-activity relationship among such peptides is of interest.

In this study, we also investigated whether chloropeptin I had synergistic antiviral activities when combined with other anti-HIV drugs. Using a 3-D analysis method, we found that chloropeptin I was synergistic when combined with the reverse transcriptase inhibitors AZT, ddI, ddC or nevirapine. Especially, the combined treatment with concentrations near the EC_{50} values of the compounds showed relatively strong synergistic interactions. This suggests that higher anti-HIV activities can be expected if chloropeptin I is used with a reverse transcriptase inhibitor.

The above results indicated that the gp120-CD4 binding inhibitor discovered here has *in vitro* anti-HIV activity. Furthermore, these findings suggest that chloropeptin I might provide a lead compound for the development of new HIV therapies, and that low molecular weight inhibitors of gp120-CD4 binding might be combined with a reverse transcriptase inhibitor to improve the management of HIV disease.

Acknowledgments

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