Table 1
Hammett constants (σ) and steric effects (E_s) of substituted aromatic rings and anti-HIV activity and cytotoxicity of synthetic compounds

Compd	R[a]	σ[b]	E_s[c]	IC_{50}[e] (μM)				CC_{50}[e] (μM)
				Lab. isolates		Primary isolates		
				IIIB (X4)	89.6 (dual)	fTOI (R5)	KYAG (R5)	
1	Br	0.23	−1.16	4	9	ND	ND	150
2	Cl	0.23	−0.97	8	10	5	>30	170
5	H	0	0	>100	>100	81	>100	350
6	F	0.06	−0.46	61	81	ND	>100	320
7	CH_3	−0.17	−1.24	23	41	16	51	210
8	OCH_3	−0.27	−0.55	>100	>100	ND	>100	340
9	CF_3	0.54	−2.40	ND	27	ND	ND	72
12	NO_2	0.78	−1.77[d]	ND	42	ND	ND	230
sCD4				0.010	0.021	0.0044	ND	ND

[a] See Schemes 1 and 2.
[b] σ = Hammett constant of a substitutent on a benzoic acid derivative.[16]
[c] E_s = steric effect of a substituent at the *para* position on the aromatic ring.[16a,17]
[d] The average value of −1.01 and −2.52, which are E_s values of the NO_2 group, −1.77, was used.
[e] Values are means of at least three experiments (ND = not determined).

(R = Cl) (σ = 0.23, E_s = −0.97), **6** (R = F) (σ = 0.06, E_s = −0.46) and **5** (R = H) (σ = 0, E_s = 0). This is the order of substituents' electron-withdrawing ability and also of their size. Methyl (σ = −0.17, E_s = −1.24) is an electron-donating group, but is almost as bulky as a bromine atom. Thus, the *p*-methyl derivative **7** has relatively potent anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B), higher than that of compound **6** (R = F) but lower than that of compound **1** (R = Br) or **2** (R = Cl). The electron-donating ability of a methoxy group is stronger (σ = −0.27), but the bulk size is smaller (E_s = −0.55), than that of a methyl group. Thus, the *p*-methoxy derivative **8** has no significant anti-HIV activity against all strains tested at concentrations below 100 μM. Two derivatives containing bulkier and more potent electron-withdrawing substituents such as trifluoromethyl (R = CF_3) (σ = 0.54, E_s = −2.40) and nitro (R = NO_2) (σ = 0.78, E_s = −1.77) at the *p*-position of the phenyl ring were evaluated. Compounds **9** (R = CF_3) and **12** (R = NO_2) showed significant anti-HIV activity against an 89.6 (dual, Sub B) strain. These are less potent than compounds **1** and **2** and this is perhaps due to the excessive size of the substituents at the *p*-position. This suggests that a certain level of the bulk size and a potent electron-withdrawing ability of the substituents are preferable for anti-HIV activity. It is estimated that a cavity around the *p*-position of the phenyl ring of CD4 mimicking compounds would be optimally filled by bromine (E_s = −1.16) or a methyl group (E_s = −1.24) at *p*-position, and that an electron-deficient aromatic ring might interact tightly with a negatively charged group such as carboxy of Glu^{370}. In isothermal titration calorimetry (ITC) experiments reported elsewhere,[10c] compound **5** (R = H) does not have significant affinity for gp120, and compound **6** (R = F) has less potent affinity for gp120 than compound **2**, consistent with the present data. In all but one of the compounds, no significant cytotoxicity was detected (CC_{50} >150 μM, Table 1), the exception being compound **9** (R = CF_3) (CC_{50} = 72 μM). Compounds **7** and **12** have relatively low cytotoxicities, compared to compounds **1** and **2**.

Fluorescence activated cell sorting (FACS) analysis was performed[15] to investigate whether these synthetic compounds interact with gp120 inducing the conformational change necessary for the approach of an anti-envelope antibody or a co-receptor to the gp120. The profile of binding of an anti-envelope CD4-induced monoclonal antibody, 4C11, to the Env-expressing cell surface (an R5-HIV-1 strain, JR-FL,-infected PM1 cells) pretreated with the above CD4 mimic analogs was examined. Comparison of the binding of 4C11 to the cell surface was measured in terms of the mean fluorescence intensity (MFI), and is shown in Figure 2. Pretreatment of the Env-expressing cells with compound **2** (MFI = 38.42) produced a remarkable increase in binding affinity for 4C11, similar to that observed in pretreatment with sCD4 (MFI = 37.90). This is consistent with the results in the previous paper[10] where it was reported that compound **2** enhances the binding of gp120 to the 17b monoclonal antibody which recognizes the co-receptor binding site of gp120. Env-expressing cells, which were not pretreated with sCD4 or a CD4 mimic compound, did not show significant binding affinity for 4C11 (Fig. 2, blank). The increase in binding affinity for monoclonal antibodies may be due to conformational changes in gp120, which were caused by the interaction of sCD4 or a CD4 mimic with gp120. It is hypothesized that such conformational changes involve the exposure of the co-receptor binding site of gp120 (the V3 loop), which is hidden internally, since the binding of gp120 to 17b is enhanced. Compound **5**, which failed to show significant anti-HIV activity, and compounds **7**, **9** and **12**, which had significant anti-HIV activity, were assessed in the FACS analysis. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **5** (MFI = 14.34) was similar to that of the blank (MFI = 11.24), suggesting that compound **5** offers no significant enhancement of binding affinity for 4C11. This result is compatible with the anti-HIV activity of compound **5**. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **7** (MFI = 38.33) was entirely similar to that of compound **2** used as a pretreatment. Pretreatment of the cell surface with compounds **9** and **12** (MFI = 29.09 and 30.01, respectively) produced a slightly lower enhancement of binding affinity for 4C11, compared to those of compounds **2** and **7** as pretreatments. However, in the ITC experiments reported elsewhere,[10c] compound **9** (R = CF_3) has a high affinity for gp120, comparable to that of compound **2**, but compound **12** (R = NO_2) does not have significant affinity for gp120, indicating that these are not consistent with the current FACS studies, possibly due to the difference in the assay systems. Although the anti-HIV activity of **7** is weaker than that of compound **2**, the level of compound **7** inducing an enhancement of binding affinity of gp120 for 4C11 is comparable to that of compound **2**. The concentration of compounds used in the FACS analysis was 100 μM, much beyond the IC_{50} values of compounds **2** and **7**. A concentration of 100 μM would be also sufficient for the expression of anti-HIV activity caused by compounds **2** and **7**.

An effect on the use of compound **2** combined with another entry inhibitor was investigated. Analysis of the synergistic effects of anti-HIV agents was performed according to the median effect principle using the CalcuSyn version 2 computer program[18] to estimate IC_{50} values of compounds in different combinations. Combination indices (CI) were estimated from the data evaluated using the MTT assay

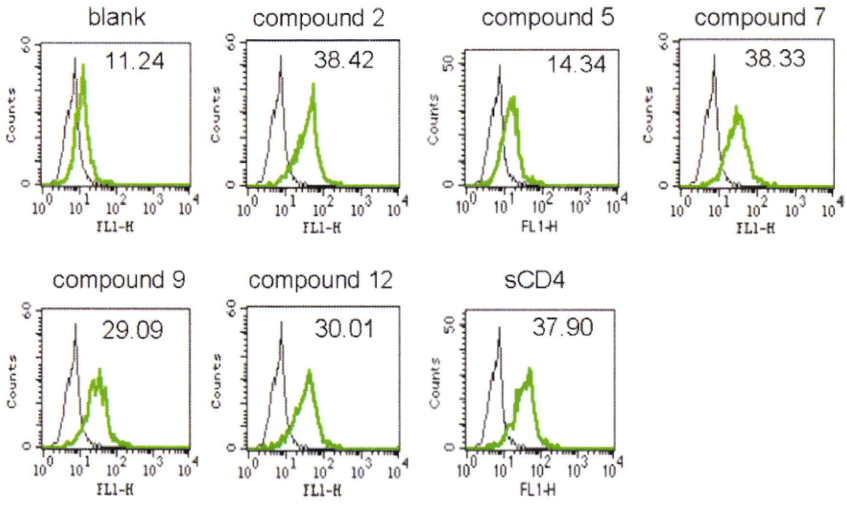

Figure 2. JR-FL (R5, Sub B) chronically infected PM1 cells were preincubated with 100 μM of a CD4 mimic or sCD4 (11 nM) for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of sCD4 or a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines). Data are representative of the results from a minimum of two independent experiments. The number at the top of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.

Table 2
Combination indices (CI) for compound **2** or sCD4 and a CXCR4 antagonist, T140, against an HIV IIIB strain

Combination	HIV strain	CI values at different IC[a]		
		IC_{50}	IC_{75}	IC_{90}
2 + T140	IIIB	0.786	0.713	0.655
sCD4 + T140	IIIB	0.705	0.528	0.400

[a] The multiple-drug effect analysis reported by Chou et al. was used to analyze the effects of combinational uses of compounds.[18] CI <0.9: synergy, 0.9 < CI < 1.1: additivity, CI >1.1: antagonism.

(Table 2).[15] Compound **2** showed a highly remarkable synergistic anti-HIV activity with a co-receptor CXCR4 antagonist, T140,[8a] against an X4-HIV-1 strain, IIIB at various IC values (IC_{50}, IC_{75} and IC_{90}). However, sCD4 exhibited a higher synergistic effect (lower CI values) with T140 (Table 2). The interaction of sCD4 or a CD4 mimic with gp120 would expose the co-receptor-binding site of gp120, and the co-receptor CXCR4 could then easily approach gp120. Thus, an inhibitory effect of a CXCR4 antagonist would be meaningful, and a significant synergistic effect might also be brought about by a combination of sCD4 or a CD4 mimic and T140.

In summary, a series of CD4 mimic compounds were synthesized and evaluated for their anti-HIV activity. Several compounds showed significant anti-HIV activity with relatively low cytotoxicity. SAR studies showed that a certain level of size and electron-withdrawing ability of the substituents at the *p*-position of the phenyl ring are suitable for potent anti-HIV activity. In addition, the treatment of Env-expressing cells with several CD4 mimicking compounds causes a conformational change, exposing the co-receptor-binding site of gp120 externally. Thus, a CD4 mimic exhibited a remarkable synergistic effect with a co-receptor antagonist. These compounds are essential probes directed to the dynamic supramolecular mechanism of HIV entry, and important leads for the cocktail therapy of AIDS.

Acknowledgments

This work was supported by Mitsui Life Social Welfare Foundation, Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare.

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11. The structure of compound **2** was built in Sybyl and minimized with the MMFF94 force field and partial charges. (see: Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490.) Docking was then performed using FlexSIS through its SYBYL

module, into the crystal structure of gp120 (PDB, entry 1RZJ). The binding site was defined as residues Val^{255}, Asp^{368}, Glu^{370}, Ser^{375}, Ile^{424}, Trp^{427}, Val^{430} and Val^{475}, and included residues located within a radius 4.4 Å. The ligand was considered to be flexible, and all other options were set to their default values. Figures were generated with ViewerLite version 5.0 (Accelrys Inc., San Diego, CA).

12. *For example, the synthesis of compound* **7**: To a solution of ethyl oxalyl chloride (0.400 mL, 3.48 mmol) in THF (20 mL) were added triethylamine (Et_3N) (0.480 mL, 3.48 mmol) and *p*-toluidine (373 mg, 3.48 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 6 h. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was extracted with EtOAc (50 mL), and the extract was washed successively with brine (20 mL), 1 M HCl (20 mL × 2), brine (20 mL), saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), then dried over $MgSO_4$. Concentration under reduced pressure gave the crude ethyl oxalamate, which was used without further purification. To a solution of the crude ethyl oxalamate (640 mg, 3.09 mmol) in THF (30 mL) were added aqueous 1 M NaOH (3.40 mL, 3.40 mmol), water (50 mL) and MeOH (20 mL) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After the addition of aqueous 1 M HCl (5 mL), MeOH and THF were evaporated under reduced pressure. The residue was acidified to pH 2 with 1 M HCl, and extracted with EtOAc (50 mL × 2). The combined organic layer was washed with brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude acid, which was used for the next reaction without further purification. To a solution of the above crude acid (514 mg, 2.87 mmol) in THF (10 mL) were added 1-hydoxybenzotriazole (484 mg, 3.16 mmol), 4-amino-2,2,6,6-tetramethylpiperidine (446 μL, 2.58 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (606 mg, 3.16 mmol) and Et_3N (0.439 mL, 3.16 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After evaporation of THF, the residue was dissolved in $CHCl_3$ (50 mL). The mixture was washed with saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude crystalline mass. The usual work-up followed by recrystallization from EtOAc–*n*-hexane gave the title compound **7** (363 mg, 1.14 mmol, 39.8%) as colorless crystals, mp = 176 °C; δ_H (400 MHz; $CDCl_3$) 1.07 (1H, m, NH), 1.16 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.44 (2H, m, CH_2), 1.91 (1H, d, *J* 3.7, C*H*H), 1.94 (1H, d, *J* 3.7, CH*H*), 2.34 (3H, s, CH_3), 4.25 (1H, m, CH), 7.17 (2H, d, *J* 8.3, ArH), 7.33 (1H, m, NH), 7.50 (2H, d, *J* 8.4, ArH), 9.18 (1H, s, NH); HRMS (FAB), *m*/*z* calcd for $C_{18}H_{28}N_3O_2$ $(MH)^+$ 318.2182, found 318.2173.
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14. *The synthesis of compound* **12**: To a solution of Et_3N (417 μL, 3.00 mmol) and 4-nitroaniline (138 mg, 1.00 mmol) in THF (1.3 mL) was added oxalyl dichloride (85.8 μL, 1.00 mmol) with stirring at 0 °C. After being stirred for 30 min at 0 °C, Et_3N (167 μL, 1.20 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine (156 μL, 0.90 mmol) were added. The reaction mixture was stirred for 6 h at 0 °C. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ (20 mL), and the mixture was washed successively with brine (10 mL), saturated $NaHCO_3$ (10 mL × 2) and brine (10 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with $CHCl_3$–MeOH (9:1) gave 42.4 mg (0.122 mmol, 13.5%) of the title compound **12** as colorless crystals, mp = 190 °C; δ_H (400 MHz; $CDCl_3$) 1.09 (1H, m, NH), 1.17 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.43 (2H, m, CH_2), 1.92 (1H, d, *J* 3.8, C*H*H), 1.95 (1H, d, *J* 3.8, CH*H*), 4.28 (1H, m, CH), 7.29 (1H, m, NH), 7.82 (2H, d, *J* 9.1, ArH), 8.28 (2H, d, *J* 9.1, ArH), 9.55 (1H, s, NH); HRMS (FAB), *m*/*z* calcd for $C_{17}H_{25}N_4O_4$ $(MH)^+$ 349.1876, found 349.1871.
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Remodeling of Dynamic Structures of HIV-1 Envelope Proteins Leads to Synthetic Antigen Molecules Inducing Neutralizing Antibodies

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A synthetic antigen targeting membrane-fusion mechanism of HIV-1 has a newly designed template with C3-symmetric linkers mimicking N36 trimeric form. The antiserum produced by immunization of the N36 trimeric form antigen showed structural preference in binding to N36 trimer and stronger inhibitory activity against HIV-1 infection than the N36 monomer. Our results suggest an effective strategy of HIV vaccine design based on a relationship to the native structure of proteins involved in HIV fusion mechanisms.

INTRODUCTION

Antibody-based therapy is one of the promising treatments for AIDS. In recent years, AIDS antibodies have been produced by immunization (*1*) and by de novo engineering of monoclonal antibodies (mAb) with molecular evolution tactics such as phage display (*2*). Despite enormous efforts, however, only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. These antibodies include gp41 Abs, 2F5 (*3*–*6*) and 4E10 (*5*–*7*), and gp120 Abs, 2G12 (*8*) and b12 (*9*). gp41 is a transmembrane envelope glycoprotein, which is divided into an endodomain and an ectodomain by the transmembrane region; the latter contains a hydrophobic amino-terminal fusion peptide, followed by amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domains with helical structures (HR1 and HR2, respectively). In the membrane fusion process of HIV-1, these subunits form a "pre-bundle" complex. The HR1 and HR2 regions are termed the N-terminal helix (N36) and C-terminal helix (C34), respectively. These helices form a six-helical bundle consisting of a central parallel trimeric coiled-coil of N36 surrounded by C34 in an antiparallel hairpin fashion. In design of immunogens that elicit broadly neutralizing antibodies, a useful strategy is to produce molecules that mimic the natural trimer on the virion surface. Previous studies show that these molecules could be proteins expressed as a recombinant form or on the surface of particles such as pseudovirions or proteoliposomes (*10*–*12*). The X-ray crystallographic study of gp41 shows that the distances between any two residues at the N-terminus of N-region are almost equal at approximately 10 Å (Figure 1A). A chemically synthetic template could be useful in connection with the design of a peptidomimetic corresponding to the native structure of gp41. To date, several gp41 mimetics have been synthesized as inhibitors or antigens and subjected to inhibition or neutralization assays (*13*–*16*). However, the templates for assembly of these helical peptides contain branched peptide linkers, which are not exactly equivalent in length (*14*). The N-terminal peptides constrained by another threefold linker showed high affinity for C-terminal peptides, although its biological advantages have not been determined (*15*). The mimicry can be estimated using the broadly neutralizing mAbs; suitable mimetics will bind neutralizing mAbs efficiently, but they will bind non-neutralizing mAbs poorly. In the present study, we designed and synthesized a novel three-helical bundle mimetic, which corresponds to the trimeric form of N36. We investigated whether mice immunized with the equivalent trimeric form of N36 mimetic can produce antibodies with stronger binding affinity for N36 trimer than for N36 monomer. This approach demonstrates the possibility of producing structure-specific antibodies by immunization of synthetic antigens corresponding to the natural form of viral proteins.

EXPERIMENTAL PROCEDURES

Conjugation of N36REGC and the Template to Produce triN36e. Compound **6** (100 μg, 0.174 μmol) and N36REGC (3.4 mg, 0.574 μmol) were dissolved in a mixture of 300 μL of 200 mM acetate buffer (pH 5.2) and 300 μL of TFE under a nitrogen atmosphere, then TCEP·HCl was added. The reaction was stirred for 72 h at room temperature and monitored by HPLC. The ligation product (triN36e) was separated as an HPLC peak and was characterized by ESI-TOF-MS, *m/z* calcd for $C_{690}H_{1160}N_{226}O_{201}S_3$ 15933.1, found 15933.8. The purification was performed by reverse phase HPLC (YMC-Pack ODS-A column, 10 × 250 mm). Elution was carried out with a 40–50% linear gradient of acetonitrile (0.1% TFA) over 50 min. Purified triN36e, obtained in 16% yield, was identified by ESI-TOF-MS. The detailed synthesis of peptides is described in the Supporting Information (SI).

CD Spectra. CD measurements were performed with a J-720 circular dichroism spectrolarimeter equipped with a thermoregulator (JASCO). The wavelength dependence of molar ellipticity [θ] was monitored at 25 °C from 190 to 250 nm. Peptides were dissolved in 20 mM acetate buffer (pH 4.0) containing 40% MeOH (*23*, *24*). The experimental helicity was calculated as reported previously (*17*–*19*).

Immunization and Sample Collection. Six-week-old male BALB/c mice were purchased from Sankyo Laboratory Service Corp. (Tokyo, Japan) and maintained under specific pathogen-free conditions in an animal facility. The experimental protocol was approved by the ethical review committee of Tokyo Medical and Dental University. Freund incomplete adjuvant and PBS

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10.1021/bc900502z
Published on Web 04/01/2010

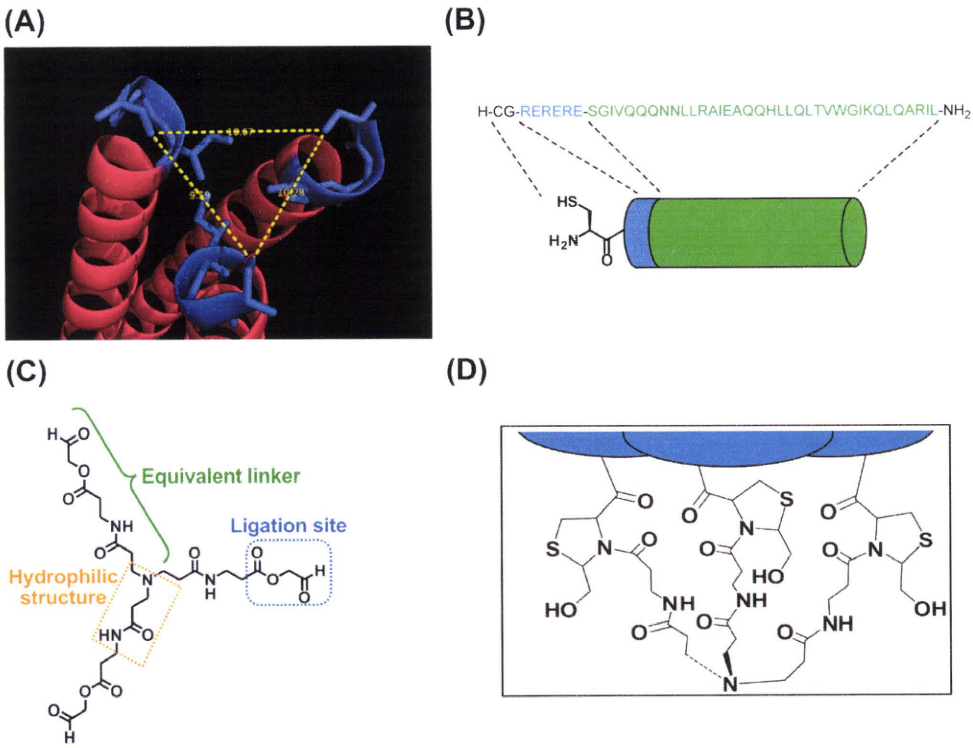


Figure 1. (A) Distances between hydrogen atoms for hydroxyl groups in N-terminal serine residues of N36 helices in trimeric form. The distances were evaluated by PyMOL (*21*). (B) Cartoon presentation of each N36 derived peptide, N36REGC. (C) Design of a C3-symmetric template. The amino acid residues are described in single letters. (D) Conjugated structure of trimeric N36 after thiazolidine ligation.

were purchased from Wako Pure Chemical Industries (Osaka, Japan). DMSO (endotoxin free) was purchased from Sigma-Aldrich (St. Louis, MO).

All mice were bled one week before immunization. One hundred micrograms of antigen was dissolved in 1 μL of DMSO. The solution was mixed with 50 μL of PBS and 50 μL of Freund incomplete adjuvant. The mixture was injected subcutaneously under anesthesia on days 0, 14, 28, 42, and 58. Mice were bled on days 21, 35, 49, and 65. Serum was separated by centrifugation (15 000 rpm) at 4 °C for 15 min and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

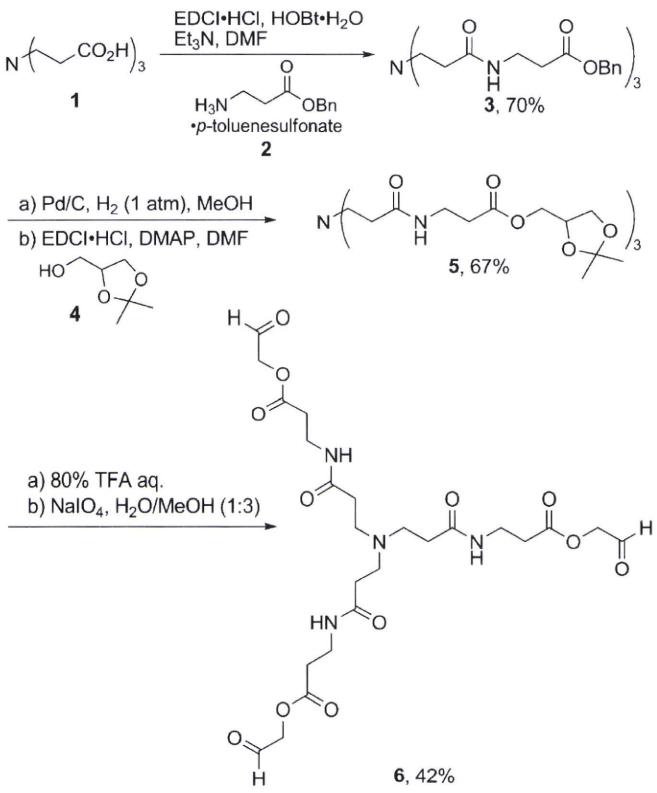
Serum Titer ELISA. Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and hydrogen peroxide (30%) were purchased from Wako. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was purchased from Sigma-Aldrich. Antimouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninty-six-well microplates were coated with 25 μL of a synthetic peptide at 10 μg/mL in PBS at 4 °C for overnight. The coated plates were washed 10 times with deionized water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed with deionized water 10 times. Mice sera were diluted in 0.02% PBST with 1% skim milk, and 50 μL of 2-fold serial dilutions of sera from 1/200 to 1/102400 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were washed 10 times with deionized water. Twenty-five microliters of HRP-conjugated antimouse IgG, diluted 1:2000 in 0.02% PBST, was added to each well. After 45 min incubation, the plates were washed 10 times and 25 μL of HRP substrate, prepared by dissolving 10 mg ABTS to 200 μLof HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities were measured at 405 nm.

Virus Preparation. The pNL4-3 construct (8 μg) was transfected into 293T cells by Lipofectamine LTX (Invitrogen, Carlsbad, CA) followed by changing medium at 12 h after transfection. At 48 h after changing medium, the supernatant was collected, passed through a 0.45 μm filter, and stored at −80 °C as HIV-1_{NL4-3} strain before use. For titration, MT-4 cells were infected with serially 3-fold diluted virus from 1/10 to 1/196830, and cultured for 7 days. HIV-1 p24 levels in supernatants were measured, and then the titer of virus solution was calculated.

Anti-HIV Assay. Virus was prepared as described above except that the transfection of pNL4-3 was performed by the calcium phosphate method. Anti-HIV-1 activity was determined on the basis of protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of AZT, N36RE, and triN36e (The starting concentrations are 100, 10, and 1 μM, respectively) were added to HIV-1-infected MT-4 cells (MOI = 0.01) by 2-fold serial dilution and placed in wells of a flat-bottomed microtiter plate (2.0×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (EC_{50}). Cytotoxicity of compounds was determined on the basis of viability of mock-infected cells using the MTT method (CC_{50}). Each experiment was performed three times independently.

Neutralizing Assay. MT4-cells (1×10^5 cells/100 μL) were incubated in 100 μL medium containing 10 μL sera from immunized or preimmunized mice for 1 h at 37 °C, then pretreated MT-4 cells were infected with HIV-1_{NL4-3} (MOI = 0.05). At 3 days after infection, cells were collected by centrifuge at 4000 rpm for 10 min at 4 °C. After discarding supernatant, pellets were lysed with 30 μL of lysis buffer (50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% NP-40), then 30 μL of 2 × SDS buffer (125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-ME, 0.004% BPB) were added and boiled for 10 min. The samples (5 μL) were subjected to SDS-page to perform Western blotting. The HIV-1 gag p24 was detected by using Western lightning ECL kit (PerkinElmer, MA) according to manufacturer's instruction after treatment of HIV-1 p24

Scheme 1. Synthesis of the Equivalently Branched Template 6



antibody (2C2; 1:2000 dilution) (*20*) and anti- mouse IgG (H+L)-HRP (Millipore, MA). The band intensity of p24 was calculated with post/pre-immunized samples by using *ImageJ* image analyzing software.

RESULTS AND DISCUSSION

The N-region of gp41 is known to be an aggregation site involving a trimeric coiled-coil conformation. In design of an N36-derived peptide (N36RE), the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase the solubility in buffer solution (Figure 1B). In order to form a triple helix corresponding precisely to the gp41 prefusion form, we designed the novel C3-symmetric template depicted in Figure 1C. This designed template linker has three branches of equal length and possesses the hydrophilic structure and ligation site for coupling with N36RE. The template was synthesized from the commercially available 3-[bis(2-carboxyethyl)amino]propanoic acid **1** as shown in Scheme 1. Coupling of **1** with β-alanine benzyl ester **2** gave the corresponding triamide **3** in 77% yield. Cleavage of three benzyl esters by hydrogenation and coupling with solketal **4** produced the corresponding triester **5**. Deprotection of the acetonides with aqueous 80% TFA followed by oxidative cleavage of diol group led to the desired template **6**. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected N36RE (N36REGC) with a three-armed aldehyde scaffold producing triN36e (Figure 1D). Thiazolidine ligation is a peptide segment coupling strategy which does not require side chain protecting groups (*22*−*26*). The reaction consists of three steps: (i) aldehyde introduction, in which a masked glycolaldehyde ester is linked to the carboxyl terminus of an unprotected peptide by reverse proteolysis; (ii) ring formation, in which the unmasked aldehyde reacts at acidic pH with the α-amino group of an N-terminal cysteine residue of the second unprotected peptide forming a thiazolidine ring; and (iii) rearrangement at higher pH in which O-acyl ester linkage is converted to an N-acyl amide linkage forming a peptide bond with a pseudoproline structure (Figure 2).

Circular dichroism (CD) spectra of triN36e and N36RE, which is a monomer form without N-terminal Cys-Gly residues, are shown in Figure 3A. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, suitable for measurement of CD spectra of membrane proteins (*27, 28*). Both spectra display double minima at 208 and 222 nm and showed high molar ellipticity as absolute values (Table 1). The results indicate that these peptides form a highly structured α-helix and that the helical content of the trimer triN36e is higher than that of the monomer N36RE. Furthermore, to assess the interaction of triN36e with C34, CD spectra of the peptide mixture with C34-derived peptide, C34RE, were measured (Figure 3B,C). The spectrum of triN36e and C34RE mixture showed high molecular ellipticity as an absolute value comparable with that of triN36e alone. This supports the conclusion that C34RE interacts with tri36e and thereby induces a higher helical form as shown previously (*29*).

Mice were immunized with these synthetic gp41 mimetics and antibody production was successfully induced (the detailed titer increase in 5 weeks' immunization is given in the Supporting Information). Two out of three mice showed induction of antibodies against either antigen (N36RE or triN36e). Antibody titers and selectivity of antisera isolated from mice immunized with N36RE or triN36e were evaluated by serum titer ELISA against coated synthetic antigens. The most active antiserum for each antigen was utilized for the evaluation of binding activity by ELISA (Figure 4). The N36RE-induced antibody showed approximately 5 times higher affinity for N36RE than for triN36e, as 50% bound serum dilutions are 3.88×10^{-4} and 2.14×10^{-3} to N36RE and triN36e, respectively. It is noteworthy that the triN36e-induced antibody showed approximately 30 times higher preference in binding affinity for triN36e antigen than for N36RE (serum dilutions at 50% bound are 3.83×10^{-3} to N36RE and 1.33×10^{-4} to triN36e). Although this evaluation was not determined with purified mAbs, it is clear that the antibodies produced exploit a structural preference for antigens. The mechanism of induction

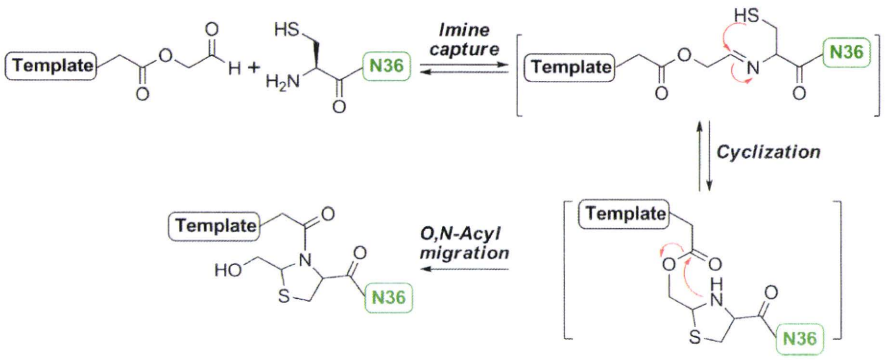


Figure 2. Reaction mechanisms of thiazolidine ligation utilized for assembly of N36RE helices on the template.

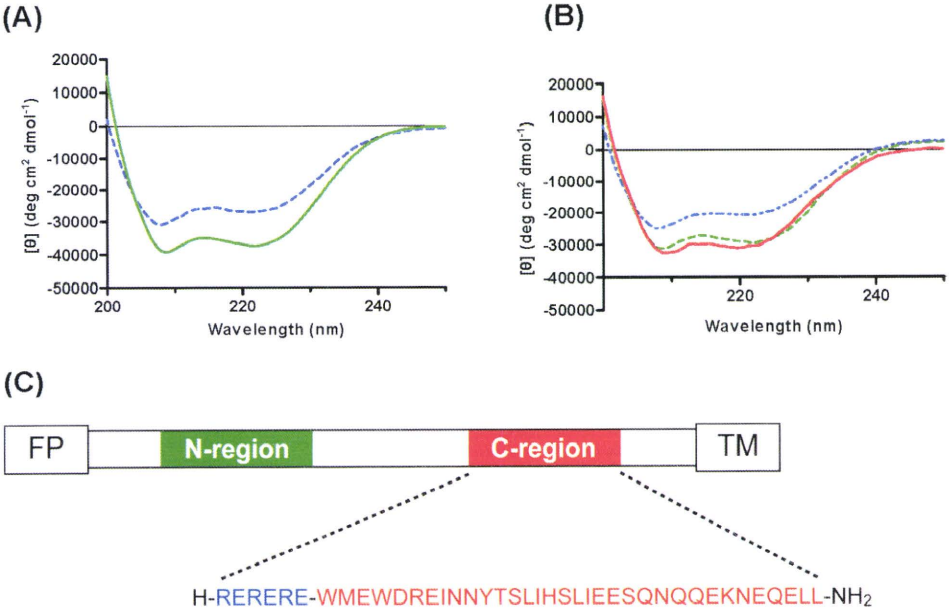


Figure 3. (A) Circular dichroism (CD) spectra of N36RE and triN36e. In the spectra, a blue dashed line and a green line show N36RE (monomer) and triN36e (trimer), respectively. Concentrations of the peptides are 10 and 3.3 μM for N36RE and triN36e, respectively. (B) CD spectra in the presence or absence of C34RE peptide. The spectra show the following: a dashed green line, triN36e; a dashed blue line, C34RE; a red line, triN36e+C34RE, respectively. The concentrations of peptides were as follows: triN36e (2.3 μM), C34-derived peptide C34RE (7 μM), and mixture of both peptides (3.5 μM each). (C) The amino acid sequence of C34RE described in single letters. FP and TM represent hydrophobic fusion peptide and transmembrane domain, respectively.

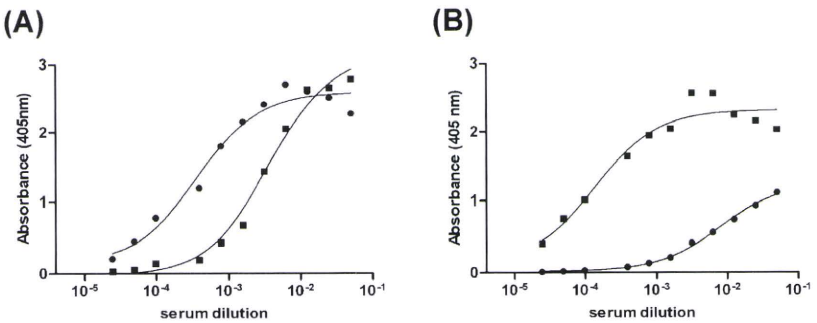


Figure 4. Serum titers of antibodies produced by N36 monomer and conformationally constrained N36 trimeric antigen. The titers were evaluated against N36RE (monomer) (A) and triN36e (trimer) (B). The plots indicate the results of sera obtained from N36RE-immunized mouse (•) and triN36e-immunized mouse (■).

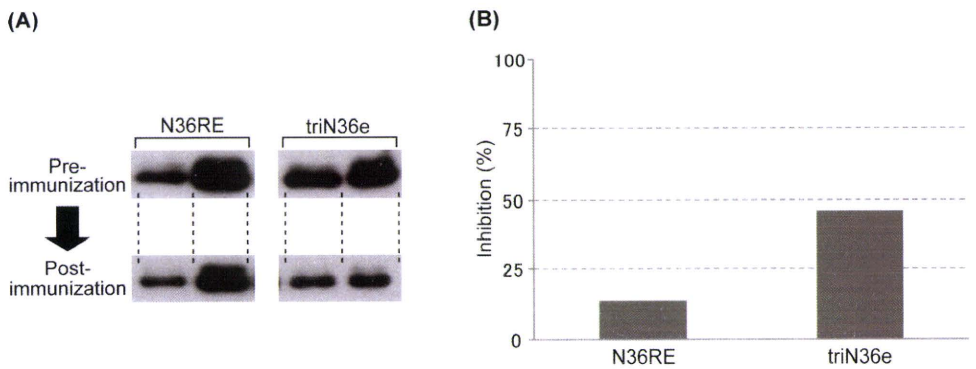


Figure 5. Determination of neutralization activity of the antibodies produced by immunization of peptidomimetic antigens. (A) Results of p24 assay to evaluate inhibition for HIV-1 infection by produced antibodies. Preimmunization sera were used as control. Experiments were duplicated. (B) Average % inhibition of p24 production calculated from the band intensities in panel (A).

of structure-specific antibody is still not clear, but the results could suggest the efficacy of producing antibodies with structural specificity and that the synthesis of structure-involving antigens is an effective strategy when higher specificity is required.

Neutralizing activity of sera against HIV-1 infection was assessed by p24 assays utilizing antisera from two mice that showed antibody production for each antigen (Figure 5). Sera

Table 1. Differences of α-Helicities between N36RE and triN36e Calculated from CD Spectra in Figure [3]

	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$	α-helicity
N36RE	−30 957	0.87	73%
triN36e	−38 998	0.96	95%

Table 2. EC_{50} and CC_{50} Values Calculated from Inhibition Assays of Peptidomimetics

	AZT	triN36e	N36RE
EC_{50} (μM)[a]	0.047	0.49	1.4
CC_{50} (μM)[b]	>50	>1	>10

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

from mice immunized with the same antigen showed similar inhibitory activity against viral infection (12.5% and 14.8% for N36RE, 40.3% and 52.1% for triN36e). A trend was observed that the sera from triN36e immunization shows higher inhibition than those from N36RE immunization. This suggests that the synthetic antigen corresponding to the N36 trimeric form induces antibody with neutralization activity superior to that of the monomer peptide antigen and implies a restricted response of B-cells upon immunization to the trimeric form of N36RE. In order to assess the compatibility of induced antibodies in HIV-1 entry inhibition, the HIV-1 inhibitory activities of peptidomimetics (N36RE and triN36e) have been evaluated by viral infection and cytotoxicity assays. A C-terminal region peptide known as Enfuvirtide (T20, Roche/Trimeris) has been used clinically as a fusion inhibitor, and its success indicates that gp41-derived peptides might be potent inhibitors, useful against HIV-1 infection (*30*). In the development of anti-HIV peptides, several mimetics such as Enfuvirtide, CD4 binding site of gp120 (*31*), and protein-nucleic acid interactions (*32*), which disrupt protein−protein interactions, have been produced. As indicated in Table 2, N36 and triN36e showed modest inhibitory activity as reported in previous studies (*33*−*35*). The potency of triN36e was three times higher than that of N36RE indicating that the active structure of monomer N36RE is a trimeric form. Cytotoxicity of the antigens was not observed at concentrations of 1 μM of triN36e and 10 μM of N36RE.

CONCLUSIONS

In summary, a mimic of HIV-1 gp41-N36 designed as a new vaccine has been synthesized utilizing a novel template with three branched linkers of equal lengths. Thiazolidine-forming ligation attached the esteraldehyde of three-branched template with N-terminal cysteine of peptides in an aqueous medium. The resulting peptide antigen successfully induces antibodies with neutralization activity against HIV-1 infection. It is of special interest that the antibody produced acquires structural preference to antigen, which showed 30 times higher binding affinity for trimer than for monomer. This indicates the effectiveness of the design based on the structural dynamics of HIV-1 fusion mechanism of an antigen which could elicit neutralizing antibodies. In a design based on the N36 region of gp41, the exposed timing of epitopes is limited during HIV-1 entry (*36*), and carbohydrates, which could make accession of antibodies to epitopes difficult, are not associated with the amino acid residues of the native protein. These two advantages could further enhance the potential of a vaccine design based on the N36 region. During preparation of the manuscript, a new HIV vaccine strategy was reported by Burton's group (*37*). The report describes the importance of antibody recognition for the trimer form of surface protein. The trimer-specific antibodies indicate broad and potent neutralization. The gp41 trimer-form specific antibody produced in this study could also obtain the corresponding properties. The elucidation of antibody-producing mechanisms and epitope recognition mode of antibodies in antiserum during HIV-1 entry will be addressed in future studies.

ACKNOWLEDGMENT

The authors deeply thank Prof. K. Akiyoshi (Tokyo Medical and Dental Univ.) for allowing access to CD spectroporalimeter.

Supporting Information Available: HPLC chromatograms and NMR charts of compounds **3**, **5**, and **6**. Results of ESI-TOF-MS, and HPLC chromatograms of peptides N36RE, N36REGC, and triN36e. Results of serum titer ELISA of antisera collected during immunization. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC900502Z

Peptide Science 2009: K. Okamoto (Ed.)
The Japanese Peptide Society (2010)

Synthesis and Evaluation of Artificial Antigen Peptide Based on the Trimeric Form of HIV Fusion Protein

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The trimer formation of the human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein gp41 is necessary for HIV infection. This membrane-fusion mechanism is mediated by the viral Env proteins (gp120/gp41) and receptor on the target cell. Our synthetic peptide antigen has a newly designed template with symmetric linkers mimicking N36 trimeric form. The immunization of the antigen successfully induced antibody against N36. Interestingly, the antibody specifically recognizes the trimeric N36. Our results indicate the strategy of HIV vaccine design based on the native structure of proteins related to HIV fusion mechanisms.

Keywords: antigen, HIV, thiazolidine ligation, vaccine

Introduction

The antibody-based therapy of HIV-1 is a promising treatment of AIDS. In recent years, antibodies for this purpose have been produced by immunization approach and de novo engineering of mAb with molecular evolution tactics such as phage display. Despite enormous efforts, only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. They include gp41 Abs 2F5 and 4E10 and the gp120 Abs 2G12 and b12 [1-3]. The gp41 is a transmembrane envelope glycoprotein. This subunit is devided by the transmembrane region into an endodomain and an ectodomain; the latter contains a hydrophobic amino-terminal fusion peptide, followed by an amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domain with a helical structure (HR1 and HR2, respectively). In membrane fusion process of HIV-1, these subunits form "pre-bundle" complex. The HR1 and HR2 regions are referred as N-terminal helices (N36) and C-terminal helices (C34), respectively. These helices forms six-helix bundle consists of a central parallel trimeric coiled-coil of N36 surrounded by C34 in an antiparallel hairpin fashion in fusion mechanism. In design of immunogens that elicit broadly neutralizing antibodies, one of the strategies is to produce molecules that mimic the mature trimer on the virion surface. These molecules can be recombinant or expressed

on the surface of particles such as pseudovirions or proteoliposomes. The degree of mimicry can be estimated using the broadly neutralizing mAbs; suitable mimics will bind to neutralizing mAbs well but to non-neutralizing mAbs poorly. Based on this approach, the chemically constrained trimeric form of N36 was designed and synthesized. The present trimeric form of N36 has an advantage in term of equivalency of helices. The helices are condensed to the template with equivalent linkers in length. Mice were immunized with the equivalent trimeric form of N36 mimic, and produced antibody indicated the stronger binding affinity for N36 trimer than for N36 monomer. This approach shows the possibility of producing structure-specific Abs by immunization of synthetic antigens corresponding to natural form of viral proteins.

Results and Discussion

Design and synthesis of trimeric antigen mimics gp41-N36 of HIV-1.

It has been shown that N36 mimic peptide-antigen involves trimeric coiled-coil of N-region in gp41, which comprises of three N36 derived peptides NP102 and a novel small molecular template for peptide assembly. Several peptide mimics have been synthesized and challenged to neutralization assays [4, 5]. The templates assembling three helical strands contain branched peptide-linkers, which is not completely equivalent in length. The N-region of gp41 is known as a trimeric coiled-coil conformation and as an aggregation site. In design of N36-derived peptide NP102, the triplet repeat of arginine and glutamic acid was fused to the N-terminus to increase solubility in buffer. The template was designed to be compatible for hydrophilicity required in thiazolidine ligation [6] with NP103. The three branched linker was designed to be equivalent in length and was elongated by an amide bond for the solubility in buffer utilizing 3-[bis(2-carboxyethyl)amino]propanoic acid as a starting material. This approach uses thiazolidine ligation for chemoselective coupling of Cys-containing unprotected NP103 with an aldehyde scaffold containing three arms to produce NP104. In this linker design, the equivalency in length would be important for the formation of triplet helix corresponding to the gp41 pre-fusion form (Fig. 1).

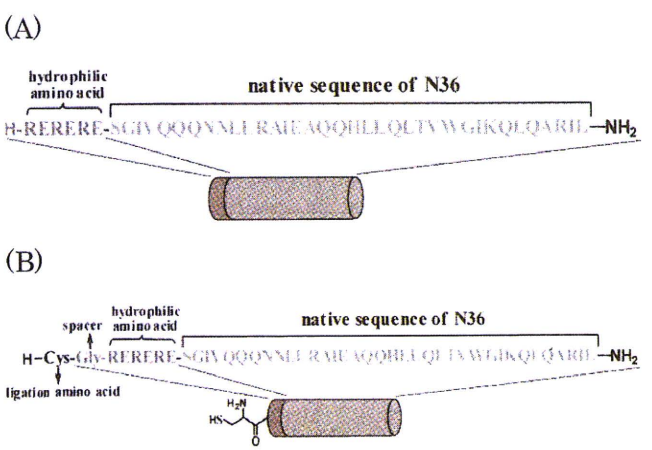

Fig. 1. Amino acid sequences of monomer helix, NP102 (A), and NP103 with unprotected cystein at N-terminal (B). The template is described in (C).

CD spectra of NP102 and NP104

CD spectra of NP104 and NP102 displayed double minima at 208 and 222 nm. The peptides were dissolved in 20 mM acetate buffer with 40% MeOH, pH4.0, which was previously reported as suitable condition for measurement of CD spectra of membrane proteins. The spectra of NP102 and NP104 peptides showed high molar elipticities ($[\theta]_{220}$ -30,957 and -38,998 deg•cm^2•dmol^{-1}). The results are indicative of a highly α-helical structure of the peptides. Furthermore, to assess the interaction of NP104 with C34, CD spectra of the peptides were measured as follows; NP104 (2.3 μM), C34 derived peptide-C34RE (7 μM) and mixture of both peptides (3.5 μM each). The spectrum of NP104 and C34RE mixture showed comparable molecular eliplicity with NP104 alone. It indicates that C34RE is associated with NP104 and induces higher helical form as shown previously. This result indicated that NP104, a N36 equivalently-constrained trimer, could form six-helix bundle with C34RE corresponding to the native structure of gp41.

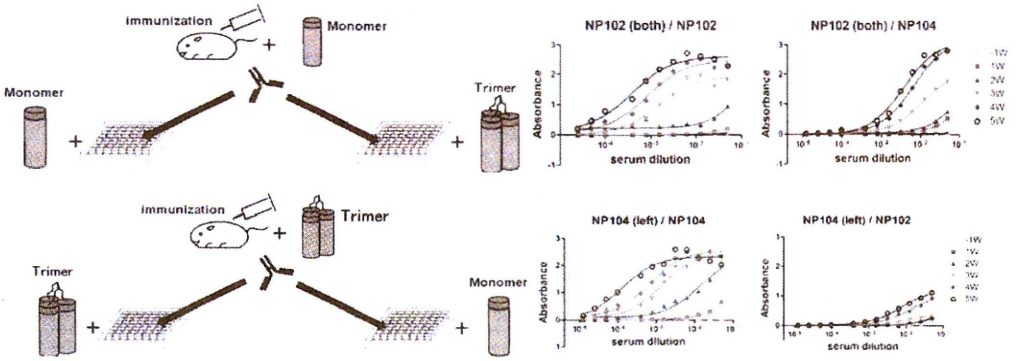

Fig. 2. Immunization and the titer ELISA monitoring induction of antibody. The right panels show specific recognition of NP102 (monomer) and NP104 (tirmer) derived antisera to monomer and trimer antigens.

Immunization to mice and antibody production.

Antibody titers and selectivity of antisera from mice immunized with NP102 and NP104 for antigens were evaluated by ELISA against coated synthetic antigens. The results indicated that each antigen has enough antigenicity. To assess the specificity of induced antisera to antigens, the binding to both antigens, NP102 and NP104, was compared (Fig. 2). The NP102-induced antiserum showed approximately 5 times higher affinity for NP102 than for NP104 (50% bound antisera dilutions are 3.84×10^{-4} and 2.14×10^{-3} to NP102 and NP104, respectively). Interestingly, the NP104 induced antiserum showed approximately 30 times higher preference in binding affinity for NP104 antigen than for NP102 (50% bound antisera dilutions are 3.83×10^{-5} and 1.33×10^{-4} to NP102 and NP104, respectively). This evaluation is not dissected with purified mAbs, however, it is clear that produced antibodies exploit structural preference for antigen. The mechanism of induction of structure-specific antibody is still not clear. The results might indicate the efficacy of producing Abs with structural-specificity, and at least the synthesis of structurally-corresponding antigens is one of the effective strategies when higher specificity is required.

p24 assay

Neutralizing activity of antisera against HIV-1 was assessed by p24 assay. Three mice are immunized with each antigen (NP102 or NP104). The results of the assays showed two immunized mice produced Abs. Sera induced by the same antigen immunization showed comparable inhibition rates (12.5% and 14.8% for NP102, 40.3% and 52.1% for NP104). The sera from NP104 immunization showed

approximately 4 times higher inhibition rates. This result indicates that the synthetic antigen corresponding to N36 trimeric form is superior to the monomer peptide antigen. The Abs producing mechanism of structural mimetics is unclear, however, this approach caused higher inhibition against HIV-1 infection.

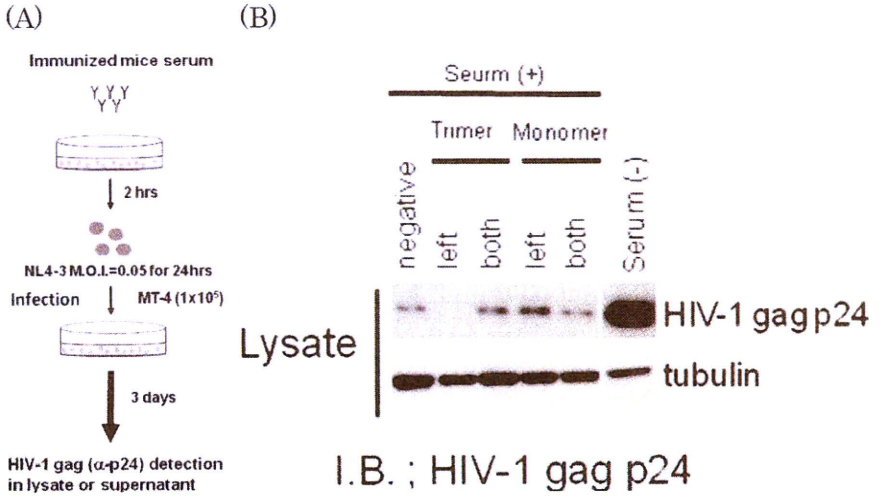

Fig.3. Experimental procedure of p24 assay (A) and results of the assay showing neutralization activity of antiserum derived from trimer-immunized mice (B).

Conclusions

In this study, we synthesized a trimeric peptide antigen mimicking N36 of HIV-1 gp41 using a novel template with three equivalent linkers centering nitrogen as a new vaccine design. The thiazolidine ligation was used to connect esteraldehyde of the three-branched template with N-term cysteine of peptides in aqueous condition. This peptide antigen successfully induced neutralizing against HIV-1 infection. Of special interest in the properties of produced Abs is that the structural-preference to antigen is 30 times higher to the trimeric form than to the monomeric form in binding. Ab-inducing mechanism or recognition mode for fusion mechanism of HIV-1 should be dissected, however, this study indicates the effectiveness of antigen design based on the structural dynamics in HIV-1 fusion mechanism.

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Peptide Science 2009: K. Okamoto (Ed.)
The Japanese Peptide Society (2010)

From Reverse to Forward Chemical Genomics: Development of Anti-HIV Agents

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To date, several anti-HIV agents have been developed based on reverse chemical genomics, in which target molecules are fixed. We found that CD4 mimic compounds have a great effect on the dynamic supramolecular mechanism of HIV entry, and that their combinational use with CXCR4 antagonists or with neutralizing antibodies shows a synergistic effect. On the other hand, based on forward chemical genomics, in which active compounds are searched according to the screening of random libraries, effective leads such as integrase inhibitors and matrix peptides have been found.

Keywords: CD4 mimic, forward chemical genomics, integrase inhibitor, matrix peptide, reverse chemical genomics

Introduction

Recently, highly active anti-retroviral therapy (HAART), which involves a combinational use of reverse transcriptase inhibitors and HIV protease inhibitors, has brought us a great success in the clinical treatment of AIDS patients. However, HAART has serious clinical problems including the emergence of multi-drug resistant HIV-1 strains. These drawbacks encouraged us to find novel drugs and increase repertoires of anti-HIV agents with various action mechanisms. The recent disclosing of the dynamic supramolecular mechanism in HIV-entry has provided potentials to find a new type of drugs. To date, we have synthesized HIV-entry inhibitors, especially coreceptor CXCR4 antagonists. In the present study, CD4 mimics in consideration of synergic effects with other entry inhibitors or neutralizing antibodies were developed. The development of the above anti-HIV agents is based on the concept of reverse chemical genomics, in which target molecules are fixed. On the other hand, based on the concept of forward chemical genomics, in which active compounds are discovered according to the screening of random libraries, effective peptide leads such as integrase inhibitors and matrix (MA) peptides were searched.

Results and Discussion

According to the concept of reverse chemical genomics, CD4 mimic compounds were developed based on NBD-556 [1, 2] (Fig. 1). Several compounds caused conformational change of an HIV surface protein, gp120. FACS analysis showed that in the presence of several compounds the anti-V3 antibody strongly binds to envelope-expressing cells. In addition, a synergistic effect of CD4 mimic compounds with an anti-V3 antibody

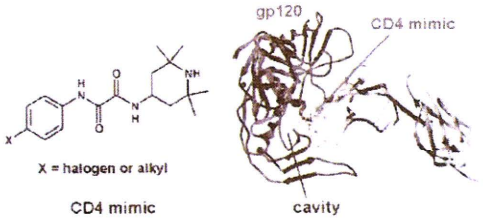

Fig. 1. Structures of CD4 mimic compounds (left) and the complex of gp120 and CD4 (right).

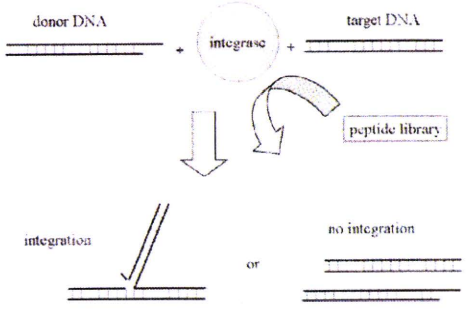

Fig. 2. Integrase inhibition assay using random peptide libraries.

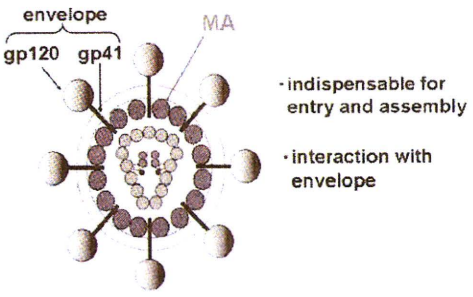

Fig. 3. Matrix (MA) in HIV particle.

or with a CXCR4 antagonist T140 was observed. According to the concept of forward chemical genomics, anti-HIV lead compounds were searched by the screening of random peptide libraries based on HIV-1 proteins. First, potent peptide leads were found by an integrase inhibition assay [3]. Second, effective leads were found in overlapping peptide libraries of MA.

As such, from a point of view on chemical biology, anti-HIV leads have been found utilizing reverse and forward chemical genomics. Furthermore, antibody-based therapy is still thought to be a promising treatment for AIDS. These anti-HIV agents might be important and useful compounds in consideration of cocktail therapy of AIDS. In addition, the present concept of chemical biology for the development of anti-HIV agents would be essential for drug discovery in medicinal chemistry.

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Peptide Science 2009: K. Okamoto (Ed.)
The Japanese Peptide Society (2010)

Caged DAG-Lactones for Study of Cellular Signaling in a Spatial- and Temporal-Specific Manner

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Protein kinase C (PKC) plays pivotal roles in cellular signal transductions. In order to elucidate PKC signaling mechanisms in detail, it is useful to develop functional molecules which can activate PKC in a spatial- and temporal-specific manner. We developed caged diacylglycerol (DAG)-lactones which can activate PKC by photoirradiation. Based on the photo-activation mechanism, the binding of DAG-lactones to PKC in cells was controlled.

Keywords: caged compound, diacylglycerol (DAG)-lactone, protein kinase C

Introduction

The signal transduction pathways attract a great interest especially in the interaction with small organic compounds in the field of chemical biology. To assess the phenomena induced by bioactive compounds, the addition of compounds could not be a sufficient way to observe the biological activity because the effects would be the whole cell scale and the signals would be multiple combined effects. To solve these problems, chemists have been developed "caged" compounds, which is not active when the pharmacophore is blocked by the photo-activatable molecules. Triggered by photo irradiation, which would be very limited ROI in the cell, the spatial- and temporal-specific effects of compounds would be observed. Several "caging" compounds have been developed and these molecules have their own advantages. In this study, we utilized the coumarin-based "caging" molecules, namely Bmc and Bhc" [1], to block the binding of DAG-lactones [2] to protein kinase Cδ [3] to assess their effects on translocation in cytoplasm.

Results and Discussion

Caged DAG-lactones 1-3 were successfully synthesized. The hydroxyl group of DAG-lactones which is a critical pharmacophore moiety was protected by a 6-bromo-7-methoxycoumarin-4-ylmethoxycarbonyl (Bmcmoc) group. The Bmcmoc group is a suitable phototrigger for alchols with high photochemical efficiency. To

evaluate the effect of caged groups on PKC binding of compounds, binding assay was performed by competition assay with [^{3}H]-PDBu. The results indicated the hydroxyl group is important for PKC binding as described previously. The loss of binding affinity was ranged from 110- to 400-folds (Fig. 1).

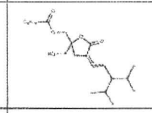
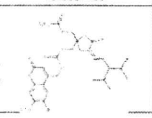
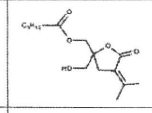
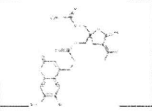
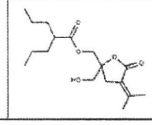
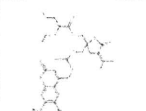
	DAG-lactones	clogP^a	K_i (nM)		Caged DAG-lactones	clog$P$	K_i (nM)
1		6.7	8.4	1C		9.1	3200
2		4.3	6.5	2C		6.7	940
3		3.2	22	3C		5.7	2500

Fig. 1. Binding assays of caged DAG-lactones 1-3. The structures of compounds are shown in columns. [a]computed logP.

Photolysis reaction of caged DAG-lactones by UV irradiation (350 nm) was monitored by HPLC analysis to find generation of DAG-lactones. The results revealed that the time to reach 90% conversion ($t_{90\%}$) of compounds **1** and **2** are about 6 min and 5 min, respectively. To further assess the properties of caged DAG-lactones, confocal laser microscopy analyses were utilized. Activation of PKC by exogenous ligands can be analyzed by its translocation in cells. The GFP-tagged PKC was expressed transiently in a CHO cell line. The addition of caged DAG-lactones showed no change in location of PKC in cells. The results correspond to the kinase activity of these compounds. On the contrary, the addition of DAG-lactones without a caging group showed clear translocation of PKC. These results indicate that photolysis of caged DAG-lactones could be a trigger of activation of PKC translocation. To assess the control of activation in spatial- and temporal manner, the area of photoirradiation was limited in a part of target cells. In this experiment, Bhc-protected DAG lactone 2, which showed faster activation of PKC, was utilized. The results showed clear translocation of PKC in the cell (Fig. 2).

It has been shown that caging technology is a powerful tool to investigate cellular functions. Our results indicate this technology could be applied to exogenous ligands of protein kinase C. The present results indicate that the activity of DAG-lactones can be controlled in a spatial- and temporal-manner by photo-removable protecting groups. This method would be a useful tool for elucidation of activation mechanism of PKC in mammalian cells.

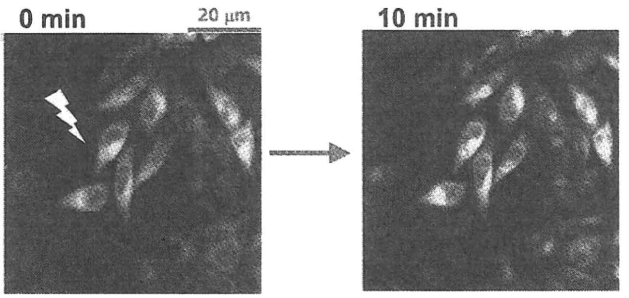


Fig. 2. The photoirradiation on the limited cell region. The circle in the center shows region of interest (ROI).

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Peptide Science 2009: K. Okamoto(Ed.)
The Japanese Peptide Society (2010)

Fluorescent-Based Orthogonal Sensing Methods for Double Evaluation in PKC Ligands Screening

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Protein kinase C (PKC) is proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. To find drug lead compounds, development of screening methods, which would be applicable to high-throughput screening, is critical. In this study, we developed two screening methods based on fluorescent labeling to PKC ligand binding domain (C1 domain) and DAG-lactone.

Keywords: DAG-lactone, liagnd-screening, fluorescent labeling, PKC C1 domain

Introduction

Protein kinase C (PKC) is a family of enzymes for phosphorylation, which is specific for Ser and Thr residues. PKC family comprises at least 11 isozymes, which play fundamental roles in signaling pathways that regulate cell cycle progression, differentiation and apoptosis [1]. PKC has also been proven to be involved in problematic diseases, such as cancer and Alzheimer's disease. Thus, it has been established as an important therapeutic target. In PKC activation that depends on such as a diacylglycerol (DAG) and phorbol ester (tumor promoter), C1 domain plays a critical role in these ligands binding. To evaluate ligand binding to C1 domain, two different approaches utilizing fluorescent sensing were developed instead of conventional method using radioisotopes. The first method utilizes the synthetic δC1b domain derivatives bearing a dansyl group, which is environmentally responsive (Fig. 1, left). The second method involves competitive ligand

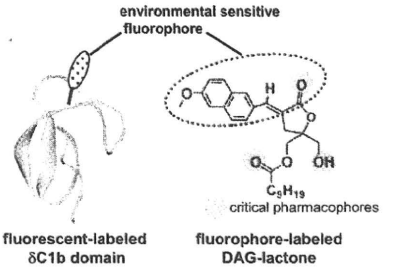

Fig. 1. Orthogonal fluorescent screening tools.

replacement by a fluorophore-labeled DAG-lactone (Fig. 1, right). DAG-lactone is a synthetic PKC ligand based on DAG. To reduce the entropic penalty of DAG, the glycerol backbone is constrained by forming a lactone ring [2]. In this study, the fluorescent screening tools, differently labeled on the acceptor and the ligand, were applied for evaluations of binding affinity of PKC ligands.

Results and Discussion

For the C1b domain of PKCδ (δC1b) (221-281), amino acids at three positions near the binding pocket were selected as insertion sites of fluorescent dye (Ser 240, Thr 242) [3]. A dansyl group was adopted as a fluorescent dye based on its sensitivity to environmental change and small molecular size. To introduce the dansyl group by Fmoc-SPPS, Fmoc-Lys(dansyl-Gly)-OH was prepared. Each fluorescent-labled δC1b domain analog was successfully synthesized. Ligand bindings of these fluorescent analogs were evaluated by utilizing [^{3}H]-phorbol 12,13-dibutylate (PDBu). The two δC1b analogs, T242K(dansyl-G) and S240K(dansyl-G), showed similar binding affinity with that of wild-type. In titration experiments of ligands, the increase of fluorescent intensity and the shift of fluorescence emission maxima showed reasonable correspondence to the binding affinity of ligands for PKC evaluated by the RI method (Fig. 2a).

As a new probe of PKC ligands binding assay based on the competitive inhibition, a fluorophore-labeled DAG-lactone was synthesized. The lactone has environmentally sensitive fluorophore on the position which is not necessary for binding to PKC C1 domain. The binding affinity of a synthetic fluorophore-labeled DAG-lactone for the PKCδ was evaluated by utilizing [^{3}H]-PDBu. The environmental sensitivity of the synthetic DAG-lactone derivative was confirmed by fluorescent measurement in various solvents. The fluorescent intensity of the fluorophore-labeled DAG-lactone was increased by binding to PKC, which indicates the hydrophobic environment of the binding pocket. According to an increase in the concentration of a test compound, the fluorescence intensity was decreased, indicating replacement of the fluorophore-labeled DAG-lactone. The fluorescent spectra in titration of PDBu are shown in Fig. 2b. The IC_{50} values were obtained from the curve-fitting of titrations of known compounds. The fluorescent-based inhibition assay showed a positive correlation between IC_{50} values and K_i values in the RI assay.

In summary, novel screening tools for PKC ligands based on fluorescent-labeling of C1b domain and DAG-lactone have been successfully developed. The combinational use of these fluorescent-labeling methods would lead to detailed and reliable evaluation of ligand compounds for PKC ligands, wchich does not require washing steps.

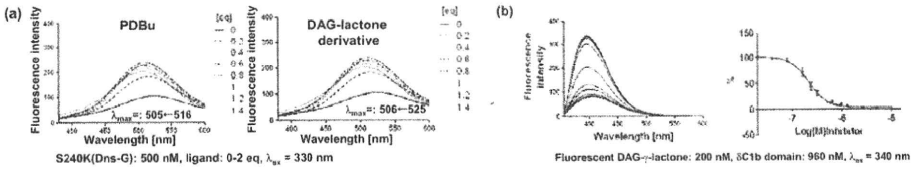

Fig. 2. Fluorescent titration of fluorescent-labeled δC1b (a) and fluorophore -labeled DAG-lactone (b).

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O-26

光機能性リガンドを用いたプロテインキナーゼ C の活性化制御

Spatio-temporal Activation of Protein Kinase C by a Photoactivatable Ligand

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プロテインキナーゼ C（PKC）はジアシルグリセロール（DAG）をセカンドメッセンジャーとするセリン・スレオニン特異的リン酸化酵素であり，がんやアルツハイマー病の治療薬創製の標的酵素として注目されている。演者らはケージド基で保護した PKC 特異的リガンドを創製し，紫外光照射による PKC 活性化の時間・空間的な制御を試みた。DAG を環化することによって結合活性を上昇させた DAG–ラクトンの重要なファーマコフォアである OH 基を 6-Bromo-7-methoxycoumarin（Bmc）（光分解性保護基）により保護したケージド DAG–ラクトン誘導体を合成した。ケージド DAG–ラクトンを緩衝液中で紫外光照射し，Bmc 基の脱保護，及び DAG–ラクトンの出現を HPLC 分析により確認した。また，その結果から分解反応の量子収率等を算出した。ケージド DAG-ラクトンの PKCδ活性化能について，試験管内での ^{3}H[PDBu]（ホルボールエステル）との競合阻害活性，リン酸化アッセイ，および CHO-K1 細胞内における GFP 融合 PKCδの細胞内局在変化（図）によって検討した。その結果，ケージド DAG ラクトンはいずれの場合も PKCδに対する結合活性，活性化能を持たず，紫外光照射によってケージド基を脱保護した場合においてのみ PKCδに対する結合活性を出現させ，活性化も行うことが確認された。以上のことから，ケージド DAG–ラクトンへの紫外光照射による脱保護，それに伴う結合活性の回復を用いて PKCδの活性化を時間・空間的に制御できる可能性が示された。

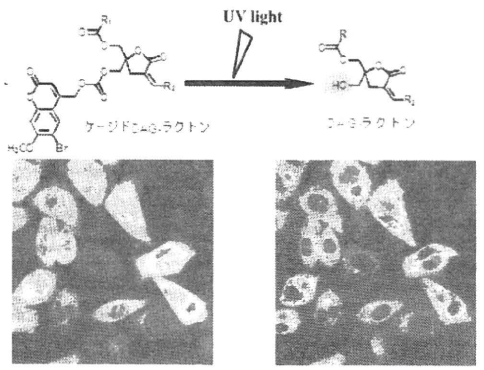

図. DAG-ラクトンのケージド化（PKC に対する結合活性に重要な水酸基を保護）と光照射による再活性化