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Novel phorbol esters exert dichotomous effects on inhibition of HIV-1 infection and activation of latent HIV-1 expression

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Two new phorbol esters, NPB-11 (12-*O*-methoxymethylphorbol-13-decanoate) and NPB-15 (12-*O*-benzyloxymethylphorbol-13-decanoate) were synthesized. The compounds exhibited potent anti-HIV-1 activity and low cytotoxicity in MT-4 cells by MTT assay even at a high concentration [50% cytotoxic concentrations (CC_{50}) were 8.32 and 4.39 μg/ml, respectively]. Two inhibitors strongly suppressed HIV-1 (IIIB strain) replication in MT-4 cells with a 50% effective concentration (EC_{50}) of 1.3 and 0.27 ng/ml, respectively. NPB-11 efficiently blocked replication of both X4 and R5 HIV-1 in PHA-activated peripheral blood mononuclear cells and MT-4 cells as revealed by p24 assay. The antiviral activity appeared to be mediated, at least partially, by the down-regulation of the expression of CD4 and the HIV-1 co-receptors, CXCR4 and CCR5. The compounds were also capable of selectively up-regulating HIV-1 expression in a variety of latently infected cell lines and inducing cell death in HIV-1 infected cells. The effect of NPBs on the induction of HIV-1 was specifically blocked by nontoxic doses of a protein kinase C blocker, staurosporine. NPB-11 blocked the spread of HIV-1 released from latently infected ACH-2 cells to MT-4 cells in a co-culture system. When combined with AZT, NPB-11 synergistically inhibited HIV-1 replication in MTT assay using MT-4 cells. These data suggest that these agents might be useful in reducing persistent viral reservoirs in patients and as adjuvant therapy in patients treated with HAART.

Keywords: phorbol ester, NPB-11, NPB-15, latent infection, anti-HIV-1 activity

Introduction

The proven efficacy of the present multi-drug regimens in substantially decreasing morbidity and mortality demands their continued use until more potent and tolerable treatment strategies become available. Stable and latently infected cells harbouring replication-competent HIV-1 have been found to persist even after many years of suppression of viraemia to <50 copies/ml (Wong *et al.*, 1997a; Finzi *et al.*, 1997; Chun *et al.*, 1997; Swingler *et al.*, 2003; Sharkey *et al.*, 2000). Latency is very important in the natural history of HIV-1 infection. In particular, latency has profound significance in the setting of highly active antiretroviral therapy (HAART), because it provides a critical mechanism for viral persistence when active replication is suppressed by drugs. Integrated viral DNA in latent reservoirs are not affected by antiretroviral drugs. The decay rate of the pool of latently infected cells slows extremely in HAART. There is a half-life of more than 43 months in the average patient and an average of at least 60.8 years of treatment would be required to eradicate the latent reservoir in most patients (Finzi *et al.*, 1999; Siliciano *et al.*, 2003; Ramratnam *et al.*, 2000; Strain *et al.*, 2003). Cessation of HAART in individuals who have had success in achieving long-term virological control at <50 copies/ml for many years promptly results in varying degrees of virological relapse in all individuals so as to rekindle productive viral infection (Wong *et al.*, 1997b; Neumann *et al.*, 1999; Davey *et al.*, 1999). The existence of a persistent and stable reservoir of HIV-1 has significant clinical implications, as the virus can continue to replicate even in the setting of optimal suppression with HAART. Since HIV-1 appears

to be ineradicable with HAART therapy alone, immune activators/modulators [such as interleukin (IL)-2 and monoclonal antibodies against CD3] have been suggested as a means for activating resting memory $CD4^+$ lymphocytes with the goal of stimulating virus production and thereby hastening the death of latently infected cells during antiviral 'coverage'. Some have interpreted this result as indicating that IL-2 does not have a substantial effect on the turnover of the latent reservoir (Chun *et al.*, 1999; Davey *et al.*, 1999). These cells are a barrier to eradication effort, and a more potent and tolerable treatment regimen is needed to eliminate the latent reservoir.

Many bioactive phorbol esters have exhibited important activities, including the ability to inhibit the replication of HIV-1 and up-regulate latent HIV-1 provirus expression. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a major active constituent of Croton oil, obtained from the seed of *Croton tiglium* L. As is well known, *Croton tiglium* (Badou) has traditionally been used as a laxative agent for oedema, pleural effusion, ascites and abdominal swelling from ancient times in Chinese folk medicine (Tsai *et al.*, 2004). TPA has been reported to inhibit the replication of HIV-1 through down-regulation of the CD4 receptor, to induce HIV-1 expression in latently infected cells (Kitano *et al.*, 1990; Chowdhury *et al.*, 1990; Kulkosky *et al.*, 2001; Korin *et al.*, 2002; Biancotto *et al.*, 2004) and to modulate the growth, differentiation, survival, function and metabolism of a variety of other primary cells and cell lines.

Furthermore, TPA has been shown to inhibit growth, stimulate apoptosis or enhance differentiation in human tumour cell lines derived from patients with melanoma or prostate, breast, colon or lung cancer (Arita *et al.*, 1994; Zheng *et al.*, 2004). TPA has been administered for the treatment of myelocytic leukaemia and relapsed/refractory haematological malignancies (Strair *et al.*, 2002; Han *et al.*, 1998a) and for depressed white blood cell counts after treatment with cytotoxic cancer chemotherapeutic drugs in China (Han *et al.*, 1998b). However, an apparently potent tumour-promoting activity has restricted wider therapeutic application. On the other hand, prostratin (12-deoxyphorbol 13-acetate) isolated from the Samoan medicinal plant *Homalanthus nutans* (Gustafson *et al.*, 1992), exhibits anti-HIV-1 activities yet up-regulates viral expression from latent proviruses in latently infected cell lines, primary cells isolated from HIV-1 infected humans and SCID-hu (Thy/Liv) mice (Gustafson *et al.* 1992; Kulkosky *et al.*, 2001; Korin *et al.*, 2002; Biancotto *et al.*, 2004). However, the concentration of prostratin required to activate HIV-1 replication in latently infected cells is approximately 1000-fold higher than TPA (Gulakowski *et al.*, 1997; Bocklandt *et al.*, 2003).

HIV-1 infected cells are highly susceptible to the apoptosis induced by various agents, including tumour promoting and anti-tumour agents such as sodium benzylideneascorbate. There are also reports of selectively induced apoptotic cell death in U1 cells expressing HIV-1 by TPA (Harada *et al.*, 1988; Matsuyama *et al.*, 1989; Aoki *et al.*, 1994). Since drug-induced apoptosis is generally associated with virus induction it is perceived as being rather harmful in the clinical use of such drug. Based on such information we attempted to discover safer phorbol agents with a potent HIV-1 inducing activity from latently infected cells as well as an inhibitory activity against cell-free HIV infection. Very recently, we succeeded in synthesizing several new agents from phorbol esters (Matsuya *et al.*, 2005). Among these, 12-*O*-methoxymethylphorbol-13-decanoate (NPB-11) and 12-*O*-benzyloxymethylphorbol-13-decanoate (NPB-15) (Figure 1) potently inhibited cell-free HIV-1 infection,

Figure 1. The chemical structure of novel anti-HIV-1 phorbol esters

(A) NPB-11, 12-*O*-methoxymethylphorbol-13-decanoate **(B)** NPB-15,12-*O*-benzyloxymethylphorbol-13-decanoate.

strongly stimulated viral expression and induced death in latently infected cells. We extensively studied these phorbol esters *in vitro* for their unique activities against HIV-1 infection in the hope of completely eradicating HIV-1.

Materials and Methods

Compounds

The synthesis and purification of NPB, that is two phorbol esters, were carried out at Lead Chemical Co., Ltd. (Toyama, Japan). These compounds were dissolved in DMSO at 1 mg/ml to exclude any antiviral or cytotoxic effect of DMSO. TPA and zidovudine (AZT) were purchased from Sigma-Aldrich (St Louis, MO, USA). TNF-α (human, recombinant) was purchased from Genzyme (Cambridge, MA, USA).

Cells and virus

T-cell lines, MT-4, CEM and Molt-4 cells, the HIV-1IIIB-infected Molt-4 cells, HIV-1 latently infected U1, J_{22}HL60, OM10.1 and ACH-2 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Cansera International Inc., Canada) and antibiotics. The HIV-1 strains NL4-3 and JR-CSF were prepared by introducing proviral constructs into 293T cells by $CaPO_4$ transfection. The titre of each viral preparation was determined according to its p24 antigen content measured by automated ELISA (Fiji Rebio Inc., Tokyo, Japan). In this assay the p24 antigen from Zeptometrix (Buffalo, NY, USA) in an Extended Range Kit was used as standard. HIV-1 IIIB Viral stocks were prepared by propagation in chronically infected Molt-4 cells.

MTT assay

To determine susceptibility to HIV-1 and cytotoxicity, the 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, Dojindo, Japan) assay using MT-4 cells was carried out as described (Pauwels *et al.*, 1988; Simizu *et al.* 1993). ACH-2 and CEM cells were also incubated with various concentrations of NPB-11 for 3 days. Cell viability was also measured using MTT reagent.

Combination assay

The synergistic effect of AZT and NPB-11 was tested with MTT assay. Appropriate concentrations of NPB-11 and AZT were added to HIV-1 IIIB infected or un-infected MT-4 cells in a tetrad test. The anti-HIV-1 activity and cytotoxicity were determined by MTT assay. The effect of combination was calculated as:

$$\text{Combination Index (CI)} = \frac{EC_{50} \text{ of NPB-11 in combination}}{EC_{50} \text{ of NPB-11}} + \frac{EC_{50} \text{ of AZT in combination}}{EC_{50} \text{ of AZT}}$$

The MT-4 cells were also treated with 10 ng/ml NPB-11 for 3 h at 37°C and were infected with culture supernatant containing 60 ng/ml of p24 antigen from NPB-11-stimulated ACH-2 cells. After washing, the cells were cultured in the presence of 10 ng/ml of NPB-11, 0.1 μM of AZT alone, or a combination of both NPB-11 and AZT for 4 days, and then the levels of p24 antigen in supernatant of MT-4 cells were determined by ELISA.

HIV-1 infection

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque density gradient centrifugation reagent. The PHA (1μg/ml)/IL-2 (100 unit/ml) activated PBMCs were treated with NPB-11 (final concentration of 200 ng/ml) for 1 h, and infected with 2 strains of HIV-1, NL4-3 and JRCSF, with an input of 20 ng/ml HIV p24 antigen equivalent virus. Duplicate plates were run as controls with virus added but no NPB-11 treatment. HIV-1 p24 antigen content was determined by auto ELISA assay.

Detection of cell surface receptor expression by flow cytometry

To detect the expression levels of cell surface receptors, PHA/IL-2 activated 1×10^6 PBMCs were treated with NPB-11 in IL-2-containing medium at 37°C for 1 day (15 h) or MT-4 cells were treated at various times. After washing, the cells were pre-treated with normal human mmunoglobulin G (IgG) at 0.1 mg/ml in 2% FBS/PBS for 30 min to block the Fc receptors and stained directly with anti-CD4 monoclonal antibodies conjugated with fluorescein isothiocyanate (DAKO A/S, Glostrup, Denmark) or indirectly with mouse-IgG monoclonal antibodies CXCR4 (12G5. R&D systems Inc, Minneapolis, MN, USA) and CCR5 (2D7. Biosciences-Pharmingen, San Diego, CA, USA) at 5 μg/ml followed by goat anti-mouse IgG-FITC (American Qualex, San Clemente, CA, USA). After washing, the cells were fixed in 1% paraformaldehyde. Cells were acquired on a FACSCalibur instrument, and the resulting data were analysed using CellQuest software (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The area of positivity was determined by using an isotype-matched mouse monoclonal antibody (DAKO).

Assay for HIV-1 binding and entry

Human MT-4 cells (4×10^5) were suspended in fresh medium in the presence or absence of various concentrations of NPB-11 at 37°C for 1 day. After washing, the cells were incubated with HIV-1 NL4-3 (200 ng of p24 Gag) for 2 h on ice or at 37°C in the absence or presence of NPB-11. The cells were washed with PBS/2% FBS and re-suspended the pellet with 500 μl of lysis buffer (PBS

containing 5% TritonX-100 and 1% BSA). Levels of p24 Gag were quantified by an automated ELISA system.

Stimulation of latent HIV-1 by NPB or TPA

Duplicate populations of 4×10^5/ml U1, ACH-2, J22HL60 and OM10.1 cells were treated with doses of 0.1, 1, 10 and 100 ng/ml of either NPB or TPA in the presence 1 μM AZT. Supernatants were drawn and analysed for p24 concentration using auto ELISA assay.

Prevention of HIV-1 spread to host cells

2.5×10^5/ml ACH-2 cells and MT-4 cells were co-cultured in the presence of 10 and 100 ng/ml of NPB-11 or 5 and 10 ng/ml of TNF-α for 2 days. The culture supernatant was collected and the amounts of p24 antigen measured by auto-ELISA. 2.5×10^5/ml ACH-2 cells were also cultured under the same conditions as control.

Testing the toxicity of NPB-11 in mice

The toxicity of NPB-11 was tested in BALB/cA 5-week-old mice by oral administration of the NPB-11 over a period of 30 days at a daily dose of 0.11, 0.33 and a 1 ng/kg range, using five or eight mice per dosage group.

Results

Anti-HIV-1 activity of novel phorbol compounds

Seventeen compounds were synthesized and studied for anti-HIV-1 activity by the conventional MTT assay using MT-4 cells (Matsuya *et al.* 2005). Through optimization of the lead compounds, NPB-11 and NPB-15 were shown to be extremely potent inhibitors of HIV-1 replication in MT-4 cells (Figure 2A). The 50% effective concentrations (EC_{50}) of NPB-11 and NPB-15 were 1.3 ng/ml and 0.27 ng/ml, respectively. The 50% cytotoxic concentration (CC_{50}) of NPB-11 was 8.3209 μg/ml, while that of NPB-15 was 4.3934 μg/ml when MT-4 cells were used. Thus, the selectivity index (ratio of CC_{50} to EC_{50}) of NPB-11 was 6401, and that of NPB-15 was 16272, indicating that these compounds are highly selective (Figure 2A). To extend this observation, human PBMCs from uninfected donors were treated with NPB-11 for 1 h before infection with JRCSF, a M-CCR5-tropic strain, or NL4-3, a T-CXCR4-tropic strain of HIV-1. The inhibitory activity of NPB-11 in PBMCs was also demonstrated by a p24 accumulation assay of culture supernatants of the cells infected with the viruses. NPB-11 reduced the antigen levels in a dose-dependent fashion at every time point (Figure 2B). NPB-11 completely inhibited replication of two HIV-1 strains at 50 ng/ml.

Down-regulation of CD4, CCR5 and CXCR4

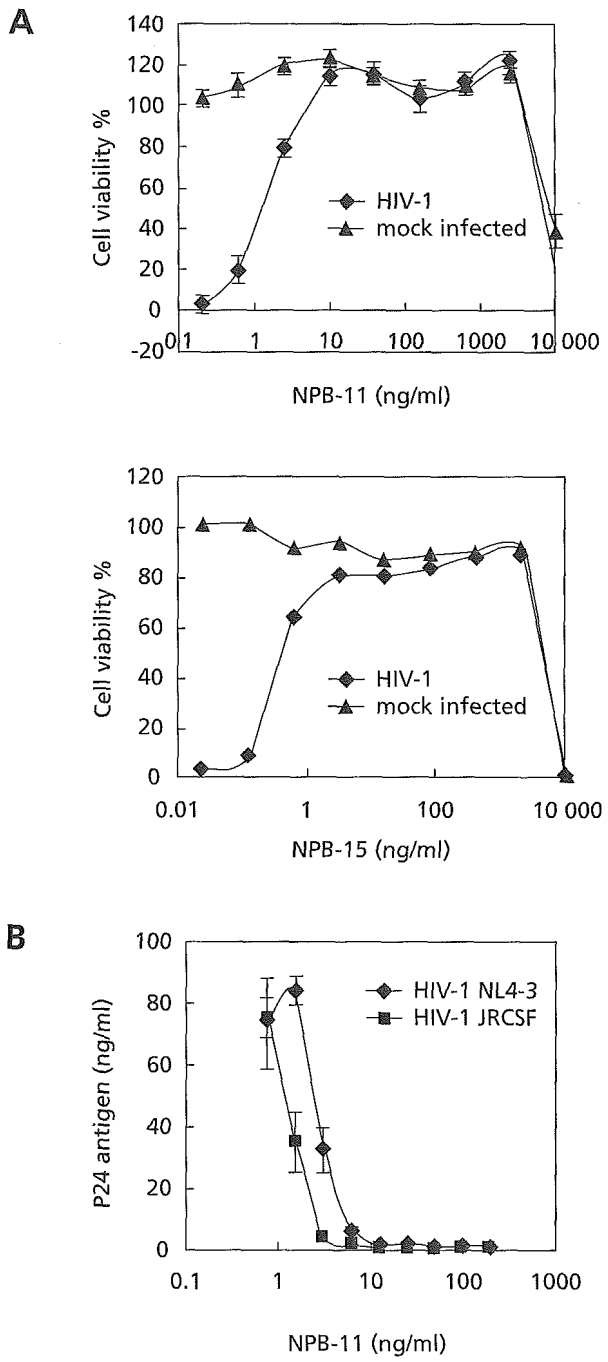
It has been shown previously that phorbol esters down-regulate the cell surface expression of both the primary receptor for HIV-1, CD4, and its coreceptors CXCR4 and CCR5 (Hamamoto *et al.*, 1989; Gulakowski *et al.*, 1997; Kulkosky *et al.*, 2001; Bocklandt *et al.*, 2003). To determine whether NPBs also have this ability, PBMCs were incubated with various concentrations of NPB-11 and then analysed for CD4, CXCR4 and CCR5 expression by FACS. Figure 3A shows that NPB-11 efficiently down-regulated CD4 with an IC_{50} of 16.04 ng/ml and maximal suppression was achieved at 100 ng/ml (19.94% of untreated control). NPB-15 also down-regulated CD4 expression in the similar concentration range (data not shown). Similarly, down-regulation of CXCR4 and CCR5 expression was observed, with maximal suppression at 12.5 and 50 ng/ml, respectively. The down-regulation of receptors also occurred after NPB-11 treatment in a dose-dependent manner in PBMCs. A time-of-addition test showed that down-regulation of CD4 and CXCR4 in MT-4 cells occurred as early as 3 h after treatment with NPB-11 and this effect was maintained for at least 4 days (Figure 3B).

Further, we investigated whether NPBs affected the entry and binding of HIV-1 in MT-4 cells. As shown in Figure 3C, a binding assay was carried out to measure the effect of NPB-11 on virion/cell surface interaction. MT-4 cells were mixed with X4 virus NL4-3 on ice for 2 h, and then the cells washed to remove unbound viruses. The result demonstrated that NPB-11 blocked virus-cell binding with an EC_{50} 32.4 ng/ml. The inhibitory effect of NPB-11 on HIV-1 entry to cells was also studied in MT-4 cells. Cells were incubated with the same concentration of viruses at 37°C for 2 h, treated with trypsin to remove bound virions, and then the intracellular p24 antigen of HIV-1 was measured. NPB-11 inhibited virus entry with an EC_{50} >200 ng/ml.

Synergistic effect of NPB-11 through combination with AZT

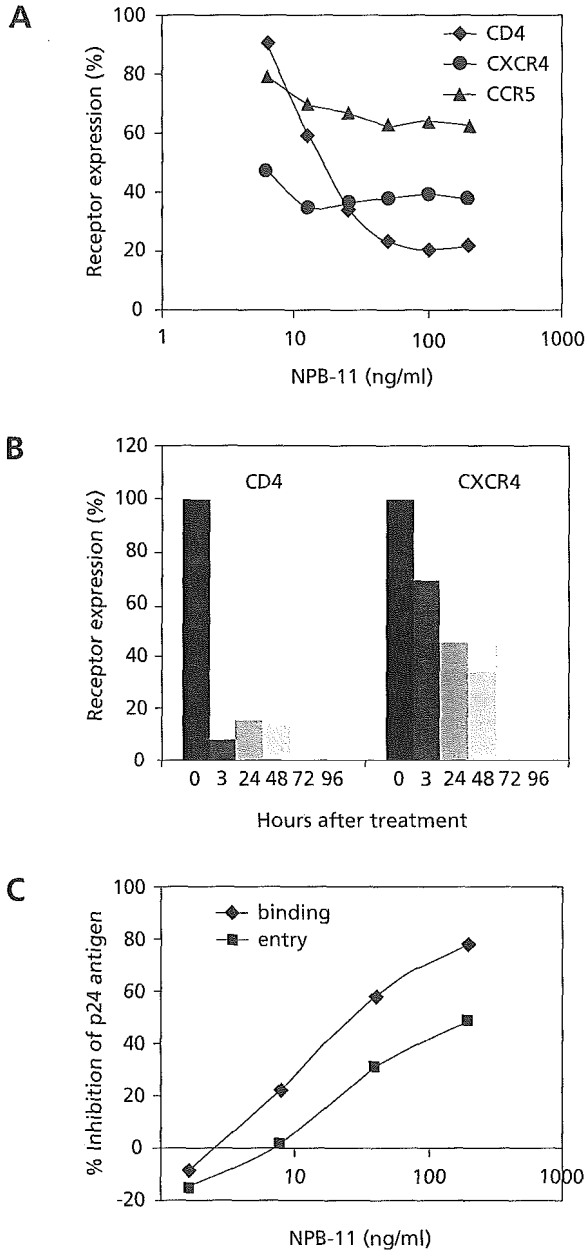
To assess the anti-HIV-1 activity of NPB-11 when combined with AZT, MT-4 cells were infected with the HIV-1IIIB strain, and the 50% effective concentration in the prevention of virus-induced cytopathic effect was determined by MTT assay. HIV-1 replication was inhibited more effectively in MT-4 cells by a combination NPB-11 and AZT than the exclusive use of each compound. Based on the EC_{50} values for NPB-11 and AZT, the combination index was found to be <1. The result suggests that the effect on anti-HIV-1 activity was synergistic when NPB-11 and AZT were used concomitantly (Figure 4A). HIV-1 LAV-infected MT-4 cells were also incubated in the presence of NPB-11 or AZT alone, as well as in combination. The concentration of cell-free p24 antigen was determined 4 days after incubation by ELISA. The viral replication was suppressed more effectively by combination

Figure 2. NPB-11 and NPB-15 efficiently inhibit HIV-1 infection in MT-4 and PBMCs



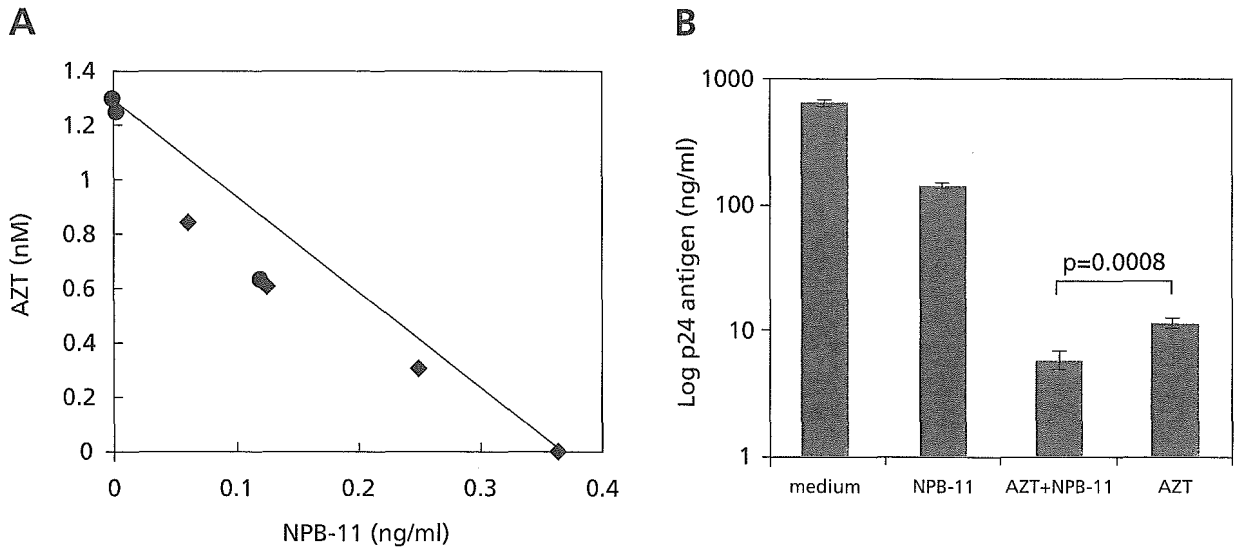
(A) Anti-HIV-1 activity of NPB-11 and NPB-15 was measured by MTT assay using MT-4 cells. HIV-1 IIIB was used in this study. **(B)** Suppression of HIV-1 infection in human PBMCs after NPB-11 treatment. Activated PBMCs were exposed to serial concentrations of NPB-11, and then infected with HIV-1 virion-containing supernatants derived from 293T cells transfected with HIV-1 expression plasmids for the 2 strains. Supernatant samples were removed on day 7 after infection and assessed for HIV-1 p24 antigen content by auto-ELISA.

Figure 3. NPB-11 inhibits the expression of CD4, CXCR4 and CCR5



(A) Dose dependent down-regulation of CD4, CXCR4 and CCR5 by NPB-11. PBMCs were treated with the indicated concentration of NPB-11 for 2 h, then analysed by antibody staining and FACS. **(B)** Effect on the time course of the expression of CD4 and CXCR4 in the presence of NPB-11. MT-4 cells were incubated with 100 ng/ml of NPB-11 and then the cells were collected 3 h, day 1, day 2, day 3 and day 4 after culture. Expression of surface markers was measured with FACS and expressed as a percentage of control in fluorescent intensity. **(C)** Inhibition of HIV-1 binding and entry into MT-4 cells by NPB-11. MT-4 cells treated with 1.6–200 ng/ml of NPB-11 were incubated for 2 h on ice or 37°C with the HIV-1 NL4-3 strain. The virions which bound to or entered in MT-4 cells were determined as values of p24 antigen in the presence of different concentrations of NPB-11. The percentage of inhibition of virus binding or entry was defined as [1-(p24 antigen with NPB-11/p24 antigen without NPB-11)]×100%.

Figure 4. NPB-11 synergistically inhibits HIV-1 infection in cooperation with AZT



(A) Inhibitory effect of NPB-11 in combination with AZT on HIV-1 replication by MTT assay. The EC_{50} values of anti-HIV-1 replication for NPB-11 and AZT were calculated by MTT assay using MT-4 cells as described in Materials and methods. **(B)** Combination effect on p24 antigen assay. MT-4 cells were infected with HIV-1LAV from activated ACH-2 culture supernatant by 10 ng/ml of NPB-11. After washing, the cells were incubated in the presence of 10 ng/ml NPB-11, 100 nM AZT alone, and both NPB-11 and AZT. The levers of p24 antigen in culture supernatant of MT-4 cells were measured on 4 day. Neither NPB-11 nor AZT was used in control culture (medium). Data indicated means and standard deviations from 3 different experiments. P value was calculated by using the paired *t*-test.

with 0.1 μM AZT and 10 ng/ml NPB-11 than either of these drugs alone (Figure 4B). The amount of p24 antigen was 5.826 ng/ml in combination treatment. By comparison, the values were 137.707 and 11.516 ng/ml, respectively, when MT-4 cells were treated with 10 ng/ml of NPB-11 or 0.1 μM of AZT alone. Furthermore, 630.382 ng/ml of p24 antigen was detected in the culture supernatant of drug-free MT-4 cells. As the result, HIV-1 replication was inhibited more significantly in combination with AZT and NPB-11 than in AZT alone (5.826 ±0.81 versus 11.516 ±0.736, P=0.0008).

NPB induction of HIV-1 expression in latently infected cells

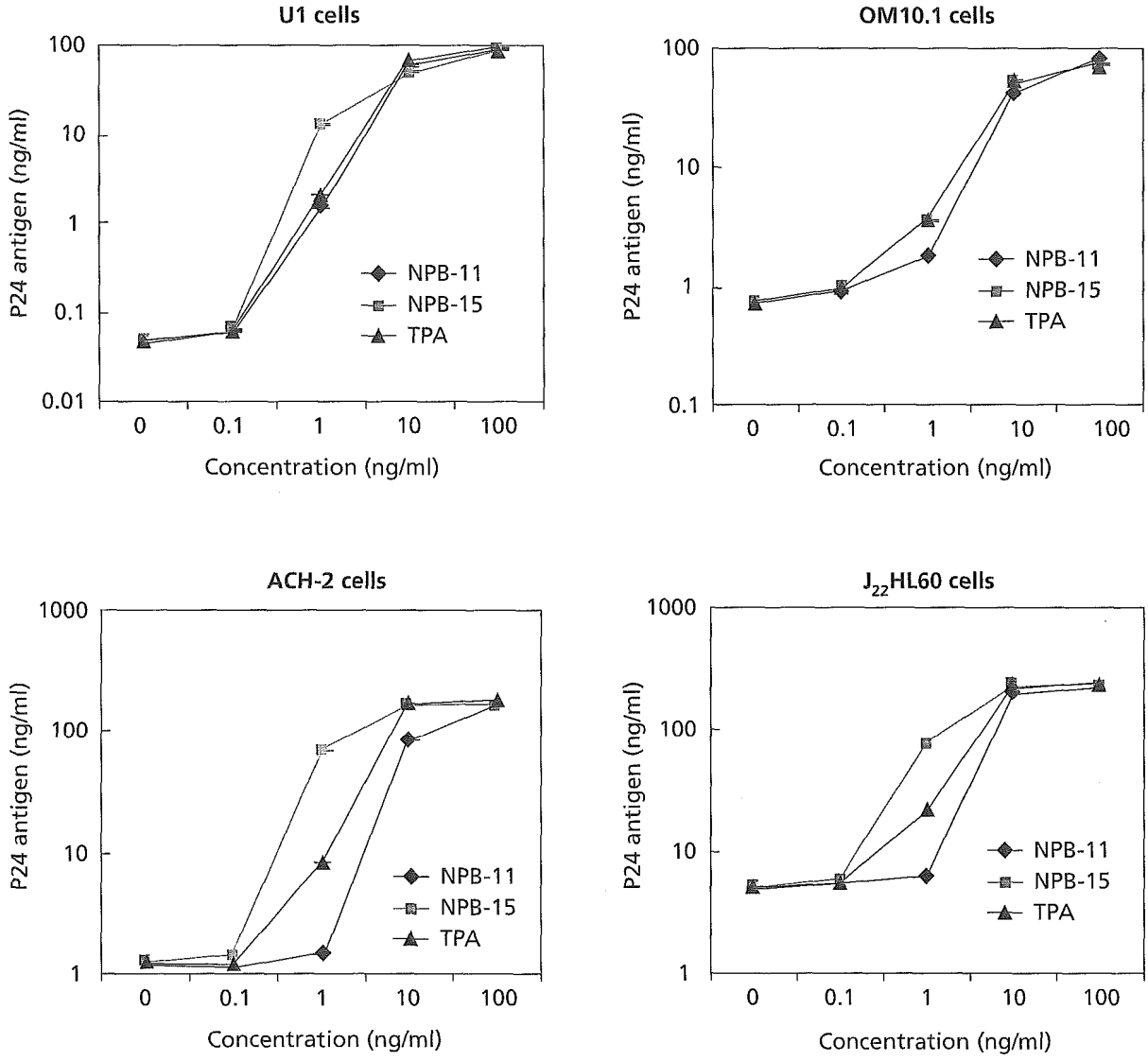
The ability of NPB to induce HIV-1 expression was investigated by measuring p24 antigen production in four different cell lines either chronically or latently infected with HIV-1; ACH-2 cells, a T cell clone that carries a single integrated provirus (Clouse *et al.*, 1989; Folks *et al.*, 1989), OM10.1 and J_{22}HL60 cells cloned from HL-60 promyelocyte cells taken from an acute or a chronic HIV-1 infection (Kitano *et al.*, 1990; Butera *et al.*, 1991) and U1 cells, a subclone of U-937 promonocytic cells chronically infected with HIV-1 (Folks *et al.*, 1987). As shown in Figure 5, the addition of a nanogram concentration of NPB to the culture medium for 3 days increased p24 expression 100 to 1000-fold over background levels. The concentration of NPB required for maximal stimulation was 100 ng/ml for NPB-11 and approximately 10 ng/ml for NPB-15. However, NPB-15 efficiently induced HIV-1 production even at 1 ng/ml in various cells. A control compound, TPA, increased p24 production to a similar extent, and the maximal stimulation was achieved in a range of 10–100 ng/ml (Figure 5). The kinetics of p24 production by NPB and TPA were very similar, with a rapid rise for the first 2 days that levelled off by day 5 (data not shown).

The effect of the PKC inhibitor staurosporine on HIV-1 induction by NPB-11 and NPB-15 were studied. The results show that the enhancement of HIV production by NPBs as well as the control compound, TPA, was inhibited significantly when 10 ng/ml of staurosporine was applied (Figure 6).

NPB-11 induces selective cell death in latently infected ACH-2 cells

Since TPA and other phorbol esters exhibit selective cytotoxity to HIV-1 infected cells (Harada *et al.*, 1988), ACH-2 and its parental cell line, CEM cells, were treated with 0.1, 1, 10 and 100 ng/ml NPB-11 for 3 days, and cell viability was compared with the MTT assay. As shown in Figure 7,

Figure 5. Novel phorbols efficiently induce HIV-1 expression from various latently infected cell lines



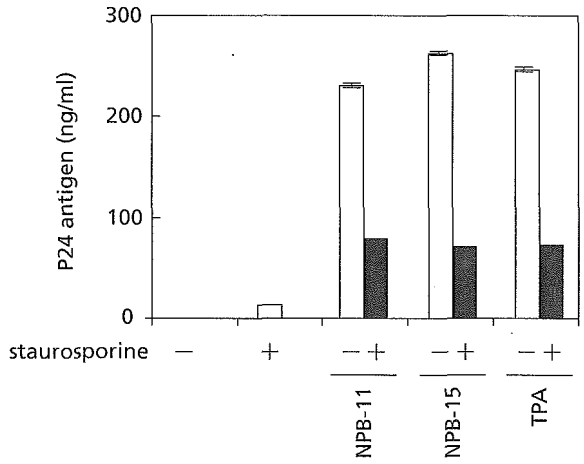
Cells were treated with 0.1, 1, 10 and 100 ng/ml NPB-11, NPB-15 or TPA. Culture media were removed 3 day after treatment and assessed for the levels of p24 antigen by auto-ELISA. Each point represents the mean of 3 trials ±1 SD.

the percentage of cell viability was markedly reduced in ACH-2 cell cultures upon treatment with NPB-11 in association with the increase of the amount of p24 antigen in culture supernatant. At 10 ng/ml of NPB-11, cell viability of ACH-2 and CEM was 35.23% versus 103.48% (Figure 7).

Prevention of virus spread to T lymphocytes by NPB-11

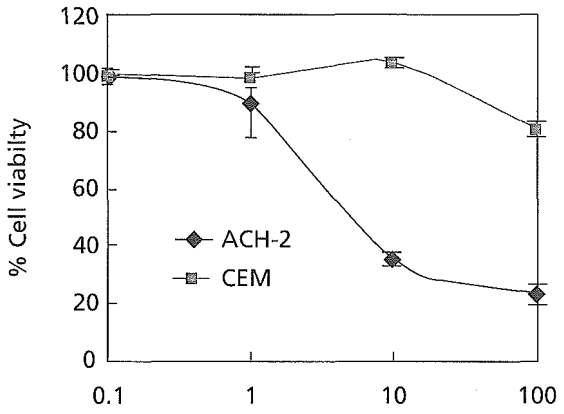
NPB possesses the ability to play a pivotal role in HIV-1 infection and replication; NPB not only inhibited infection of treated T-lymphocyte cell lines and PBMCs, but also was able to stimulate HIV-1 expression in latently infected U1, J_{22}HL60, OM10.1 and ACH-2 cells. This suggests the possibility that the latently infected cells are preferentially killed upon stimulation with NPB-11 and the spread of the induced virus to T-cells is blocked by the compound. To address this question, a co-culture experiment using MT-4 cells and ACH-2 cells (in a ratio 1:1) was carried out. The concentration of cell-free p24 antigen was determined by ELISA to monitor the effects of NPB-11 on preventing

Figure 6. Staurosporine A inhibits the HIV-1 inducing ability of phorbols in ACH-2 cells



ACH-2 cells were incubated with 10 ng/ml staurosporine for 3 h and then the staurosporine treated and untreated cells were stimulated with 10 ng/ml of NPB-11, NPB-15 or TPA. The levels of p24 antigen in supernatant samples were determined 3 days after culture.

Figure 7. HIV-1 latently infected cells are more vulnerable to the cytotoxic effect of NPB-11



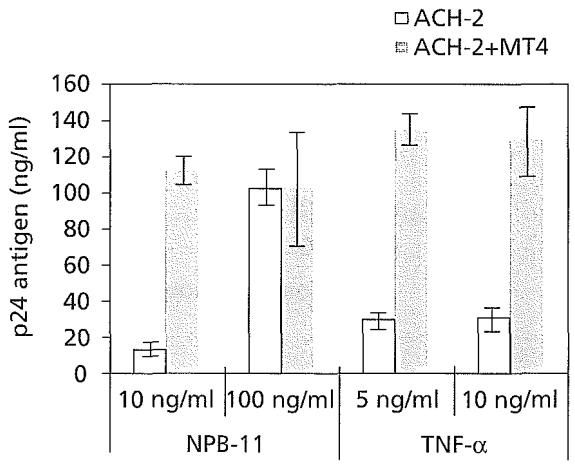
2.5x10^5/ml ACH-2 or CEM cells were incubated with 100, 10, 1 and 0.1 ng/ml NPB-11 for 3 days. Cell viability was determined by MTT assay.

HIV-1 spread. In the presence of 100 ng/ml NPB-11 for 2 days, equivalent levels of p24 antigen were revealed in the supernatant of the co-culture and ACH-2 cells alone. In contrast, in the presence of 5 or 10 ng/ml TNF-α, the amounts of p24 antigen in the supernatant of the co-culture were 4.2–8.2 fold higher than in ACH-2 cells alone (Figure 8). These results strongly suggest that infectious virions released from induced ACH-2 cells rapidly entered MT-4 cells and replicated, contributing to the generation of p24 antigen in the supernatant. However, this viral spread was efficiently blocked by a higher concentration of NPB-11 (100 ng/ml).

Discussion

Using the MTT assay we have determined that two novel phorbol ester compounds, NPB-11 and NPB-15, efficiently protect MT-4 cells from the cytopathic effect of HIV-1 IIIB (Figure 2A). HIV-1 infected and uninfected MT-4 cells treated with a very low concentration of NPB grew at equivalent rates, but the overall growth rates in both groups were somewhat suppressed relative to control cells not treated with NPB. The NPB-11 exhibited lower cytotoxicity over a wide concentration range, even at high concentration (2500 ng/ml) than NPB-15. Similarly, NPB-11 also inhibited viral replication more completely (EC_{90}: 4.5 ng/ml) (Figure 2A). Furthermore, NPB-11 was found to be more stable against degradation in plasma than

Figure 8. NPB-11 inhibits the spread of HIV-1 to T-cells after being induced from latently infected ACH-2 cells



2.5x10^5/ml ACH-2 and MT-4 cells were co-cultured in the presence of 10 and 100 ng/ml NPB-11, or 5 and 10 ng/ml TNF-α for 2 days. Then the levels of p24 antigen in the supernatant were measured with auto-ELISA. 2.5x10^5/ml ACH-2 cells alone were also incubated with the same stimulus for 2 days.

NPB-15, indicating that the former may be more relevant than the latter given the clinical use of these drugs. Thus, we paid more attention to NPB-11. Potent inhibition of HIV-1 replication by NPB-11 in PBMCs was further

confirmed with p24 antigen ELISA. This finding was observed for two strains of HIV-1 with the CXCR4 or CCR5 co-receptors. In addition, microscopic observation (data not shown) revealed that syncytia were blocked in co-cultures of Molt-4 chronically infected with HIV-1 IIIB by 10 ng/ml NPB-11. The anti-HIV effect of NPBs is consistent with the down-regulation of CD4 and co-receptor expression. However, this may not be the sole mechanism for the anti-HIV activity of NPBs given their very low EC_{50} values obtained in the MTT assay and the relatively inefficient effect of these compounds on the down-modulation of CD4/co-receptors. Indeed, the effect of NPB-11 was marginal, if any, in blocking HIV-1 binding and entry into the cells, as shown in Figure 3C. Since the NPB effects were efficiently blocked by the PKC inhibitor staurosporine, it is likely that PKC is involved in the anti-HIV as well as the HIV inducing effects of these compounds. The preliminary data also show that NPBs can induce TNF-α (Zhong, unpublished data), so it is possible that this cytokine plays an important role in the viral activation by NPBs. However, it is not clear at this moment how TNF would be involved in the NPB anti-HIV-1 activity. Because NPBs have dual effects on both the inhibition of infection and the activation of HIV-1, future studies will be needed to disentangle the complicated induction and down-regulation of molecules in the host to address the multiple mechanisms of action of the NPB compounds.

HIV-1 persists even after long periods of HAART mainly due to the presence of cellular reservoirs that include memory $CD4^+$ T lymphocytes, blood monocytes and macrophages/cells of the macrophage lineage. These cellular reservoirs have become understood to be a serious obstacle to ultimately successful antiretroviral treatment of HIV-1 infection. We examined the ability of the compounds NPB-11 and NPB-15 to stimulate latent virus and extended these studies by comparing this property in TPA. NPBs have similar, if not superior, potential to TPA for inducing viral expression in various reservoir cells. NPBs can induce latent viral expression from T lymphocyte and promonocyte cell lines. HIV-1 release is potently stimulated by NPBs in a variety of cells, even at concentrations lower than 10 ng/ml. The ability of NPB-11 to selectively kill HIV infected cells was also studied, using ACH-2 and the parental cell line, CEM. NPB-11 activated the latently infected ACH-2 cells to release virus together with efficiently inducing cell death. In contrast, the same concentration did not induce death in the parental CEM cells. Based on these results, NPB-11 might reduce the size of the latent HIV-1 reservoir through the preferential killing of HIV-1 infected cells. However, the NPB compound exerts multiple effects, and the spread of released viruses should be demonstrably suppressed prior to actually administering this type of drug to patients. Otherwise, there is the possibility that these drugs would paradoxically increase the size of the latent reservoir. Therefore, the ability of NPB-11 to block the spread of induced viruses to host T-cells needs to be examined. Latently infected ACH-2 and uninfected MT-4 cells were co-cultured in a 12-well plate in the presence of NPB-11 or TNF-α. This condition should activate latently infected ACH-2 cells, which would in turn lead to the release of infectious viruses and spread to infect T-cells. However, MT-4 cells were refractory to infection with the HIV-1 released from induced ACH-2 cells (at 100 ng/ml NPB-11). By contrast, when those co-culture cells were exposed to TNF-α and a lower concentration of NPB-11, MT-4 cells were permissive to HIV-1 replication based on the increase of p24 antigen. It is interesting to address the reason why NPB-11 does not show blocking effect at 10 ng/ml, the concentration of which is clearly inhibitory in the MTT assay using MT-4 cells. Although the exact mechanism remains to be determined it is possible that 10 ng/ml of NPB-11 is not sufficient to inhibit cell to cell infection of HIV-1 in the co-culture system.

Latently infected cells are a permanent source of virus reactivation and may lead to a rebound of viral load after interruption of HAART, resulting in an increase in the pool size of the latent reservoir. The results presented here show a noticeable increase in the effect of NPB-11 when used in combination with an anti-HIV agent. Using MTT assay and p24 antigen monitoring, we observed a synergistic effect of NPB-11 in combination with AZT on the modulation of HIV-1 infection, and cytopathic effects when MT-4 cells were used.

Preliminary pharmacotoxic studies were conducted and the data are summarized as follows; 21 out of 21 mice survived, with no obvious ill effects at a 0.11 (5 mice), 0.33 (8 mice) and 1 (8 mice) mg/kg oral administration of NPB-11 for up to 1 month. Treated mice (BALB/cA) did not exhibit weight loss compared with control mice not administered NPB-11. There were no apparent adverse effects on the brain, pituitary gland, thymus, lung, heart, liver, spleen, kidney, adrenal gland or testis (data not shown).

Collectively, these results show NPBs efficiently inhibit cell-free HIV-1 infection in human PBMCs and T-cell lines, block virally-induced syncytia formation and prevent viral spread. The inhibition of HIV-1 replication is partially explained by down-regulation of the CD4 receptor and expression of the HIV-1 co-receptors CXCR4 and CCR5, in target host cells. In addition, the synergistic effect of NPB-11 in combination with AZT in the modulation of HIV infection was confirmed. Conversely, NPBs up-regulate expression of viral gene products from latent proviral templates, and appear to have stimulatory effects on the expression of two strains of latent HIV-1 from monocyte and T lymphocytes. It is thought that for HIV-1 to be

stimulated to escape from latency, both the tissue-bound and blood-borne monocyte/macrophage must be targeted, because they both may harbour a significant reservoir of HIV-1 in patients on HAART. Such a compound would be the first capable of true viral eradication, by completely eliminating the latent reservoir. The novel compounds described here block HIV-1 viral spread yet up-regulate latent HIV-1 provirus expression, features that could perhaps be effectively exploited as an adjuvant inductive therapy in HAART patients. Further testing will be required to determine whether the initial promise reported here will be borne out. Such studies will be undertaken at the earliest opportunity.

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Highly potent anti-HIV-1 activity isolated from fermented *Polygonum tinctorium* Aiton

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Abstract

A water-soluble extract of fermented *Polygonum tinctorium* Aiton (Polygonaceae) called *Sukumo*, exhibited a potent inhibitory activity against HIV type 1 in vitro. The extract potently suppressed acute HIV-1 (III_B) infection in MT-4 cells with EC_{50} values of 0.5 μg/ml but exhibited low cytotoxicity to MT-4 cells even at a high concentration (CC_{50} > 1000 μg/ml). It also inhibited giant cell formation in co-cultures of HIV-infected cells and uninfected Molt-4 cells. *Sukumo* extract was found to interact with both the viral envelope glycoprotein and cellular receptors, thus blocking virus-cell binding and virus-induced syncytium formation. There was a good correlation between the extract's anti-HIV-1 activity and its inhibitory effects on HIV-1 binding. It also suppressed replication of herpes simplex virus type 1 in Vero cells with an EC_{50} of 11.56 μg/ml. On the other hand, there was no appreciable activity against influenza A virus, poliovirus or SARS corona virus when tested at concentrations ranging from 3.2–400 μg/ml as shown by microscopic image analysis for cytopathic effect (CPE). Physico-chemical studies revealed that the anti-HIV activity in the extract was essentially maintained after boiling at 100 °C in 1N HCl or 1N NaOH, and after treatment with 100 mM $NaIO_4$. The inhibitory activity of the extract was also not reduced after pronase digestion. The active factor in the extract is likely to be a novel compound(s) having a polyanionic substructure and a molecular weight of 10,000–50,000.

Keywords: *Polygonum tinctorium*; *Sukumo* extract; HIV-1; HSV-1; Viral entry

1. Introduction

One of the logical targets of the viral life cycle at which to inhibit HIV-1 replication is the step in the process where the infectious virion enters its host cell (Moore and Stevenson, 2000; Lin et al., 2002). Therefore, the identification of HIV entry inhibitors, which can serve as novel anti-HIV drugs, is urgently needed.

Retroviral infection is initiated by the attachment of the virion to the cell surface, which even occurs before glycoproteins on the viral envelope interact with specific receptors on the host cell to trigger fusion. A great variety of polyanionic compounds have been described which act as virus adsorption inhibitors. This class of compounds also comprises the cosalane analogues, containing the polycarboxylate pharmacophore, as well as the sulfated polysaccharides extracted from sea algae (Nakashima et al., 1987a, 1992; Santhosh et al., 2001; Witvrouw and De Clercq, 1997). All of these compounds are assumed to exert their anti-HIV activity by shielding the positively charged sites in the V3 loop region of

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the viral gp120 envelope glycoprotein, and interrupting virus attachment to the negatively charged heparan sulfate proteoglycans on cell surface, and inhibiting the specific binding to the CD4 receptor of $CD4^+$ cells. Some of these compounds can also interfere with later events in receptor-mediated fusion by virtue of attachment to gp120. These compounds probably do not penetrate into cells because of their mass and highly anionic charge, but rather, act as antiviral agents by impeding the attachment and subsequent entry of virus particles into the cell.

A number of sulfated polysaccharides, including dextran sulfate and heparin, have been reported to have potential as antiviral drugs, since they inhibit the replication of a variety of viruses in vitro (Baba et al., 1988; Bartolini et al., 2003; Nakashima et al., 1989; Ylisastigui et al., 2000). The extent of inhibition appeared to be dependent on both the viral strain and host cell type. Dextran sulfate interferes with the association of gp120 with CXCR4 while having no detectable effect on gp120-CD4. The interaction between polyanions and X4 or X4R5 gp120 was readily detectable, whereas weak or undetectable binding was observed with R5 gp120 (Moulard et al., 2000). Cosalanes inhibited the binding of gp120 to CD4 as well as the fusion of the viral envelope with the cell membrane and is more potent against R5 HIV-1 RF in CEM-SS cells than against vs X4 HIV-1 IIIB in MT-4 cells (Santhosh et al., 2001).

Polygonum tinctorium has been used extensively in Chinese and Japanese folk medicine for the treatment of many infectious diseases and is believed to have effects such as detoxification, anti-pyrexia and anti-nociception. Extracted constituents of this medicinal plant, such as tryptanthrin, has been shown to possess anti-fungal, cancer chemopreventive and anti-bacterial activities (Honda and Tabata, 1979; Koya-Miyata et al., 2001; Kataoka et al., 2001; Miyake et al., 2003), while pigment (PtP) has an anti-anaphylactic activity (Kim et al., 1998). In this study, we report for the first time the potent anti-HIV-1 and HSV-1 activity of an aqueous extract from the fermented leaves of *Polygonum tinctorium* (*Sukumo*). This extract was found to be highly selective against HIV-1 and HSV-1 in vitro. *Sukumo* extract suppresses production of HIV-1 by inhibiting the viral entry process through binding to the virus envelope and thus preventing HIV-induced syncytium formation with an exceedingly broad therapeutic window. Based on the results of physico-chemical analysis of the anti-viral active factor, it is putatively a novel polyanionic high-molecular-weight compound containing a phenolic substructure in aqueous extract of *Sukumo*.

2. Materials and method

2.1. Compound

Sukumo was collected from the leaves of *Polygonum tinctorium* (Tokushima, Japan) and fermented for 3 months, which was provided and identified by Dr. Matsuda. Voucher specimens were deposited at the Institute of Hemorheological Function of Food Co. Ltd., Hyogo, Japan. *Sukumo* powder (100 g) was refluxed three times with 99.9% ethanol, then with water (1 l). The aqueous solution was clarified by filtering through a 0.2 μm filter. The high-molecular compounds were precipitated from the aqueous extracts of *Sukumo* by 66.6% ethanol, which were collected by centrifugation (10,000 rpm, 30 min) in a yield of 26.8% (26.8 g). Anti-HIV activity of the *Sukumo* extract was tested and stored at 4 °C before use.

2.2. Cells and virus

MT-4, Molt-4 cells and Molt-4 cells chronically infected with the HIV-1 (III_B) strain (Molt-4/IIIB) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., Canada) and antibiotics (100 μg/ml penicillin/100 μg/ml streptomycin). 293T, Vero and stably expressing CD4-CCR5 of Hos cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing the same supplements. X4/HIV-1 (III_B) was prepared by propagation in Molt-4/IIIB cells. HIV-1 molecular clones of the X4 HIV-1 strain NL4-3 and the R5 HIV-1 strain JRCSF were prepared by transfection of 293T cells with NL4-3 or JRCSF plasmids carrying full-length proviral DNA. The culture supernatants were clarified by 0.45 μm filters and frozen at −80 °C. Herpes simplex virus type 1 was propagated in Vero cells (kindly provided by Dr. Shaku). Cell-free virus stock was prepared by sonication of HSV-infected Vero cells in 9% skim milk and stored at −80 °C until use.

2.3. Antiviral assay

To determine the anti-HIV-1 activity and cytotoxicity of the *Sukumo* extract, MT-4 cells were either infected with HIV-1 (III_B) strains at a multiplicity of infection (MOI) of 0.01 or un-infected (mock infection). Cell viability was quantified with MTT (Dojindo, Kumamoto, Japan) assay for MT-4 cells. EC_{50} values were calculated in infected cells for the anti-HIV-1 effect and CC_{50} values were calculated in un-infected cells for drug cytotoxicity (Ichiyama et al., 2003). Peripheral blood mononuclear cells (PBMCs) from HIV-1-seronegative donors were isolated by Ficoll-Hypaque density gradient centrifugation. PHA (1 μg/ml, Sigma-Aldrich)/IL-2 (100 U/ml, Shionogi, Osaka, Japan) activated PBMCs were infected for 2 h with 20 ng of HIV-1 p24 Gag (X4/NL4-3 or R5/JRCSF) in the presence or absence of the *Sukumo* extract (0.64–400 μg/ml), washed three times with PBS and cultured for 7 days in RPMI-1640 medium/10% FBS plus 100 U/ml IL-2 with or without the *Sukumo* extract. HIV-1 p24 Gag of culture supernatant was determined by automated enzyme-linked immunosorbent assay (EILSA) (Fuji Rebio Inc., Tokyo, Japan). In this assay the p24 antigen from Zeptometrix (Buffalo, New York) was used as the standard. Chronically HIV-1 (III_B)-infected Molt-4 cells were

co-cultured with HIV non-infected Molt-4 cells (ratio = 1:1) at 37 °C for 1 day in the presence of a test compound at graded concentrations. Cell–cell fusion was analyzed by confocal microscopic assessment of syncytium formation.

Sukumo extract were also tested as an inhibitor of HSV-1 replication in Vero cells using a standard plaque assay. Two hour after treatment with *Sukumo* extract, Vero cells were subjected to a 1-h infection with about 50 PFU of HSV-1 in the absence or presence of serially diluted *Sukumo* extract and then cultured with 199 medium supplemented with 1% FBS, human γ-globulins (164 μg/ml, Sigma-Aldrich) and antibiotics, with or without compounds cultured for 4 days. The cells were stained with Giemsa solution. The numbers of viral plaques were calculated as a percentage of the tested control in order to determine the percent inhibition. The EC_{50} value is the concentration of compound that inhibits viral replication by 50% relative to control.

2.4. Virus binding and entry assay

Human MT-4 cells (4×10^5) were suspended in fresh medium in the presence or absence of various concentrations of *Sukumo* extract at 37 °C for 1 h. After washing, the cells were incubated with HIV-1 NL4-3 (200 ng of p24 Gag) for 2 h on ice or at 37 °C in the absence or presence of extract. The cells were washed with PBS/2% FBS and the pellet resuspended with 500 μl of lysis buffer (PBS containing 5% TritonX-100 and 1% BSA). Levels of p24 Gag were quantified by an automated ELISA system.

2.5. Flow cytometric analysis

MT-4 or Hos/CCR5 cells (5×10^5) were pretreated with normal human IgG (Zymed, South San Francisco) at 0.1 mg/ml in PBS containing 2% FBS buffer for 30 min on ice to block the Fc receptors and then were treated by anti-CXCR4 antibodies (5 μg/ml, 12G5. R&D Systems Inc.) or anti-CCR5 antibodies (3.5 μg/ml, 2D7. Biosciences-Pharmingen, San Diego) in the presence or absence of *Sukumo* extract at 37 °C for 2 h. Cells were washed with PBS/2% FBS and strained with FITC-conjugated anti-mouse IgG (0.02 mg/ml, American Qualex) for 30 min on ice. Pretreated MT-4 cells also were stained with FITC-conjugated anti-human CD4 or monoclonal mouse antibodies of isotype IgG (negative control for flow cytometry) (1:50 dilution, DAKO). The cells were fixed in 1% paraformaldehyde-PBS solution and analyzed on FACS Calibur (Becton Dickinson), a flow cytometer with CELLQUEST software (Becton Dickinson).

2.6. Single-cycle infectivity assay

Pseudotyping vesicular stomatitis virus protein G (VSV-G) onto HIV cores from an env-defective reporter virus was carried out as follows. Plasmid DNA (10 μg) encoding envelope from VSV-G was co-transfected with pNL-E env(−) nef(−) (20 μg) into 293T cells using the calcium phosphate method. The virus titer was determined based on the level of p24 Gag. Time course assays were conducted to determine which steps in viral infection (entry and post-entry) were inhibited by *Sukumo* extract. Three treatment schedules were applied for HIV-1 infection with 293T cells, which were infected with pseudotyped HIV-1/VSV-G viruses (5 ng/ml of p24 Gag). The amount of p24 Gag in culture supernatant was determined to assess HIV-1 replication. *Sukumo* extract was used at serial concentrations in a range of 0.16–100 μg/ml and added at different times. The levels of p24 Gag were determined after 3 days of incubation by an auto-ELISA system.

2.7. Sukumo extract binding assay

The binding assay was used to determine the affinity of *Sukumo* extract for virions by viral replication assay. Separation of *Sukumo* extract and virus was carried out on a chromatography of gel filtration system with a column of Sephacryl S-500 (1 by 18 cm) (Amersham Pharmacia Biotech, Sweden). The column was equilibrated and the compounds were eluted from column with PBS. The samples were separated into the following three samples; *Sukumo extract control*: up to 150 μl of *Sukumo* extract (16 mg/ml), with 450 μl of RPMI-1640 medium containing 10% FBS added; *virus control*: up to 150 μl of PBS, with 450 μl of cultured supernatant containing HIV-1 NL4-3 added; *Sukumo extract–virus mixture*: up to 150 μl of *Sukumo* extract, with 450 μl of cultured supernatant containing HIV-1 NL4-3 added. A volume of 500 μl of each sample was injected onto the analytical column after incubation at 37 °C for 1 h and one fraction of eluant was collected (1 ml) on ice. The elution peak of the *Sukumo* extract control fractions at a wavelength of 492 nm and anti-HIV-1 IIIB activity by MTT assay were monitored to determine the elution position of *Sukumo* extract (Fig. 4A). The levels of p24 Gag in the eluted fractions were measured with auto-ELISA to determine the elution position of virus (Fig. 4B). Viral infectivity was analyzed for the eluted fractions of the virus control and *Sukumo* extract–virus mixture; the selected fractions 6 and 7 were clarified by 0.2 μM filter. MT-4 cells (4×10^5/ml) were infected by a mixture of the eluted fractions. Two hours after infection, the cells were washed and added to fresh RPMI-1640/10% FBS medium, cultured for 4 days and the p24 Gag of supernatant was measured.

2.8. Separation of compound

Anion exchange chromatography was carried out on a DEAE-Sephacel column, which had been equilibrated with phosphate buffer (pH 7.2). The bound sample was eluted by stepwise increases of the NaCl concentrations in phosphate buffer. The eluted fractions were analyzed for anti-HIV activity using the MTT assay method. The *Sukumo* extract was also separated with 15% SDS-PAGE. The gel was stained

by silver reagent and cut as described in Fig. 6B. *Sukumo* was re-extracted with RPMI-1640 medium from the SDS-gel fractions and collected supernatants for anti-HIV-1 activity.

3. Results

3.1. Spectrum of anti-viral activity of Sukumo extract

The anti-HIV-1 activity of *Sukumo* extract was first investigated by conventional MTT assay using MT-4 cells. *Sukumo* extract completely inhibited HIV-1 (III_B strain) replication in MT-4 cells at a concentration as low as 3.9 μg/ml. Its 50 and 90% effective concentrations (EC_{50} and EC_{90}) were 0.5494 and 2.1378 μg/ml, respectively. The 50% cytotoxic concentration (CC_{50}) was found to be >1000 μg/ml (Fig. 1A), and the selectivity index (ratio of CC_{50} to EC_{50}) of *Sukumo* extract was >1820, indicating that this compounds is very potent and selective.

Sukumo extract was also evaluated for the inhibition of wild-type herpes simplex virus-1 replication in infected Vero cells, using a standard viral plaque assay (Fig. 1B). *Sukumo* extract, and exhibited anti-viral activity with an EC_{50} value of 11.56 μg/ml. However, no inhibitory activity was observed against influenza A virus, poliovirus and SARS virus when *Sukumo* extract was tested at concentrations ranging from 3.2 to 400 μg/ml (data not shown).

3.2. Anti-HIV-1 activity of Sukumo extract

Sukumo extract inhibited a variety of HIV-1 isolates, including a laboratory adapted isolate IIIB strain, laboratory molecular clones X4 type NL4-3 and R5 type JRCSF in a variety of cells, including Molt-4, Jurkat, PM1, and CD4-CCR5 expressing Hos cells (data not shown). The inhibitory activity of *Sukumo* extract against X4 HIV-1 (NL4-3) and R5 HIV-1 (JRCSF) replication in PBMCs was also demonstrated

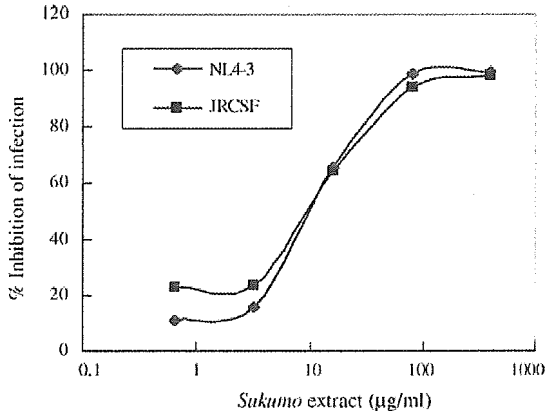


Fig. 2. Anti-HIV-1 activity of *Sukumo* extract in PHA-stimulated PBMCs. PHA-stimulated PBMCs were infected for 2 h at 37 °C in the absence or presence of 0.64–400 μg/ml *Sukumo* extract followed by washing. 1×10^6/ml infected cells per well were seeded in 24-well plate and were incubated for 7 days in the absence or presence of appropriate concentrations of compound. Quantity of HIV-1 p24 Gag was measured by auto-ELISA system.

by p24 assay of culture supernatants of the cells infected with the viruses exhibiting EC_{50} values of 12.02 and 11.5 μg/ml, respectively (Fig. 2). The p24 Gag levels of untreated samples of HIV-1 NL4-3 and JRCSF were 27.914 and 14.096 ng/ml, respectively. HIV-1 replication in MT-4 cells appeared to be more sensitive to *Sukumo* extract than in PBMCs.

3.3. Inhibition of HIV-1 binding and entry to the cells

In the various steps of the HIV-1 life cycle, we next investigated at which step *Sukumo* extract exerts its effect as an HIV-1 antagonist. To determine whether the viral binding to cells is a target of *Sukumo* extract, a binding assay was carried out to measure the effect of *Sukumo* extract on virions/cell surface interactions. MT-4 cells were mixed with X4 virus NL4-3 on ice for 2 h, and then the cells washed to remove the unbound viruses. The results demonstrate that

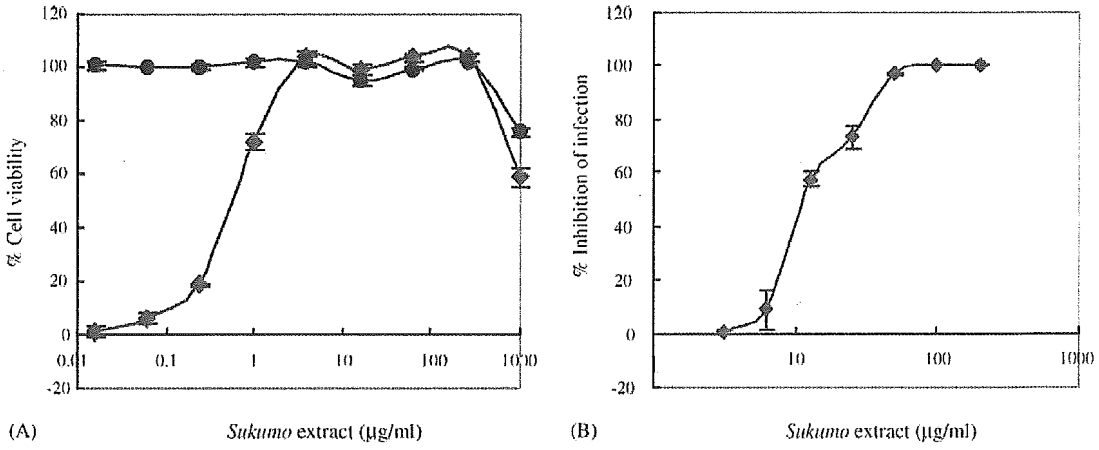


Fig. 1. Anti-viral activity of *Sukumo* extract. (A) Anti-HIV-1 activity of *Sukumo* extract in MT-4 cells was measured by MTT assay. HIV-1 (III_B) was used in this study ((●) mock infected and (◆) HIV-1)) and the EC_{50} values for inhibition of *Sukumo* extract against HIV-1 replication were determined. (B) Anti-HSV-1 activity of *Sukumo* extract in Vero cells was determined by plaque assay. The results shown are mean ± S.D. of triplicates.

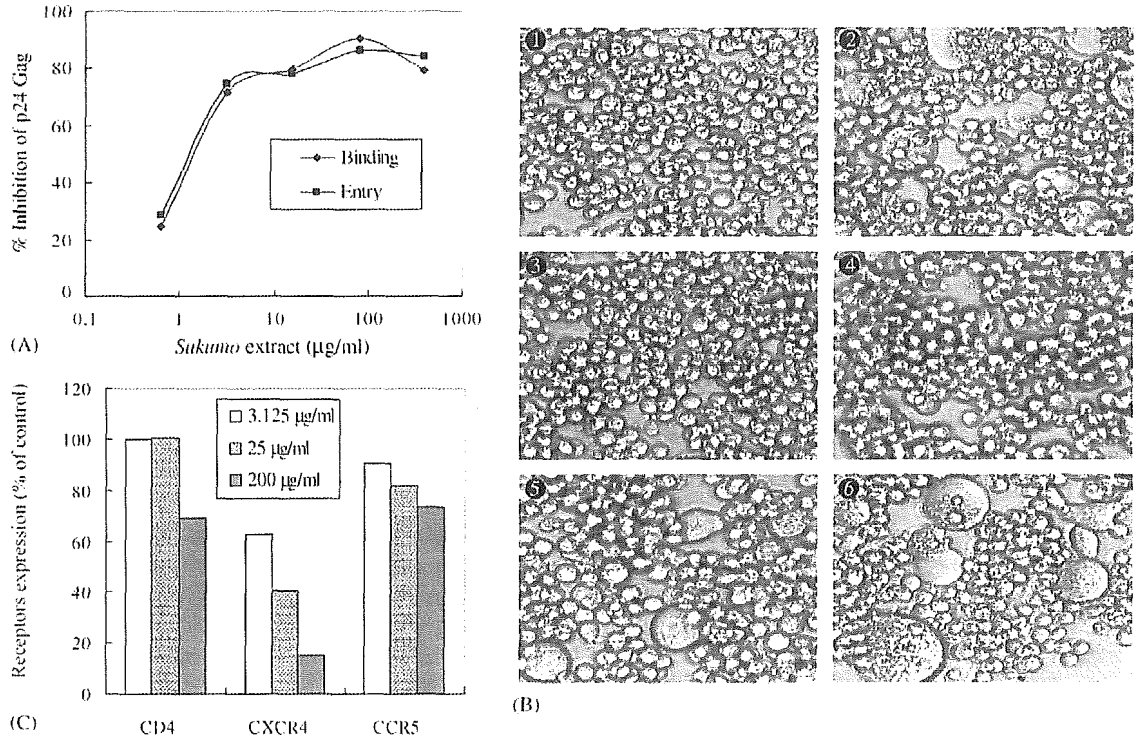


Fig. 3. Inhibition of HIV-1 entry and syncytia formation by *Sukumo* extract. (A) Inhibition of HIV-1 binding and entry into MT-4 cells by *Sukumo* extract. The cells were incubated for 2 h on ice or 37 °C with HIV-1 NL4-3 strain in the presence of various concentrations (0.64–200 μg/ml) of *Sukumo* extract. The levels of p24 antigen of virions bound to or entered in MT-4 cells were measured in the presence of different concentrations of *Sukumo* extract. The percentage of inhibition of virus binding or entry was defined as [1 − (p24 Gag with *Sukumo*/p24 Gag without *Sukumo* extract)] × 100%. (B) Inhibition of cell–cell fusion by *Sukumo* extract. (1) Molt-4 cells; (2) Molt-4 and Molt-4/IIIB cells co-culture; (3) 200 μg/ml *Sukumo* extract; (4) 25 μg/ml *Sukumo* extract; (5) 3.125 μg/ml *Sukumo* extract and (6) 0.39 μg/ml *Sukumo* extract. (C) Down-modulation of CD4, CXCR4 and CCR5 expression in MT-4 or CCR5 expressing Hos cells after treatment with *Sukumo* extract. Cells were exposed to an anti-CD4, anti-CXCR4 (12G5) or anti-CCR5 (2D7) antibody in the presence of 3.125, 25 and 200 μg/ml *Sukumo* extract, or to a negative control antibody, followed by labeling with a FITC-conjugated anti-mouse Ig probe and analyzed by flow cytometry. These results are representative of multiple experiments and microscopic fields.

Sukumo extract blocked virus-cell binding with an EC_{50} of 2.02 μg/ml (Fig. 3A). The inhibitory effect of *Sukumo* extract on HIV-1 entry to cells was also studied in MT-4 cells. Cells were incubated with the same virus at 37 °C for 2 h, treated with trypsin to remove bound virions, and then the intracellular p24 Gag of HIV-1 was measured. *Sukumo* extract inhibited viral entry with an EC_{50} of 1.84 μg/ml (Fig. 3A). The binding and entry of HIV-1 NL4-3 in MT-4 cells was efficiently inhibited by *Sukumo* extract in a dose-dependent manner. A similar result was also observed with R5 type HIV-1 JRCSF on Hos/CD4-CCR5 cells (data not shown). These experiments show that there is a good correlation between the anti-HIV activity and the inhibitory activity against virus binding/entry induced by the *Sukumo* extract.

Sukumo extract also completely prevented syncytium formation through co-culture of Molt-4 and HIV-1-converted Molt-4 cells at a concentration of 25 μg/ml and efficiently prevented it even at 3.125 μg/ml (Fig. 3B). These data indicate that *Sukumo* extract exerted its effect at an initial step of HIV-1 infection, such as viral entry and membrane fusion in the target cells.

We then analyzed changes in CD4 and CXCR4 expression on MT-4 cells and CCR5 expression on Hos/CD4-CCR5 cells upon treatment with different concentrations of *Sukumo* extract. Only a high concentration of *Sukumo* extract (200 μg/ml) caused down-expression of CD4 (68.87% of control). When *Sukumo* extract was used at the concentrations of 200, 25 and 3.125 μg/ml, the levels of CXCR4 expressed were only 15.33, 40.64 and 62.81% of control, respectively. In contrast, the levels of CCR5 expression on the surface of Hos cells were 73.25, 82.01 and 90.6% of control at the same concentration range (Fig. 3C).

3.4. Interaction of Sukumo extract with the HIV-1 envelope

To determine the *Sukumo* extract and HIV-1 interaction, we applied a chromatographical analysis using a Sephacryl S-500 for separation of the virus particles and the *Sukumo* extract based on differential molecular size. When *Sukumo* extract was fractionated, the main anti-HIV-1 activity was eluted in fractions 10–14, as revealed by the MTT assay

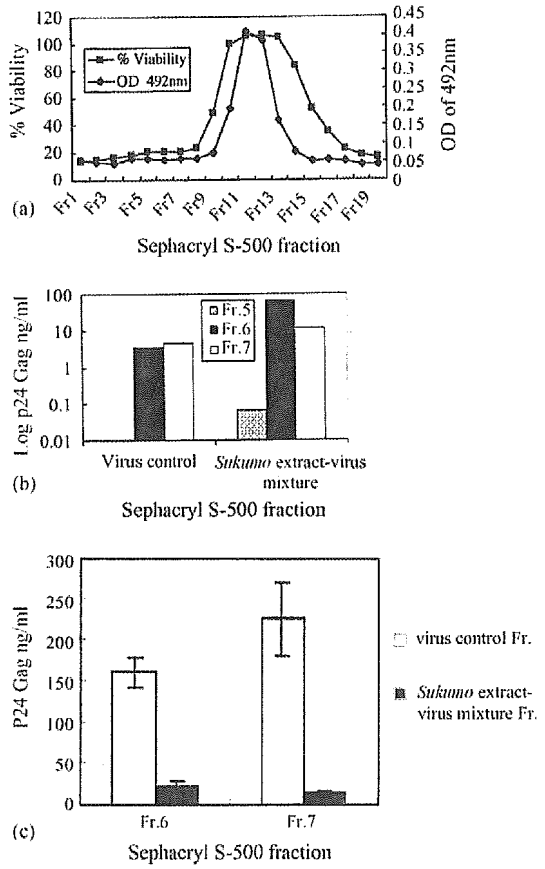


Fig. 4. Specific binding of *Sukumo* extract to HIV-1 virions, resulting in viral entry blockade and inhibition of HIV-1 replication in MT-4 cells. Experiments were carried out with a Sephacryl S-500 column in which three samples of *Sukumo* extract control, virus control (HIV NL4-3) and *Sukumo* extract–virus mixture were separated with a chromatograph column, respectively. (A) Anti-HIV-1 activity and absorbance of the wavelength of 492 nm from chromatogram fractions of *Sukumo* extract control eluate. Absorbance of *Sukumo* extract control fractions was measured at the wavelength of 492 nm (♦); the activity of each fraction was tested against HIV-1 replication by MTT assay, and then the viability of cells was calculated (■). (B) The quantity of HIV-1 p24 Gag was measured by auto-ELISA p24 Gag assay from chromatogram fractions 5–7 of virus control eluate and *Sukumo* extract–virus mixture eluate. (C) Infectivity of HIV-1 NL4-3 from chromatogram fractions of the virus control and *Sukumo* extract–virus mixture. The eluted fractions 6 and 7 were selected and infected into MT-4 cells for 2 h at 37 °C. After washing, the cells were incubated for 4 days and p24 Gag of culture supernatant was measured by auto-ELISA.

(Fig. 4A). On the other hand, HIV-1 was eluted in fractions 6 and 7 as shown by p24 assay (Fig. 4B left). When an excess amount of *Sukumo* extract was mixed with HIV-1 and separated with Sephacryl, the viral peak was detected in fractions 6 and 7 once again (Fig. 4B right), while anti-HIV activity was still observed in fraction 10–14 (data not shown). To see whether these fractions contained an infective capacity of HIV-1, the amount of p24 Gag was assessed in the supernatant of MT-4 cells after infection. We used the same volume (150 μl) of eluted fractions 6 and 7 from viral control, which contained 0.53 and 0.69 ng of p24 antigen, or those from the *Sukumo* extract–virus mixture which contained 10.24 and 1.78 ng of p24 antigen to infect 4×10^5 MT-4 cells, respectively. As shown in Fig. 4C, fractions 6 and 7 obtained from the viral control exhibited high HIV-1 activity (161.35 and 226.32 ng/ml in p24 level) 4 days after infection while fractions 6 and 7 from the *Sukumo* extract–virus mixture had p24 levels as low as 21.6 and 13.76 ng/ml, respectively. These results strongly suggests that the *Sukumo* extract specifically bound to viral particles and was efficiently trapped by viral particles so that viral infectivity was significantly abrogated due to the blockage of entry into the cells.

3.5. Effect of Sukumo extract on VSV-G pseudotyped HIV-1 replication

Although all the data provided evidence that HIV-1 entry could be a primary anti-viral target of *Sukumo* extract, there still remained the possibility that *Sukumo* extract exerts its effect on a late step of viral replication. To address this, a time course assay was performed using a single cycle infection with VSV-G pseudotyped HIV-1 and 293T cells. P24 Gag in the supernatant was measured 3 days post-infection. The result showed that a dose-dependent anti-viral activity of *Sukumo* extract was observed when it was added at the time B (entry) step. In contrast, the inhibition was not seen when *Sukumo* extract was added at the time A (pre-treatment) or time C step (post-entry) at any concentrations studied (range 0.16–100 μg/ml) (Fig. 5). A similar result was also observed when Hos/CD4-CCR5 cells were used (data not shown). These results suggest that *Sukumo* extract does affect an early step, not a post-entry step, of the viral life cycle.

Based on these studies, we conclude that there is persuasive evidene that *Sukumo* extract is a binding inhibitor that interferes with virion/cells interactions and that this inhibition is likely mediated through binding to the HIV-1 viral envelope.

3.6. Physico-chemical characterization of anti-viral factors in Sukumo extract

The anti-viral factor was extracted from *Sukumo* using organic solvent and water. Inhibitory activity was found in the aqueous extract. Crude *Sukumo* extract was fractionated by DEAE-Sephacel column chromatography. The main fractions which had anti-viral activity was eluted from the column by 1.0–2.0 M NaCl (Fig. 6A). The *Sukumo* extract was also separated by using SDS-PAGE and anti-HIV-1 activity was detected in fractions 3–7 of the SDS-gel extracts corresponding to a molecular weight of 10,000–50,000 (Fig. 6B). Gas chromatography analysis of the acid hydrolysates of the *Sukumo* extract revealed the carbohydrate contents of Ara:Xyl:Man:Gal:Glc were 5.1:1:2.1:3.3:2.6 (Table 1) and SDS-PAGE/PAS staining yielded a bright red band (Zacharius et al., 1969) (data not shown). Elemental analysis revealed that the sulfer content is 1.14% in the

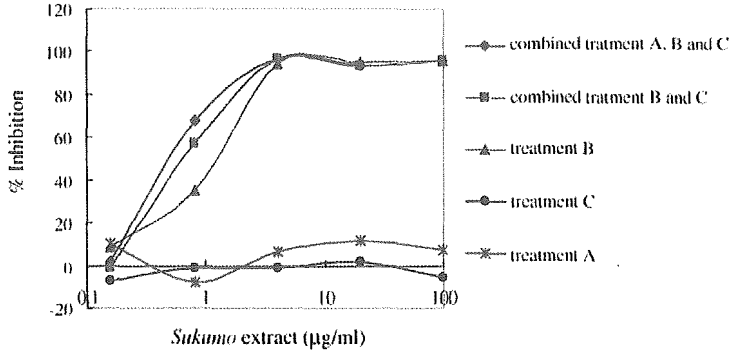
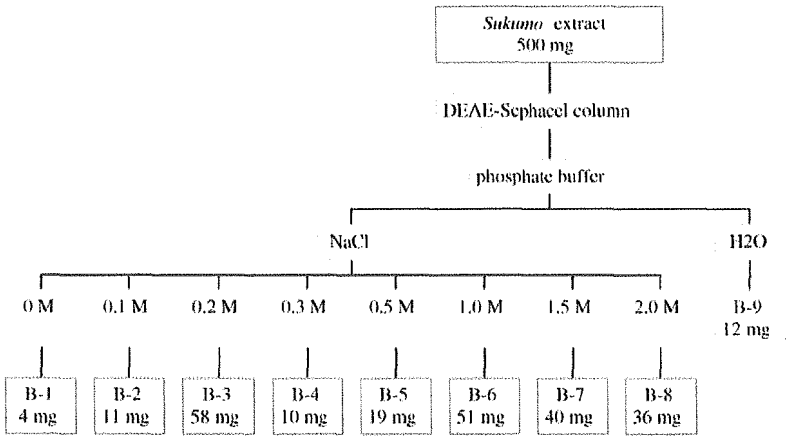
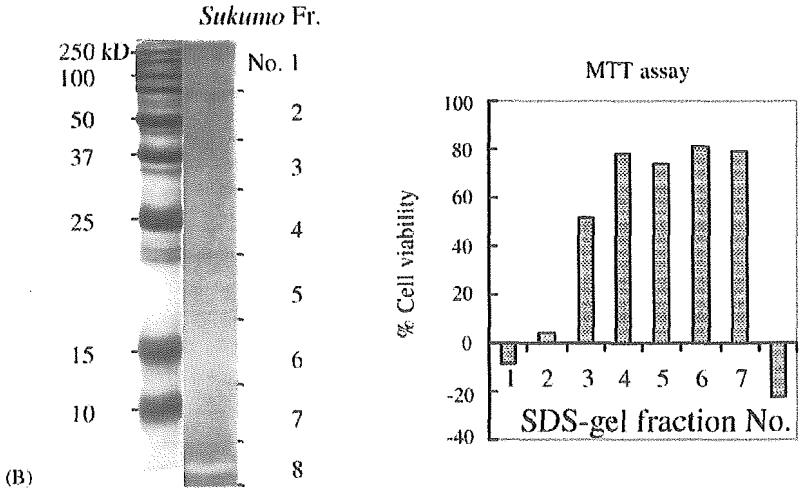


Fig. 5. Effect of *Sukumo* extract on VSV-G pseudotyped HIV-1 replication. 293T cells were infected with the HIV-1 NL-E strain lacking env and nef with VSV-G envelope of pesudotyped virus. 0.16–100 μg/ml *Sukumo* extract was used and anti-HIV-1 activity was determined 3 days later by measuring p24 Gag. Treatment A (a pre-entry step): the cells were incubated with *Sukumo* extract for 2 h at 37 °C and washed before exposure to virus, and then the cells were infected and incubated in the absence of *Sukumo* extract. Treatment B (an entry step): the cells were exposed to virus in the presence of *Sukumo* extract for 2 h, then both *Sukumo* extract and unabsorbed viruses were removed by washing. The cells were further incubated in the absence of *Sukumo* extract. Treatment C (a post-entry step): the cells were infected with virus for 2 h, unabsorbed virus were removed and further incubated in presence of *Sukumo* extract.



(A)

Fr.	EC_{50} μg/ml
B-1	>100
B-2	>100
B-3	>100
B-4	>100
B-5	>100
B-6	0.7405
B-7	0.6155
B-8	0.8818
B-9	9.7729



(B)

Fig. 6. Physico-chemical characterization of *Sukumo* extract. (A) *Sukumo* extract was analyzed with a DEAE-Sephacel column. The anti-HIV-1 activity of each eluting fraction was tested by MTT assay. The antiviral factor was eluted in 1.0–2.0 M NaCl. (B) *Sukumo* extract was analyzed by SDS-PAGE. 0.4 mg of *Sukumo* extract was separated with 15% SDS-PAGE. The gel was stained with silver reagent for protein analysis. The anti-HIV-1 activities of extracts from SDS-gel fractions were tested by MTT assay.

Table 1
Compound properties of *Sukumo* extract

Amino acid (%)	Carbohydrate[a]	Molar ratio	Element	Percentage (%)
0.002	Arabinose	5.1	H	4.14
	Xylose	1	C	37.16
	Mannose	2.1	N	6.66
	Galactose	3.3	S	1.14
	Glucose	2.6		
	Rhamnose	Trace		

[a] The *Sukumo* extract was hydrolyzed with 1 M H_2SO_4 at 100 °C for 6 h, and then the solution was applied on Supelco SP-2380 column and was analyzed with Shimadzu gas cells chromatograph GC-14B.

Sukumo extract (Table 1). The anti-viral factor in *Sukumo* extract was stable under a wide range of pH conditions. As shown in Table 2, the anti-viral activity of *Sukumo* extract was not reduced after treatment with 6N H_2SO_4, 1N HCl or $NaIO_4$, but the value of EC_{50} (1.095 μg/ml) was somewhat decreased when it was treated with 1N NaOH. The activity was also not lost after being heated at 121 °C for 20 min and was not inactivated by protease (trypsin, proteinase K and pronase) digestion. Finally, we addressed whether the anti-HIV-1 activities of *Sukumo* extract, heparin and dextran sulfate were abrogated after they were treated with acid. When *Sukumo* extract was boiled at 100 °C in 6N H_2SO_4 and 1N HCl for 6 h the 50% effective concentration (EC_{50}) against HIV-1 was essentially unaffected. In sharp contrast, when heparin was boiled at 100 °C in 6N H_2SO_4 for 6 h or dextran sulfate in 1N HCl for 2 h, the 50% effective concentrations against HIV-1 were >940 and >1000 μg/ml by MTT assay, while those of the untreated samples were 8.2095 and 0.7707 μg/ml, respectively (Table 2).

Table 2
Effect of various physico-chemical treatments on anti-HIV-1 (III_B) activity of *Sukumo* extract in MT-4

Treated with	Compound[a] (EC_{50} μg/ml)		
	Sukumo extract	Heparin	Dextran sulfate (MW 500,000)
Untreated	0.5891	8.2095	0.7016
121 °C 20 min	0.5166	ND[f]	ND[f]
Trypsin[b]	0.6686	ND	ND
Proteinase K[b]	0.5727	ND	ND
Pronase[b]	0.4736	ND	ND
NaOH[c]	1.0954	ND	ND
$NaIO_4$[d]	0.5832	ND	ND
H_2SO_4 treated[e]	0.4155	>940	ND
HCl treated[e]	0.7707	ND	>1000

[a] The 50% effective concentration was determined by MTT assay using HIV-1 (III_B) strain and MT-4 cells.

[b] The *Sukumo* extract was digested by trypsin (Sigma) (0.5–1 mg/ml at a final concentration), proteinase K (100 ng/ml) and pronase (Fluka) (0.2 mg/ml) at 37 °C for 30 h. The digestions were terminated by boiling the solution for 20 min at 100 °C.

[c] The *Sukumo* extract was boiled at 100 °C for 6 h in the presence of 1N NaOH.

[d] The *Sukumo* extract was incubated at 4 °C for 40 h in the presence of 100 mM $NaIO_4$. After treatment, the *Sukumo* extract was precipitated with 2 volumes of ethanol and resuspended in 1 volume of H_2O.

[e] Each compound was boiled at 100 °C for 6 h in the presence of 6N H_2SO_4 or for 2 h in the presence of 1N HCl. After treatment, pH was adjusted to 7.5.

[f] ND: not determined.

4. Discussion

Sukumo extract potently and selectively inhibited HIV-1 replication in vitro. The compound was also evaluated for activity against various virus species with or without an envelope including vesicular stomatitis virus G protein enveloped HIV-1 pseudotyped type virus. Whereas *Sukumo* extract was active against herpes simplex virus, it was devoid of any activity against influenza A virus, SARS virus and a non-enveloped poliovirus.

Based on the current knowledge of HIV, several stages of the viral life cycle are potentially vulnerable to inhibitors. These can be divided into the entry steps and post-entry steps. In this study, we have demonstrated by several different techniques that *Sukumo* extract inhibits the HIV-1 infectious process at the cell entry step. The data presented in Fig. 3 indicate that *Sukumo* extract is able to block viral binding to target cells and inhibits virus-induced cell–cell fusion. Furthermore, a time-course experiment showed that the full protective activity of *Sukumo* extract was achieved when the compound was present during the 2-h virus adsorption period, but none of the effect was seen when the compound was incubated with the cells prior to viral infection. Also, the extract did not suppress the viral replication after the virus had entered the cells. Thus, *Sukumo* extract interferes with an early event of the virus replication cycle, most presumably the viral adsorption step.

Two classes of cell surface molecules, CD4 and chemokine receptors, as well as CCR5 or CXCR4, are often viewed as HIV coreceptors which mediate HIV-1 entry. We found that the down-modulation of HIV-1 receptor CD4 or co-receptor CCR5 in target cells was induced by the *Sukumo* extract. However, the inhibitory activity was rather weak. In addition, this activity of *Sukumo* extract was lost if the cells were washed prior to addition of antibody, indicating that the compounds can only weakly associate with the cell surface. Therefore, the results cannot perfectly explain why the *Sukumo* extract is able to block virus entry of HIV so efficiently, especially the R5 HIV-1 virus.

The effect of *Sukumo* extract on the viral binding process was assessed directly, using a chromatography method (Fig. 4). The results show that *Sukumo* extract was bound to HIV-1 and was separated along with the larger virus particle fraction from a gel filtration column. From this study we hypothesized that *Sukumo* extract exerts its anti-HIV activity by binding to the viral envelope glycoprotein. This results in prevention of virus attachment to the cell surface receptor or co-receptor, whereby interference with early adsorption and entry into the HIV replicative cycle. These findings are