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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 (Polygonaceae) called Sukumo, exhibited a potent inhibitory activity against HIV type 1 in vitro. The extract potently suppressed acute HIV-1 (IIIB) infection in MT-4 cells with EC50 values of 0.5 μg/ml but exhibited low cytotoxicity to MT-4 cells even at a high concentration (CC50 > 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 EC50 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 NaIO4. 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.

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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 HIV envelope glycoprotein.
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; Yisastigui 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 (Santosh 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; Koyama-Miyata et al., 2003; Kataoka et al., 2003; Miyake et al., 2003), while pigment (PiP) 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 (IIIB) strain (Molt-4/IIIB) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Canseco 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 (IIIB) 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. Anti-HIV assay

To determine the anti-HIV-1 activity and cytotoxicity of the Sukumo extract, MT-4 cells were either infected with HIV-1 (IIIB) 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. EC50 values were calculated in 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 with 2b 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 (ELISA) (Fuji Rebio Inc., Tokyo, Japan). In this assay the p24 antigen from Zeptometrix (Buffalo, New York) was used as the standard. Chronically HIV-1 (IIIB)-infected Molt-4 cells were
co-cultured with HIV non-infected MoTL-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 was 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^{6}) 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 re-suspended 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 with anti-CCR5 antibodies (3.5 μg/ml, 2D7. Biosciences-Pharmingen, San Diego) or anti-CCR5 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 stained with FITC-conjugated anti-mouse IgG (0.02 mg/ml, American Qualex) for 30 min on ice. Pre-treated 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 enve(−) 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/JSV-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 (1640/10% FBS 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 with 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 (IIIB strain) replication in MT-4 cells at a concentration as low as 3.9 µg/ml. Its 50 and 90% effective concentrations (EC50 and EC90) were 0.5494 and 2.1378 µg/ml, respectively. The 50% cytotoxic concentration (CC50) was found to be >1000 µg/ml (Fig. 1A), and the selectivity index (ratio of CC50 to EC50) of Sukumo extract was >1820, indicating that this compound 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 EC50 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 by p24 assay of culture supernatants of the cells infected with the viruses exhibiting EC50 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
Y. Zhong et al. / Antiviral Research 66 (2005) 119–128

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 extract/p24 Gag without Sukumo extract)] × 100%. (B) Inhibition of cell–cell fusion by Sukumo extract. (1) Molt-4 cells; (2) Molt-4 and Molt-4IIIB 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 EC50 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 EC50 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.
The eluted fractions 6 and 7 were selected and infected into MT-4 cells for matogram fractions of the virus control and extract–virus mixture eluate. (C) Infectivity of HIV-1 NL4-3 from chromatogram fractions 5–7 of virus control eluate and (B) The quantity of HIV-1 p24 Gag was measured by auto-ELISA p24 Gag cation by MTT assay, and then the viability of cells was calculated. (A) Anti-HIV-1 activity and absorbance of the wavelength of 492 nm were measured by auto-ELISA. The amount 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 HeLa/CD4-CR5 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 evidence 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-Sephard 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 hydrolyses 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 sulfur content is 1.14% in the extract (Fig. 4A). On the other hand, HIV-1 was eluted in fractions 6 and 7 once again (Fig. 4B right). When an excess concentration of Sukumo extract–virus mixture was mixed with HIV-1 and separated by DEAE-Sephadex column chromatography. The main fractions which had anti-viral activity was eluted 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.

![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.](image-url)
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 pseudotyped 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.

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.
The solution for 20 min at 100 °C or for 2 h in the presence of 1N HCl. After treatment, pH was adjusted to 7.5.

Table 2

Effect of various physico-chemical treatments on anti-HIV-1 (IIIB) activity of Sukumo extract in MT-4

| Treated with | Compound a (EC50 μg/ml) | Heparin (IU/ml) | Dextran sulfate (MW 100,000) |
|--------------|-------------------------|-----------------|----------------------------|
| Untrated     | 0.5891                  | 8.2095          | >1000                       |
| 121 °C 20 min| 0.5166                  | ND              | ND                         |
| Trypsin b    | 0.6806                  | ND              | ND                         |
| Protease K c | 0.5727                  | ND              | ND                         |
| Pronase d    | 0.4736                  | ND              | ND                         |
| NaOH e       | 1.0954                  | ND              | ND                         |
| NaIO4 f      | 0.5832                  | ND              | ND                         |
| H2SO4 treated g | 0.4155              | ND              | ND                         |
| HCl treated h | 0.7707                  | ND              | >1000                       |

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

The Sukumo extract was digested by trypsin (Sigma) (0.5-1 mg/ml at a final concentration), protease 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.

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

The Sukumo extract was incubated at 4 °C for 40 h in the presence of 100 mM NaIO4. After treatment, the Sukumo extract was precipitated with 2 volumes of ethanol and reconstituted in 1 volume of H2O.

Each compound was boiled at 100 °C for 6 h in the presence of 6N H2SO4 or for 2 h in the presence of 1N HCl. After treatment, pH was adjusted to 7.5.

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 envelope 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...
consistent with the hypothesis that Sukamo extract interferes with viron rather than cell function. It might also explain why Sukamo extract is less toxic to target cells in vitro.

The biochemical features of water extract of Sukamo prepared from Polygonum tinctorium that selectively inhibited the replication of HIV-1 were studied. The anti-viral activity was extracted from Sukamo in a variety of ways, using water and organic solvents (hexane, chloroform, acetone and ethanol). Inhibitory activity was found in the aqueous extracts, whereas the extracts by organic solvents did not show any anti-HIV activity. Indigo, a staining ingredient and tryptanthrin, a low molecular weight component from Polygonum tinctorium, also did not exhibit any anti-HIV activity (data not shown).

The main fraction of anti-HIV activity was eluted from the DEAE-Sephadex column, a negative ion-exchange column at higher molar (1.0–2.0) NaCl. This result indicate that the active factor(s) is highly anionic. It was also confirmed that the anti-HIV compound(s) consist of phenolic substructure by FeCl3–K3Fe(CN)6 staining (Barton et al., 1952) (data not shown) and a polysaccharide containing sulfur atom by sugar analysis and elemental analysis, respectively (Table 1). The factor was estimated to be a high molecular weight compound of 10,000–50,000 by Sephadex G-75 gel-filtration analysis (data not shown) and a SDS-gel of Sukamo extract (Fig. 6B). No protein was detected in the water extract of Sukamo with SDS-PAGE/silver staining. The data confirm our observation that the inhibitory activity of Sukamo extract was not inactivated by protease digestion or heating at 121°C for 20 min. Furthermore, boiling of the Sukamo extract in the presence of 1N HCl, 6N H2SO4 and 1N NaOH for 6 h did not result in any loss of this activity. Similarly, it was not inactivated by NaIO4 treatment (Nakashima et al., 1987b), which breaks down carbohydrates (Table 2). This suggests that the sugar backbone is not essential for the anti-HIV activity of the Sukamo extract. The pharmaceutical value of the Sukamo extract is likely to be further enhanced by its stability over a wide range of pH values, as shown by the heating at 121°C 20 min and treatment with acid and alkaline conditions. Since the anti-HIV-1 activity of Sukamo was higher than that of fresh leaves (data not shown), the possibility that the active substances were derived from bacteria could not be excluded.

We compared difference between representative sulfated polysaccharides and Sukamo extract for their susceptibility to acid treatment. The anti-HIV-1 effect was clearly abrogated by this treatment in the case of dextran sulfate and heparin, but not Sukamo extract (Table 2). The anti-HIV-1 activity of heparin was completely destroyed by 6N H2SO4 treatment as in the case of 1N HCl treatment of dextran sulfate. Unlike epigallocatechin gallate, a polyphenolic substance from green tea (Suzutani et al., 2003; Yamaguchi et al., 2002), Sukamo extract did not exert any anti-HIV-1 activity on the post-virus entry process. Further work on the characterization of the Sukamo extract and its potency as an anti-viral candidate drug is in progress.

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