Synergism of Alkaline Extract of the Leaves of Sasa senanensis Rehder and Antiviral Agents

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Abstract. Background: Previous studies have shown a much greater antiviral activity of alkaline extract of the leaves of Sasa senanensis Rehder (SE) against human immunodeficiency virus (HIV), compared to lignin precursors, tannins and flavonoids, suggesting its possible application to oral diseases. Systematic comparative study with herpes simplex virus (HSV) has been limited compared to that with HIV. In the present study, we investigated whether combination of SE with other popular antiviral agents further enhances their individual activity. Materials and Methods: Cell viability of mock-infected, HIV-infected and HSV-infected cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The antiviral activity was evaluated by the selectivity index, defined as the ratio of 50% cytotoxic concentration to 50% effective concentration. Synergy between SE and antiviral agents was evaluated by MacSynerg and CompuSyn software. Results: SE showed potent anti-HIV activity, although its activity was two-orders lower than that of azidothymidine, 2’,3’-dideoxycytidine dextran sulfate and curdlan sulfate. Combination of SE with these antiviral agents produced synergistic effects. Using a newly established MTT assay system for anti-HSV activity, SE and acyclovir were found to have synergistic anti-HSV activity. Conclusion: The present study suggests the possible efficacy of the clinical application of SE combined with antiviral agents.

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bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium, AZT, and ddC were from Sigma Chemical Co. (St Louis, MO, USA); dimethyl sulfoxide (DMSO), and DS (5 kDa) were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan); CRDS (79 kDa) from Ajinomoto Co. Inc., (Tokyo, Japan); and ACV from Tokyo Chemical Industry Co. (Ltd, Tokyo). All antiviral agents were dissolved in DMSO, and cytotoxicity induced by DMSO was subtracted from data.

SE was manufactured by Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan. One milliliter of SE contained 66.1 mg freeze-dried powder.

**Assay for anti-HIV activity.** Human T-cell leukemia virus I (HTLV-I)-bearing CD4+ positive human T-cell line, MT-4 (supplied by Dr. Naoki Yamamoto), was cultured in RPMI-1640 medium supplemented with 10% FBS and infected with HIV-1_HIV at a multiplicity of infection (MOI) of 0.01. HIV- and mock-infected (control) MT-4 cells (3x10^5 cells/96-microwell) were incubated for 5 days with different concentrations of products and the relative viable cell number was determined by MTT assay. The concentration that reduced the viable cell number of the uninfected cells by 50% (CC50) and the concentration that increased the viable cell number of the HIV-infected cells up to 50% that of the control (mock-infected, untreated) cells (EC50) were determined from the dose–response curve. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated by the following equation: SI=CC50/EC50 (19).

**Assay for anti-HSV activity.** Plaque assay was performed according to the modification of our previous article (20). Confluent monolayer Vero cells [3x10^5 cells in 6-well tissue culture plate (NUNC Labware Product-Sigma Aldrich Inc, Tokyo, Japan)] were infected with HSV-1 (F strain) (supplied by the National Institute of Infectious Diseases, Tokyo, Japan) at 300 plaque-forming unit (PFU) per well for 1 h at 37°C in the absence or present of the test agents during infection. After allowing 1 h for virus adsorption, the infected cells were washed once with minimum essential medium (MEM), and overlaid with 2 ml MEM-10% fetal calf serum (FCS) containing 0.5% Bacto agar (Difco, Bacto Laboratory Pty Ltd, Mt. Pritchard, Australia) without or with test agents. After incubation for 3 days, the agar overlay was removed, and the attached cells were fixed with 50% ethanol and stained with 0.25% crystal violet. The number of visible plaques was then counted under light microscopy.

We also performed the MTT assay to quantify the anti-HSV activity of agents. Vero cells (10,000 cells) were inoculated on a 96-well plate (NUNC). After 24 h, the cells were infected with HSV-1 (strain F) (MOI=0.01). HSV-1 was mixed first with test sample and stood for 20 min, then mixed with ACV and added to the cells. After incubation for 4 or 5 days, the cells were added with MTT reagent (BioAssay Systems, Hayward, CA, USA) and incubated for 4 h. Cells were dissolved with 10%SDS in 0.01 M HCl, and the absorbance at 595 nm was measured.

**Statistical treatment.** Experimental data are the mean±standard deviation (SD). The statistical differences between control and treated groups were evaluated by Student’s t-test. A value of p<0.05 was considered to be significant. Significance of synergy between SE and four anti-HIV agents was evaluated by MacSynergy II, an analytical tool which describes and quantifies drug interaction. (21). CompuSyn software program (CompuSyn, Inc., Paramus, NJ, USA) was used to calculate the combination index (CI), where the combination effect was judged “synergistic” when CI was less than 1 (22).

**Results**

**Anti-HIV activity.** We first compared the anti-HIV activity of SE and four representative anti-HIV agents: AZT, ddT, CRDS and DS (Figure 1). Using uninfected cells, the viable cell number was reduced dose-dependently. From the dose–response curve, we determined the CC50 of SE, AZT, ddC, CRDS and DS as 1.42%, 64.10 μM, 2451 μM, >1000 μg/ml and >1000 μg/ml, respectively (Figure 1).

When cells were infected with HIV, all the cells were killed by its cytotoxic effect. By addition of increasing concentrations of antiviral agents, the viability of cells returned to the level of the control (uninfected and untreated). From the dose–response curve, we determined the EC50, the concentration that restored cell viability to 50% of the control level. The EC50 of SE, AZT, ddC, CRDS and DS was 0.0178%, 0.0028 μM, 0.206 μM, 0.098 μg/ml and 0.021 μg/ml, respectively (Figure 1).

**Combination effects of SE and anti-HIV agents.** We found that the combination treatment with 32 μM AZT and 0.00064, 0.0032, 0.016 or 0.08% SE, and that with 160 μM AZT and 0.0064 or 0.0032% SE had synergistic anti-HIV activity (Figure 2A). When 0.016% SE was present, 6.4 μM AZT was as effective as 32 μM AZT (without SE) (i.e. requiring only 1/5th the amount of AZT), and 32 μM AZT had anti-HIV activity comparable with 160 or 800 μM AZT (without SE) (i.e. requiring only 1/5-1/25th the amount of AZT) (Figure 2A). This indicates that supplementation with SE can reduce the concentration of AZT needed to treat HIV-infected cells to 1/5th or 1/25th.

Combination treatment with 1,600 μM ddC and 0.016% SE, and that with 8,000 μM ddC and 0.00064% SE had synergistic anti-HIV activity (Figure 2B). When 0.016% SE was present, 320 μM ddC had anti-HIV activity comparable with 8,000 μM AZT (without SE) (requiring only 1/25th the amount of AZT) (Figure 2B).

Combination treatment with 1,600 ng/ml CRDS and 0.00064, 0.016, 0.08 or 0.4% SE, and that with 8000 ng/ml CRDS and 0.016 or 0.4% SE had synergistic anti-HIV activity (Figure 2C). In the presence of 0.00064% SE, 1600 μM CRDS had anti-HIV activity comparable with 8,000 μM CRDS (without SE) (i.e. requiring only 1/5th the amount of CRDS) (Figure 2C).
Combination treatment with 2.56 ng/ml DS and 0.016% SE, that with 12.8 ng/ml DS and 0.032 or 0.016% SE, or that with 64 ng/ml DS and 0.08 or 0.4% SE produced synergistic anti-HIV activity (Figure 2D). In particular, the combination of 2.56 ng/ml DS and 0.032% SE increased the cell viability of HIV-infected cells to nearly 50% (99.9% synergy), while single treatment of DS or SE had no anti-HIV activity (Figure 2D).

Anti-HSV activity. Plaque assay demonstrated that a high concentration of SE (above 0.3%) exhibited cytotoxicity against Vero cells (Figure 3A, left column), and that infection with HSV-1 (300 PFU) induced cytopathic effect, killing all cells (Figure 3A, right column). Mixing of HSV-1 with SE for 20 min rapidly reduced the infectivity, restoring the cellular viability completely (Figure 3A, right column).

To quantify the anti-HSV activity of SE, we newly established the MTT assay for anti-HSV activity. Infection of HSV-1 significantly reduced the cellular viability to 25% or 10% after 4 and 5 days, respectively (Figure 3B). The addition of more than 0.03 to 0.01% SE nearly completely eliminated...
the cytopathic effect of HSV-1 infection. ACV (300 nM) was also partially effective. The CI calculated using an isobologram analysis indicated that addition of 0.003 or 0.01% SE together with ACV produced synergy of anti-HSV activity (CI=0.8 and 0.5, respectively) (Figure 3B). When the incubation time was prolonged to 5 days, synergic anti-HSV activity was observed at a wider range of SE concentrations (0.001, 0.003, 0.01, and 0.03%; CI=0.8, 0.6, 0.5 and 0.3, respectively) (Figure 3B).

Discussion

The present study demonstrated for the first time that SE can synergistically enhance the anti-HIV activity of four established chemotherapeutic agents (AZT, ddC, CRDS, DS). Such synergistic action was more pronounced when SE was used with nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), such as, AZT and ddT (23), rather than with sulfated polysaccharides such as CRDS or DS that prevent viral adsorption through binding to the viral envelope glycoprotein (24). This is reasonable since LCC, a putative active component of SE, was found to have very strong affinity to cells (25), similar to sulfated polysaccharides, but had a different site of action from NRTIs.

Sub-Saharan Africa has the highest number of patients with HIV but a low ability to purchase expensive drugs, therefore it is important to establish therapeutic methods that further enhance the therapeutic potential of conventional anti-HIV drugs. In 2014, this area had 25.8 million HIV-positive patients, 1.4 million newly-infected patients, and 790 thousand fatalities (26). The present study demonstrated that the addition of SE can reduce the amount of anti-HIV agent
necessary for antiviral action (only 4-20% are needed under optimal conditions). Such supplementation with SE may achieve more economical use of the same supplies.

We have established a new simplified method for quantification of anti-HSV activity with MTT reagent. Using this method, we found that SE and ACV also had synergistic anti-HSV activity. Repeated experiments revealed that the SI value of SE for anti-HSV activity was 6-7 (unpublished data), approximately one order lower than that for anti-HIV activity (SI=80) (Figure 1A). This low SI may be explained by the difference of cell types we used. MT-4 cells cultured in suspension can divide 32-times over 5 days, while the growth of adherent cells, such as Vero, are much slower due to contact inhibition, possibly reducing the difference of viable cell number of mock-infected cells and that of HSV-infected cells, thus yielding an unexpectedly lower SI value.

Using this anti-HSV assay method, we found that SE had much higher anti-HSV activity than other molecular polyphenols (unpublished data). Further study is underway to identify the active components in SE, and searching more potent natural and synthetic anti-HIV substances.

**Conflicts of Interest**

The first Author (HS) was supported by Daiwa Biological Research Institute Co., Ltd., Kanagawa, Japan. The Authors wish to confirm that such financial support has not influenced the outcome or the experimental data.
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