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Inhibition of human immunodeficiency virus type 1 (HIV-1) replication by daphnodorins

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Abstract

Three flavans, daphnodorins A, B and C isolated from *Dahpne odora* THUNB. were tested for their abilities to inhibit human immunodeficiency virus type 1 (HIV-1(IIIB)) replication in MT-4 cells. The effective concentrations (EC_{50}) of daphnodorins A, B and C against HIV-1-induced cytolysis were 0.26 ± 0.08, 1.8 ± 0.6 and 3.6 ± 0.5 μg/ml, respectively. Also these three compounds showed inhibitory effects of p24 antigen in human peripheral blood lymphocytes. As compared with 2′, 3′-dideoxycytidine 5′-triphosphate (DDC-TP), daphnodorin A and daphnodorin C had relatively weak inhibitory effects on the reverse transcriptase of HIV-1, while daphnodorin B did not show any inhibitory effect at concentrations up to 1000 μg/ml. These three compounds showed marked inhibitory effects on syncytium formation between HIV-1(IIIB)-infected and uninfected MOLT-4 (clone 8) cells at 3–30 μg/ml without inducing cytotoxicity. The concentrations of the compounds blocking syncytium formation were consistent with the effective concentrations (EC_{50}) against HIV-induced cytolysis of MT-4 cells. These results, differing from reverse transcriptase inhibitors, suggest that the daphnodorins exert their anti-HIV-1 activity through inhibition of early events of viral replication including adsorption of the virions to the cells or the subsequent entry.

Key words: Anti-HIV agent; HIV

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[1] Dr. M. Kozawa passed away on May 31, 1993.

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1. Introduction

An important therapeutic strategy for the treatment of acquired immune deficiency syndrome (AIDS) has been to develop compounds that interfere with replication of human immunodeficiency virus type 1 (HIV-1) (Mitsuya et al., 1991). Recently, in addition to 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxycytidine (DDC), several drugs including the benzodiazepine derivative TIBO (Pauwels et al., 1990) and 6-substituted acyclouridine derivative HEPT (Miyasaka et al., 1989) have been shown to efficiently inhibit the HIV-1-induced cytopathic effect in vitro by blocking HIV-1 reverse transcriptase activity. Clinically, AZT improves symptoms and prolongs of the survival of patients with AIDS (Fischl et al., 1987; Fischl et al., 1989). However, chemotherapy with AZT is limited by serious side effects (Richman et al., 1987) and the appearance of resistant mutant viruses. Continuous efforts must be made to find effective chemotherapeutic agents against HIV-1.

In traditional Chinese medicine, the roots of *Daphne odora* THUNB. have been used to treat stomach ache, bruises and venomous snake bites, and the leaves have been used to treat abcesses and neuralgic pain (Ching, 1977). Three flavans, daphnodorin A, daphnodorin B (Baba et al., 1986) and daphnodorin C (Baba et al., 1987), isolated from the root and the bark of *Daphne odora* THUNB., inhibit gastric H^+, K^+-ATPase and acid secretion (Murakami et al., 1992), and have antifungal activities against *Pyricularia oryzae* (Inamori et al., 1987). In this study, we found that daphnodorins possessed anti-HIV-1 activities. Differing from the inhibitors of reverse transcriptase, daphnodorins show inhibitory activity against syncytium formation between HIV-1-infected and uninfected MOLT-4 (clone 8) cells.

2. Materials and methods

2.1. Compounds

Daphnodorin A, B (Baba et al., 1986) and C (Baba et al., 1987) were isolated from the root and the bark of *Daphne odora* THUNB. The purity of these compounds was $> 99\%$, as analyzed by thin-layer chromatography. DDC was purchased from Sigma Chemical Co. All other chemicals were obtained commercially and were of reagent grade.

2.2. Cells and virus

MT-4 cells (Miyoshi et al., 1982) and MOLT-4 (clone 8) (Minowada et al., 1972) were provided from Dr. N. Yamamoto (Tokyo Medical and Dental University). Cells were cultured in RPMI 1640 (GIBCO) medium supplemented with 10% fetal bovine serum and kanamycin (100 μg/ml). HIV-1(IIIB) (Gallo et al., 1984) was obtained from the culture supernatants of MOLT-4 (clone 8) cells chronically infected with the virus. Titers of HIV-1 stocks were determined in MT-4 cells, and virus stocks were stored at -80°C until use.

2.3. Antiviral assay

Anti-HIV activities of the compounds were evaluated as described previously (Katagiri et al., 1992). MT-4 cells were exposed to HIV-1(HTLV-IIIB) at a m.o.i. of 0.002 and were cultured for 6 days in the presence of various concentrations of the drug. On day 5, the cell suspension was diluted with a 3-fold volume of fresh culture medium. The viability of the control cells was > 95% on day 6 (trypan blue exclusion assay). Control cells were treated similarly but not exposed to the virus. Cell proliferation was assessed by the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2 *H*-tetrazolium hydroxide] method (Weislow et al., 1989). The effective concentration (EC_{50}) represents the concentration of the drug that increases formazan production in infected cultures to 50% of that of untreated, uninfected cell controls. The inhibitory concentration (IC_{50}), represents the toxic concentration of the drug that reduces formazan production in uninfected cultures to 50%, which was determined by simple linear interpolation from the data. The therapeutic index (TI) was determined by dividing the IC_{50} by the EC_{50}.

For anti-HIV activity of the compounds in peripheral blood lymphocytes (PBL), HIV-1 p24 antigen in the culture supernatant was measured (Baba et al., 1991). Phytohemagglutinin-stimulated PBL (10^6/ml) were infected with HIV-1 at a m.o.i. of 0.02. The cells were incubated for 2 h, washed twice and then cultured in the presence of various concentrations of the compounds for 7 days. On day 4, the cell suspensions were diluted with a 5-fold volume of fresh culture medium. The concentration of p24 in the supernatant was evaluated using p24 capture enzyme-linked immunosorbent assay (Abbott Lab.). The effective concentration, EC_{50}, represents the concentration of the drug that inhibits p24 antigen production in infected cultures to 50% of that of untreated, uninfected cell controls. The inhibitory concentration, IC_{50}, was also obtained by XTT method.

2.4. Reverse transcriptase assay

Recombinant HIV-1 and avian myeloblastosis virus (AMV) reverse transcriptases (RT) were purchased from Eiken Chemical Co. and Seikagaku Kogyou Co., respectively. The RT reaction mixture contained 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 150 mM KCl, and 2 mM dithiothreitol, and 0.1 mM dTTP. The template/primer [poly(A)oligo$(dT)_{10}$] and substrate [^{3}H]dTTP (90 Ci/mmol) were at concentrations of 50 μg/ml and 0.11 μM, respectively. After addition of the enzyme (2 units) and varying concentrations of the inhibitors, the reaction mixtures were incubated for 1 h at 37°C. The reaction was terminated by addition of an equal volume of 10% trichloroacetic acid. The reaction product was collected on glass filter discs, which were washed three times with 10% trichloroacetic acid and counted in a scintillation counter. Inhibition of HIV-1 RT activity of compounds was expressed by the concentrations resulting in 50% inhibition (IC_{50}).

2.5. HIV-1-infected cell fusion assay

Syncytium formation assay was performed as described previously (Nakashima et al., 1987) with slight modification. Chronically HIV-1(IIIB)-infected MOLT-4 (clone 8)

cells (4×10^4 cells/50 μl) were transferred to 96-well microtiter plates. Then, MOLT-4 (clone 8) cells (4×10^4 cells/50 μl) and an appropriate concentration of test compound (100 μl) were added to each well. The mixed cells were cultured at 37°C in 5% CO_2 atmosphere. After a 24 h incubation, the number of syncytia were counted under a microscope. Cytotoxic activity of the compounds against the infected and the uninfected MOLT-4 (clone 8) cells (each 4×10^4 cells) were also examined by XTT method after a 24 h incubation in the presence of various concentrations of the drugs.

3. Results

3.1. Effects of daphnodorins on HIV-induced cytolysis

The inhibitory effects of daphnodorins A, B, and C (Fig. 1a, b, c) were evaluated against HIV-1-induced cytopathogenicity in MT-4 cells (Table 1). As a reference compound, 2′, 3′-dideoxycytidine (DDC) was used. Daphnodorins A, B and C protected

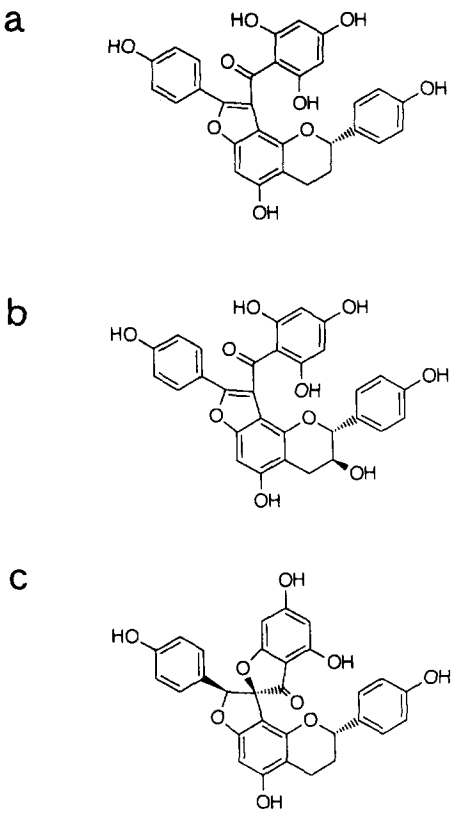

Fig. 1. Chemical structures of daphnodorin A (a), daphnodorin B (b) and daphnodorin C (c).

Table 1
In vitro antiviral activity of daphnodorin A, B, and C against HIV-1 cytopathogenicity in MT-4 cells

Compound	EC_{50} [a], μg/ml	IC_{50} [b], μg/ml	TI
Daphnodorin A	0.26 ±0.08	61 ± 12	235
Daphnodorin B	1.8 ±0.6	> 100	> 56
Daphnodorin C	3.6 ±0.5	38 ± 1	11
DDC	0.013 ± 0.009	38 ± 7	2923

[a] Cell proliferation was assessed by the XTT method (Weislow et al., 1989). The 50% effective concentration (EC_{50}), represents the concentration of compound that increased formazan production in infected cultures to 50% of untreated, uninfected cell controls.
[b] The 50% inhibitory concentration (IC_{50}), represents the toxic concentration of drug that reduced formazan production in uninfected cultures to 50%, as determined by simple linear interpolation from the data.

of MT-4 cells from the cytopathic effects of HIV-1(IIIB). Daphnodorin A inhibited virus-induced cytopathogenicity in MT-4 cells by 50% at a concentration of 0.26 ± 0.08 μg/ml (EC_{50}). The EC_{50} of daphnodorin B and daphnodorin C against HIV-1(IIIB) was 1.8 ± 0.6 and 3.6 ± 0.5 μg/ml, respectively, while the EC_{50} of DDC was 0.013 ± 0.009 μg/ml. The cytotoxic effects of daphnodorins on MT-4 cells were also examined, and the IC_{50} (50% inhibitory concentration) of daphnodorin A and C was 61 ± 12 and 38 ± 1 μg/ml, respectively. Daphnodorin B had no growth inhibitory effect on MT-4 cells at a concentration of 100 μg/ml. Therapeutic index (TI) values of daphnodorin A, daphnodorin B and daphnodorin C were 235, > 56, and 11, respectively, whereas the TI value of DDC was 2923.

We also evaluated the anti-HIV-1 acitivities of the compounds in peripheral blood lymphocytes by quantitative detection of HIV-1 p24 antigen in the culture supernatant (Table 2). The EC_{50} of daphnodorin A, B and C was 5.7 ± 1.7, 7.3 ± 2.0 and 1.9 ± 1.0 μg/ml, respectively, while the EC_{50} of DDC was 0.00033 ± 0.00013 μg/ml.

We examined the effects of daphnodorins on the production of HIV-1(IIIB) from chronically-infected MOLT-4 (clone 8) cells (data not shown). After a 24 h incubation of the cells in the presence of the compounds, the daphnodorins had no effect on the p24 antigen production in the infected cells at concentrations of 0.1 to 30 μg/ml. These results indicate that daphnodorins have no specific inhibitory effect on the late events of HIV replication.

Table 2
In vitro antiviral activity of daphnodorin A, B, and C against HIV-1 p24 antigen production in peripheral blood lymphocytes

Compound	EC_{50} [a], μg/ml	IC_{50} [b], μg/ml	TI
Daphnodorin A	5.7 ± 1.7	74 ± 5	13
Daphnodorin B	7.3 ± 2.0	67 ± 3	9.2
Daphnodorin C	1.9 ± 1.0	65 ± 6	34
DDC	0.00033 ± 0.00013	43 ± 2	130000

[a] The 50% effective concentration (EC_{50}) represents the concentration of compound that inhibited p24 production in acutely HIV-1-infected cultures to 50% of untreated, infected cell controls.
[b] IC_{50} was obtained by XTT method as described in footnote [b] to Table 1.

Table 3
Effects of daphnodorins on HIV-1 and AMV reverse transcriptases

Compound	IC_{50} [a], μg/ml	
	HIV-1 RT	AMV RT
Daphnodorin A	34 ± 3	820 ± 210
Daphnodorin B	> 1000	> 1000
Daphnodorin C	42 ± 5	> 1000
DDC-TP	0.30 ± 0.10	1.5 ± 0.3
Dextran sulfate	39 ± 10	91 ± 13

[a] 50% Inhibitory concentration required to inhibit RT activity by 50%, expressed as mean ± S.D. ($n = 3$).

3.2. *Effect of daphnodorins on reverse transcriptase activity*

We measured the inhibitory effects of daphnodorins on RT activity of HIV-1 (Table 3). Dideoxynucleosides inhibit RT activity after phosphorylation to their 5′-triphosphate kinases (Mitsuya and Broder, 1986, 1987). Triphosphorylated DDC (DDC-TP) and dextran sulfate (MW 6000) inhibited the HIV-1 RT activity by 50% at concentration of 0.3 and 39 μg/ml, respectively. The IC_{50} of daphnodorin A and daphnodorin C was 34 and 42 μg/ml, respectively, while daphnodorin B had no inhibitory effect on RT activity at concentrations up to 1000 μg/ml. Daphnodorin A showed a relatively weak inhibitory effect on avian myeloblastosis virus (AMV) RT activity, whereas daphnodorin B and C did not show inhibitory effects on AMV RT activity at concentrations up to 1000 μg/ml.

3.3. *Effect of daphnodorins on syncytium formation*

As shown in Fig. 2, DDC did not block the gp120/CD4-mediated syncytium formation observed under the condition used, whereas dextran sulfate showed a marked inhibitory effect on the syncytium formation without cytotoxic effects. At daphnodorin A concentrations from 0.03 to 1 μg/ml, a 31–44% inhibitory effect on syncytium formation was observed. Also, daphnodorin A markedly inhibited syncytium formation at concentrations from 3 to 30 μg/ml without cytotoxic effects; however, daphnodorin A had a marked cytotoxic effect at 100 μg/ml. Similar concentration-dependent inhibitory effects of daphnodorins B and daphnodorin C were obtained at concentrations from 1 to 30 μg/ml and from 0.1 to 30 μg/ml, respectively, without cytotoxic effects. Effect of daphnodorins on binding of anti-CD4 antibody to CD4 on the cell surface was examined (Yamamoto et al., 1992) and no inhibitory effect was obtained (data not shown).

4. Discussion

Daphnodorins were found to be potent inhibitors of HIV-1 in acutely HIV-1-infected cells. Daphnodorins did not inhibit p24 production in chronically HIV-1-infected cells.

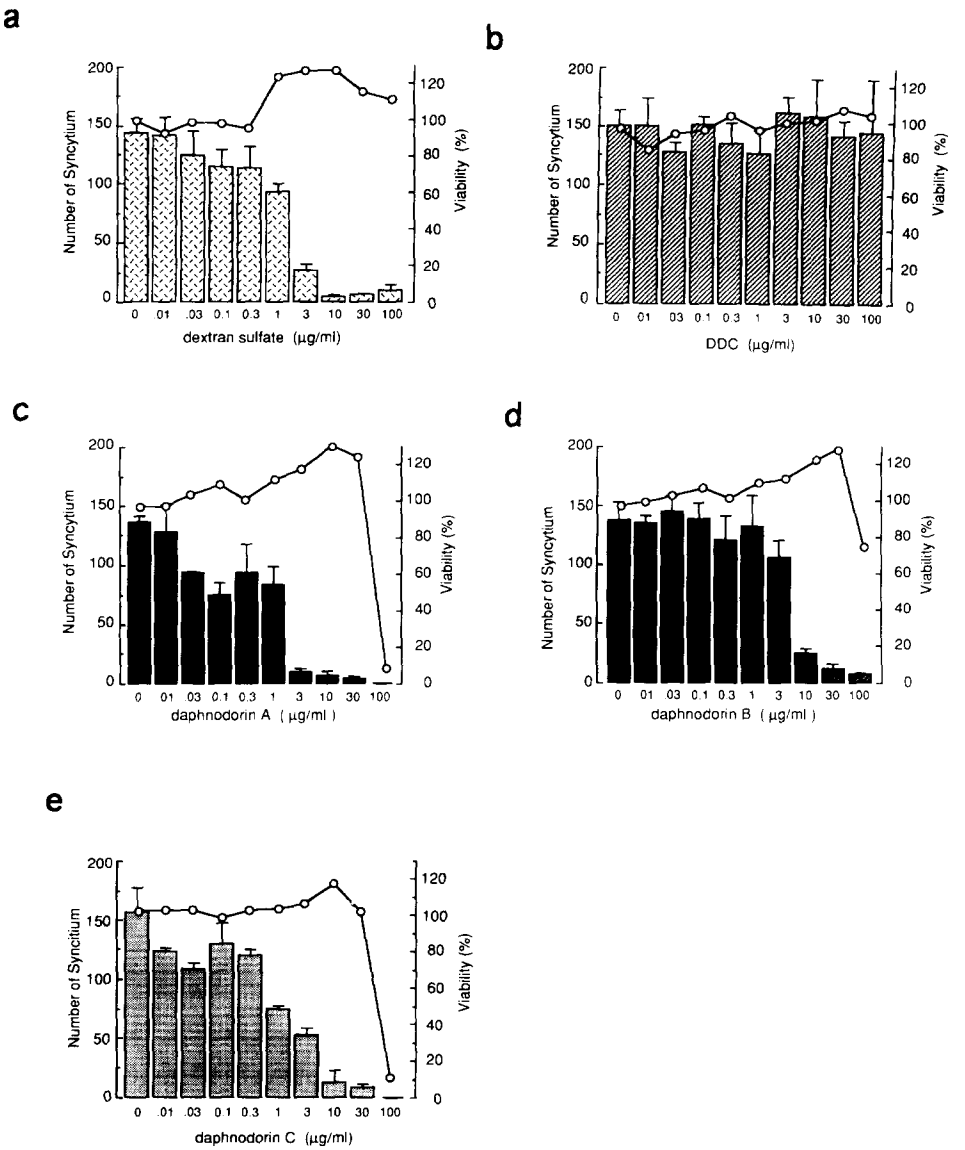

Fig. 2. Effect of daphnodorins on syncytium formation between uninfected MOLT-4 (clone 8) and infected MOLT-4 (clone 8) cells. MOLT-4 (clone 8) and chronically HIV-1(IIIB) infected MOLT-4 (clone 8) cells were co-cultured for 24 h and the number of syncytia was counted. Each bar represents the mean ± S.D. Survival cells were evaluated by the XTT assay (○). Each point represents the mean ($n = 3$). Dextran sulfate, a; DDC, b; daphnodorin A, c; daphnodorin C, c; daphnodorin C, d.

These results indicate that daphnodorins did not inhibit the replication of HIV-1 through inhibition of viral protein production, viral assembly, and release, as suggested for ribavirin (McCormick et al., 1984) and interferon-α (Ho et al., 1985). Daphnodorins A

and C had relatively weak inhibitory effect on HIV-1 RT activity compared with DDC-TP. Daphnodorin B had no inhibitory effect on HIV-1 and AMV RT at concentrations up to 1000 μg/ml. These results indicate that daphnodorins did not inhibit the replication of HIV-1 through inhibition of HIV-1 RT activity.

Syncytium formation has been used to quantitate the ability of HIV-1-infected cells to form multinucleated giant cells (syncytia) through the interaction of surface gp120 of the infected cells with the surface CD4 receptor of the infected indicator cell line (Lifson et al., 1987). Daphnodorins had marked inhibitory effects on syncytium formation between uninfected and infected MOLT-4 (clone 8) cells. The effective concentrations of daphnodorins were in accord with the concentrations of anti-HIV-1 activity (EC_{50}) against HIV-1-induced cytolysis. These results suggest that the in vitro antiviral effect of daphnodorins occurred at an early stage of the viral replicative cycle. It is possible that daphnodorins block the interaction between viral gp120 and CD4. Such inhibitory effects have been described for sulfated polysaccharides (Ito et al., 1987) and synthetic CD4 derivatives (Traunecker et al., 1988). Sulfated polysaccharides, including heparin, dextran sulfate, and pentosan polysulfate, inhibit HIV-1 replication by inhibiting virus adsorption (Baba et al. 1988; Mitsuya et al., 1988; Schols et al., 1990). Daphnodorins may interact with the virus adsorption, and/or the subsequent virus-cell fusion step. Further experiments are required to resolve the exact mechanism of anti-HIV action of the daphnodorins.

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