Lignified Materials as Medicinal Resources. V. Anti-HIV (Human Immunodeficiency Virus) Activity of Some Synthetic Lignins

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A class of synthetic lignins (dehydrogenation polymers of *p*-coumaric acid, ferulic acid, and caffeic acid) inhibited cytopathogenicity of HIV-1 and HIV-2 infection. The ratio of cytotoxic to anti-HIV (human immunodeficiency virus) doses depended strongly on conditions during polymer preparation. The activity increased when polymers were treated with reducing agent $NaBH_4$, whereas it decreased when treated with oxidizing agent ceric ammonium nitrate. The polymers inhibited expression of HIV-specific antigen in the infected cells and also inhibited HIV-binding to the cells, but not completely, even at doses that almost completely inhibited the HIV-induced cytopathogenic effect. These results suggest that lignin structure, regardless of whether synthetic or natural, may inhibit HIV replication by an unidentified process, and thus prove to be a new class of anti-HIV agents possibly effective in the treatment of AIDS (acquired immunodeficiency syndrome).

Keywords lignin; AIDS; human immunodeficiency virus; anti-HIV activity

Inhibition of human immunodeficiency virus (HIV) replication by lignified materials was recently demonstrated in our collaborative study with Dr. Nonoyama's group at Tampa Bay Research Institute, Florida. Thus, lignin fraction extracted from cones of the Japanese white pine *Punus parviflora* SIEB. *et* ZUCC. inhibited HIV-1 replication in chronically infected CR10/HIV-1 cells and in acute cytotoxic HIV-1 infected CEM cells.[1] Partial hydrolysis experiments suggested the importance of the polymerized phenolic portions of lignins for induction of anti-HIV activity. To confirm the dependence on the lignin skeleton, we synthesized several synthetic lignins[2] and obtained a preliminary result that they inhibited expression of HIV-1 p24 antigen (to be submitted). In this study, we confirmed the efficacy of these synthetically polymerized phenylpropenoic acids in inhibiting HIV replication, and extended our study to a more detailed structure–activity relationship among synthetic lignins and related natural products. Reports of anti-HIV activity of some other natural lignins have recently been published.[3-5]

Anti-HIV activity was evaluated by efficiency in protecting against HIV-induced cytopathogenic effect[6] using CD4-positive MT-4 cells infected with each of two HIV strains, HIV-1 (HTLV-IIIB) and HIV-2_{ROD}. Inhibition of the expression of the HIV-specific antigen was evaluated by laser flow cytofluorography after treating the cells with fluorescein isothiocyanate (FITC)-conjugated human immunoglobulin G (IgG).[7,8] Inhibition of adsorption of HIV on MT-4 cells was also evaluated.[9]

Materials and Methods

Dehydrogenation polymers (DHPs) were synthesized at pH 8 with 1.5 molar eq of H_2O_2 by the bulk method described in a previous paper.[2] Some of them were synthesized with 0.5, 1.0, 1.5, and 2.0 eq molar amounts of H_2O_2, and termed as DHP-CA (caffeic acid 0.5 eq H_2O_2), DHP-CA (1.0 eq H_2O_2), DHP-CA (1.5 eq H_2O_2), and DHP-CA (2.0 eq H_2O_2), respectively. No preparations were dialyzable, and molecular weight ranged from several thousands to several tens of thousands.[2] Alkali-lignin and lignin sulfonic acid were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Hydrolyzable tannins (gemin D, nobotanin B, camelliin B, and trapanin B) listed in Table I were supplied by Professor T. Okuda of Okayama University).

Treatment of DHP-CA with the Oxidant, Ceric Ammonium Nitrate (CAN) One concentration of CAN (Katayama Chemical Industry Co., Osaka), 76 mg (0.5 molar eq), 152 mg (1.0 molar eq) or 228 mg (1.5 molar eq), was added to each of three solutions of 50 mg of DHP-CA (1.5 eq H_2O_2) in 5 ml of CH_3CN. Each solution was stirred at 0 °C for 10 min and mixed with 20 ml of H_2O, followed by dialysis against 5 l of distilled H_2O. The outer H_2O was renewed every 2 h during 48 h dialysis. The fractions in the tubes treated with 0.5, 1.0, and 2.0 molar eq were lyophilized to yield, respectively, 57.6, 57.4, and 58.7 mg of pale brownish powder.

Treatment of DHP-CA with the Reductant, Sodium Borohydride ($NaBH_4$) To each of two solutions of 50 mg of DHP-CA (1.5 eq H_2O_2) in 50 ml of H_2O, $NaBH_4$ (Wako Pure Chemical Co., Ltd., Osaka) was added to one at 10.5 mg (1.0 molar eq) and to the other at 21.0 mg (2.0 molar eq). The solutions were stirred at 0 °C for 1 h and dialyzed against 5 l of distilled H_2O. The outer H_2O was renewed every 2 h during 48 h dialysis. The fractions in the tubes, treated with 1.0 and 2.0 molar eq of $NaBH_4$, were lyophilized to give, respectively, 48.4 and 41.2 mg of pale brownish powder.

Inhibition of Cytopathogenicity of HIV-Infection Test samples of solution were added to CD4-positive MT-4 cells (2.5×10^4/well) infected with HTLV-IIIB (7×10^5 PFU/ml) or with HIV-2_{ROD} (1×10^6 PFU/ml) at a multiplicity of infection (MOI) of 0.01. To test cytotoxicity, a sample of the solution was added to MT-4 without infection. The wells were incubated in a CO_2 incubator at 37 °C for 5 d and the surviving cells were determined by the MTT method.[6] The effect of each sample was evaluated by the 50% and 90% inhibitory concentrations (EC_{50} and EC_{90}), 50% cytotoxic concentration (CC_{50}), and selectivity indices ($SI_{50} = CC_{50}/EC_{50}$ and $SI_{90} = CC_{50}/EC_{90}$).

Inhibition of Expression of HIV-Specific Antigen A test sample solution (500 μl of 1.6—100 μg/ml saline) was added to 500 μl of MT-4 cell suspension (3×10^5/ml) infected with HTLV-IIIB (7×10^5 PFU/ml) in each well, and incubated at 37 °C for 5 d. The reaction mixture was then placed in a test tube and contrifuged. The precipitated cells were subjected to laser flow cytofluorographic analysis for HIV-specific antigen (anti-HIV human polyclonal antibody), as previously described.[7,8] The percentage population of antigen-positive fluorescent cell (F-cell) was calculated as follows:

$$\frac{(\%\text{ of F-cell in test well}) - (\%\text{ of F-cell in mock MT-4})}{(\%\text{ of F-cell in HIV-Infected MT-4}) - (\%\text{ of F-cell in mock MT-4})} \times 100$$

Inhibition of HIV-Binding to MT-4 Cells As previously reported,[9] MT-4 cells were exposed to HIV-1 stock (MOLT-4/HTLV-IIIB concentrated 100 times by ultracentrifugation at 35000 rpm for 1 h) in various concentrations of test samples. After incubation at 37 °C for 1 h, the cells were washed twice to remove unbound virus. The cells were then

processed for indirect immunofluorescence using a human polyclonal anti-HIV-positive serum as the first antibody and an FITC-conjugated rabbit anti-human IgG as the second antibody. After immunofluorescence staining, the cells were washed twice with phosphate buffered saline (PBS), resuspended in 0.37% paraformaldehyde in PBS, and analyzed by laser flow cytometry.[9] The binding inhibitory activity ratio (*BI*) was calculated as follows:

$$BI=\left(1-\frac{\%\,MF(\mathrm{VS})-\%\,MF(\mathrm{CS})}{\%\,MF(\mathrm{V})-\%\,MF(\mathrm{C})}\right)\times 100$$

where *MF*, mean fluorescence; VS, HIV-infected cells treated with test sample; CS, control cells (not exposed to HIV) treated with test sample; V, HIV-infected cells without test sample; C, control cells (not exposed to HIV and not treated with test sample).

Results

Inhibition of HIV-Induced Cytopathogenic Effect All the DHPs examined inhibited the cytopathogenic effect of HIV-1 (HTLV-IIIB) infection against MT-4 cells, whereas their precursors were all inactive, caffeic acid (CA) was highly cytotoxic (Table I). Dose-dependent inhibitions are shown in Fig. 1. Among the polymers, DHP-*p*-coumaric acid (DHP-*p*CA) had the smallest EC (EC_{50} 3.9 μg/ml), and judging from the SI ($SI_{50}>26$), DHP-*p*CA was also among the most promising of the preparations examined in this study; the pine cone extract (fr. VI) ($SI_{50}>3$), alkali-lignin ($SI_{50}>17$), lignin sulfonate ($SI_{50}>13$), the water extract of slash pine wood chips (inactive) (Exp. 2). Four hydrolyzable tannins showed some anti-HIV activity ($SI_{50}=13-15$) (exp. 2). Most of the other 82 tannin-related compounds previously examined were inactive.[10]

To establish the most appropriate experimental condition for the synthesis of DHPs, caffeic acid was polymerized with various amounts of hydrogen peroxide as the polymerization reagent. The results shown in Table I (exp. 3) indicate greater activity when more hydrogen peroxide was used for the polymerization. SI_{50} value increased from 7 to 100 when the H_2O_2 dose was increased from 0.5 to 2.0 eq.

Synthetic lignins (DHPs) showed an electron spin resonance (ESR) signal ($g=2.003$) in the solid state, possibly due to semiquinones,[2] so we tested the dependence of anti-HIV activity on structural modification produced by an oxidizing or reducing agent. Preparations of DHP-CA

TABLE I. Inhibition of Cytopathogenic Effects Induced by HIV-1 (Exp. 1—3) and HIV-2 (Exp. 4) by Synthetic/Natural Lignins and Tannin-Related Materials

Substance	CC_{50} (μg/ml)	EC_{50} (μg/ml)	EC_{90} (μg/ml)	SI_{50}	SI_{90}
Experiment 1 (HIV-1)					
DHP-*p*CA (1.5 eq H_2O_2)	>100.0	3.9	6.1	>26	>17
DHP-FA (1.5 eq H_2O_2)	>100.0	5.4	9.4	>18	>11
DHP-CA (1.5 eq H_2O_2)	83.1	7.1	9.4	12	9
*p*CA	>100.0	a)	b)		
FA	>100.0	a)	b)		
CA	2.1	a)	b)		
Experiment 2 (HIV-1)					
DHP-*p*CA	>100.0	1.3	2.5	>75	>40
Pine cone fr. VI	>100.0	34.9	b)	>3	
Alkali-lignin	>100.0	5.8	9.7	>17	>10
Lignin sulfonate	>100.0	7.9	12.2	>13	>8
SP-water extract	>100.0	a)	b)		
Gemin D	26.7	2.0	b)	13	
Nobotanin B	33.7	2.4	15.0	14	2
Camelliin B	74.3	4.8	b)	15	
Trapanin B	39.1	2.7	b)	14	
Experiment 3 (HIV-1)					
DHP-CA (0.25 eq H_2O_2)	88.6	13.3	21.3	7	4
DHP-CA (0.5 eq H_2O_2)	92.2	6.4	13.0	14	7
DHP-CA (1.0 eq H_2O_2)	132.3	2.2	4.0	61	33
DHP-CA (1.5 eq H_2O_2)	176.6	2.3	4.7	76	38
DHP-CA (2.0 eq H_2O_2)	157.9	1.6	2.8	100	56
0.5 eq CAN-DHP-CA (1.5 eq H_2O_2)	249.4	3.6	12.4	69	20
1.0 eq CAN-DHP-CA (1.5 eq H_2O_2)	187.7	5.2	66.9	36	3
1.5 eq CAN-DHP-CA (1.5 eq H_2O_2)	316.8	5.5	13.8	58	23
1.0 eq $NaBH_4$–DHP-CA (1.5 eq H_2O_2)	157.4	1.7	2.3	95	68
2.0 eq $NaBH_4$–DHP-CA (1.5 eq H_2O_2)	207.3	2.1	3.1	99	67
Experiment 4 (HIV-2)					
DHP-CA (0.25 eq H_2O_2)	129.1	22.7	b)	6	
DHP-CA (0.5 eq H_2O_2)	107.0	13.8	42.4	8	3
DHP-CA (1.0 eq H_2O_2)	118.0	4.1	10.0	29	12
DHP-CA (1.5 eq H_2O_2)	174.6	5.1	13.3	34	13
DHP-CA (2.0 eq H_2O_2)	115.9	3.2	5.7	36	20
0.5 eq CAN-DHP-CA (1.5 eq H_2O_2)	166.5	5.3	13.9	31	12
1.0 eq CAN-DHP-CA (1.5 eq H_2O_2)	285.4	7.8	19.7	37	15
1.5 eq CAN-DHP-CA (1.5 eq H_2O_2)	289.8	13.6	28.9	21	10
1.0 eq $NaBH_4$–DHP-CA (1.5 eq H_2O_2)	154.6	3.2	8.1	49	19
2.0 eq $NaBH_4$–DHP-CA (1.5 eq H_2O_2)	208.0	2.9	7.3	73	29

The relative activity within an exp. was fairly reproducible although absolute values varied somewhat from one exp. to another. *a)* ≥50% inhibition could not be achieved with doses up to 100 μg/ml. *b)* ≥90% inhibition could not be achieved with doses up to 100 μg/ml.

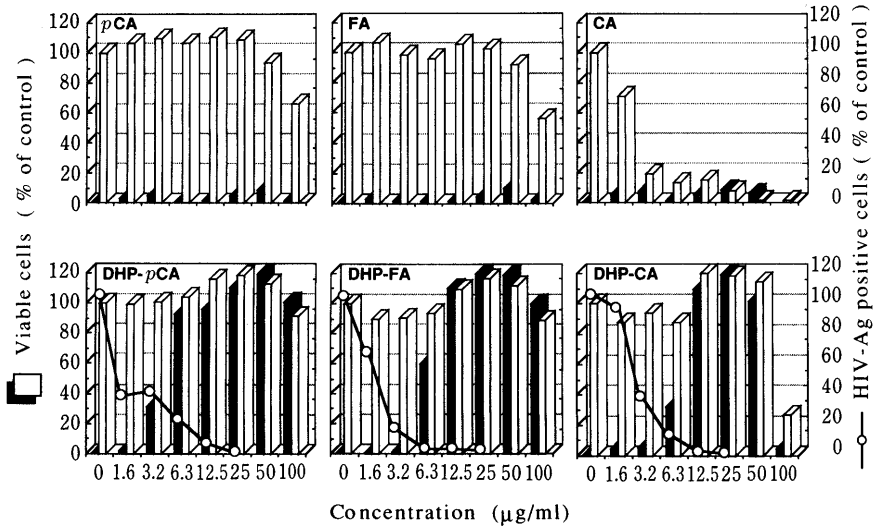

Fig. 1. Inhibition of HIV-1-Induced Cytopathogenic Effect and HIV-Specific Antigen Expression by DHPs of Phenylpropenoic Acids

Viable HIV-1-infected (black bars) and mock-infected (open bars) MT-4 cells in the presence of the test compound are expressed as the percentage of mock-infected controls with no test compound. The number of HIV-1 antigen-positive cells (open circles), determined by indirect immunofluorescence and laser flow cytometry, is expressed as the percentage of virus-infected and compound-free positive control cells.

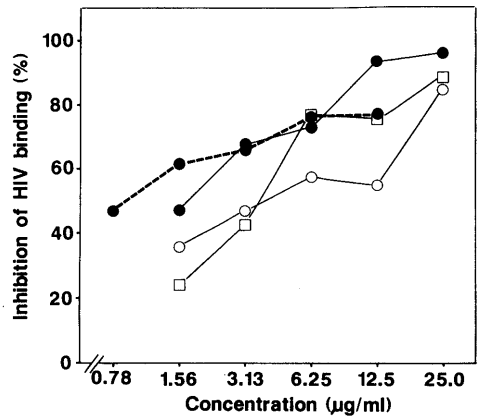

Fig. 2. Inhibition of HIV-1-Binding to MT-4 Cells by DHPs of Phenylpropenoic Acids

MT-4 cells were exposed to HIV-1 virions in the presence of the indicated concentrations of DHP-*p*CA (○), DHP-FA (□) or DHP-CA (●) synthesized with 1.5 eq H_2O_2, or DHP-CA (2.0 eq H_2O_2) (indicated by broken line).

(1.5 eq H_2O_2) treated with CAN or $NaBH_4$ were tested for anti-HIV-I activity. The results shown in Table I (exp. 3) indicate that reduction with $NaBH_4$ slightly potentiated activity, whereas oxidation with CAN reduced activity.

The inhibitory effects of DHP-CA derivatives on HIV-2_{ROD} replication shown in Table I (exp. 4) are somewhat less than those for HIV-I replication, however, the order of efficacy among the tested preparations seems to be the same as that for HIV-I inhibition.

Inhibition of Expression of HIV-Specific Antigen As indicated by open circles in Fig. 1, DHP-*p*CA, DHP-ferulic acid (DHP-FA), and DHP-CA dose-dependently reduced expression of HIV-specific antigen. The 50% inhibition concentrations were calculated to be 0.8, 1.8, and 2.6 μg/ml, respectively.

HIV-Binding Assay by Laser Flow Cytofluorography As seen in Fig. 2, DHP-*p*CA, DHP-FA, and DHP-CA dose-dependently inhibited HIV-1 binding to MT-4 cells, the 50% inhibition concentrations being 3.8, 3.6, and 1.7 μg/ml, respectively. However, inhibition of HIV-binding was incomplete even at doses that almost completely inhibited the cytopathogenic effect of HIV and expression of HIV-antigen, even if DHP-CA (2.0 eq H_2O_2) was used (Fig. 2).

Discussion

It was confirmed in the present study that these dehydrogenation polymers synthesized from *p*CA, FA, and CA inhibited HIV proliferation more effectively than natural lignins and tannin-related materials. It seems that enough H_2O_2 should be used to prepare more potent synthetic polymers. Anti-HIV activity of the preparations once synthesized was significantly potentiated dose-dependently by treatment with the reducing agent, $NaBH_4$, whereas it was reduced by treatment with the oxidant, CAN. These results lead us to expect further improvement in the activity of this class of the lignin family.

There are some processes proposed for anti-HIV activity of chemotherapeutics. One is selective inhibition of reverse transcriptase, as exemplified by azidothymidine (AZT) and dideoxyinosine (DDI). The lignins studied here have a polymeric lignin skeleton which is completely different from the antimetabolic nucleic acid bases. An alternative mechanism may be the inhibition of the adsorption of HIV onto the cell surface, as exemplified by tannins[10] and polyanions such as dextran sulfate.[11,12] It is not likely that only this mechanism could be involved in the anti-HIV activity of the lignins concerned, since these polymers did not completely inhibit HIV-binding to the cells, even in a dose range that was effective for anti-HIV activity (Fig. 2). It is, therefore, possible that lignins might inhibit proliferation of HIV by an unidentified mechanism.

It could be concluded that lignins, either synthetic or natural, are definitely promising candidates as anti-HIV drugs. Further studies are being pursued in our laboratory. Finally, both synthetic and natural lignins inhibited proliferation of another RNA (ribonucleic acid) virus, the influenza virus, after infection was established in MDCK

cultured cells,[13,14] whereas lignins only inhibited adsorption of herpes simplex viruses (DNA (deoxyribonucleic acid) virus) to CV-1, Vero, and A549 cells.[15]

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