Antiviral Activities of Glycyrrhizin and Its Modified Compounds against Human Immunodeficiency Virus Type 1 (HIV-1) and Herpes Simplex Virus Type 1 (HSV-1) in Vitro

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Chemically modified compounds of glycyrrhizin have been synthesized and evaluated for their inhibitory effect on the replication of human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus type 1 (HSV-1). Among them, the 11-deoxy compound having a heteroannular diene structure at the C and D rings proved as active against HIV-1 as glycyrrhizin in MT-4 and MOLT-4 cells. It completely inhibited HIV-1-induced cytopathogenicity in both cell lines at a concentration of 0.16 μM. The compound was also effective against HSV-1 with a 50% inhibitory concentration of 0.5 μM.

Keywords glycyrrhizin analog; HIV-1; HSV-1; antiviral activity

It has been reported that glycyrrhizin, the major saponin isolated from licorice root (Glycyrrhiza spp.), is inhibitory to the replication of human immunodeficiency virus type 1 (HIV-1) in vitro, the etiologic agent of the acquired immune deficiency syndrome (AIDS). 1-3 This finding has been developed to the clinical use of a glycyrrhizin preparation, Stronger Neomorphagen C (SNMC), to protect HIV-1 carriers from the crisis of AIDS. 4-6 In addition, it was demonstrated that glycyrrhizin inhibited the in vitro growth and cytopathogenicity of several deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses including herpes simplex virus type 1 (HSV-1), Newcastle disease virus, vesicular stomatitis virus, and poliovirus type 1. 7,8 Baba and Shigeta, two of the present authors, reported the antiviral activity of glycyrrhizin against varicella-zoster virus (VZV) in cell culture. 9

On the basis of these results, some chemically modified compounds of glycyrrhizin were investigated for their inhibitory effect on the replication of HIV-1 and HSV-1. The functional groups of glycyrrhetinic acid, the aglycone part of glycyrrhizin, at the 11 and 20 positions, and the carboxyl at the 5' position of the sugar portion were modified. The αβ-unsaturated carbonyl system of the C-ring of the glycyrrhetinic acid part was converted into a heteroannular diene system at the C and D rings. By the above modifications, the following compounds (3-7) were prepared starting from glycyrrhizin (1) to test their antiviral activity.

Materials and Methods

Apparatus Melting points were taken on a Yanaco micro melting point apparatus and are uncorrected. Optical rotations were determined in MeOH with a JASCO model JZO ORD/CD polarimeter. Infrared (IR) spectra were recorded with a JASCO IR-700 spectrophotometer and ultraviolet (UV) spectra with a Hitachi 2-20 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-400 NMR spectrometer using 10-20 mg of 0.45 ml dimethyl sulfoxide (DMSO-d6) as a solvent and tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in ppm downfield from this standard. Fast atom bombardment (FAB) mass spectral analysis was performed with a JEOL DX-300 spectrometer. A high performance liquid chromatograph (HPLC) consisting of a Spectra-Physics SP-8700 solvent pumping system with bellows damper (NB-III), ERC-7520 (refraction index detector) or SSC-Y-1000 (UV-detecto) and a Rhodyne model 7125 injection valve was used. Chromatograms were recorded on a SIC-7000B (System Instruments) integrator. Experiments were performed on a column (25 cm x 10 mm i.d.) (Senshu Scientific) packed with Nucleosil 100-5 C4 (octadecyl silica, 5 μm) (Machery, Nagel and Co., Duren, G. F. R.). Column chromatography was performed using a Kieselgel 60 column (70-230 mesh, Merck). Thin layer chromatography (TLC) was conducted on precoated Kieselgel 60 F254 plates (0.2 mm thick, Merck) with CHCl3-MeOH-H2O (65:35:10, lower layer). The molecular composition of the compound given by the chemical formula was determined by elemental analysis.

Preparation of Test Compounds Commercially available glycyrrhizin (1) isolated from licorice root was used as the starting material for chemical modification.

18-OH-11-deoxy-3β,11β-diol-30-oic Acid 3-O-β-D-Glucuronopyranosyl(1→2)β-D-glucopyranoside (11f-Hydroglycyrrhizin) (2) To a mixture of NaBH4 (7.56 g, 0.2 mol) dissolved in αq 1.8 NaOH (50 ml) and tetrahydrofuran (THF) (100 ml) at 80 °C, glycyrrhizin (I) (8.23 g, 0.01 mol) dissolved in THF (100 ml) was added dropwise and allowed to react for 24 h under reflux in a H2O bath. The reaction mixture was stirred with acetone (50 ml) at room temperature (r.t.), and dil. HCl was added to adjust to pH 9-10. The filtrate of the reaction mixture was concentrated to ca. 100 ml, and then precipitated by the addition of acetone (700 ml) and MeOH (300 ml). The crude product was refluxed with boiling 75% EtOH (200 ml) to remove insoluble substances. From the filtrate 11β-Hydroxyglycyrrhizin Na salt (7.6 g) was separated (yield 85%). 11β-Hydroxyglycyrrhizin (2) was obtained by treating the Na-salt with Dowex 50 W-X2 Type II in H2O, mp 271-277 °C (dec.), [α]D +80.0° (MeOH), C21H28O12.5H2O, IR νmax cm-1: 1050 (C-OH), 1080 (C-O-C), 1710 (COOH), 2890 (CH2), 3450 (OH). (→)FAB MS m/z: 823 (M+1)7

11-C-NMR: 179.0 (s, C-30), 170.5 (s), 170.0 (s, C-6, C-6′), 148.5 (s, C-13), 121.5 (d, C-12), 104.1 (d), 103.3 (d, C-1, C-1′), 61.2 (d, C-11). Olean-11,13(18)-diene-3β-O-β-D-Glucuronopyranosyl(1→2)β-D-glucopyranoside (3) 11β-Hydroxyglycyrrhizin Na salt (5 g) dissolved in H2O (100 ml) was treated with dil. HCl to adjust to pH 7-8. The solution was heated at 100 °C for 6 h. After cooling, the reaction mixture was acidified to pH 2-3 and then extracted twice with n-ButOH (100 ml). On evaporation, compound 3 was obtained. Yield: 4.2 g (92.7%); mp 247-250 °C (dec.), [α]D +120.0° (MeOH), C24H32O15.5H2O, 2H2O·UV 805 nm: 243, 251, 260, IR νmax cm-1: 1040 (C-OH), 1080 (C-O-C), 1640 (C=C-C), 1720 (COOH), 2930 (CH1), 3400 (OH). (→)FAB MS m/z: 805 (M+1)7, 797 (→)H-NMR (in CD3OD) δ (TMS): 6.35 (3H, dd J=16 Hz, vinyl-H), 5.99 (1H, d, J=12 Hz, vinyl-H), 1.3-C-NMR: 178.9 (s, C-30), 170.2 (s), 169.9 (s, C-6, C-6′), 135.1 (s, C-30), 134.6 (C-13), 127.8 (d), 126.3 (C-11, C-12), 104.6 (d), 103.5 (d, C-1, C-1′), 88.5 (d, C-3). Kitagawa et al.19 isolated compound 3, mp 249-251 °C, [α]D +120.0° (MeOH), from Dong-bei licorice (Glycyrrhiza uralensis root) as one of the minor saponins and called licorice saponin C-2.

Olean-11,13(18)-diene-3β-O-β-D-Glucuronopyranosyl(1→2)β-D-glucopyranoside (4) Trimethyl ester penta-acetate of compound 3 (500 mg) dissolved in dry THF (20 ml) was added to a solution of NaBH4 (OCH3)2CH2OH (70% in toluene) (2.9 ml) in dry THF (20 ml) under vigorous stirring at 60 °C in an N2 atmosphere. The reaction was

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Results and Discussion

When the inhibitory effect of glycyrrhizin (1) and its derivatives (3–7) was examined for HIV-1 replication in MT-4 cells, glycyrrhizin (1) and compound 3 were found to have achieved more than 80% protection of the cells from the virus-induced destruction at a concentration of 0.62 and 0.16 μM, respectively (Table I). In particular, compound 3 almost completely suppressed HIV-1-induced cytopathogenicity at this concentration. Compounds 4 and 5 were slightly inhibitory to HIV-1 replication at subtoxic concentrations (Table I), yet other compounds (6 and 7) did not show any activity at their non-toxic concentrations (data not shown).

In MOLT-4 (clone 8) cells, compound 3 also exhibited the selective inhibition at a concentration between 0.16 and 0.31 μM (Fig. 1). The compound slightly reduced the number of viable infected cells at a concentration of 1.24 μM. The HIV-1 antigen expression in MOLT-4 (clone 8) was suppressed by compounds 1 and 3 at concentrations higher than 0.16 μM (Fig. 2). In contrast, compounds 4 and 5 were totally inactive against the antigen expression. When we examined the effect of compound 3 on HIV-1 RT activity, no inhibition was observed at the concentrations active against HIV-1-induced cytopathogenicity in MOLT-4 cells.

In the next experiment, we investigated the inhibitory effect of glycyrrhizin and its homologs on HSV-1 replication in HEF cells. As shown in Table III, glycyrrhizin and compound 3 proved inhibitory to HSV-1 replication, their 50% inhibitory concentrations (IC₅₀) for HSV-1 cytopathogenicity. The activity was also monitored by the viability and HIV-1 antigen expression in MOLT-4 (clone 8) cells infected with HIV-1 in the absence or presence of the test compounds. In this experiment, MOLT-4 (clone 8) cells were infected with HIV-1 at a multiplicity of infection of 0.002 and incubated at 37°C. On day 3 after infection, viral antigen expression was measured by indirect immunofluorescence using a polyclonal antibody. For the viability test, half of the culture medium was replaced by fresh culture medium containing appropriate concentrations of the compounds. The cells were incubated for another 24 h, and the number of viable cells was counted microscopically in a hemocytometer by the trypan blue exclusion method. The cytotoxicity of the compounds was determined by measuring the viability of mock-infected MT-4 and MOLT-4 (clone 8) cells in parallel with the virus-infected cells.

C. Reverse Transcriptase (RT) Assay

The effect of the compounds on HIV-1 RT activity was evaluated with the enzyme obtained from disrupted virions which had been partially purified and concentrated from the supernatant of MOLT-4-HTLV-IIIa cells. The assay was performed for 30 min at 37°C in a 50 μl reaction mixture containing 50 mM Tris-HCl (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 1 μCi of [methyl-³H]-dTTP (30 Ci/mmol), 0.01 OD unit of poly(rA)- oligo (dT), test compound and ca. 0.1 μg of enzyme. The reaction was stopped with 200 μl of 5% trichloroacetic acid, and the precipitated material was washed as described.

D. Anti-HSV-1 Assay

Assay of the compound for HSV-1 replication was carried out in confluent HEF monolayer cells in a flat-bottomed microtiter tray. The HEF cells were infected with 50 plaque forming units (PFU) of HSV-1 and incubated at 37°C in the presence of various concentrations of the test compounds. After 5–7 days, HSV-1-induced cytopathogenicity was recorded microscopically.

**Biological Activity: A. Cells and Viruses**

MT-4 and MOLT-4 (clone 8) were used for anti-HIV-1 assays. The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin G, and 100 μg/ml streptomycin. The HIV-1 was obtained from the culture supernatant of MOLT-4-HTLV-IIIa cells. For anti-HSV-1 test, we employed a KOS strain-exposed human embryonic fibroblast (HEF) cells growing in Eagle’s minimum essential medium (MEM) supplemented with 10% heat-inactivated newborn calf serum (NCS) and antibiotics.

**B. Anti-HIV-1 Assay**

The activity of the compounds against HIV-1 replication in MT-4 was based on the inhibition of virus-induced cytopathogenicity, as previously described. The MT-4 cells were infected at 2 x 10⁵ cells per ml and infected with HIV-1 at 1000 50% cell infective doses per ml. Immediately after infection, 100 μl of the cell suspension was put into each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After 5 days of incubation at 37°C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method, as described.

The activation of the viability and HIV-1 antigen expression in MOLT-4 (clone 8) cells infected with HIV-1 in the absence or presence of the test compounds. In this experiment, MOLT-4 (clone 8) cells were infected with HIV-1 at a multiplicity of infection of 0.002 and incubated at 37°C. On day 3 after infection, viral antigen expression was measured by indirect immunofluorescence using a polyclonal antibody. For the viability test, half of the culture medium was replaced by fresh culture medium containing appropriate concentrations of the compounds. The cells were incubated for another 24 h, and the number of viable cells was counted microscopically in a hemocytometer by the trypan blue exclusion method. The cytotoxicity of the compounds was determined by measuring the viability of mock-infected MT-4 and MOLT-4 (clone 8) cells in parallel with the virus-infected cells.
Table 1. Inhibitory Effect of Glycyrrhizin and its Modified Compounds on HIV-1 Replication in MT-4 Cells

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<tr>
<th>Concentration (nm)</th>
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<th>5</th>
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</table>

a) Number of viable cells is determined by the MTT method and expressed as the percentage of mock-infected control. N.D., not determined.

![Chart 1](image)

Fig. 1. Inhibitory Effect of Compound 3 on HIV-1 Replication in MOLT-4 Cells
The viability of HIV-1-infected cells (■) and mock-infected cells (□) was assessed by the trypan blue exclusion method. The number of viable cells is expressed as the percentage of the mock-infected control.

HIV-1 antigen positive cells were detected by indirect immunofluorescence using polyclonal antibody on day 3 after infection.

Fig. 2. Inhibitory Effect of Glycyrrhizin (■) and Compounds 3 (□), 4 (○) and 5 (×) on HIV-1 Antigen Expression in MOLT-4 Cells

Genicinity being 3.6 and 0.5μm, respectively. Other compounds (4–7) did not affect the replication of HSV-1 nor the cell morphology of mock-infected HEP cells (data not shown). Based on the anti-HIV-1 activity of glycyrrhizin in vitro, the glycyrrhizin preparation, SNMC has been clinically applied to HIV-1 carriers by i.v. administration, and indeed, their immunological parameters have been improved. The present study indicates that a glycyrrhizin analog compound 3 also seems applicable to the treatment of AIDS. Furthermore, compound 3 might be safer than glycyrrhizin in long-term administration because, it is less capable of exerting pseudoaldosteronism, as suggested by our earlier investigations. The RT assay suggests that, like glycyrrhizin, the anti-HIV-1 activity of compound 3 is not due to the inhibition of RT activity. The mechanism of action of the compound remains to be elucidated. It has been demonstrated that glycyrrhizin partially prevents the adsorption of HIV-1 particles to CD4+ cells. Inhibition of protein kinase C (PKC) activity by glycyrrhizin has also been discussed in relation to its anti-HIV-1 effect. However, it is still not known whether the inhibition of virus adsorption and the inhibition of PKC activity are definitely related, though the binding of HIV-1 to CD4 lymphocytes induces rapid phosphorylation of CD4 receptors which involve PKC. On the other hand, comparative study with other glycyrrhizin analogs (4–7) has revealed that the presence of a carboxyl group at the 20-position in compound 3 is essential for the anti-HIV-1 activity, while the hetero-annular diene C/D ring system in compound 3 is convertible with the αβ-unsaturated carbonyl structure in C-ring of glycyrrhizin. These results suggest...
that the carboxyl group at 20-position may play a crucial role in inhibiting the virus adsorption.

Considering the current extreme prevalence of AIDS, the finding of effective chemotherapeutic agents is highly desired. In this regard, compound 3 should be further pursued for its potential as an anti-AIDS agent.

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