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Mechanism of inhibitory effect of glycyrrhizin on replication of human immunodeficiency virus (HIV)

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Summary

Glycyrrhizin (GL) achieved a dose-dependent inhibition of the replication of human immunodeficiency virus type 1 (HIV-1) in MOLT-4 (clone No. 8) cells within the concentration range of 0.075 to 0.6 mM. Within this concentration range, GL also effected a dose-dependent reduction in the protein kinase C (PKC) activity of MOLT-4 (clone No. 8) cells. A well-known PKC inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), also proved inhibitory to HIV-1 replication in MOLT-4 (clone No. 8) cells. PKC inhibition may thus be considered as one of the mechanisms by which GL inhibits HIV-1 replication. In addition, GL may also owe its anti-HIV-1 activity, at least in part, to an interference with virus-cell binding, since the compound at 1.2 mM partially inhibited the adsorption of radiolabeled HIV-1 particles to MT-4 cells. At this concentration GL also suppressed giant cell formation induced by co-culturing MOLT-4 (clone No. 8) cells with MOLT-4/HTLV-III$_B$ cells, whereas the PKC inhibitor H-7 failed to do so.

Glycyrrhizin; Human immunodeficiency virus; Protein kinase C; Virus adsorption

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Introduction

Acquired immune deficiency syndrome (AIDS) is a pandemic immunosuppressive disease caused by human immunodeficiency virus type 1 (HIV-1). A number of compounds have been found effective as inhibitors of HIV-1 replication in vitro (De Clercq, 1987), and clinical trials with these compounds have been advocated or even initiated. We have previously demonstrated that glycyrrhizin (GL), a substance present in the aqueous extract of liquorice root (*Glycyrrhiza radix*) inhibits HIV-1 replication in vitro (Ito et al., 1987). Initial clinical studies suggest that when administered at a relative high dose (800–1600 mg/day) to AIDS patients GL increases the number of T4 lymphocytes and decreases HIV-1 antigen levels (IV International Conference on AIDS, Stockholm, Sweden, 1988, Abstract No. 3534). GL is not inhibitory to the HIV-1-associated reverse transcriptase (RT) (Ito et al., 1987) and further details on its mode of action against HIV-1 are still lacking.

Protein kinase C (PKC) is an enzyme that is known to be activated by some tumor promotors (Nishizuka, 1986). Recently, Fields et al. (1988) showed that phosphorylation of the CD4 molecule by PKC might be a crucial step in the binding of HIV-1 to the CD4 receptor, and a PKC inhibitor, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride (H-7), was found to inhibit HIV-1 replication in peripheral blood lymphocytes. We have now investigated the effect of GL on PKC activity at drug concentrations needed to inhibit HIV-1 replication in vitro. Also, the effects of GL on HIV-1 adsorption to the cells and virus-induced fusion were examined.

Materials and Methods

Chemicals

GL was supplied by Minophagen Pharmaceutical Co., Tokyo, Japan. It was dissolved in 0.01 M phosphate buffered saline (PBS) and adjusted to pH 7.2 with 1 N sodium hydroxide. Protein kinase C (PKC) inhibitors [such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), *N*-(2-aminoethyl)-5-isoquinolinesulfonamide dihydrochloride (H-9) and *N*-(2-guanidinoethyl)-5- isoquinolinesulfonamide hydrochloride (HA 1004)] were purchased from Seikagaku Kogyo Co., Tokyo, Japan. [γ-^{32}P]ATP (2–10 Ci/mmol) and [^{3}H]12-*O*-tetradecanoylphorbol-13-acetate (TPA) were obtained from New England Nuclear. Dextran sulfate (5000 Da), diolein and H-1 histone (type III-S) were obtained from Sigma. Phosphatidylserine was purchased from Avanti Polar-Lipid Inc.

Cells and virus

The human leukemic T-cell line, MOLT-4 (clone No. 8) (Kikukawa et al., 1986), and the HTLV-I (human T-cell lymphotropic virus type 1) carrying cell line MT-4 (Harada et al., 1985) were used in this study. The cells were cultured and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin G and 100 μg/ml streptomycin.

HIV-1 was obtained from the culture supernatant of MOLT-4/HTLV-III_B cells (Harada et al., 1985), and the method for preparing HIV-1 particles, radiolabeled with [5-^{3}H]uridine (30 Ci/mmol, Amersham, UK) has been previously described (Baba et al., 1988).

Anti-HIV-1 assays

Activity of the compounds against HIV-1 replication was based on the inhibition of virus-induced cytopathogenicity in MOLT-4 (clone No. 8) cells, as determined by the trypan blue exclusion procedure (Ito et al., 1987). Anti-HIV-1 activity was also determined by monitoring viral antigen expression in MOLT-4 (clone No. 8) cells. Briefly, methanol-fixed cells were incubated with a 1:1000 dilution of anti-HIV-1 human serum (IF titer 1:4096) for 30 min at 37°C. Then, the cells were washed with PBS for 15 min, incubated with the fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dakopatts A/S, Copenhagen, Denmark) for 30 min at 37°C and washed again with PBS. More than 500 cells were counted under a fluorescent microscope and the percentage of fluorescent-positive cells was calculated. In both assays, MOLT-4 cells were infected with HIV-1 at a multiplicity of infection of 0.002.

Preparation of cytosolic and particulate cell fractions

MOLT-4 (clone No. 8) cells were cultured with various concentrations of GL for 1–3 days. The cells were washed 3 times with PBS, sonicated for 30 s at 4°C in buffer A (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 0.01% leupeptin, 50 mM 2-mercaptoethanol) and centrifuged at 100000 × *g* for 1 h at 4°C. The supernatant was referred to as the cytosolic fraction. The pellet was rehomogenized in buffer A containing 0.1% Triton X-100 and centrifuged at 30000 × *g* for 15 min at 4°C. The supernatant was referred to as the particulate fraction.

DEAE-cellulose chromatography

Cytosolic and particulate fractions were applied onto a DE-52 column (0.9 × 1.8 cm) equilibrated with buffer A without 0.25 M sucrose (buffer B). PKC preparations were eluted batchwise with 3 ml of 0.2 M NaCl in buffer B, according to the method described by Kraft and Anderson (1983). This batch elution procedure yielded more than 90% of the total PKC activity that could be detected by a linear NaCl (0–0.5 M) gradient elution. The protein content was determined by the method of Bradford (1976).

PKC activity assay

PKC activity was assayed, according to the method of Kikkawa et al. (1982), by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H-1 histone after 3 min incubation at 37°C in a reaction mixture (0.25 ml) containing 5 M Tris-HCl, pH 7.5, 1.25 M magnesium acetate, 50 μg of H-1 histone, 2.5 nM [γ-^{32}P]ATP, 125 nM $CaCl_2$, 10 μg of phosphatidylserine and 0.2 μg of diolein. PKC activity was determined by subtracting the activity measured in the absence of phosphatidylserine and diolein from that measured in their presence.

[^{3}H]TPA binding assay

[^{3}H]TPA binding was assayed by the method described by Hoshijima et al. (1986), using phosphatidylserine-Affi-Gel (Wise et al., 1982).

Virus adsorption assay

Virus attachment to the cells was measured using radiolabeled HIV-1 particles, as previously described (Baba et al., 1988). Briefly, 2×10^6 MT-4 cells were suspended in medium (final volume 200 μl) containing the indicated concentrations of the compounds. Ten μl of [5-^{3}H]uridine-labelled virus suspension (11 000 cpm) were added, and the samples were incubated for 60 min at 37°C. The cells were collected and washed three times with PBS to remove unadsorbed virus particles. Cell-associated acid-insoluble material was analyzed for radioactivity.

Results

Inhibitory effects of GL on HIV-1-induced cytopathogenicity and virus antigen expression in MOLT-4 (clone No. 8) cells

The effects of GL on the HIV-1-induced cytopathogenicity and virus antigen expression in MOLT-4 (clone No. 8) cells were assessed on day 5 after infection.

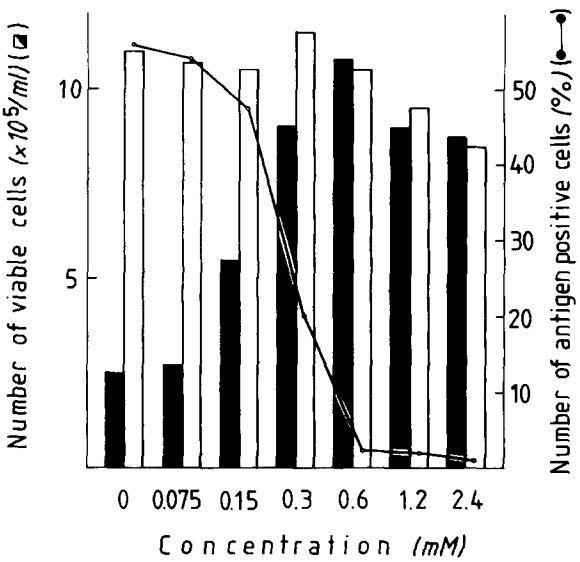

Fig. 1. Inhibitory effect of GL on HIV-1-induced cytopathogenicity and virus antigen expression in MOLT-4 (clone No. 8) cells. Mock-infected (□) and HIV-1-infected (■) cells were adjusted to 2×10^5 cells/ml and incubated in the presence of varying concentrations of GL. On the 3rd day after infection, half of the medium of all cultures was replaced. Viability of the cells was assessed by trypan blue exclusion method on the 5th day after infection. Also determined on the 5th day after infection was the percentage of HIV-1-specific antigen positive cells (●——●). The latter was monitored by indirect immunofluorescence.

Fig. 1 shows that GL inhibited HIV-1-induced cytopathogenicity and virus antigen expression at the concentrations of 0.15–2.4 mM in a dose-dependent manner. Similar results were obtained previously in MT-4 cells (Ito et al., 1987).

Effect of GL on PKC activity in MOLT-4 (clone No. 8) cells

We then examined whether GL could suppress PKC activity in the cytosolic and particulate fractions of MOLT-4 (clone No. 8) cells. When the cells were incubated in the presence of varying concentrations of GL for 3 days, the compound caused a dose-dependent inhibition of both the cytosolic and particulate PKC activities within the concentration range 0.15–1.2 mM (Fig. 2A). When the cells were incubated with GL at a concentration of 1.2 mM for 1, 2 or 3 days, inhibition of PKC activity increased in function of the incubation time, irrespective of whether PKC activity was measured in the cytosolic or particulate fractions (Fig. 2B). At the concentration used (1.2 mM), GL did not affect the growth of the MOLT-4 (clone No. 8) cells.

Effect of GL on cell-free PKC activity and [^{3}H]TPA binding to PKC

It is well known that TPA is a tumor promotor which binds to PKC as a target enzyme (Nishizuka, 1986). We therefore examined the effect of GL on cell-free PKC activity as well as [^{3}H]TPA binding to PKC partially purified from MOLT-4 (clone No. 8) cells. As shown in Table 1, GL caused a dose-dependent inhibition

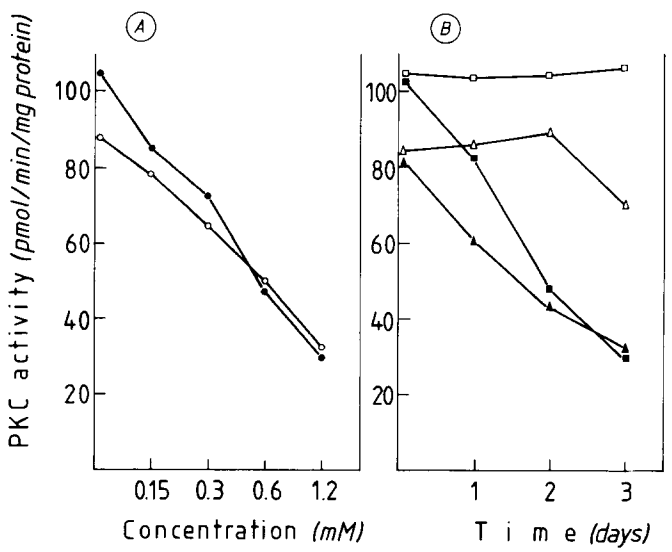

Fig. 2. Inhibitory effect of GL on PKC activity of MOLT-4 (clone No. 8) cells. (A) Dose-dependent curve: after the cells were incubated in the presence of varying concentrations of GL for 3 days, the cytosolic (●——●) and particulate (○——○) fractions of MOLT-4 (clone No. 8) cells were assayed for PKC activity. (B) Time-dependent curve: after the cells were incubated for 1, 2 or 3 days in the presence of (▲,■) or absence (△,□) of 1.2 mM GL, the cytosolic (■,□) and particulate (▲,△) fractions of MOLT-4 cells were assayed for PKC activity.

TABLE 1

Inhibitory effect of GL on cell-free PKC activity and [^{3}H]TPA binding to PKC

GL (mM)	PKC activity (pmol/min/mg/protein)	[^{3}H]TPA bound to PKC (pmol/mg protein)
0	152.4	70.6
0.075	126.9	21.8
0.15	39.7	4.1
0.3	0	3.8
0.6	0	4.2

PKC was partially purified from the cytosolic fraction of MOLT-4 (clone No. 8) cells by DEAE-cellulose chromatography. PKC was exposed to varying concentrations of GL for 10 min and was then assayed for PKC activity and [^{3}H]TPA binding.

of both cell-free PKC activity and [^{3}H]TPA binding within the concentration range of 0.75–0.3 mM. At the latter concentration PKC activity was completely suppressed.

Effect of other PKC inhibitors on HIV-1 replication

H-7 and two other PKC inhibitors (H-9 and HA 1004) were evaluated for their inhibitory effects on HIV-1 replication. H-7 is known as the most potent and selective PKC inhibitor among these agents, K_i (μM) values of H-7, H-9 and HA 1004 for PKC being 6.0, 18 and 40, respectively (Nakashima et al., 1987a). H-7 suppressed HIV-1-induced cytopathogenicity and viral antigen expression in

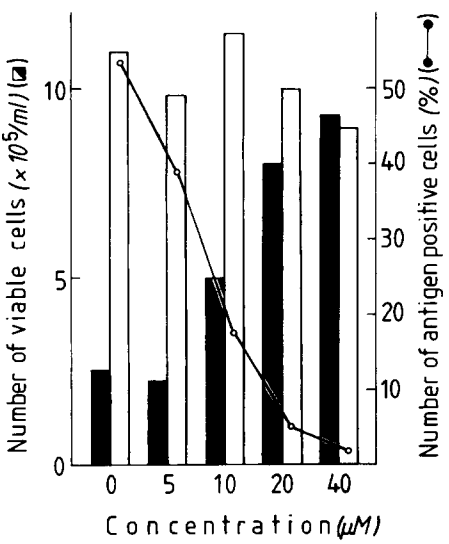

Fig. 3. Effect of H-7 on HIV-1-induced cytopathogenicity and virus antigen expression in MOLT-4 (clone No. 8) cells. HIV-infected MOLT-4 (clone No. 8) cells were incubated in the presence of H-7 for 4 days. On the 3rd day, half of the medium of all cultures was replaced. Further details are as described in the legend to Fig. 1.

MOLT-4 (clone No. 8) cells (measured 4 days after infection) at a concentration of 10–40 μM (Fig. 3). Within this concentration range, H-7 did not affect the growth of MOLT-4 (clone No. 8) cells. In contrast with H-7, neither H-9 nor HA 1004 inhibited HIV-1 replication at a concentration up to 40 μM (data not shown). At a concentration of 80 μM all three compounds showed a toxic effect on the MOLT-4 cells. At a concentration of 10–40 μM H-7 also suppressed the CPE of HIV-1 in MT-4 cells (data not shown).

Effect of GL on HIV-1 adsorption to the cells

When [5-^{3}H]uridine-labeled HIV-1 particles were incubated with an MT-4 cell suspension in the absence of any compound, cell-associated radioactivity increased with the incubation time (Fig. 4). If dextran sulfate (5 μM) had been added to the cell suspension, no increase in cell-associated radioactivity was observed. In the presence of 1.2 mM GL, i.e. at a concentration that achieves complete protection of MT-4 cells against HIV-1 cytopathogenicity (Ito et al., 1987), cell-associated radioactivity was suppressed by approximately 62% and 40% after 60 min and 120 min incubation, respectively (Fig. 4). These results indicate that GL partially prevents the adsorption of HIV-1 to MT-4 cells. This conclusion was confirmed using indirect immunofluorescence and laser flow cytofluorographic (FACSTAR, Bec-

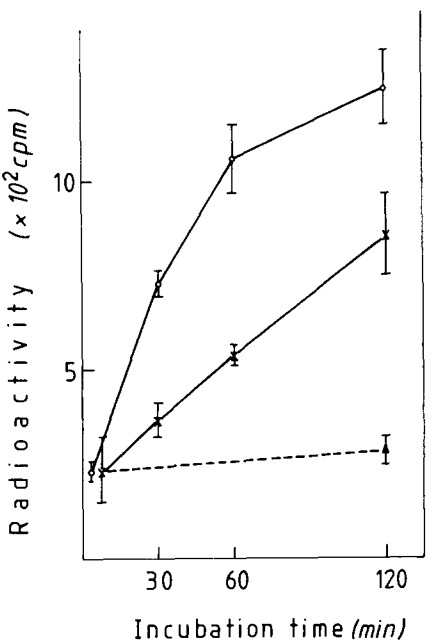

Fig. 4. Effect of GL on the adsorption of radiolabeled HIV-1 particles to MT-4 cells. Suspensions of MT-4 cells with either 1.2 mM GL (X) or 5 μM dextran sulfate (positive control) (▲) or no compound (○) and [5- ^{3}H]uridine-labeled HIV-1 particles were incubated at 37°C. After 0, 30, 60 or 120 min, the cells were thoroughly washed to remove unadsorbed virus particles, and cell-associated radioactivity was determined.

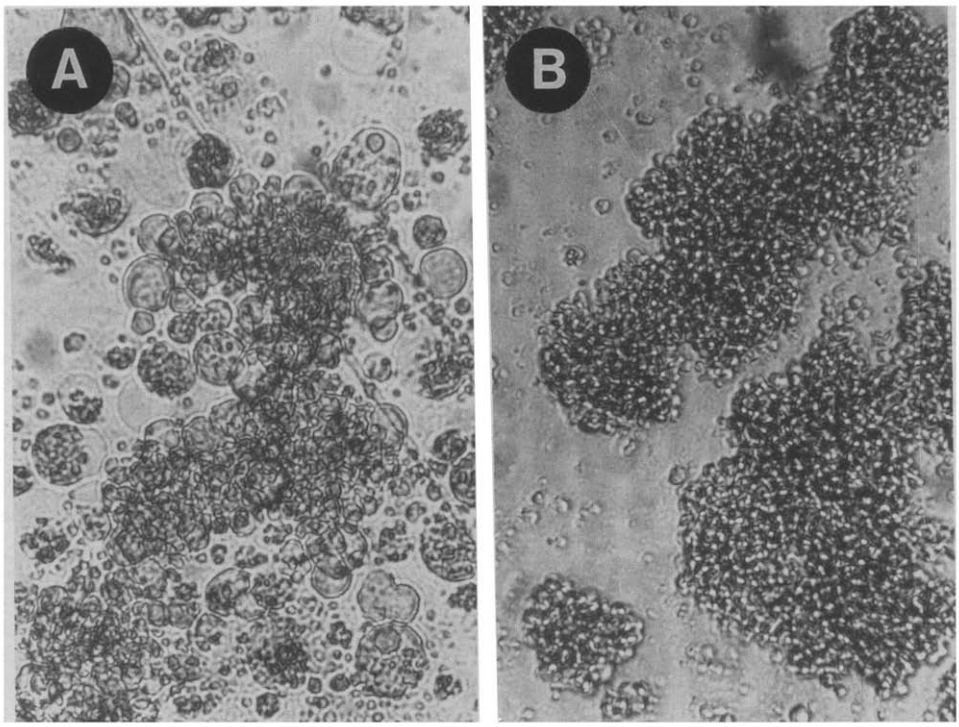

Fig. 5. Effect of H-7 and GL on giant cell formation on co-culturing MOLT-4 (clone No. 8) with MOLT-4/HTLV-III$_B$ cells. MOLT-4 (clone No. 8) cells and MOLT-4/HTLV-III$_B$ cells were co-cultured at a ratio of 4:1 for 3 days in the presence of 20 μM H-7 (panel A) or 1.2 mM GL (panel B).

ton Dickinson) analysis of MT-4 cells which had been exposed to HIV-1 virions in the presence or absence of the compounds (Schols et al., in press).

Effect of GL and H-7 on giant cell formation

When MOLT-4 (clone No. 8) cells and persistently HIV-1-infected MOLT-4/HTLV-III$_B$ cells are co-cultured, the cells fuse, thereby forming multinucleated giant cells. This giant cell formation is completely inhibited by dextran sulfate at a concentration of 25 μM (data not shown). When the effects of GL and H-7 (Nakashima et al., 1987b) were compared in this assay, GL inhibited giant cell formation at a concentration of 0.6–1.2 mM, whereas H-7 at a concentration of 20 μM failed to do so (Fig. 5).

Discussion

GL and glycyrrhetinic acid (GA) exhibit an inhibitory effect on several DNA and RNA viruses including HIV-1 in vitro (Baba and Shigeta, 1987; Ito et al., 1987; Pompei et al., 1979). The mode of antiviral action of GL and GA has not been

fully resolved, however. Based on their kinetic studies with GL against varicella-zoster virus, Baba and Shigeta assumed that GL inhibited an early stage of virus replication (Baba and Shigeta, 1987).

We have now examined the effect of GL on PKC activity and found that GL is an inhibitor of PKC whether occurring in its cell-associated or cell-free form (Fig. 2, Table 1). Although the precise role of PKC in the replication process of HIV-1 has not been clarified, PKC seems to be required for the binding of HIV-1 particles to the cellular CD4 receptors, since CD4 is phosphorylated by PKC (Fields et al., 1988). In fact, the adsorption of HIV-1 to the cells was partially suppressed by GL, as monitored by the use of radioactive virus particles (Fig. 4).

An important question that remains to be addressed is whether the inhibition of virus adsorption and the inhibition of PKC activity are causally linked. GL consists of one molecule of GA and two molecules of glucuronic acid, and thus contains three carboxyl groups. Being a polyanionic substance GL may be expected to share some common features with other polyanionic substances such as suramin and dextran sulfate. Suramin also partially inhibits the adsorption of HIV-1 to MT-4 cells (Schols et al., in press). Dextran sulfate has been shown to completely block adsorption of HIV-1 to the cells (Baba et al., 1988). Furthermore, dextran sulfate interferes with giant cell formation based on the interaction between the HIV-1 gp120 and cellular CD4 receptor, when MOLT-4 (clone No. 8) cells are co-cultured with MOLT-4/HTLV-III_B cells. GL also inhibits this fusion process. However, the PKC inhibitor H-7 does not (Fig. 5).

Taking these results together, it thus appears that the effect of GL on HIV-1 replication may result, on the one hand, from a specific inhibition and, on the other hand, from a non-specific inhibition of virus adsorption to the cells. Unlike dextran sulfate, GL did not achieve complete inhibition of virus adsorption at a concentration that completely inhibited HIV-1-induced cytopathogenicity (Fig. 4). It is inferred therefore that the inhibitory effect of GL on virus adsorption is further complemented by an inhibitory effect on PKC, and that both effects contribute to the inhibition of HIV-1 replication by GL.

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