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Inhibitory effect of glycyrrhizin on the in vitro infectivity and cytopathic activity of the human immunodeficiency virus [HIV (HTLV-III/LAV)]

Masahiko Ito[1], Hideki Nakashima[2], Masanori Baba[1,3], Rudi Pauwels[3], Erik De Clercq[3], Shiro Shigeta[1] and Naoki Yamamoto[2]

[1]*Department of Bacteriology, Fukushima Medical College, Fukushima 960, Japan;* [2]*Department of Virology and Parasitology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan;* [3]*Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

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Summary

Glycyrrhizin (GL), one of the plant extracts, was investigated for its antiviral action on the human immunodeficiency virus [HIV (HTLV-III/LAV)] in vitro, using cytopathic effect and plaque forming assay system in MT-4 cells (a HTLV-I-carrying cell line). Cloned Molt-4 cells (clone No. 8), which are sensitive to HIV and fuse to giant cells after infection, were also used as a parameter for cytopathic effect of HIV. GL completely inhibited HIV-induced plaque formation in MT-4 cells at a concentration of 0.6 mM, the 50% inhibitory dose being 0.15 mM. GL completely inhibited the cytopathic effect of HIV and the HIV-specific antigen expression in MT-4 cells at a concentration of 0.3 and 0.6 mM, respectively. Furthermore, GL inhibited giant cell formation of HIV-infected Molt-4 clone No. 8 cells. GL had no direct effect on the reverse transcriptase of HIV. Its mechanism of anti-HIV action remains to be elucidated.

HIV; HTLV-III/LAV; Glycyrrhizin; AIDS

Introduction

Acquired immune deficiency syndrome (AIDS) is a pandemic immunosuppressive disease which results in life-threatening opportunistic infections and malignancies. A retrovirus, designated human immunodeficiency virus [HIV (HTLV-III/LAV)], has been isolated and identified as the etiologic agent of this disease

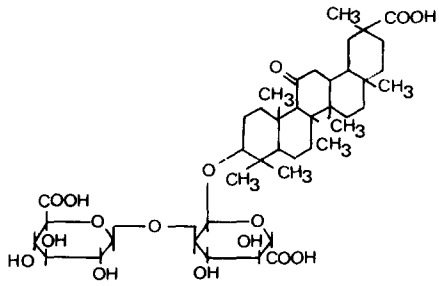

Fig. 1. Structure of glycyrrhizin (GL).

[4,17]. Recently, some compounds have been evaluated for their inhibitory effects on HIV replication in vitro, e.g. ribavirin [13], phosphonoformic acid [18], recombinant interferon-α [10], 3′-azido-2′,3′-dideoxythymidine [14] and 2′,3′-dideoxynucleosides [15]. Also, clinical trials have been initiated with most of these compounds, the results of these trials cannot yet be fully interpreted [5].

Glycyrrhizine (GL) is one of the aqueous extracts of licorice root *(Glycyrrhiza radix)*, which is known as an anti-inflammatory substance in Chinese medicine. This compound consists of one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid (Fig. 1). GA has proved inhibitory to the replication of several DNA and RNA viruses in vitro [16]. In addition, some therapeutic and prophylactic effects on chronic active viral hepatitis have been claimed for GL in Japan [6], and the combination of GL with glycine and cysteine has been widely used in Japan in the treatment of chronic viral hepatitis.

In view of the severity of the AIDS situation and the limited clinical efficacy of the compounds which has so far been reported with any compound, all possible therapeutic approaches towards this disease should be considered. In the present study, we examined the in vitro inhibitory effect of GL on HIV, using MT-4 cells which is highly permissive to HIV replication [8].

Materials and methods

Cells

The HTLV-I-carrying cell line, MT-4, and clone No. 8 of the human leukeumic T-cell line Molt-4 [11], 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 (culture medium).

Drugs

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. A nuceloside analogue, 3′-azido-2′,3′-dideoxythymidine [AZT (MW 267.24)] and its 5′-triphosphate (AZT-TP) were kindly provided by Dr. A. Matsuda and Dr. T. Ueda (Hokkaido University, Japan).

Virus and virus infection

HIV was obtained from the culture supernatant of the Molt-4/HTLV-III cell line as previsouly described [9]. The titer of the virus preparation was 6×10^4 plaque-forming units (PFU) per ml, and the MT-4 cells and Molt-4 clone No. 8 cells were infected with HIV at a multiplicity of infection (MOI) of 0.002, as described [9]. Briefly, the cells were incubated with the virus for 1 h at 37°C. After virus adsorption, infected cells were washed and resuspended in fresh medium, adjusted to a concentration of 3×10^5 cells/ml and cultured in the presence or absence of various concentrations of GL at 37°C in a CO_2 incubator.

Plaque forming assay

To evaluate the direct inhibitory effect of GL on HIV infection in MT-4 cells, a plaque assay using agarose overlay with varying concentrations of GL was performed as previously described [8]. Briefly, to fix MT-4 cells onto culture vessels, 35 mm polystyrene tissue culture dishes were coated with poly-L-lysine [PPL (MW 120,000); Sigma Chemical Co., St. Louis, Mo]. A suspension of 2.25×10^6 MT-4 cells in 1.5 ml was put onto each PLL-coated dish and incubated for 1 h at room temperature. The dishes were gently washed with PBS to remove unadherent cells. An appropriate dilution (100 μl) of the virus stock was slowly added and incubated with the cells for 1 h at room temperatue. After the virus adsorption period, 1 ml of agarose overlay consisting of RPMI 1640 medium with 10% FCS, antibiotics and 0.6% agarose (Sea Plaque Agarose, Marine Colloid Corp., Rockland, Me) was poured into each dish. The dishes were incubated at 37°C for 3 days, and 1 ml of agarose overlay containing neutral red was added. The dishes were incubated for another 3 days and the number of visible plaques was counted. All experiments were carried out in triplicate.

Assay for HIV-induced cytopathic effect

HIV-induced cytopathic effect (CPE) was monitored by the decrease in viability of MT-4 or Molt-4 clone No. 8 cells infected with HIV. The viable cells were detected by the trypan blue exclusion method.

Assay for HIV-specific antigen expression

Virus-specific antigen expression in HTLV-III-infected MT-4 cells or Molt-4 clone No. 8 cells was determined by indirect immunofluorescence (IF). Briefly, methanol-fixed cells were incubated with 1:1000 diluted seropositive anti-HIV 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 miscroscope and the percentage of fluorescent-positive cells was calculated.

Reverse transcriptase (RT) assay

Supernatants of the Molt-4/HTLV-III were concentrated 100 times by sucrose gradient ultracentrifugation. The viral pellets were disrupted by addition of 5 mM

Tris-HCl (pH 8.1), 0.5 M KCl, 0.1 mM dithiothreitol (DTT) and 0.1% Triton X-100. The assay for RT activity was performed at 37°C for 1 h with 10 μl of disrupted HIV in a final volume of 50 μl containing 50 mM Tris-HCl (pH 8.4), 2 mM DTT, 100 mM KCl, 10 mM MgCl, 0.01% Triton X-100. 1 μCi [^{3}H-*methyl*]thymidine triphosphate (57 Ci/mmol; Amersham, Buckinghamshire, UK) and 50 μg/ml poly(rA)·poly(dT) (P-L Biochemicals Inc., Milwaukee, Wis.). The reaction was stopped with 5% trichloroacetic acid (TCA), and precipitates were collected on glass fiber filters and counted in a liquid scintillation counter [9]. The assays were carried out in triplicate.

[^{3}H-methyl]thymidine uptake by MT-4 cells or normal pheripheral blood mononuclear (PBM) cells

MT-4 cells were adjusted to 3×10^5 cells/ml in fresh medium and cultured in the presence of varying concentrations of GL in a 96-flat bottom microtiter tray for 3 days. One million of PBM cells were cultured with 1 μg/ml of polyclonal mitogen, phytohemagglutinin (PHA) or concanavalin A (Con A) and varying concentrations of GL for 3 days. The cells were incubated with 1 μCi of [^{3}H-*methyl*]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) per well for the last 18 h of the 3-day incubation period. The cells were collected on glass fiber filters and counted in a liquid scintillation counter. The experiments were carried out in triplicate.

Assay for combined effect of GL with AZT

The combined inhibitory effects with AZT on HIV-induced CPE in MT-4 cells was examined by checkerboard combinations of various GL and AZT concentrations, and analyzed by the isobologram method, described previously [2]. In this experiment, the 50% inhibitory dose (ID_{50}) of either GL or AZT for cell destruction was used for calculating the fractional inhibitory concentration (FIC).

Results

Inhibitory effect of GL on HIV plaque formation

When the inhibitory effect of GL on HIV plaque formation in MT-4 cells was examined, GL brought about 34.2% plaque reduction at a concentration of 0.06 mM, and GL completely inhibited plaque formation at a concentration of 0.6 mM. The inhibitory effect of GL on HIV plaque formation was dose-dependent. Its ID_{50} was 0.15 mM (Fig. 2).

The ID_{50} of GL for DNA synthesis, as measured by [^{3}H-*methyl*]thymidine incorporation, in uninfected MT-4 cells was 2.6 mM (data not shown). The selectivity index (SI), based on the ratio of the ID_{50} for host cell DNA synthesis to the ID_{50} for HIV plaque formation, was 17.3. These results indicate that GL effected a selective inhibition of the replication of HIV in MT-4 cell cultures.

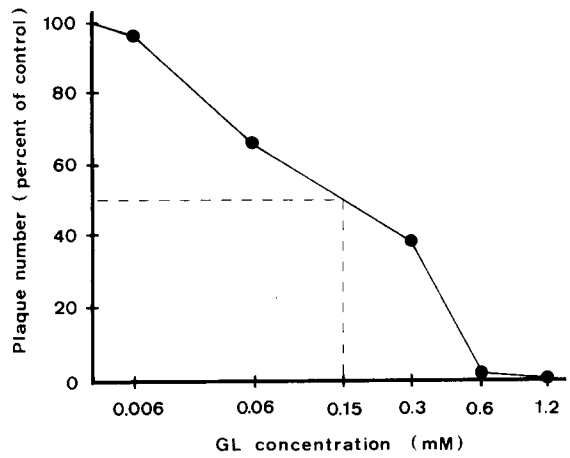

Fig. 2. Inhibitory effect of GL on HIV plaque formation in MT-4 cells. Results represent the average values for two experiments each performed in triplicate (see Materials and Methods for details).

Inhibitory effect of GL on cytopathic effect of HTLV-III

When MT-4 cells were infected with HTLV-III at a MOI of 0.002, viability of the cells gradually decreased because of viral CPE, and on day 3 after infection, the number of viable cells was only 3.2×10^5 cells/ml, as compared to 1.2×10^6 cells/ml in the uninfected controls (Fig. 3). GL completely protected MT-4 cells from viral CPE at a concentration of 0.3 mM, and at this concentration GL was not inhibitory to the uninfected MT-4 cells (Fig. 3). At a concentration of 2.4 mM, GL had a 50% inhibitory effect on the growth of uninfected cells (data not shown).

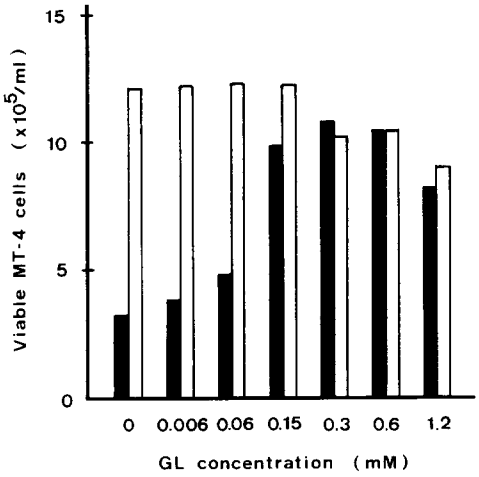

Fig. 3. Inhibitory effect of GL on HIV-indued CPE in MT-4 cells. MT-4 cells (3×10^5) were exposed to HIV (MOI: 0.002) and cultured in the presence and absence of varying concentrations of GL (■). Control uninfected cells were exposed to the same concentrations of GL (□). The total number of viable cells was determined after 3 days.

TABLE 1

Inhibitory effect of GL on the expression of HIV-specific antigens in MT-4 cells

GL concentration (mM)	Percentage of IF-positive cells
1.2	0.6
0.6	1.0
0.3	23.3
0.06	69.3
0.006	70.9
0	73.8

More than 500 cells were counted, and the percentage of IF-positive cells was calculated on day 3 after HIV infection.

Inhibitory effect of GL on HIV-specific antigen expression

When MT-4 cells were infected with HIV and examined for IF, 73.5% of the cells became positive for viral antigen on day 3 after infection (Table 1). A dose-dependent inhibition of IF was observed if the HIV-infected MT-4 cells had been incubated in the presence of varying concentrations of GL. At the concentration of 0.6–1.2 mM, viral antigen expression was almost completely suppressed.

Effect of GL on RT activity of HIV

The effect of GL and AZT-TP on the activity of cell-free HIV RT are shown in Table 2. AZT-TP achieved a 97% reduction of RT activity at a concentration of 18.7 μM, whereas GL had no inhibitory effect on HIV RT activity even at a concentration of 12 mM, the highest concentration tested (Table 2).

Effect of Gl on normal lymphocyte blast formation

When the effect of GL on lectin-induced reactivity of normal lymphocytes was examined, its ID_{50} was 2.5 and 2.4 mM for the PHA and ConA response, respectively.

TABLE 2

Effect of GL and AZT-TP on the cell-free RT activity of HIV

Compound concentration (mM)		RT activity ($\times 10^{-4}$ cpm)	
GL	AZT-TP	GL	AZT-TP
12	–	270 ± 21.4[a]	–
6	–	297 ± 17.1	–
1.2	3.74	247 ± 3.0	0.8 ± 0.2
0.6	1.87	264 ± 2.8	0.7 ± 0.1
0.12	0.374	241 ± 14.5	1.0 ± 0.3
–	0.187	–	1.3 ± 0.3
–	0.0187	–	7.9 ± 1.5
–	0.00187	–	56.5 ± 0.6
0	0	256 ± 14.2	155 ± 9.7

[a]Mean ± standard deviation.

TABLE 3

Effect of GL on cell viability and HIV-specific antigen expression of Molt-4 clone No. 8 cells

Time after infection	GL concentration (mM)	
	0	1.2
Day 3		
Viable cells ($\times 10^5$/ml)	18.1	15.0
Viability (%)	89.8	91.4
IF-positive cells (%)	4.7	3.0
RT (cpm)	1470	1536
Virus titer (pfu/ml)	< 500	< 500
Day 6		
Viable cells ($\times 10^5$/ml)	42	34.0
Viability (%)	82.4	83.4
IF-positive cells (%)	11.8	7.1
RT (cpm)	10624	5369
Virus titer	2500	< 500
Day 9		
Viable cells ($\times 10^5$/ml)	37.8	75.7
Viability (%)	38.6	84.4
IF-positive cells (%)	99.0	5.8
RT (cpm)	20344	4369
Virus titer (pfu/ml)	15500	1000

HTLV-III-infected Molt-4 clone No. 8 cells (3×10^5 cells/ml) were cultured with or without 1.2 mM GL. Cell numbers, viability, percentage of IF-positive cells, RT activity and virus titer were determined on days 3, 6 and 9 after infection. RT activity in the culture fluid is expressed as CPM per 10^4 viable cells. Virus titer of the culture fluid was evaluated by plaque formation in MT-4 cells.

Inhibitory effect of GL on giant cell formation and CPE in HIV-infected Molt-4 clone No. 8 cells

Like MT-4 cells, Molt-4 clone No. 8 cells are also sensitive to the CPE of HIV. Upon infection, Molt-4 clone No. 8 cells fuse thereby forming giant cells, but the time needed for the cytopathic effect to develop is much longer in MT-4 cells. When GL (1.2 mM) was added to HIV-infected Molt-4 clone No. 8 cells, viability of GL-treated and untreated cells was, on day 3, 91.4% and 89.8%, respectively, on day 6, 83.4% and 82.4%, respectively, and, on day 9, 84.4 and 38.6%, respectively (Table 3). On day 9, the number of IF-positive cells was 99% for the control group, as compared to 5.8% for the GL-treated group. A marked difference between the drug-treated and untreated cultures was also observed for RT activity and titer of HIV in the cell culture fluid, especially on day 9 (Table 3). Giant cell formation of Molt-4 clone No. 8 cells was observed on day 6 and 9 after HIV infection (Fig. 4B). However, no such phenomenon was apparent if the HIV-infected cells had been incubated in the presence of 1.2 mM GL (Fig. 4A).

Combined effects of GL and AZT

The combined effect of GL and AZT were assessed by the minimum FIC index

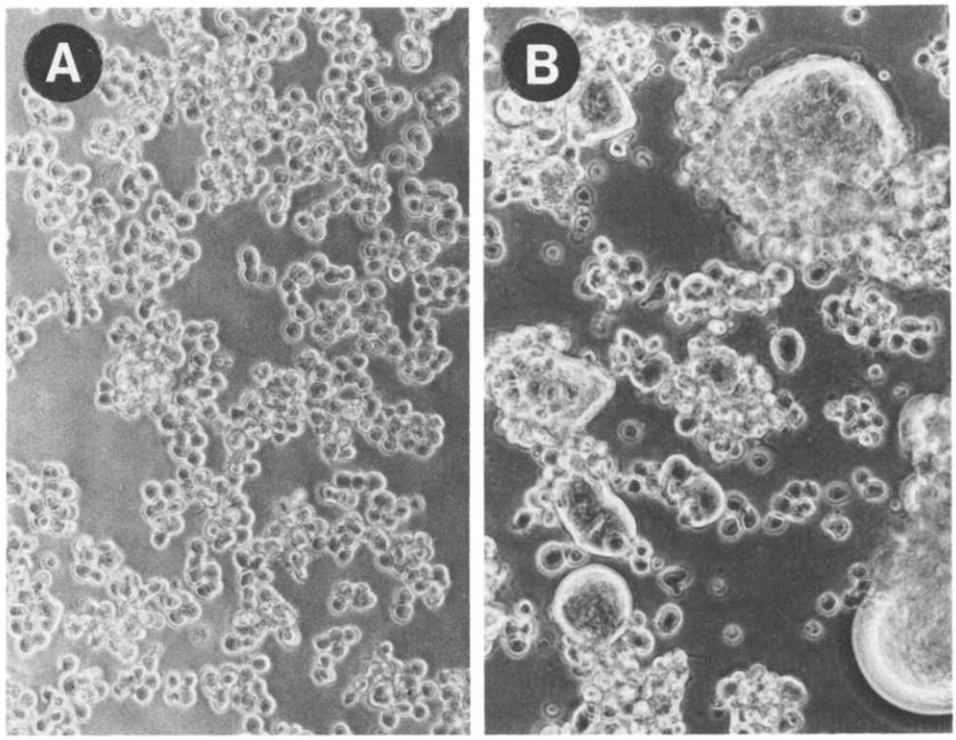

Fig. 4. Giant cell formation of Molt-4 clone No. 8 cells following infection with HIV and incubation for 7 days in the absence (panel B) or presence (panel A) of GL (1.2 mM). There are no giant cells observed in Fig. 4A, whereas several giant cells are present in Fig. 4B. (× 140).

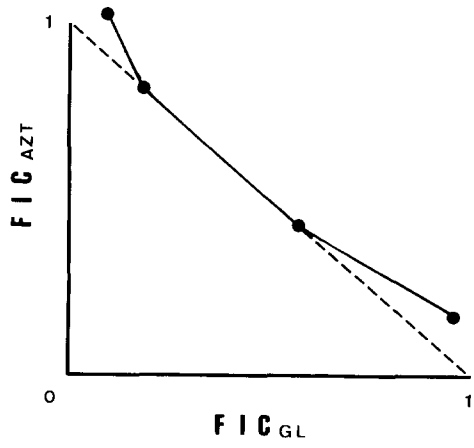

Fig. 5. Isobologram representation of the inhibitory effects of GL in combination with AZT on HIV-induced CPE in MT-4 cells. The minimum FIC value was 1.0. Broken line is the unity line.

obtained by the isobologram (Fig. 5) procedure. In general, when the minimum FIC index is equal to 1.0, the combination can be judged as additive, when less than 1.0, it is synergistic, and when more than 1.0, it is antagonistic. The minimum FIC index calculated for the combination of GL and AZT was 1.0 (Fig. 5) which means that the combination resulted in an additive effect.

Discussion

The number of patients with AIDS reported every year has dramatically increased since the first report concerning AIDS was presented in 1981 [7,19]. The investigation presented here demonstrates that GL has an inhibitory effect on HIV replication in vitro. The ID_{50} of GL for HIV (0.15 mM) was not as low as that of other nucleoside derivatives, i.e. AZT, which have been reported to inhibit HIV at a concentration of about 1 μM [5]. This difference in potency may be related to differences in the mechanism of action of GL and the nucleoside analogues which are assumed to be targeted at RT. In fact, GL was not directly inhibitory to the HIV RT (Table 2). Furthermore, combination of GL with AZT resulted in an additive inhibitory effect (Fig. 5), which suggests that GL may be directed at a target other than RT.

MT-4 cells were destroyed shortly after HIV infection, whereas it took a much longer time for HIV to develop its cytopathic effect in Molt-4 clone No. 8 cells. Molt-4 clone No. 8 cells tend to fuse and form giant cells, when infected with HIV [11]. It is well established that the other types of cells infected with HIV also tend to form syncytia. Recently, it has been demonstrated that the cell fusion plays an important part in the transmission of HIV from infected CD4 (T4/Leu3) positive cells to uninfected cells [12]. GL rapidly suppressed the formation of giant cells in cultured Molt-4 clone No. 8 cells (Fig. 4).

It has been shown previously [2,16], that GL has an inactivating effect on HSV and VZV particles. We also examined whether GL directly inactivated HIV. However, no such effect could be demonstrated (data not shown). In addition, it has also been reported that GL exhibits various biological effects including interferon inducing activity in vivo [1]. However, in the supernatant of MT-4 cell cultures treated with various concentrations of GL we could not detect any interferon activity (data not shown).

Whether GL has any clinical potential, i.e. in the therapy of AIDS, remains to be determined. The in vivo bioavailability of GL, its half-life upon systematic administration, its ability to penetrate the blood-brain barrier and other pharmacokinetic parameters are subjects of future study. Investigations have been started on the inhibitory effects of GL on tumor induction by Moloney sarcoma virus in newborn NMRI mice, which seems to be a representative model for retrovirus infection in vivo. The acute toxicity of GL for mice is very low, its 50% lethal dose (LD_{50}) being 805 mg/kg following intraperitoneal injection and 405 mg/kg following intravenous injection (unpublished data). Also, the fact that in humans intravenous injection of GL (at 200 mg/day) for 2 weeks does not show any noticeable

side effect points to the safety of this drug. AIDS is such a serious disease that an effective chemotherapy would be of crucial importance. The results presented here suggest that GL should be further evaluated for its efficacy in the treatment of retrovirus infections, including AIDS.

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