ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1992, p. 1890–1893
0066-4804/92/091890-04$02.00/0

Vol. 36, No. 9

Mechanism of Action of the Antiherpesvirus Biflavone Ginkgetin

KYOKO HAYASHI,[1]* TOSHIMITSU HAYASHI,[2] AND NAOKATA MORITA[2]

Department of Virology, School of Medicine[1] and Faculty of Pharmaceutical Sciences,[2] Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

Received 16 March 1992/Accepted 29 June 1992

Screening of plant extracts found that a biflavone from *Cephalotaxus drupacea*, which was found to be ginkgetin, is active against herpes simplex virus type 1 (HSV-1). This compound caused dose-dependent inhibition of virus replication with a 50% cytotoxic activity at 12.8 μg/ml and 50% anti-HSV-1 activity at 0.91 μg/ml, the therapeutic index being 14.1. Ginkgetin also showed inhibitory effects against HSV type 2 and human cytomegalovirus with therapeutic indices of 13.8 and 11.6, respectively. Ginkgetin had a weak virucidal activity against HSV-1 at more than 5 μg/ml. Both adsorption of HSV-1 to host cells and virus penetration into cells were unaffected by this agent. Ginkgetin suppressed viral protein synthesis when added at various steps of HSV-1 replication and exerted strong inhibition of transcription of the immediate-early genes.

For several years, we have screened natural compounds for antiviral activity. Ginkgetin, which is a biflavone originally isolated from *Ginkgo biloba*, has been obtained as an active component from *Cephalotaxus drupacea* Siebold and Zuccarini. The chemical structure was determined by Baker et al. (1). In 1971, Khan et al. (9) reported the isolation of ginkgetin from the leaf of *C. drupacea*.

So far, many flavonoids extracted from various plants have been reported to possess antiviral activity (2, 3, 7, 8, 15). The purpose of this study was to determine the effects of ginkgetin on herpes simplex virus type 1 (HSV-1) replication in vitro.

MATERIALS AND METHODS

Preparation of ginkgetin. Fresh leaves and twigs (6.5 kg) of *C. drupacea* Siebold and Zuccarini were cut into small pieces and extracted with methanol (MeOH) at room temperature. The chloroform-soluble part of the MeOH extract (65.3 g) was chromatographed on a silica gel column by using a stepwise gradient of $CHCl_3$-MeOH as an eluent to give 11 fractions. Fraction 6 (9.71 g) eluted with $CHCl_3$-MeOH (9:1) was further column chromatographed on silica gel and Sephadex LH-20 successively to afford yellow crystals (199 mg). This crystalline compound was identified as ginkgetin (Fig. 1) by comparison of its spectral data with those reported in the literature (10).

Cells and viruses. HeLa and Vero cells were cultured in Eagle's minimal essential medium supplemented with 6% fetal bovine serum for growth of virus and plaque assay. Human embryonic lung (HEL) cells were grown in minimal essential medium containing 10% bovine serum. HSV-1 strain HF was propagated at a low multiplicity of infection and plaque assayed on HeLa or Vero cell monolayers, HSV type 2 (HSV-2) strain UW-268 was propagated and plaque assayed on Vero cells, and human cytomegalovirus (HCMV) strain Towne was plaque assayed on HEL cells.

Cytotoxicity assay. To specify the degree of cell proliferation, the doubling times of HeLa and Vero cells were determined by seeding at a density of 2×10^5 cells per 35-mm dish and by counting viable cells every 24 h for 96 h. For cell growth inhibition studies, 2×10^5 cells per 35-mm dish were seeded and cultured for 72 h at 37°C in the presence of increasing amounts of ginkgetin. After the medium was removed, the cells were trypsinized and viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as dose-response curves, from which the 50% inhibitory dose (ID_{50}) was obtained.

Antiviral activity. HeLa or Vero cell monolayers in 24-well culture plates were washed and infected with HSV-1 or HSV-2 at 0.2 PFU per cell, adsorbed for 1 h at room temperature, and refed with maintenance medium (minimal essential medium plus 2% fetal bovine serum) containing various concentrations of ginkgetin. The cultures were incubated for 24 h at 34°C in 5% CO_2, harvested, and disrupted by three cycles of freezing and thawing. HEL cells infected with HCMV at 0.2 PFU per cell were treated with ginkgetin for 48 h. The harvested cultures were disrupted by three cycles of freezing and thawing. Virus yields were determined by plaque assay. Each concentration was assayed in two independent experiments. The antiviral activity was expressed as the 50% effective dose (ED_{50}) for viral replication, which was the lowest drug concentration that reduced plaque numbers by 50% in the treated cultures compared with untreated ones.

Virucidal activity. In order to determine the inactivation of virus by the drug, ginkgetin was diluted in the medium to provide final concentrations ranging from 0.2 to 50 μg/ml. HSV-1 was added to the solutions, which were then incubated for 9 h at 37°C. Samples were harvested in a small quantity of the mixtures every 3 h and plaque assayed.

Effect on virus adsorption to cells. HeLa cells grown to confluence in 60-mm dishes were pretreated with ginkgetin for 3 h at 37°C. After being washed with phosphate-buffered saline (PBS) (pH 7.2), the cells were inoculated with HSV-1 at 1 PFU per cell for 1 h at 37°C. The inocula were recovered, and the unadsorbed viruses in these samples were titrated by plaque assay for comparison with those recovered from untreated control cultures.

Assay for rate of virus penetration. Virus penetration into host cells was measured by the method reported by Huang and Wagner (6) and modified by Highlander et al. (4).

* Corresponding author.

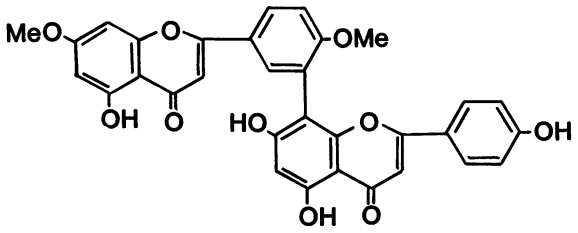

FIG. 1. Structure of ginkgetin.

Briefly, HeLa cell monolayers were pretreated with ginkgetin for 3 h at 37°C and then inoculated with HSV-1 and incubated at 4°C for adsorption. After 1 h, the cultures were shifted to 37°C for penetration into cells. Every 30 min, unpenetrated viruses were inactivated by treatment with citrate buffer (pH 3.0) for 1 min. The monolayers were overlaid with 0.5% methylcellulose, incubated for 2 days, and stained with crystal violet to count the number of plaques.

Detection of viral DNA. Vero cells infected with HSV-1 at 10 PFU per cell were harvested every 1 h for 8 h postinfection (p.i.). The procedures for the detection of viral DNA by hybridization with a biotin-labeled probe have been described elsewhere (11).

Preparation of anti-HSV serum. Rabbit antiserum was prepared by the procedure described by Showalter et al. (14) with a slight modification. Virus-infected HeLa cells showing maximum cytopathic effect were harvested and disrupted by freezing-thawing followed by centrifugation. A 0.5-ml volume of the supernatant (10^8 PFU/ml) per animal was used as an immunogen. The samples (a 1:1 emulsion of antigen in Freund's complete adjuvant) were injected in five subcutaneous sites along the back. Booster immunizations were at 7, 14, and 21 days in Freund's incomplete adjuvant. The rabbits were bled at day 45.

SDS-PAGE analysis of radiolabeled proteins. To analyze the effect of ginkgetin on host cell protein synthesis, Vero cells were radiolabeled for 4 h in the medium containing the compound. In the infection experiments, Vero cells were infected with HSV-1 at 10 PFU per cell and radiolabeled at the times indicated. After the monolayer was washed and replenished with methionine-free minimal essential medium (Flow Laboratories, Irvine, United Kingdom), 10 μCi of Tran ^{35}S-label (70% [^{35}S]methionine and 20% [^{35}S]cysteine; 1,000 Ci/mmol; ICN Biomedicals, Inc.) per 60-mm dish was added and incubated at 37°C. For labeling of immediate-early (IE) proteins of HSV-1, Vero cells were incubated in the presence of 50 μg of cycloheximide per ml from 2 h before infection (−2 h) to 2 h p.i., infected with virus from −1 h to 0 h, and further incubated for 1 h with the medium supplemented with drug and Tran ^{35}S-label in the presence of 5 μg of actinomycin D per ml. Infected cells were harvested and extracted with cell lysis buffer (0.05 M Tris-HCl [pH 7.0], 0.15 M NaCl, 1% sodium dodecyl sulfate [SDS], 1% Triton X-100) and then centrifuged at 120,000 × *g* for 1 h at 4°C. An aliquot of the cell lysates was treated with rabbit antiserum and protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) overnight at 4°C with rocking. The immunoprecipitates and the cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide). After electrophoresis, the gels were soaked in 1 M sodium salicylate for 30 min, dried, and exposed to X-ray films (13).

TABLE 1. Effect of ginkgetin on the cell growth and replication of virus[a]

Virus	Host cell	Cytotoxicity (ID_{50}; μg/ml)[b]	Antiviral activity (ED_{50}; μg/ml)	Therapeutic index (ID_{50}/ED_{50})
HSV-1	HeLa	12.8 ± 0.36	0.91 ± 0.064	14.1 ± 0.57
HSV-1	Vero	11.5 ± 0.71	0.76 ± 0.050	15.1 ± 1.84
HSV-2	Vero	11.5 ± 0.71	0.83 ± 0.022	13.8 ± 1.20
HCMV	HEL	19.0 ± 2.80	1.75 ± 0.360	11.6 ± 0.71

[a] Each value is the mean ± standard deviation of two independent experiments.
[b] For the cytotoxicity assay, cells were treated with ginkgetin for 72 h at 37°C. In the assay for anti-HSV-1, anti-HSV-2, and anti-HCMV activities, infected cells were harvested after incubation at 34°C for 24, 24, and 48 h, respectively.

RESULTS

Effects of ginkgetin on cell growth and virus replication. The length of time of cell doubling was 23 h for HeLa and Vero cells. Uninfected or HSV-infected cells were cultured for 72 h in the medium supplemented with 0.2 to 100 μg of ginkgetin per ml. Drug concentrations below 2 μg/ml showed no cytotoxicity. The ID_{50}s for HeLa, Vero, and HEL cells were 12.8, 11.5, and 19.0 μg/ml, respectively (Table 1). In HSV-1-infected HeLa cells, a dose-dependent inhibition of virus replication was observed, showing about 2.1 and 3.0 log units of reduction of virus yield at 2 and 5 μg/ml. The ED_{50} for anti-HSV-1 activity was 0.91 μg/ml. Therefore, the in vitro therapeutic index, calculated by dividing the ID_{50} by the ED_{50}, was 14.1. The replication of HSV-1 in Vero cells was also inhibited by ginkgetin to almost the same degree that it was in HeLa cells, the ED_{50} and the therapeutic index being 0.76 μg/ml and 15.1, respectively. Furthermore, ginkgetin exerted anti-HSV-2 and anti-HCMV activities, for which the therapeutic indices were 13.8 and 11.6, respectively.

Direct virucidal activity of ginkgetin. A dose-dependent effect by direct incubation with ginkgetin was observed (Table 2). Ginkgetin reduced infectivity by approximately 60 and 80% at 5 and 50 μg/ml, respectively. A 50% reduction in the control preparation without ginkgetin was noted.

Effect of ginkgetin on virus adsorption and penetration. Ginkgetin did not interfere with the attachment to cell membranes at concentrations of 0.2 to 50 μg/ml (data not shown). The kinetics of penetration was determined by inactivating the unpenetrated viruses with a low-pH citric acid buffer at various times after temperature shift from 4 to 37°C (Fig. 2). Pretreatment of HeLa cells with 1, 5, or 50 μg/ml did not inhibit virus penetration.

Effects of ginkgetin on protein synthesis. Ginkgetin did not

TABLE 2. Direct virucidal activity of different concentrations of ginkgetin on HSV-1

Ginkgetin concn (μg/ml)	HSV-1 infectivity (%) after the following incubation times (h):			
	0	3	6	9
0	100 ± 7.8[a]	67 ± 7.8	54 ± 4.2	50 ± 3.6
0.2		64 ± 10.6	53 ± 7.8	51 ± 5.0
1		60 ± 9.2	52 ± 1.4	47 ± 5.0
5		67 ± 4.2	53 ± 8.5	39 ± 4.2
50		65 ± 6.4	26 ± 5.0	19 ± 2.8

[a] The plaque titer of inocula (approximately 10^6 PFU/ml) was expressed as 100%. Each value is the mean ± standard deviation of duplicate experiments.

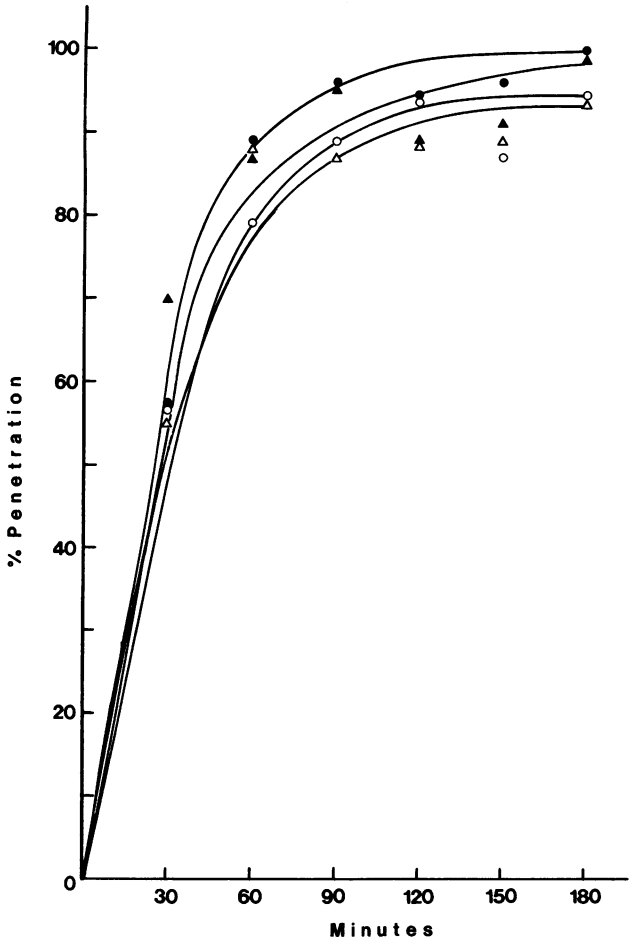

FIG. 2. Effect of ginkgetin on HSV-1 penetration. Dilutions of virus (ca. 100 PFU) were adsorbed for 1 h at 4°C on HeLa cells pretreated for 3 h with ginkgetin at concentrations of 0 (●), 1 (○), 5 (▲), or 50 (△) μg/ml. After being washed with PBS, the cultures were shifted to 37°C and treated with 40 mM citric acid buffer (pH 3.0) for 1 min at 30-min intervals. Surviving virus plaques were counted after 2 days. The results were expressed as percent penetration, with the maximum penetration of virus on untreated cells taken to be 100%. Each point is the mean of duplicate experiments.

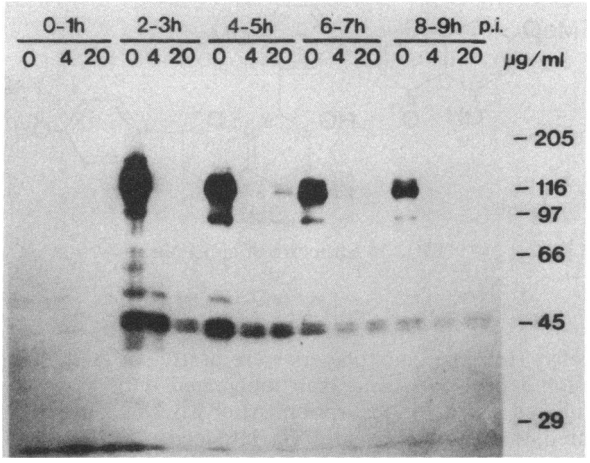

FIG. 3. Analysis by SDS-PAGE of the proteins synthesized in HSV-infected Vero cells untreated or treated with 4 or 20 μg of ginkgetin per ml. The cells were pulse-labeled with Tran ^{35}S-label at 0 to 1, 2 to 3, 4 to 5, 6 to 7, or 8 to 9 h p.i., and the immunoprecipitated proteins were analyzed as described in Materials and Methods. Ginkgetin was added at the same times of labeling, that is, at 0, 2, 4, 6, or 8 h p.i. The numbers on the right indicate apparent molecular weights (in thousands).

inhibit cellular protein synthesis at concentrations of 4 or 20 μg/ml (data not shown). In contrast, when Vero cells were infected with HSV-1 and ginkgetin was added at different times after infection, viral protein synthesis was strongly inhibited (Fig. 3). The compound inhibited the bulk of the protein synthesis even when it was added 8 h p.i.; 4 μg/ml was sufficient for ginkgetin to cause inhibition. Viral DNA was detected from 5 h p.i., and late structural proteins began to be synthesized from about 6 h p.i. under these conditions (data not shown). The effect of ginkgetin on the viral IE proteins was unclear from the data obtained in Fig. 3. IE proteins were detected by virus infection of cells in the presence of cycloheximide, which was followed by treatment with actinomycin D (Fig. 4). When infected cells were treated with ginkgetin during transcription of viral DNA to mRNA (−2 to 2 h p.i.), an almost complete blocking of protein synthesis was observed at 4 and 20 μg/ml. However, the expression of IE proteins of HSV-1 was suppressed to a

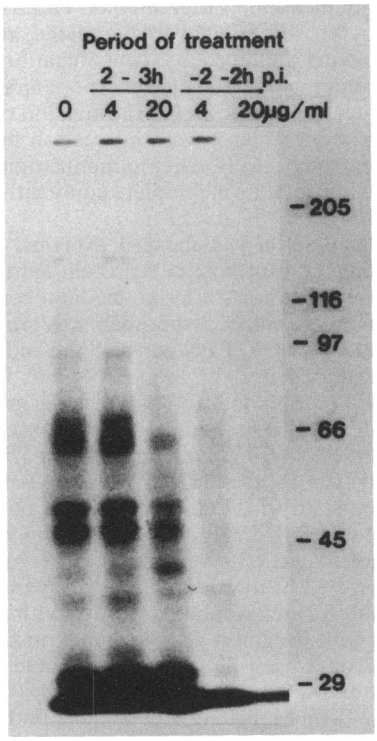

FIG. 4. Analysis by SDS-PAGE of HSV-1 IE proteins synthesized in virus-infected cells untreated or treated with 4 or 20 μg of ginkgetin per ml from 2 to 3 h p.i. or from 2 h before infection (−2 h) to 2 h p.i. Vero cells were incubated in the presence of cycloheximide from −2 to 2 h p.i., infected with virus during −1 to 0 h, and then cultured for 1 h (i.e., during 2 to 3 h p.i.) in the medium containing Tran ^{35}S-label and actinomycin D. An aliquot of the cell lysates was subjected to SDS-PAGE as described in Materials and Methods.

lesser extent when infected cells were treated during translation (2 to 3 h).

DISCUSSION

The results obtained in the present study show that ginkgetin isolated from *C. drupacea* Siebold and Zuccarini exerts a dose-dependent inhibitory effect in vitro on the replication of HSV-1. This activity was demonstrated by virus yield reduction at concentrations that were below the cytotoxic dose. Ginkgetin also inhibited the replication of HSV-2 and HCMV.

In order to elucidate the mode and mechanism of its action in the inhibition of HSV-1 replication, the effect of ginkgetin was studied under various conditions. It was shown that direct inactivation of virus was observed at considerably high concentrations and might be attributed partially to the antiviral effect of ginkgetin. Attachment of the virus to cells and penetration were unaffected by ginkgetin.

Studies on the times at which ginkgetin is added to HSV-infected cell cultures indicate that the agent suppresses the protein synthesis at various steps of viral replication. There was no clear evidence for selective reduction of synthesis of any particular proteins.

IE (alpha) genes, by definition, are transcribed in infected cells in the absence of viral protein synthesis. Expression of the IE genes is obligatory for sequential transcription of the viral genome (16). In the present study, selective enhancement of IE proteins was achieved through accumulation of IE transcripts by cycloheximide treatment. Transcription of the IE genes was strongly suppressed by ginkgetin, while translation of the transcripts was not affected as much, suggesting that this compound blocks a step of transcription rather than protein synthesis in the early events of viral replication. Some flavones are known to block RNA synthesis (3, 7). In this experiment, ginkgetin inhibited protein synthesis even when added at 4 to 8 h p.i., when proteins of the β and γ groups are synthesized (5, 12). Thus, ginkgetin might also interfere with the synthesis of virus mRNA in the later steps of viral replication.

In conclusion, ginkgetin is an inhibitor of HSV-1 replication in vitro at concentrations that have little or no effect on the growth of host cells. The agent showed efficacy in the inactivation of virus.

ACKNOWLEDGMENT

This work was supported by a grant from the Shorai Foundation for Science and Technology.

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