Geldanamycin, a Ligand of Heat Shock Protein 90, Inhibits the Replication of Herpes Simplex Virus Type 1 In Vitro

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Geldanamycin (GA) is an antibiotic targeting the ADP/ATP binding site of heat shock protein 90 (Hsp90). In screening for anti-herpes simplex virus type 1 (HSV-1) candidates, we found GA active against HSV-1. HSV-1 replication in vitro was significantly inhibited by GA with an 50% inhibitory concentration of 0.093 µM and a concentration that inhibited cellular growth 50% in comparison with the results seen with untreated controls of 350 µM. The therapeutically effective concentration of GA was over 3.700 (comparable to the results seen with acyclovir). GA did not inhibit HSV-1 thymidine kinase. Cells infected with HSV-1 demonstrated cell cycle arrest at the G1/S transition; however, treatment with GA resulted in a cell cycle distribution pattern identical to that of untreated cells, indicating a restoration of cell growth in HSV-1-infected cells by GA treatment. Accordingly, HSV-1 DNA synthesis was suppressed in HSV-1+ cells treated with GA. The antiviral mechanism of GA appears to be associated with Hsp90 inactivation and cell cycle restoration, which indicates that GA exhibits broad-spectrum antiviral activity. Indeed, GA exhibited activities in vitro against other viruses, including severe acute respiratory syndrome coronavirus. Since GA inhibits HSV-1 through a cellular mechanism unique among HSV-1 agents, we consider it a new candidate agent for HSV-1.

MATERIALS AND METHODS

Compounds. GA was purchased from Sigma (St. Louis, Mo.). It was dissolved in dimethyl sulfoxide at 10 mg/ml as a stock solution and further diluted in the medium prior to use. Acyclovir was kindly provided by the Department of Chemical Drugs of the Chinese State Drug Administration (Beijing, China).

Cell, viruses, and plasmids. African green monkey kidney cells (Vero) were obtained from the Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, China, and grown as specified in Eagle’s minimum essential medium with 10% heat-inactivated fetal bovine serum plus antibiotics (penicillin, 250 U/ml; streptomycin, 250 µg/ml). HSV-1 (SM44 strain) was propagated in Vero cells in the exponential growth phase were infected with HSV-1 at a multiplicity of infection (MOI) of 0.1 PFU per cell. After 1 h of absorption at 37°C, the cells were washed three times with phos-
phosphate-buffered saline (PBS) and incubated at 37°C in the maintenance medium (Eagle’s minimum essential medium plus 5% fetal bovine serum) with or without GA. The viral titer of the samples (containing cells and the supernatant) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution. The 50% inhibitory concentration (IC50) was determined using a plaque assay (32) after a 1:10,000 dilution.

**TK assay.** The TK assay method was described previously (14). Briefly, GA at a final concentration between 0 and 50 μM was mixed with HSV-1 TK (2 U/assay) in 100-μl reaction buffer containing 150 mM Tris-HCl (pH 7.5), 2 mM ATP, 2 mM MgCl2, 5 mM dithiothreitol, and 10 μM methyl-[3H]thymidine and then incubated at 37°C for 30 min. A 50-μl sample was spotted onto a Whatman DE 81 paper filter followed by washing with H2O and ethanol. The dried circles were assayed (for radioactivity levels) in 10 ml of BetaFluor (National Diagnostic, Atlanta, Ga.).

**Effect of GA on viral adsorption.** Cell suspensions were diluted in a precooled medium to a density of 4 × 10^6 cells per ml and incubated in melting ice for 2 h. Precooled (4°C) HSV-1 and GA were added to the cells with HSV-1 at a MOI of 1 PFU/cell and GA at concentrations between 0 and 20 μM. The mixture was incubated in ice for 1 h followed by three washing with ice-cold PBS. The cell pellet was diluted serially with cold PBS and added to new Vero cell monolayers which were then covered with maintenance medium—0.5% methylcellulose and incubated for plaque assay.

**Measurement of viral penetration.** The method for measurement of viral penetration was described before (7). Vero cell monolayers were placed on ice for 2 h followed by infection with HSV-1 (1 PFU/cell). After 1 h of adsorption at 4°C, the free viruses were removed by washing with cold PBS followed by the addition of maintenance medium with or without GA. Viral penetration was initiated by shifting the incubation from 4 to 37°C. Unpenetrated viruses were inactivated at each indicated time point by treatment with citrate buffer (pH 3.0) for 1 min. The monolayers were then overlaid with maintenance medium and incubated for plaque assays.

**Determination of viral release.** Vero cell monolayers were exposed to HSV-1 at a MOI of 0.1 PFU per cell for 1 h, and then the monolayers were washed three times with PBS and grown in cultures with maintenance medium at 37°C. After 30 min of incubation, the cells were treated with citrate buffer (pH 3.0) to inactivate the unpenetrated viruses and were incubated in maintenance medium with or without GA. The supernatant was collected 24 h later, and the monolayer was washed three times with PBS followed by the freezing and thawing process. Quantitative assay of the HSV-1 in the medium as well as in the cells was done separately by plaque assays after a 1:10,000 dilution of the samples.

**Cell cycle analysis.** Vero cell monolayers were infected with HSV-1 (0.1 PFU/cell) for 1 h followed by three washings in PBS. The cells were then grown in cultures at 37°C in the absence or presence of GA (1 μM) and harvested 24 h later. Cell cycle distribution was measured using a Cycle TEST kit (Becton Dickinson, San Jose, Calif.). Levels of DNA luminescence of individual cells were examined with a FACScan flow cytometer and Cellfit software (Becton Dickinson).

**Southern blot analysis.** A total of 5 × 10^6 Vero cells were infected (using the protocol mentioned above) with HSV-1 at a MOI of 0.1 PFU/cell. After washing, the cells were incubated in the maintenance medium with or without GA (1 μM) for 12 and 24 h. DNA was extracted from the cells with the reported method (12). Briefly, cells were lysed with 0.2 M Tris-HCl (pH 8.5) containing 100 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1% sodium dodecyl sulfate (SDS), and 100 μg of protease K/ml. The resulting products were extracted with phenol and chloroform:isoamylalcohol (24:1 [vol/vol]) and precipitated with ethanol followed by centrifugation at 7,800 g for 30 min. The pellet was resuspended in 10 mM Tris-HCl (pH 8.5) containing 100 mM NaCl plus 0.015 M sodium citrate, 0.1% SDS, 5× Denhardt’s solution, 100 μg of denatured salmon sperm DNA/ml, 50% formamide) at 68°C for 20 min. DNA content was determined spectrophotometrically at 260 nm (A260). Total DNA (10 μg) was loaded on a 1% agarose gel followed by transfer to a nitrocellulose filter which was prehybridized in a prehybridization buffer (5 × SSC [1× SSC is 0.15 M NaCl] plus 0.015 M sodium citrate, 0.1% SDS, 5× Denhardt’s solution, 100 μg of denatured salmon sperm DNA/ml, 50% formamide) at 68°C for 4 h. Hybridization was done for 24 h at 42°C with agitation in the prehybridization buffer plus 5 × 10^7 cpm of deoxycytidine 5’-[α-32P]triphosphate-labeled HSV-1 tk DNA. The nitrocellulose filter was rinsed three times with 0.1× SSC (containing 0.1% SDS) at 22°C (room temperature) and twice at 65°C. The filter was then exposed to X-ray film (Kodak).

**FIG. 1.** Inhibition of HSV-1-induced CPE in Vero cells. (A) Untreated Vero cells; (B) CPE in HSV-1-infected (24 h) Vero cells; (C) HSV-1-infected Vero cells treated with GA (1 μM, 24 h).
RESULTS

Inhibition of HSV-1 replication by GA in vitro. As shown in Fig. 1, GA significantly inhibited HSV-1-induced cytopathic effect (CPE) in Vero cells. Viral titration showed that GA exhibited a dose-dependent inhibition of virus yields (Fig. 2). The IC$_{50}$ of GA was 0.093 μM, and the inhibition rate increased to over 90% when the GA concentration was increased to 0.5 μM. The cytotoxic effect of GA on Vero cells was measured with a conventional MTT method, and the CC$_{50}$ was 350 μM. The therapeutic index of GA for HSV-1 infection in Vero cells was 3,763 (comparable with that known for the HSV-1 drug acyclovir) (Table 1).

Effects of GA on HSV-1 TK activity, viral adsorption, penetration, and release. To understand the antiviral mechanism of GA, the direct effect of the presence of GA on HSV-1 TK was measured in a cell-free system; the results showed no difference in TK activity levels in the presence or absence of GA ($P > 0.05$). GA treatment did not change viral adsorption and penetration (data not shown). With respect to the reduction of the intracellular viral titer caused by the presence GA, however, release of HSV-1 into the medium by the host cells decreased to a greater degree (Fig. 2), indicating an intracellular obstruction of viral replication.

Restoration by GA of the cell cycle in HSV-1-infected Vero cells. It has been documented that HSV-1 infection caused an arrest of cell cycle at the G$_1$/S transition in host cells, indicating a disruption in regulation of cell cycle (5, 16, 37). To explore the effect of GA in Vero cells infected with HSV-1, cell cycle analysis was done with naive cells, GA-treated cells, and HSV-1-infected cells as well as with GA-treated HSV-1$^+$ cells. As shown in Fig. 3, Vero cells treated with GA (1 μM) for 24 h (Fig. 3B) showed a cell cycle profile identical to that of naive Vero cells (Fig. 3A); Vero cells 24 h after infection of HSV-1 displayed a significant accumulation of the cells at the G$_1$/S transition (Fig. 3C); however, treatment of the HSV-1$^+$ Vero cells with GA (at 1 μM for 24 h) resulted in a cell cycle pattern (Fig. 3D) identical to that seen with the naive Vero cells. Similar results were found with HSV-1-infected HeLa cells (Fig. 3E, F, G, and H).

Inhibition of HSV-1 DNA replication by GA in Vero cells. Furthermore, HSV-1 DNA replication was examined to learn whether rescuing the cell cycle from HSV-1 infection by GA would suppress the viral DNA synthesis in host cells. Using a Southern blot technique with HSV-1 tk sequence as a probe, we found that compared to the results seen with the untreated HSV-1-infected Vero cells, GA (1 μM) substantially reduced

### TABLE 1. Comparison of the anti-HSV-1 activity characteristics of GA and acyclovir in vitro

<table>
<thead>
<tr>
<th>Drug</th>
<th>CC$_{50}$ (μM)$^a$</th>
<th>IC$_{50}$ (μM)$^b$</th>
<th>CC$<em>{50}$/IC$</em>{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>350 ± 12</td>
<td>0.093 ± 0.011</td>
<td>3,763</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>4,400 ± 84</td>
<td>0.848 ± 0.015</td>
<td>5,189</td>
</tr>
</tbody>
</table>

$^a$ Vero cells were infected with HSV-1 followed by treatment with GA or acyclovir; antiviral effect was evaluated by plaque assay (see Materials and Methods).

$^b$ Means (± SDs) were derived from three independent replicates.
the number of HSV-1 tk copies in HSV-1\(^+\) cells, indicating an inhibition of the viral DNA synthesis in these cells (Fig. 4). Inhibitory effect was detectable 12 h after exposure of the HSV-1\(^+\) cells to GA and was sustained for more than 24 h (Fig. 4).

**Broad-spectrum antiviral activity of GA.** It was our hypothesis that GA should have activities against other viruses if it acts through the inactivation of Hsp90 and restoration of the progression of cell cycle. GA was then tested in vitro for ac-

**FIG. 3.** Restoration of the progression of cell cycle by the presence of GA in Vero cells infected with HSV-1. (A) Untreated Vero cells; (B) GA-treated (1 \(\mu\)M, 24 h) Vero cells; (C) HSV-1-infected (24 h) Vero cells; (D) HSV-1-infected Vero cells treated with GA (1 \(\mu\)M, 24 h); (E) untreated HeLa cells; (F) GA-treated (1 \(\mu\)M, 24 h) HeLa cells; (G) HSV-1-infected (24 h) HeLa cells; (H) HSV-1-infected HeLa cells treated with GA (1 \(\mu\)M, 24 h). \(x\) axis, intensity of DNA luminescence; \(y\) axis, cell count.

**FIG. 4.** GA-induced reduction of HSV-1 DNA synthesis in Vero cells. Vero cells infected with HSV-1 were treated with GA (1 \(\mu\)M) for 12 h or 24 h. Synthesis of HSV-1 DNA (tk) was analyzed by Southern blotting. GAPDH served as an internal control. The 24-h GAPDH control displayed a result similar to that seen at 12 h and is therefore not shown in this figure to avoid redundancy. Lane 1, normal Vero cells; lane 2, Vero cells infected with HSV-1; lane 3, HSV-1-infected Vero cells treated with solvent dimethyl sulfoxide; lane 4, HSV-1-infected Vero cells treated with GA at 1 \(\mu\)M.
Taking over the cell proliferation machinery and suspending trudor virus needs a strategy for the creation of a cellular HIV-1 (24), and hepatitis C virus (40) has also been reported. Hsp90 in viral proliferation in studies of vaccinia virus (11), cation for viral assembly and initiation of DNA synthesis virus (HBV), duck HBV, or avian HBV in the formation of a could be employed when they are needed (24, 33, 34). In cyclin T1, and Cdk9) are required for viral replication and (1, 5, 8, 21, 38, 42); second, others (such as CDK2, CDK4, pRb, and cyclin D) could be used to induce a cell cycle arrest (24, 34, 35). It could be a new mechanism for antiviral agents different from those inhibiting HSV-1 DNA polymerase activity (for example, acyclovir). Hsp90, which plays a key role as a cellular protein in this mechanism, is a novel molecular target for antiviral re-

was our assumption that a cellular operation related to HSV-1 considering that GA is a natural ligand for molecular chaper-

neutron virus type A and B. As shown in Table 2, GA exhibited broad-spectrum activity against viruses (except influ-

neur virus types A and B). The IC_{50} ranged between 0.5 and 4 μM. HIV-1 and SARS coronavirus exhibited the highest sen-

sitivity to GA in this panel.

DISCUSSION

Antiviral activity of GA against HSV-1 infection was demon-

strated in vitro. The inhibitory effect of GA on HSV-1 was seen to occur in a dose-dependent fashion. Since GA was cytotoxic in tumor cell lines (13, 18), one of our major concerns was its toxicity. In the present study, we found that GA had a low toxicity to Vero cells and exhibited a therapeutic index (C_{50}/IC_{50}) comparable with acyclovir. Since GA inhibited neither HSV-1 TK activity nor viral adsorption or penetration, it provoked our curiosity regarding its antiviral mechanism. Considering that GA is a natural ligand for molecular chaper-
one Hsp90 that accumulates during HSV-1 infection (15), it was our assumption that a cellular operation related to HSV-1 replication might be the target of GA.

One of the important functions of Hsp90 is to protect its client proteins, such as p53, pRb, cyclins and cyclin-dependent kinases, from their degradation (6, 22, 39, 42). These proteins contribute to viral replication in the infected cells in at least two ways. First, some of these client proteins (such as p53, pRb, and cyclin D) could be used to induce a cell cycle arrest (1, 5, 8, 21, 38, 42); second, others (such as CDK2, CDK4, cyclin T1, and Cdk9) are required for viral replication and could be employed when they are needed (24, 33, 34). In addition, Hsp90 binds with viral components. Hsp90 had direct interaction with the reverse transcriptase of human hepatitis B virus (HBV), duck HBV, or avian HBV in the formation of a ribonucleoprotein complex, which was required early in replication for viral assembly and initiation of DNA synthesis through a protein-priming mechanism (9, 10). The role of Hsp90 in viral proliferation in studies of vaccinia virus (11), HIV-1 (24), and hepatitis C virus (40) has also been reported.

To obtain productive viral replication, theoretically, the in-

truder virus needs a strategy for the creation of a cellular environment that is optimally conductive to its replication (20). Taking over the cell proliferation machinery and suspending the cell cycle at the G1/S or G2/M phases are parts of the strategy. We did find a cell cycle arrest at the G1/S transition in HSV-1-infected cells. This phenomenon was consistent with observations from studies of other viruses (17, 20, 30). We also found that HSV-1-disturbed cell cycles could be rescued by GA treatment. We assumed that through inactivation of Hsp90, GA enhanced the degradation of the client proteins and therefore abolished the operation of the cell cycle check-

point established by HSV-1. The cell cycle was then restored to a normal proliferative phase. This change was in favor of the growth of host cells but not of that of the intruder virus. Of course, one could argue that if what we mentioned above were true, HSV-1 replication should be suppressed by GA; using Southern blotting, we did find a significant reduction of the synthesis of HSV-1 DNA in the GA-treated cells. One could also argue that if this was true, GA should exhibit activity against other viruses. Indeed, we found GA to be active against HSV-2, VSV, Cox B3, HIV-1, and SARS coronavirus. As a matter of fact, the results indicating that GA interrupted the assembly of the Hsp90-dependent Cdk9/cyclin T1 heterodimer (which was required for HIV-1 transcription) (24) are consistent with our finding in this study. Data on the inhibitory effect of GA on SARS coronavirus (Chinese patent application 03146591, July 2003) will be published separately.

The exact antiviral mechanism of GA is still a matter of discussion. Since GA also has an inhibitory effect on cellular DNA polymerase alpha (41) (which is in the same class of DNA polymerases as HSV-1 polymerase), direct inhibition of HSV-1 DNA synthesis could be part of the explanation. The point we have made in this study agrees with the suggestion that cell cycle regulators (such as cyclin-dependent protein kinases) are targets for antiviral drugs (17, 34, 35). It could be a new mechanism for antiviral agents different from those inhibiting HSV-1 DNA polymerase activity (for example, acyc-

lovir). Hsp90, which plays a key role as a cellular protein in this mechanism, is a novel molecular target for antiviral re-

search. As a matter of fact, our data obtained with radicicol, another known Hsp90 inhibitor (37), were supportive of this point since it also inhibited HSV-1 replication in vitro at an IC_{50} of 0.1 μM (data not shown). Viral inhibition through the regulation of cellular machinery could be a new antiviral ap-

proach with the consequential benefits of avoiding induction of resistant viral strains and having a broad-spectrum antiviral activity. In cases of the emergence of a new virus, such as SARS coronavirus, agents in this category are of particular value because they might inhibit the replication of the new virus before the viral target proteins are characterized.

Taken together, these data indicate that since GA (an anti-
biotic extracted by fermentation) has a significant inhibitory effect against HSV-1 in vitro and restrains HSV-1 replication through a cellular mechanism unique among HSV-1 drugs, there is justification for development of this antibiotic as a new agent for HSV-1 infection.

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REFERENCES


TABLE 2. Antiviral activities of GA in vitro

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC_{50} (μM)</th>
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<tbody>
<tr>
<td>HSV-2</td>
<td>4.0 ± 1.84</td>
</tr>
<tr>
<td>VSV</td>
<td>1.52 ± 1.68</td>
</tr>
<tr>
<td>Cox B3</td>
<td>3.35 ± 2.77</td>
</tr>
<tr>
<td>HIV-1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>SARS coronavirus</td>
<td>0.91 ± 0.31</td>
</tr>
<tr>
<td>Influenza virus type A</td>
<td>NA^{a}</td>
</tr>
<tr>
<td>Influenza virus type B</td>
<td>NA^{a}</td>
</tr>
</tbody>
</table>

^{a} Means (± SDs) were derived from at least three independent experiments.

^{b} NA, not active.


