Anti-adenovirus Activities of Shikonin, a Component of Chinese Herbal Medicine in Vitro

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Radix Lithospermi erythrorhizon is a common prescription compound in traditional Chinese medicine. Shikonin is a major component of Radix Lithospermi and has various biological activities. We have investigated the inhibitory effect of shikonin on the growth of adenovirus type 3 (AdV3) in vitro. The antiviral function of shikonin against AdV3 and its virus inhibition ratio were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (MTT). The expression of hexon protein in AdV3 was determined by immunofluorescence assay using laser scanning confocal microscopy (LSCM) and Western blot analysis. In addition, the rate of apoptosis in cells infected by AdV3 was determined by flow cytometry. Shikonin (0.0156—1 μg) inhibited growth of AdV3 in a concentration-dependent manner with a virus inhibition rate of 23.8—69.1%. Expression of hexon protein in AdV3 was higher in the virus control group than in the shikonin-treated groups as determined by immunofluorescence assay and Western blotting (p<0.05). The rate of shikonin-treated HeLa cell apoptosis had a statistically significant decrease with increasing concentration of drug (p<0.05). Our data demonstrate that shikonin possesses anti-AdV3 capabilities and that the potential antiviral mechanism might involve inhibiting the degree of apoptosis and hexon protein expression of AdV.

Key words anti-adenovirus type 3; shikonin; in vitro

Adenovirus (AdV) is characterized by a linear double-stranded DNA genome that ranges at 26—46 kb in size, encoding 30—40 proteins. AdV is an important etiological agent that can cause a variety of nonlethal infectious diseases in humans, and lethal disseminated adenovirus infection in immunosuppressed patients.1 AdV infection in humans can lead to many diseases including acute upper and lower respiratory infection, fulminating conjunctivitis, acute hemorrhagic cystitis, rheumatoid arthritis, cerebritis, cerebral meningitis, and infant gastroenteritis.2—5 The human AdVs serotype presently contains 52 members. The reported structure and etiology of AdV are becoming increasingly diverse. Currently, there are few antiviral drugs for AdV. Only ribavirin and cidofovir have been evaluated in small clinical studies, yet with variable results.

Radix Lithospermi is an important medicinal plant that grows mainly in the Xinjiang and Xizang provinces in China and is commonly used in traditional Chinese medical prescriptions due to its multiple pharmacological actions. Shikonin, a naphthoquinone pigment extracted from the Radix Lithospermi,6,7 has been used as a red dye for centuries and is reported to possess several medicinal properties such as a multifunctional antibacterial,3—10 anti-inflammatory,11—13 and anti-tumorigenic effects14—16 and the ability to promote wound healing activity,17—20 and can also be used in food additives and cosmetics.

To date, few studies have been performed to examine the antiviral function of shikonin. There are the present study was performed to investigate the anti-AdV3 function of shikonin from Radix Lithospermi in vivo. This is the first report on the anti-AdV activities of shikonin.

MATERIALS AND METHODS

Reagents Ribavirin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, U.S.A.). All other chemicals used were of analytical reagent grade. Ribavirin (50 mg/ml) was used as positive control in the present investigation.

Cell Lines The HeLa cell line used in this study was purchased from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.) and preserved by the Department of Microbiology of Harbin Medical University.

Virus Strains Human AdV type 3 (AdV3) was obtained from ATCC (Manassas, VA, U.S.A.) and propagated in HeLa cells. AdV3 stocks were stored at −80 °C until use.

Plant Material Shikonin (Fig. 1) was purchased from Calbiochem Co., CA, U.S.A. The purity of shikonin (Molecular weight (MW) 288.3) used in this study as measured by high-performance liquid chromatography (HPLC) system was 99%.

Cell Culture HeLa cells were cultured in RPMI-1640...
(Invitrogen Corp., CA, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU penicillin ml⁻¹, and 100 μg streptomycin ml⁻¹. Conventional methods were used to culture and passage the cells. For the antiviral assay, the medium was supplemented with 2% (v/v) FBS and penicillin–streptomycin.

**Inoculation of Virus** AdV3 was inoculated into a culture flask containing 70% confluent monolayer of HeLa cells at 37 °C and incubated for 2 h. RPMI-1640 maintenance medium containing 2% FBS was added into the culture flask and the mixture was allowed to incubate at 37 °C in 5% CO₂. At the indicated days, the cytopathic effect (CPE) of HeLa cells reached >75%. The culture was subjected to three cycles of freezing and thawing then the virus was harvested.

**Determination of AdV3 Toxicity** The viral toxicity was determined for Hela cells by a cell culture infectious dose of 50% (CCID₅₀) assay. Ten-fold serial dilutions were prepared in RPMI-1640 maintenance medium and inoculated into a confluent monolayer of HeLa cells and in 96-well plates. Plates were evaluated for CPE after 72 h of infection. CCID₅₀ was calculated by Reed–Muench method.

**Determination of Shikonin Toxicity to HeLa Cells** Different concentrations of shikonin were added to 96-well plates containing a confluent monolayer of HeLa cells. Shikonin toxicity was determined by conventional MTT method.²¹ Optical density values were read by microplate reader at two wavelengths (540, 690 nm).

**Anti-AdV3 Effect of Shikonin Determined by CPE and MTT** Hela cells in 96-well plates were inoculated with AdV3 at 100 CCID₅₀ at 37 °C for 2 h then washed to remove any unbound virus. To test shikonin under various atoxic conditions, different concentrations of shikonin were added into the 96-well plates. For each shikonin concentration, eight duplicate wells, a cell control group, a virus control group, and a ribavirin (50 μg/ml) control group were tested simultaneously. When the CPE of virus control group reached >75%, cell survival rate was detected by MTT method. All experiments were repeated three times.

**Immunofluorescence Assay** The effect of shikonin on AdV3 was determined by laser scanning confocal microscope (LSCM). The cells were inoculated with mouse anti-human AdV3 hexon protein monoclonal antibody (Sigma Chemical Co., MO, U.S.A.) at 37 °C for 1 h and rinsed with phosphate buffered saline (PBS) with 0.2% Tween 20 (PBST) three times for 10 min each. The mixture was then incubated with fluorescein isothiocyanate (FITC)-goat anti-mouse immunoglobulin G (IgG) antibody (Invitrogen) at 37 °C for 30 min and rinsed three times for 10 min each with PBST solution. A cell control group, a virus control group, and a ribavirin (50 μg/ml) control group were tested simultaneously. Cell pictures were obtained by LSCM (ECLIPSE TE2000-E, Nikon, Japan) and 30 cells in every sample in a field were randomly selected to test cell fluorescence intensity.

**Western Blotting Analysis** At 2 d postinfection, cell monolayers were lysed and mixed with 6× loading buffer solution and denatured at 100 °C for 5 min. The proteins were separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis then transferred onto nitrocellulose membrane. The nitrocellulose membrane was blocked with 1% bovine serum albumin at 4 °C overnight. The membrane was then reacted with rabbit-anti-β-actin polyclonal antibody (Invitrogen) or mouse anti-human AdV3 hexon protein monoclonal antibody (Sigma), respectively, at 37 °C for 2 h and rinsed with 0.05% TBST three times for 10 min each. The membrane was incubated with alkaline-phosphatase-labeled goat-anti-rabbit IgG or alkaline-phosphatase-labeled goat-anti-mouse IgG (Invitrogen) at 37 °C for 1 h. The lysine was then rinsed twice for 10 min with TBST solution, once for 10 min with TBS solution, and finally stained using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

**Immunohistochemistry Analysis** A streptavidin–biotin–peroxidase complex (SABC) immunohistochemical method was used to observe the effects of shikonin on AdV3. Changes in cell morphology were detected by SABC kits (R&D Co., U.S.A.) according to the manufacturer’s protocol. After hematoxylin counterstaining, AdV3-infected cells were observed with the aid of a light microscope. Cells whose nucleus had a buffy granular appearance were apoptotic cells.

**Cell Surface Staining and Flow Cytometry** To assess the rate of cell apoptosis, HeLa cells from AdV3 infection with shikonin treatment were double-stained with FITC-conjugated anti-Annexin V and PI simultaneously (BD Pharmingen). After washing the cells twice with PBS containing 1% FCS and suspending them in 300 μl of PBS, the cells were analyzed in FACS Calibur Flow Cytometer (BD Bioscience) with Cell Quest software. Viable cells were gated by forward and side scattering.

**4,6-Diamidino-2-phenylindole Dihydrochloride Hydrate (DAPI) Staining** The HeLa cells were exposed to AdV3 at 100 CCID₅₀ for 2 h then to various concentrations of shikonin. The cells were washed, fixed, and incubated in a fluid containing 1 mg/ml DAPI (Vector Laboratories, Burlingame, CA, U.S.A.).²² Cell pictures were obtained by LSCM. The experiments were repeated three times.

**Statistical Analysis** The data were processed using SAS (version 9.13) software. The drug median toxic concentration (TC₅₀) and the median inhibitory concentration (IC₅₀) were calculated by linear-regression method. A dependability analysis of the medicine dosage and its cytological effect (cell survival rate, inhibition ratio) was carried out to establish whether there was a dose–effect relationship. Variance analysis was used for the group comparison. The following calculations were made:

<table>
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<tr>
<th>Parameter</th>
<th>Formula</th>
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<tbody>
<tr>
<td>HeLa cell survival rate (%)</td>
<td>medicine group absorbance value/cell control group absorbance value × 100%</td>
</tr>
<tr>
<td>inhibition ratio (%)</td>
<td>100% − survival rate</td>
</tr>
<tr>
<td>virus inhibition ratio</td>
<td>(medicine treatment group absorbance value − virus control group absorbance value)/(medicine treatment group absorbance value) × 100%</td>
</tr>
<tr>
<td>therapeutic index (TI)</td>
<td>(TC₅₀)(IC₅₀)</td>
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Data are presented as mean±S.D. Statistical analyses were performed using one-way analysis of variance. Differences between the means of each group in each assay were tested using Dunnet’s tests. If the mean values of at least one group differed from others with p<0.05, they were considered statistically significant.

**RESULTS**

**AdV3 Toxicity Assay** The suitable challenging dose of virus was identified by calculating the CCID₅₀ using the
Reed–Muench method. CPE was observed with the aid of an inverted microscope. Compared with the cell control group, HeLa cells infected with AdV3 became round and clumped together, showing a typical “bunches of grapes” morphological change. When 50% of the cells exhibited a CPE, they were regarded as CPE+ cells. If the cells did not exhibit any CPE after 72 h, they were regarded as CPE− cells. The CCID50 of AdV3 in HeLa cells was 10^4.95.

Toxic Activity of Shikonin on HeLa Cells The atoxic range of shikonin was assessed by measuring the TC0 and TC50 using the MTT method. The toxic activity of shikonin on HeLa cells was reduced with decreasing concentration of shikonin, with a concomitant gradual increase in the cell survival rate. TC50 of shikonin was 28.91 μM. TC0 of shikonin was 1.08 μM (Fig. 2).

Anti-AdV3 Activity of Shikonin Assessed by CPE and MTT The virus exerted the following CPE on HeLa cells as evidenced by the control group: cells developed a vacuole, swelled, and became round; they fused together, exhibited the typical “bunches of grapes” change, and finally became necrotic and exfoliated. In the shikonin-treated groups, however, the cells appeared mostly normal, with some of the cells exhibiting the CPE characteristics: atrophy-like, shrinkage-like, string-like, overlapping, or shedding (Fig. 3). The virus inhibition rate was positively correlated with the concentration of shikonin (0.0156—1 μM) and its scope was 23.8—69.1% in a concentration-dependent manner (Fig. 4). Hela cells treated with >1 μM shikonin exhibited chromatin condensation.

Effect of Shikonin on AdV3 as Revealed by LSCM and Western Blotting The potential mechanism that shikonin affected AdV3 was investigated by assessing the changes of expression in hexon protein of AdV3. Fluorescence intensity revealed that expression of hexon protein was increased in the virus control groups compared with shikonin-treated group (p<0.05; Figs. 5, 6). Western blotting further showed that expression of hexon proteins was negatively correlated with the concentration of shikonin, confirming the LSCM results (p<0.05; Fig. 7).

Effect of Shikonin on AdV3 as Revealed by Immunohistochemistry and Flow Cytometry To investigate potential anti-ADV3 mechanism of shikonin in vivo, the proportion of apoptotic HeLa cells were evaluated. As expected, the apoptotic population of HeLa cells treated with different concentrations of shikonin was significantly decreased in a concentration-dependent manner compared with virus control (p<0.05) (Fig. 8).

Effect of Shikonin on AdV3 as Revealed by 4′-6-Diamidino-2-phenylindole (DAPI) Staining DAPI staining was used to assess the apoptosis of HeLa cells. In the virus control group, the cell number was reduced and the cell shape became round up, and apoptotic bodies were found (Fig. 9).

DISCUSSION
AdVs, respiratory viruses with a double-stranded DNA genome, replicate in the nuclei of mammalian cells and infect billions of people worldwide and cause many different kinds of diseases. AdV is one of the main etiological agents of diseases that lead to respiratory infection in children.23) In infants aged between 6 months and 3 years, AdV can not only cause viral pneumonia but also give rise to long-term lung damage.24) Some AdVs can also cause cell transformation, and even carcinogenesis.25) The infectivity of AdV is...
very high, especially in densely populated areas and among immune-deficient patients (such as the elderly, and those with bone marrow transplantation, leukemia, and AIDS). 26—28) Droplet- and touch-borne infection with AdV may result simultaneously in a regional prevalence and cause endemic outbreak. 29,30)

There remains a lack of specific drugs for the treatment of such viral diseases, and thus the search for effective and safe antiviral medicines that have few (if any) side effects is imperative. Traditional Chinese medicine has shown intriguing potential in this area since it is associated with abundant resources, multitargeted mechanisms of activity, few side effects, and no drug resistance. Therefore screening of and research for traditional Chinese antiviral medicines, with particular emphasis on high efficiency and low toxicity, have become a hot spot in the field of drug development.

Shikonin is a major component of Radix Lithospermi, which is a type of traditional Chinese medicine. It has been identified in the roots of several plants of the Boraginaceae family, especially Alanna tinctoria, Lithospermum erythrorhizon, and Arnebia euchroma. 31) In the past 20 years, pharma-
Ecological studies have certified that shikonin has antibacterial and antitumor effects in addition to many other pharmacologic actions. It is associated with few side effects. However, there have been few studies on the antiviral effects of shikonin.\(^3\)\(^2\),\(^3\)\(^3\) The antiviral, toxic, and side effects profile of shikonin on host cells was investigated by the MTT method to detect cytoactivity. Our data show that shikonin (0.0156—1 \(\mu M\)) inhibited the growth of AdV3. The rate of virus inhibition was 23.8—69.1% in a concentration-dependent manner. As shown by the CPE method, most of the HeLa cells maintained normal cellular shape with normal epithelium, and only a small proportion of the cells presented pathological changes such as shrinking, clustering, overlapping, or exfoliation in the shikonin groups. There was a positive correlation between virus inhibition ratio and drug concentration (as assessed by the MTT and DAPI method). Thus the methods used here revealed that shikonin has clear anti-AdV3 effects.

Virion of AdV contains 252 shell tablets where 20 shell surface angles form 12 pexons, a substrate (base). In addition, there are some non-angle shell tablets except for plexon, called hexon. Hexons are the most important caspid proteins and homologous trimers of hexon protein, including the main genus/sub-genus-specific and the secondary species-specific antigenic determinants.\(^3\)\(^4\) Hexons are the most abundant coat proteins making up the triangular facets of the capsid. Previous researchers reported that the hexon protein of AdV was the major protein on the surface of the AdV nucleocapsid that docks at the nuclear pore complex (NPC). AdVs that replicate in the nucleus must use the NPC to introduce their genome into the nucleoplasm.\(^3\)\(^5\) The mechanism of anti-AdV3 effect was investigated by immunofluorescence and Western blotting assay, yielding data regarding the protein expression of hexon. We found that protein hexons were higher in the virus control group than in the shikonin-treated groups. The findings demonstrate that shikonin inhibits expression of hexon protein.

Apoptosis is an initiative process of cell death regulated by gene and a mechanism with which growth and development of body, and differentiation and death of cells are associated. Apoptosis is a common feature to life and is associated with normal growth and environmental stability. Apoptosis is also an anti-viral mechanism of host cells by which a cell may inhibit viral infection and proliferation. Some studies have already indicated that AdV has a cytotoxic effect on human tumor cells, inducing apoptosis. The mechanism on this anti-AdV3 effect was investigated by immunofluorescence and Western blotting assay, yielding data regarding the protein expression of hexon. We found that protein hexons were higher in the virus control group than in the shikonin-treated groups and that AdV3 induced apoptosis of infected host cells. However, shikonin inhibited viral infection and proliferation, and then significantly reduced apoptosis induced by AdV3 infection. Therefore the data suggest that shikonin could resist AdV3 through inhibiting apoptosis in a concentration-dependent manner.

The focus of this study was mainly the anti-AdV3 effects of shikonin in vitro. However, when entering an organism, this medicine may play some unknown biological role that is involved in a series of complex pharmacokinetics processes (e.g., it may bind with plasma proteins or tissue proteins, or infiltrate liver enzyme metabolism). Thus its antiviral activity needs to be confirmed through animal experiments and eventual clinical trials.

In conclusion, we used shikonin (which accounts for a major portion of Radix Lithospermi and according to previous reports has a significant drug effect) to test the effectiveness of Radix Lithospermi as a putative anti-adenoviral medicine. Observation of HeLa cell morphology by light microscope, MTT, DAPI, and other methods revealed the anti-AdV3 effect of shikonin. Immunofluorescence assay and detection of cell apoptosis were used to clarify the possible mechanisms on the antiviral effect of this medicine. We suggest that the antiviral effect of shikonin is closely correlated with inhibition of expression of hexon protein and cell apoptosis infected with AdV3. The aim of this study was to provide the bases for novel hypotheses and experiments effectively to control AdV and other infective diseases. Our data provide initial evidence to search for highly-efficient and low-toxic antiviral drugs.

**Acknowledgments** Thanks are due to Ms. Weijun Lu and Ms. Yuehui Zhao for their assistance with laboratory techniques. Financial support was provided by National Natural
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