Urolithin A inhibits enterovirus 71 replication and promotes autophagy and apoptosis of infected cells in vitro

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Abstract
Hand, foot, and mouth disease (HFMD) is a serious threat to the health of infants, and it can be caused by enterovirus 71 (EV71). The clinical symptoms are mostly self-limiting, but some infections develop into aseptic meningitis with poor prognosis and even death. In this study, urolithin A (UroA), an intestinal metabolite of ellagic acid, significantly inhibited the replication of EV71 in cells. Further evaluation showed that UroA was better than ribavirin in terms of its 50% cytopathic concentration (CC50), 50% inhibitory concentration (IC50), and selectivity index. Moreover, UroA inhibited the proliferation of EV71 by promoting autophagy and apoptosis of infected cells. Therefore, UroA is a candidate drug for the treatment of EV71 infection.

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Materials and methods

Cells and viruses

African green monkey kidney cells (Vero cells), rhabdomyosarcoma cells (RD cells), human neuroblastoma SK-N-SH cells (SK-N-SH cells), and Madin-Darby canine kidney cells (MDCK cells) were purchased from the American Type Culture Collection (ATCC) and cultured in modified Eagle’s medium (BasalMedia, China) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Solarbio, China). Human lung adenocarcinoma A549 cells (A549 cells) were purchased from the ATCC and cultured in F-12 medium (BasalMedia, China) supplemented with 10% inactivated FBS and 1% penicillin-streptomycin. EV71 strain BrCr (VR-1775) was purchased from the ATCC and passaged in Vero cells. Influenza A virus strain A/Puerto Rico/8/34(H1N1) was purchased from the ATCC and passaged in MDCK cells.

Compounds and antibodies

UroA, UroB, UroC, and ribavirin were purchased from MedChemExpress (MEC; Shanghai, China). The purity was at least 98%, and all of the compounds were dissolved in DMSO (Solarbio, China).

The EV71-VP1 antibody was purchased from Abnova, and the β-tubulin antibody was purchased from BioPM (Wuhan, China). Antibodies against LC3B, p62, caspase-3 and cleaved-caspase-3, were purchased from Cell Signaling Technology (CST; USA). The influenza NP antibody was purchased from SinoBiological (Beijing, China). Goat anti-rabbit and anti-mouse HRP-labeled antibodies were obtained from BioPM.

Cell counting kit (CCK)-8 assay

The cytotoxic effects of each compound were assayed by CCK (Beyotime Biotechnology, Shanghai, China), and the 50% cytotoxic concentration (CC50) was determined using GraphPad Prism 5.0.

CPE inhibition assay for anti-EV71 activity

RD cells (3 × 10⁴ cells/well) were placed in 96-well culture plates and incubated for 24 h. The medium was then removed, and the cells were infected with EV71 (MOI = 0.1) in serum-free medium for 1 h at 37 ºC. Then, the unbound viruses were removed, various concentrations of the compound were added, and the cells were incubated for another 48 h. The cells were then stained with 0.1% crystal violet at room temperature for 20 min, washed with phosphate-buffered saline (PBS), and dried. The OD value of each well was measured at 570 nm, and the 50% inhibitory concentration (IC50) was calculated using GraphPad Prim 5.0 software. The selectivity index (SI) was calculated as the ratio CC50/IC50.

TCID50 assay

The virus was serially diluted tenfold and added to Vero cells in 96-well plates, and the cells were cultured at 37 ºC for 48 h. Median tissue culture infectious dose (TCID50) values were determined based on CPE. Further calculations were conducted using the Reed-Muench method.

qRT-PCR assay

Total cellular RNA was isolated using a TransZol Up Kit, reverse transcribed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix, and analyzed using PerfectStarTM Green qPCR SuperMix, following the instructions. All reagents were purchased from Transgen Biotech, Beijing, China. The sequences of the primers used for qPCR analysis are as follows: sense primer 5′-GCAGCCCAAAAAGAACCTCAG-3′ and antisense primer 5′-ATTTCAAGC GCTTGGAGTCG-3′ targeting a conserved region of the EV71-VP1 gene; sense primer 5′-GACAAGTCTTGTATCGTG GAA-3′ and antisense primer 5′-CCAGGAATGAGCTT GACA-3′ targeting a conserved region of the human GAPDH gene. All primers were synthesized by Sangon Biotech, Shanghai, China.

Western blot assay

Total cellular proteins were extracted using 1× RIPA buffer (CST) and denatured by adding SDS loading buffer and boiling for 4 min at 100 ºC. Twenty µg of protein was resolved by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad, USA). The membranes were blocked with 2% bovine serum albumin (Sigma Aldrich, Germany) and then incubated overnight with the appropriate primary antibody at 4 ºC. The membranes were washed with 1× TBST and incubated with a 1:5000 dilution of anti-rabbit or anti-mouse horseradish-peroxidase-conjugated antibody for 1.5 h. Following incubation, the membranes were washed extensively with 1× TBST. Immunoreactive bands detected using ECL reagent (Advanta, USA) were analyzed using Image Lab (Bio-Rad, USA).

Results

Screening of antiviral drugs

The cytotoxicity of the three urolithins was determined by CCK-8 assay in RD cells. The results showed that the...
maximum nontoxic concentration of UroA, UroB, and UroC was 50, 25, and 25 µM, respectively (Fig. 1A).

RD cells were infected with EV71 and maintained with UroA, UroB, and UroC at nontoxic concentrations (25 µM) in cell culture medium to detect the antiviral effects of the three urolithins. The total protein of the cells was extracted at 24 h postinfection. Expression of the EV71-VP1 protein was detected by Western blot assay. The results showed that UroA had strong antiviral effect (Fig. 1B).

**UroA inhibits EV71 replication**

The antiviral effects of UroA on intracellular viral RNA levels, intracellular viral VP1 protein levels, and the viral titer in the cell culture medium were further evaluated. The results showed that UroA effectively inhibited EV71 RNA replication (Fig. 2A) and EV71 VP1 expression (Fig. 2B) and reduced the viral titer (Fig. 2C) in the cell culture medium. The results demonstrated a clear dose-dependent antiviral effect of UroA (Fig. 2D).

**UroA is an effective anti-EV71 compound**

The antiviral effects of UroA were compared with those of ribavirin for further evaluation. First, a CCK-8 assay was used to evaluate the viability of RD cells incubated with a series of concentrations of ribavirin and UroA for 48 h. The CC50 was calculated for both compounds. The results indicated that UroA is less cytotoxic than ribavirin (Fig. 3A). The anti-EV71 activity of ribavirin and UroA at different non-cytotoxic concentrations was then assessed at 48 hpi using a CPE inhibition assay (Fig. 3B and Table 1). The SI was calculated as the CC50/IC50 ratio. The SI of UroA was much higher than that of ribavirin (Table 1). The results suggest that UroA is superior to ribavirin.

Three types of antiviral effects of UroA were evaluated: prevention, treatment, and direct viricide (Fig. 4). The results showed that UroA exhibited significant anti-EV71 effects under treatment, but neither prevention nor direct viricide.

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**Fig. 1** Effects of UroA, UroB, and UroC on the proliferation of EV71. (A) A CCK-8 assay was performed to determine the cytotoxicity of UroA, UroB, and UroC. (B) RD cells were placed in six-well culture plates and infected with EV71 (MOI=0.1) for 1 h. The infected cells were treated with the three urolithins at a concentration of 25 µM for 24 h. A Western blot assay was used to detect the intracellular viral VP1 protein.

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UroA promotes autophagy and apoptosis of infected cells

Autophagy and apoptosis are self-defense mechanisms of host cells during EV71 [3, 6, 31, 35]. A Western blot assay showed that the level of LC3-II, a marker for autophagy, increased in the presence of 25 µM UroA during EV71 infection for 12 h. The level of p62, which functions as a bridge between LC3B and ubiquitinated substrates to be degraded [26], decreased for 12 h (Fig. 5A). Cleaved-caspase-3, which is associated with apoptosis, also increased in this process (Fig. 5B). These results showed that autophagy and apoptosis were activated for 12 h after EV71 infection with UroA treatment.

UroA also exhibits an antiviral effect in SK-N-SH cells

EV71 infection can affect the nervous system of children [12]. We therefore examined the effects of UroA on the proliferation of EV71 in SK-N-SH cells, which are human neuroblastoma cells. The nontoxic concentration of UroA was determined by CCK-8 assay (Fig. 6A), and the anti-EV71 effect was assessed by Western blot assay (Fig. 6B). The results showed that UroA could significantly inhibit the proliferation of EV71 in SK-N-SH cells. The antiviral effects of UroA and ribavirin were also compared in SK-N-SH cells (Fig. 6C). The results showed that UroA had a stronger antiviral effect in these cells than ribavirin.

UroA has no effect on the proliferation of influenza virus

The effect of UroA on the proliferation of influenza virus was examined. A549 cells infected with influenza A virus strain A/Puerto Rico/8/34(H1N1) were treated with a nontoxic concentration of UroA for 24 h, and intracellular viral NP protein was detected by Western blot assay (Fig. 7A and B). The result indicated that UroA does not inhibit influenza virus replication.
EV71 belongs to the genus Enterovirus of the family Picornaviridae, and it is one of the main pathogens causing HFMD in infants. The development of antiviral drugs is a slow process, especially those intended for use in infants and young children, and safe and effective drugs against EV71 infection are urgently needed [26, 27].

Ellagic acid, a natural polyphenolic compound that is widely distributed in fruits and nuts, has various biological functions. However, its low absorption efficiency restricts its application as a drug [18]. Urolithins are metabolites of ellagic acid that are also biologically active but can reach appropriate concentrations in the blood after absorption through the intestinal tract [8, 23]. In the present study, UroA, UroB, and UroC were screened for their antiviral effects. The results showed that UroA had a significant antiviral effect (Fig. 1B), which was confirmed by examining viral protein expression, the levels of viral RNA, and virus titers (Fig. 2A-C). In addition, the antiviral effect of UroA was found to be dose-dependent (Fig. 2D). The cytotoxicity and anti-EV71 activities of UroA was also investigated in terms of CC50, IC50, and SI. The results showed that UroA has better safety and stronger antiviral activity than ribavirin. Thus, it has potential as a candidate drug for treating EV71 infection (Table 1). The functions of UroA in the prevention and treatment of infection and its direct viricidal effect on EV71 were also tested. The results showed UroA to be effective in the treatment of EV71 infection, but it had little preventative effect and no direct viricidal effect of EV71 (Fig. 4).
Viral infection induces different cellular stress responses in infected cells, including autophagy and apoptosis, which are self-defense mechanisms of host cells that are activated during infection [17]. Autophagy and apoptosis induced by viral infection can have both positive and negative effects of the proliferation on the virus [3, 31]. In viral infection, autophagy initially triggers an innate immune response that induces interferon production and clears aggressive viruses [20]. However, the autophagy induced by EV71 provides support for viral replication in vitro and in vivo [13].

Fig. 4 Analysis of the prevention, treatment, and direct killing effect of UroA on the virus. The proliferation of the virus was detected under three conditions: RD cells were pretreated with UroA at a non-toxic concentration of 25 µM and then infected with EV71; EV71-infected RD cells were treated with UroA (25 µM); and EV71 and UroA (25 µM) were mixed and incubated at 37 °C for 1 h and then infected with RD cells. After 24 h of infection, intracellular viral VP1 protein was detected by Western blot assay.

Fig. 5 UroA promotes autophagy and apoptosis of EV71-infected cells. (A) The effect of UroA (25 µM) on the autophagy of infected cells was detected using Western blot assay. (B) The effect of UroA (25 µM) on the apoptosis of infected cells was examined using Western blot assay.
In the present study, the effects of UroA on the autophagy and apoptosis of EV71-infected cells were examined. The results revealed that LC3-II levels increased in the presence of UroA. In addition, the level of p62, a selective autophagy receptor [4], decreased at 12 h and then returned to normal at 24 h. (Fig. 5A). EV71 induces cell apoptosis by activating caspase-3 [14]. However, virus-infected cells are eliminated through apoptosis to prevent the generation of progeny virions and inhibit the proliferation of the virus [9, 22]. As shown in Fig. 5B, after 12 h, the level of cleaved-caspase-3 was higher and that of the EV71-VP1 protein was significantly lower in treated group than in infected control cells. After the breakdown of infected cells during treatment with UroA, the surviving cells continued to grow. Accordingly, the change in expression of cleaved-caspase-3 protein was the opposite at 24 h. The results showed that UroA promoted cell autophagy and apoptosis for 12 h after EV71 infection (Figs. 5A and B), which might facilitate viral clearance, as suggested previously [10]. This may also be the reason why there was significantly more autophagy and apoptosis of UroA treated cells than of infected control cells at 12 h after EV71 infection.

Some clinical case reports have shown that EV71 infection causes nerve damage, and the replication of EV71 in
nerve cells has been demonstrated [12, 15, 16, 33]. In the present study, the effects of UroA on the proliferation of EV71 in SK-N-SH cells were examined. The results showed that UroA could significantly inhibit the proliferation of EV71 (Fig. 6B), and the antiviral effects of UroA were superior to those of ribavirin in SK-N-SH cells (Fig. 6A and C).

We also tested the effect of UroA on influenza virus infection. A Western blot assay showed that UroA did not inhibit influenza virus replication (Fig. 7A and B), suggesting that UroA does not have a broad-spectrum antiviral effect. However, the effect of UroA on the proliferation of other viruses still needs to be tested.

In conclusion, UroA exerted more-potent antiviral activity against EV71 in vitro and exhibited less cytotoxicity than ribavirin. The anti-EV71 mechanism of UroA may be related to the promotion of the autophagy and apoptosis of infected cells. Therefore, UroA could be used as a potential drug candidate and is worthy of further study.

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Declarations

Conflicts of interest The authors declare no conflict of interest.

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