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Capparilosside A shows antiviral and better anti-inflammatory effects against influenza virus via regulating host IFN signaling, in vitro and vivo

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ABSTRACT

Aims: This study aimed to evaluate the efficacy and mechanisms of Capparilosside A, a chemically synthesized compound, against virus and inflammation induced by influenza virus.

Main methods: The inhibitory activity of Capparilosside A against influenza virus was determined by plaque assay and cytopathic effect inhibition assay. Quantitative real-time PCR, enzyme-linked immunosorbent assay and Bio-Plex methods were used to quantify cytokine and chemokine expression profiles. Effects of Capparilosside A were also evaluated in a mouse model of influenza virus infection.

Key findings: We successfully synthesized Capparilosside A, which could inhibit replication of a variety of viruses, including influenza viruses H1N1 and H3N2, PIV3 and ADV in vitro. Capparilosside A could also inhibit progeny virus replication at concentrations of 2 and 1 mg/mL. Simultaneously, it significantly reduced the expressions of IL-6, IP-10, MIG and RANTES/CCL-5 stimulated by A/PR/8/34 (H1N1) at a range of doses, even 0.5 mg/mL. Similar anti-inflammatory activity was detected in cells induced by avian influenza virus H9N2 or lipopolysaccharide. In addition, Capparilosside A clearly inhibited inflammatory response induced by mouse lung-adapted influenza strain PR8/H1N1. Furthermore, Capparilosside A strongly inhibited phosphorylated STAT1 levels and IFN-β and IL-29 expressions induced by PR8/H1N1. Capparilosside A also inhibited IP-10 and CCL-5/RANTES expressions induced by exogenous human recombinant IFN-β.

Significance: Capparilosside A not only shows broad-spectrum antiviral efficacy, but more effectively impairs the upregulations of pro-inflammatory factors in host cells induced by influenza virus. The potential antiviral mechanism of Capparilosside A is through inhibiting the activation of the host IFN signaling pathway.

1. Introduction

The pathogenesis of influenza virus infection includes two main aspects: direct damage caused by viral replication and indirect damage caused by influenza-induced cytokine storms. Cytokine dysregulation contributes to the pathogenesis of H5N1 and H7N9 viruses [25,52] by inducing an imbalance of the host regulatory network and causing more severe complications such as acute respiratory distress syndrome and multiple organ dysfunction syndrome (MODS), which eventually lead to high mortality rates [5,9].

Currently, the most important methods for preventing and controlling influenza infection are antiviral treatments [12]. Neuraminidase inhibitors (NAIs) and M2 ion channel protein inhibitors (M2 inhibitors) have been approved by the United States Food and Drug Administration for clinical treatments of influenza. NAIs, such as oseltamivir and peramivir, target on the influenza virus neuraminidase (NA) protein and therefore reduce viral spread by cleaving glycosidic linkage of neuraminic acids and therefore inhibiting NA activity. M2 inhibitors, such as amantadine and rimantadine, target on the influenza virus M2 protein and inhibit viral replication by interfering with M2

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protein ion channel activity. Since these drugs directly target on the virus, high variability of influenza viruses allows the developments of drug resistance and subsequently renders the ineffectiveness of antiviral drugs. For example, amantadine and rimantadine have become largely ineffective owing to the high prevalence of resistant viruses in nature; thus, these drugs have been withdrawn from the market [8,14,17,47]. Furthermore, a gradual increase in NAI resistance has been reported [28,42].

High-dose glucocorticoid, which has been clinically used to treat excessive inflammatory responses induced by influenza virus, is considered to promote viral replication, delay virus clearance and fail to reduce mortality [29,30]. Thus, currently available antiviral drugs have several disadvantages: high side effects [21,24], increased drug resistance rates [1,8,15,20,24,28,39] and single-target effects (limited efficacy). Thus, recent anti-influenza drug development has increasingly focused on host immunoregulation [3], particularly anti-inflammatory and immunomodulatory drugs [41]. In this regard, traditional Chinese medicine (TCM) offers potential candidates for developing such drugs, as they can inhibit both viral replication and inflammation induced by influenza virus infection via host immunoregulation. Such medicines include Lianhuaqingwen capsules [16], baicalin [11], Jin Yin Hua (*Lonicera japonica*), Lian Qiao (*Forsythia suspensa*), Ban Lan Gen (*Isatis indigotica* root) [49], and so on. In particular, Ban Lan Gen (*I. indigotica* root), which is widely used for treatment of viral infectious diseases in China [19,37,48], has been confirmed that it not only directly inhibits influenza virus replication, but more significantly prevents virus-induced inflammation by regulating excessive host immune responses. There are a variety of active ingredients derived from *I. indigotica* root with antiviral and anti-inflammatory efficacy, such as polysaccharide [33], indirubin [35,38], lignans [32], and so on. Therefore, potential development of a new antiviral drug from Ban Lan Gen (*I. indigotica* root) warrants further investigation.

We have derived another active ingredient from *I. indigotica* root and named it Cappariloside A. The structure of Cappariloside A was confirmed as indole alkaloid by nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS) spectra analysis in combined with data from Calis et al. [22]. Indole alkaloids have broad-spectrum antiviral activity [2,13,43,45]. Furthermore, they also demonstrate efficacy against influenza virus-induced inflammation [2,35]. Arbidol, an antiviral drug currently approved for use in China and Russia, has a similar structure to Cappariloside A and demonstrates antiviral and anti-inflammatory effects by regulating the expression of host interferon proteins [7,34].

We have proved that natural Cappariloside A derived from *I. indigotica* root has anti-influenza activity in vitro. However, the content of Cappariloside A in *I. indigotica* root is very low. Thus we chemically synthesized Cappariloside A for this study. We hypothesized that Cappariloside A may share similar mechanisms with Arbidol or other indole alkaloids in antiviral treatment. Here, we evaluated the efficacy of Cappariloside A in both in vitro and in vivo models of influenza virus infection.

2. Materials and methods

2.1. Cells

Madin-Darby canine kidney (MDCK) cells, VERO cells (African green monkey kidney cells line), LLC-MK2 cells (rhesus monkey kidney cells line), 16HBE cells (human bronchial epithelial cell line), A549 cells (human lung adenocarcinoma cells line) and RAW264.7 cells (murine macrophage cell line) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MDCK, VERO and LLC-MK2 cells were cultured in modified Eagle's medium (MEM) containing 10% fatal bovine serum (FBS), and 16HBE, A549 and RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Cells were cultured in a humidified incubator with a 5% CO_2 atmosphere at 37 °C.

2.2. Viruses

Influenza virus A/PR/8/34 (H1N1) (hereafter referred to as PR8/H1N1), A/Aichi/2/68 (H3N2), B/Lee/1940, parainfluenza virus (PIV) 3, respiratory syncytial virus (RSV), adenovirus (ADV), herpes simplex virus type 1 (HSV-1), enterovirus (EV) 71, rhinovirus (RHV) and coxsackievirus (Cox) A16 were purchased from the ATCC. Influenza A/GZ/GIRD07/09 (H1N1 pdm2009) was isolated from fever patients in Guangzhou, China between June and October 2009 and identified by genome sequencing. A/Duck/Guangdong/1994 (H7N3) and A/Chicken/Guangdong/1996 (H9N2) were kind gifts from Dr. Chen Jianxin (South China Agriculture University, Guangzhou, China). All viruses were propagated in the allantoic cavity of 9-day-old embryonated chicken eggs for 48 h at 35 °C and then 12 h at 4 °C. Viruses were then harvested and preserved at −80 °C prior to use. Viral titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) assay in confluent MDCK cells in 96-well microtiter plates.

2.3. Evaluation of antiviral efficacy

2.3.1. Plaque assay

Viral titers were determined by standard plaque assay using MDCK cells. In brief, MDCK cells were grown in MEM and seeded onto six-well plates. Diluted influenza virus was added to confluent MDCK cell monolayers for 1 h. Then, the inoculum was removed and a mixture of agarose (2%, w/v) containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK; 1 μg/mL) (GBCBIO Technologies Company, Guangzhou, China) was added onto MDCK cells monolayers. After 72 h of incubation, plates were fixed with formaldehyde (4%, v/v) overnight and then the agarose was discarded. Plaques were counted after staining with crystal violet (0.2%, w/v).

2.3.2. Cytopathic effect (CPE) inhibition assay

MDCK cells (2×10^4 cells/well, 100 μL) were seeded into each well of 96-well plastic plates and cultured at 37 °C under 5% CO_2 for 24 h. For the anti-influenza activity assay, MDCK cells were inoculated with 100 $TCID_{50}$ of influenza virus at 34 °C for 2 h. Infected cells were then washed and cultured for 48 h at 37 °C under 5% CO_2 in the presence of 100 μL Cappariloside A (5–300 μM) or oseltamivir (5–400 μM) in MEM supplemented with 2 μg/mL TPCK-trypsin (Sigma, USA). After incubation, CPE in virus-infected cells was observed by light microscopy. The 50% inhibitory concentration (IC_{50}) of virus-induced CPE was detected by the Reed-Muench method. Each value was an average from three independent experiments. The selectivity index (SI) was calculated from the ratio of TC_{50}/IC_{50}.

2.4. Analysis of anti-inflammatory efficacy

2.4.1. Cytotoxicity assays

The cytotoxic effects of Cappariloside A on 16HBE and RAW264.7 cells were assessed using the MTT assay as described previously. In brief, 16HBE and RAW264.7 cells (5×10^4 cells/well) were seeded onto 96-well plates for overnight incubation. Cells were then cultured in the presence or absence of Cappariloside A at varying concentrations for 48 h. The culture medium was subsequently removed, and cells were washed with phosphate-buffered saline (PBS). Filtered MTT solution (0.5 mg/mL) was added to each well, and plates were incubated for 4 h at 37 °C. After 4-h incubation, supernatants were aspirated and formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (DMSO). We measured absorbance at a wavelength of 570 nm with a Multiskan Spectrum machine (Thermo Fisher, USA).