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Original article

Oroxylin A suppresses influenza A virus replication correlating with neuraminidase inhibition and induction of IFNs

Jing Jin[a], Shuo Chen[a], Dechuan Wang[b], Yuanjin Chen[a], Yuxu Wang[a], Min Guo[a], Changlin Zhou[a,*], Jie Dou[a,*]

[a] State Key Laboratory of Natural Medicines, School of Life Science and Technology, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, Jiangsu, PR China
[b] Department of Organic Chemistry, School of Science, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, Jiangsu, PR China

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ABSTRACT

Because it is highly contagious, the influenza A virus (IAV) has the potential to cause pandemics in humans. The emergence of drug-resistant strains requires the development of new chemical therapeutics. Oroxylin A (OA) is a flavonoid which has been shown to have antioxidant and antitumor effects. However, intensive studies in which OA fights against different influenza virus strains and the underlying antiviral mechanisms have not been reported. In our study, the antiviral activities in cells and in mice, the preliminary mechanisms of OA were investigated. Our data show that it can inhibit A/FM/1/47 (H1N1), A/Beijing/32/92 (H3N2) and oseltamivir-resistant A/FM/1/47-H275Y (H1N1-H275Y) viruses in MDCK cells in a dose-dependent manner with inhibitory rates of 70.9%, 59.5% and 23.2%, respectively, at 50 μM doses. Orally administered OA effectively protected mice from H1N1 virus-induced death, body weight loss and lung injury, with a survival rate of 60.0% at 100 mg/kg/d dose. In addition, the H1N1 M1 gene transcription and protein synthesis were suppressed by 43.7% and 33.2%, respectively, in the late biosynthesis stage. This resulted in inhibition of viral replication. Furthermore, we found that OA has a neuraminidase (NA) inhibitory effect with IC_{50} values for H1N1-H275Y and A/Anhui/1/2013-R294 K (H7N9-R294 K) of 241.4 μM and 203.6 μM, respectively. Interferons (IFNs) produced by the virally infected cells play important roles in antiviral defense, therefore, IFN levels in the blood were also tested in mice. We found that IFN-β and IFN-γ in the OA 100 mg/kg/d group were markedly increased by 24.5 pg/mL and 859.9 pg/mL, respectively, compared with those in the model group. This indicated that OA could induce the secretion of IFNs. The potent inhibition of virus replication and NA inhibitory activity, as well as the promotion of IFN production suggest that OA could be a drug candidate to fight against IAVs including oseltamivir-resistant strains.

1. Introduction

Oroxylin A (OA, Fig. 1A) is a flavonoid that is derived from the root of *Oroxylum indicum*, a traditional Chinese medicinal herb exhibiting many biological activities [1], including apoptotic activity in cancer cells and adipocytes [2,3]. Previous studies showed that OA has effects on LPS-induced acute hepatic injury in mice [4]. Furthermore, OA has been reported to have a protective effect on LPS-induced acute lung injury in mice [5]. However, its effect on influenza virus-induced infection has not been studied in depth. Many flavonoids such as baicalin and baicalein have been shown to have anti-influenza virus activity [6,7], and both of these flavonoids have neuraminidase (NA) inhibitory activity. In addition, baicalin is reported to have the significant effect of inducing IFN-γ secretion in mice serum [8,9] to assist the host in fighting the virus. OA belongs to the flavonoid families and is similar to baicalin in structure. Therefore, we carried out experiments to explore its antiviral activity.

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family [10]. It is a prominent plague for humans, and it can result in mild clinical symptoms or severe morbidity and even mortality [11]. There are two important proteins on the surface of the virus, hemagglutinin (HA) and NA, which mediate the entry of virus and release of progeny viruses, respectively. Currently, NA inhibitors are one important class of the two licensed anti-IAV drugs commonly used in the clinic. Vaccines are beneficial in controlling IAVs, but their usage is restricted because of their lag time and side effects. Moreover, the rapid emergence of drug-resistant strains due to accumulation of mutations in viral genes and the occurrence of repeated outbreaks has drawn attention to the development of novel anti-IAV therapeutics [12–14] with effective strategies.

In this study, we tested OA for treatment of IAV infections, and

* Corresponding authors.
E-mail addresses: cl_zhou@cpu.edu.cn (C. Zhou), doujie@cpu.edu.cn (J. Dou).

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found that it is highly effective in virus-infected cultured cells and mice. OA alleviates the cytopathic effect (CPE) in virus-infected cells and reduces M1 viral protein expression in a dose-dependent manner. It can prolong survival and ameliorate lung histopathological symptomatic changes of infected mice. OA also inhibits the NA activities of wild-type and oseltamivir-resistant virus strains. Moreover, the IFN levels in mouse blood serum were also increased after the administration of OA. This serves a central innate antiviral defense mechanism, and this response represents the early host defense event that occurs prior to the onset of the adaptive immune response. But the level of Interferon-γ-induced protein 10 kDa (IP-10/CXCL10), a newly reported biomarker of respiratory infection, was not much regulated by OA. Suppression of the H1N1 M1 mRNA and protein synthesis, inhibition of NA activity and promotion of the production of IFNs all contribute to the efficacy of OA in fighting viral infections, suggesting that OA could be a promising antiviral chemical.

2. Method

2.1. Chemicals and regents

Oseltamivir phosphate (OS) and oseltamivir carboxylate (OC) were purchased from MedChem Express (Shanghai, China). OA was synthesized at the School of Science, China Pharmaceutical University. The structure of the purified compound was confirmed by ^{1}H NMR (Bruker Avance 300) and the purity was determined to be > 95%. The compound was dissolved in dimethyl sulfoxide (DMSO) to a 100 mM concentration for use as stock solution that was diluted to the required concentrations for *in vitro* studies.

2.2. Cells and viruses

Madin-Darby Canine kidney (MDCK) cells and Human lung epithelial (A549) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and kept in 5% CO_2, 37 °C, in DMEM or RPMI-1640 medium supplemented with 10% foetal bovine serum.

CHO-K1 cells containing recombinant plasmids pcDNA3.1(+) stablely expressing NA protein sequences of A/Vienam/1203/2004 (H5N1), A/Anhui/1/2013 (H7N9) and A/Anhui/1/2013-R294 K (H7N9-R294 K) were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum at 37 °C in the presence of 5% CO_2.

IAV subtypes A/FM/1/47 (H1N1), A/Beijing/32/92 (H3N2) and H1N1 oseltamivir-resistant mutant strain A/FM/1/47-H275Y (H1N1-H275Y) which was sequenced to confirm possessing a histidine-to-tyrosine substitution at position 275 (H275Y) of NA, were maintained at the Department of Microbiology, School of Life Science and Technology, China Pharmaceutical University. All viruses were propagated in 10-day-old embryonated chicken eggs and stored at −80 °C.

2.3. Determination of $TCID_{50}$

MDCK and A549 cells were seeded in 96-well plates, cells were infected with serial dilution of H1N1, H1N1-H275Y and H3N2 viruses for 2 h after cells were grown to a single layer. Infected cells were incubated at 37 °C for 48 h and the cell viabilities were determined with a cell-counting kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions. The $TCID_{50}$ was determined using the Reed-Müench method.

2.4. Cytopathic effect (CPE) reduction assay

Cells were planted in 96-well plate at 37 °C, 5% CO_2 for 24 h to obtain a mono-cell-layer. After removing the medium, cells were washed with PBS for twice, 100 $TCID_{50}$ H1N1 virus was added and the plate was incubated for 2 h. Serial concentrations of OA with replicates were added to the cells for incubation at 37 ° C for 48 h. The viabilities of MDCK and A549 cells were determined with CCK-8 and the microscopic observations directly observing CPE were taken 24 h post infection. The inhibitory rate was calculated using the following equation: Inhibitory rate (%) = [(mean optical density of test group – mean optical density of model)/(mean optical density of cell control – mean optical density of model)] × 100. Effective concentrations (EC_{50}) of test chemicals were determined by fitting the curve of percent CPE versus the compound concentrations using GraphPad Prism 5. Cytotoxicity was also determined with procedures similar to that in CPE reduction assay without virus infections. Cytotoxic concentrations (CC_{50}) of chemicals on MDCK or A549 cells were determined by procedures similar to those for EC_{50} determination.

2.5. Inhibition of OA on IAV at different stages of viral replication

A time of drug-addition experiment was carried out as previously described [7] to explore the mechanism of OA in IAV life cycle. The three protocols pre-infection, co-infection and post-infection were operated and CCK-8 was used to calculate the inhibitory rate to make a comparison.

2.6. Western blot

Single layer of MDCK cells in 12-well plates were infected with 1×10^3 $TCID_{50}$ of H1N1 virus and treated with OA (10, 20, 40 μM) and OC (10 μM) as control for inhibition experiment or timed-addition experiment in which OA (40 μM) was added at different exposure periods. The former experiment was operated similar to the CPE reduction assay except that proteins were extracted at 24 h post infection. In the latter experiment, tested chemical was added at 0–2, 2–5, 5–8, 8–10 h, OC was added at time interval of 8–10 h, and cells were collected 10 h post infection. All protein lysates were applied to immunoblotting with specific primary M1 antibody (Catalog No. GTX125928, GeneTex) and secondary antibodies. The blots were detected on a Bio-Imaging system and the band intensities of proteins were quantified using ImageJ and normalized to β-actin levels.

2.7. Quantitative RT-PCR (qRT- PCR)

MDCK cells were seeded in 12-well plates for 24 h to obtain single layers for 1×10^3 $TCID_{50}$ H1N1 infection. OA (40 μM) was added 2 h post infection, OC (10 μM) was used as control. Cells were collected 10 h post infection. And extraction of RNA for qRT-PCR was performed using TaKaRa PrimeScript RT Master Mix and TaKaRa Premix Ex Taq Kits according to manufacturer's specifications. Each sample was run in triplicate alongside the endogenous control (GAPDH) to normalize reactions. The relative RNA level of M1 gene was calculated based on the $2^{-\triangle\triangle Ct}$ method.

Primers used to detect the viral mRNAs are:
M1 forward primer, 5′-TTCTAACCGAGGTCGAAAC-3′
M1 reverse primer, 5′-AAGCGTCTACGCTGCAGTCC-3′
GAPDH forward primer, 5′-CACTCACGGCAAATTCAACGGCAC-3′
GAPDH reverse primer, 5′-GACTCCACGACATACTCAGCAC-3′

2.8. The inhibition of IAV A/FM/1/47 (H1N1) in infected mice

Six-week-old ICR mice were divided into 6 groups: normal control, model group (virus control with placebo), OA-treated groups (25, 50 and 100 mg/kg/d), and OS-treated group (100 mg/kg/d). Mice were anesthetized by inhalation of diethyl ether followed by intranasal infection with a 50 μL suspension of 8 × LD_{50} (50% murine lethal dose) of the H1N1 viruses diluted in saline. OA was dissolved in a small quantity of dimethyl sulfoxide (DMSO) and mixed with a 0.5% CMC-Na solution. The final concentration of DMSO was 5%. OA and OS were given by oral gavage. All the drug-treated groups received doses once a