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Rupestonic acid derivative YZH-106 suppresses influenza virus replication by activation of heme oxygenase-1-mediated interferon response

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ABSTRACT

Given the limitation of available antiviral drugs and vaccines, there remains to be a pressing need for novel anti-influenza drugs. Rupestonic acid derivatives were reported to have an anti-influenza virus activity, but their mechanism remains to be elucidated. Herein, we aim to evaluate the antiviral activity of YZH-106, a rupestonic acid derivative, against a broad-spectrum of influenza viruses and to dissect its antiviral mechanisms. Our results demonstrated that YZH-106 exhibited a broad-spectrum antiviral activity against influenza viruses, including drug-resistant strains *in vitro*. Furthermore, YZH-106 provided partial protection of the mice to Influenza A virus (IAV) infection, as judged by decreased viral load in lungs, improved lung pathology, reduced body weight loss and partial survival benefits. Mechanistically, YZH-106 induced p38 MAPK and ERK1/2 phosphorylation, which led to the activation of erythroid 2-related factor 2 (Nrf2) that up-regulated heme oxygenase-1 (HO-1) expression in addition to other genes. HO-1 inhibited IAV replication by activation of type I IFN expression and subsequent induction of IFN-stimulated genes (ISGs), possibly in a HO-1 enzymatic activity-independent manner. These results suggest that YZH-106 inhibits IAV by up-regulating HO-1-mediated IFN response. HO-1 is thus a promising host target for antiviral therapeutics against influenza and other viral infectious diseases.

1. Introduction

Influenza A virus (IAV) infection remains to be a global public health threat with significant morbidity and mortality. Although the currently available antiviral drugs, including inhibitors of the viral M2 proton-selective ion channel, neuraminidase and RNA-dependent RNA polymerase (RdRp), are effective in control of IAV infection, their antiviral efficacy diminishes with the rapid emergence of drug-resistant viruses [1]. In addition, emerging pathogenic strains, such as H7N9 IAV emerged 2013 in China, present a tremendous challenge to currently available antiviral therapeutics [2,3]. These highlight an urgent need for development of novel anti-IAV drugs to overcome pandemic influenza, especially by newly emerged influenza viruses or strains resistant to available antiviral drugs.

The innate immune response plays a crucial role in defending virus infection. Unlike the adaptive immune system that recognizes invading microorganisms by using antigen specific receptors generated through somatic gene rearrangement of T and B lymphocytes, the sensing of the innate immune system is

Abbreviations: IAV, influenza A virus; HO-1, heme oxygenase-1; Nrf2, erythroid 2-related factor 2; ISGs, IFN-stimulated genes; RdRp, RNA-dependent RNA polymerase; TLRs, toll-like receptors; RLR, RIG-I-like receptors; MKP5, MAPK phosphatase 5; IRF3, interferon regulatory factor 3; HIV, human immunodeficiency virus; HCV, hepatitis C virus; HBV, hepatitis B virus; EBOV, Ebola virus; OC, oseltamivir carboxylate; AH, amantadine hydrochloride; RBV, ribavirin; OP, oseltamivir phosphate; MRC-5, human fetal lung fibroblast-like cells; MOI, multiplicity of infection; PFU, plaque-forming units; TC_{50}, 50% toxicity concentration; CPE, cytopathic effect; $TCID_{50}$, 50% tissue culture infective doses; SI, selectivity index; NA, neuraminidase; MUNANA, 4-methylumbelliferyl-a-D-N-acetylneuraminic acid; HA, hemagglutination; P.I., post-infection; NBF, neutral buffered formalin; NS1, non-structural protein l; ARE, antioxidant response element; AP-1, activator protein-1; IFIT1, IFN-induced protein with tetratricopeptide repeats 1; IFITM3, IFN-inducible transmembrane protein 3; OAS1, 2′-5′-oligoadenylate synthetase 1; PKR, double-stranded RNA-dependent protein kinase; BVR, biliverdin reductase; SnPP, tin protoporphyrin

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mediated by groups of germline encoded pattern recognition receptors, such as Toll-like receptors (TLRs) [4], RIG-I-like receptors (RLR) [5], which recognize the structural features unique to microbes, such as viral nucleic acids. Activation of the pattern recognition receptors by viral ligands induces interferons (IFNs), inflammatory cytokines and chemokines that control the multiplication and spreading of viruses before the onset of a more specific and powerful adaptive immune response. In particular, type I IFN has been demonstrated to play essential roles in defending IAV infection through induction of tens to hundreds of IFN-stimulated genes (ISGs) that inhibit the virus replication via multiple distinct mechanisms [6,7]. However, in order to colonize the hosts, IAV also evolves multiple mechanisms to evade or inhibit host innate immune responses. For instance, IAV NS1 protein, a nonstructural protein of the virus, inhibits IFN-α/β production and antiviral functions of ISGs [8]. Additionally, recent work suggested that IAV also inhibits IFN-α/β production by inducing the expression of MAPK phosphatase 5 (MKP5) [9]. Accordingly, approaches that induce or restore IFN-α/β production and antiviral response in hosts infected by IAV are likely to inhibit the virus replication, regardless of its drug-resistance profiles.

Heme oxygenase-1 (HO-1) is the anti-inflammatory and antioxidant enzyme catalyzing the metabolism of heme into CO, iron, and biliverdin [10]. HO-1 plays a crucial role in the maintenance of cellular homeostasis. Recently, Sotiria Tzima and colleagues showed that HO-1 interaction with interferon regulatory factor 3 (IRF3) was required for IFN-β induction in response to Sendai virus infection [11], suggesting that HO-1 also plays an important role in innate antiviral immune response. In agreement with this notion, other studies also revealed that the up-regulation of HO-1 suppressed infection of many different species of viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), enterovirus and Ebola virus (EBOV) [12–16]. Recent studies also provided evidence showing that the enzymatic activity of HO-1 may be required for host restriction of some viruses, such as HCV and EBOV [16,17]. Given the known function of HO-1 in the innate antiviral immune responses against the variety of viruses, it can be envisaged that therapeutic up-regulation of HO-1 might be an effective strategy to inhibit the infection of a broad-spectrum of viruses, including IAV.

As a traditional Chinese medicine, *Artemisia rupestirs* L. is widely used as an anti-inflammatory, anti-cancer, anti-bacterial and antiviral herbal medicine in China. It is also a key component of Compound Yizhihao Granule, which is used to treat the common cold in clinic [18]. Rupestonic acid, extracted from *Artemisia rupestris* L., is a sesquiterpene with inhibitory activities against influenza viruses [18,19]. However, to date, the antiviral mechanism of rupestonic acid has not been illuminated. In the present study, we showed that YZH-106, a rupestonic acid derivative, inhibited a broad-spectrum of influenza viruses, including drug-resistant strains of IAV. Interestingly, we further demonstrated that YZH-106 was likely not to directly target viral components, but inhibited IAV replication by activation of HO-1-mediated type I IFN response.

2. Materials and methods

2.1. Compounds

YZH-106 (N-[(3-*p*-fluoro-phenyl-isoxazol-5-yl)-methyl] rupestonic acid amide) with greater than 98% purity was originally developed and supplied by Xinjiang Technical Institute of Physics and Chemistry, Chinese Academy of Sciences. For *in vitro* assay, oseltamivir carboxylate (OC; Medchem, Princeton, NJ, USA), amantadine hydrochloride (AH; Sigma-Aldrich, St Louis, MO, USA) and ribavirin (RBV; Sigma-Aldrich) were used as reference compounds. 2 mM stock solutions of YZH-106, OC and AH were prepared in DMSO. 2 mM stock solutions of RBV were dissolved at in culture medium. These drugs were diluted to final working solutions as indicated in experiments. For *in vivo* experiment, YZH-106 was dissolved in absolute ethyl alcohol with the mass ratio of 1:10, and further solubilized in four-fold Kolliphor-EL (BASF, AG, Ludwigshafen, German). YZH-106 stock solutions were diluted with double distilled water. Similarly, oseltamivir phosphate (OP; Chinese National Institutes for Food and Drug Control, Beijing, China) used as a control drug in mice studies was dissolved in double distilled water.

2.2. Viruses, cells and viral infection

Experiments involving viral infection were performed under BSL-2 condition. IAV A/Fort Monmouth/1/1947(H1N1) strain was obtained from the America Type Culture Collection (ATCC). Clinical isolated influenza virus strains A/TianjinJinnan/15/2009(H1N1, oseltamivir-resistant), A/Wuhan/359/1995(H3N2), A/FujianTongan/196/2009(H3N2, amantadine-resistant), BY/FujianXinluo/54/2006 were kindly provided by Professor Yuelong Shu at the Institute for Viral Disease Control and Prevention, China Centers for Disease Control and Prevention, Beijing, China. Viral stocks of these strains were prepared by propagating them in 10-day-old embryonated chicken eggs for 48 or 72 h.

Madin-Darby canine kidney cells (MDCK) and human fetal lung fibroblast-like cells (MRC-5) were obtained from ATCC, mouse macrophage RAW264.7 cells were obtained from Cell Resource Center at Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China. MDCK cells were grown in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 1% MEM Non-Essential Amino Acids Solution (Invitrogen), 10% FBS (Gibco, Grand Island, NY, USA) and 1% Penicillin-Streptomycin (10,000 U/mL) (Invitrogen). MRC-5 cells and RAW264.7 cells were cultured in MEM and Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) respectively, containing 10% FBS and 1% Penicillin-Streptomycin.

For infections of MDCK cells, cells were washed with PBS and infected with influenza virus at indicated multiplicity of infection [MOI, plaque-forming units (PFU)/cell] in serum-free medium for 2 h at 37 °C. Then, the viral inoculum was removed and replaced by maintenance medium supplemented with 2 μg ml^{-1} TPCK-treated trypsin (Worthington, Lakewood, Colorado, USA) and 0.08% BSA (Beijing Yuan Heng Golden Horse biological technology development Co., Ltd., China). As for RAW264.7 and MRC-5 cells, the maintenance medium were supplemented with 2% FBS.

2.3. Cytotoxicity test

The cytotoxicity of compounds was evaluated by a tetrazolium dye (MTT) assay using a modification of standard methods [20]. Briefly, MDCK cells (2.5×10^4 per well) grown in 96-well plate were treated with serial two-fold dilutions of YZH-106 or control drugs for 72 h. Then, 10 μl of 5 mg ml^{-1} MTT (Promega, Madison, WI, USA) completely dissolved in PBS was added to cells. After 4 h incubation, the medium per well was aspirated and replaced by 150 μl of DMSO. The plates were shaken for 10 min and measured by scanning absorbance at 450 nm on Enspire (Perkin Elmer, Waltham, MA, USA). 50% toxicity concentration (TC_{50}) of drugs were determined using Reed and Muench method [21].

2.4. Cytopathic effect (CPE) assays

MDCK cells were seeded into 96-well plates at a density of 2.5×10^4 cells per well. After 24 h, cells were washed with PBS and