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Inhibition of the infectivity and inflammatory response of influenza virus by Arbidol hydrochloride *in vitro* and *in vivo* (mice and ferret)

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ABSTRACT

Influenza virus infections are the main contagious respiratory disease with high levels of morbidity and mortality worldwide. Antiviral drugs are indispensable for the prophylaxis and treatment of influenza and other respiratory viral infections. In this study, the Arbidol hydrochloride (ARB), which has been licensed in Russia and China, is used to investigate its anti-viral and anti-inflammatory efficacy *in vitro* and *in vivo*. The antiviral results *in vitro* showed that ARB had a better inhibition on Influenza virus A/PR/8/34 (H1N1), A/Guangdong/GIRD07/09 (H1N1), A/Aichi/2/68 (H3N2), A/HK/Y280/97 (H9N2) with IC_{50} ranging from 4.4 to 12.1 μM. The further mechanisms study demonstrated that ARB is able to inhibit hemagglutinin-mediated hemolysis at concentration of 3.91-15.63 μg/mL. The anti-inflammatory efficacy *in vitro* indicated that IL-6, IP-10, MCP-1, RANTES and TNF-α levels were diminished by ARB at concentrations of 22.6 and 18.8 μM.

The *in vivo* results in mice model displayed that the survival rates of mice administered 25 mg/mL and 45 mg/mL ARB were 40% and 50% respectively. And also, ARB can inhibit the decrease of body weight at 45 mg/mL and inhibit the increase of mice lung index at 25 mg/mL and 45 mg/mL comparing to virus group. In ferret model, the ARB-treated ferrets showed a fever that peaked at 2 dpi and gradually decreased beginning at 3 dpi while relatively high temperatures were observed until 4 dpi in the virus group. The ARB-treated group scored 0–1 in the activity level at 2 dpi and 3 dpi at all time points. The transcription levels of cytokines in the respiratory tract of ferrets were detected at 3 dpi. Several proinflammatory cytokines induced by influenza (IL-10, TNF-α, IL-8 and IL-6) were down-regulated by post-treatment with ARB. The histopathological results of ferret lung displayed that ARB can alleviate the influenza virus induced lung lesions.

Our results clarified the activity of ARB in both suppressing virus propagation and modulating the expression of inflammatory cytokines *in vitro* and *in vivo*, it can be as an effective drug to treat the influenza virus infection.

1. Introduction

Influenza A viruses (IAV) are classified by subtype based on two surface proteins hemagglutinin (HA) and neuraminidase (NA). Until now, there are 16 known HA subtypes and 9 known NA detected in IAV throughout the world [1]. Due to the *anti*genic *drift*

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and shift, the emergence of new influenza virus subtypes resulted in global epidemics and pandemics, which caused the contagious respiratory disease with high levels of morbidity and mortality, such as 2009 pandemic influenza A virus and avian influenza H7N9 virus [2,3].

The influenza vaccination is the best choice for the prevention and control influenza outbreaks. However, the influenza seasonality and antigenic drift lead to the restriction and ineffectiveness of vaccine in the corresponding year [4].

In the past decades, anti-influenza virus drugs have been developed, and among them, amantadine derivatives (amantadine and rimantadine) that blocking the virus-specific proton channel M2 and neuraminidase inhibitors (oseltamivir, zanamivir and peramivir) were FDA-approved for the prophylaxis and treatment of influenza virus infection. Because of long-term using with single target, the development of drug resistance caused the limitation in reducing symptom burden, as well as improving survival of hospitalized influenza patients [5,6].

Except target the virus protein, host immune response also plays an important role in the disease. Clinical and *in vivo* studies showed that "cytokine storm" were associated with disease exacerbation, such as excessive expression of TNF- α, IL-1 β, IL6, IL8, IP-10 and MCP-1 et al., especially the highly pathogenic influenza infection [7,8]. So, the virus proteins and the host immune system are both important in the drug development.

Arbidol (ARB), ethyl-6-bromo-4- [(dimethylamino)methyl]-5-hydroxy-1-methyl-2-[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate, which has been licensed in Russia and China for treatment of influenza A and B virus, exerts broad antiviral effect against influenza virus, respiratory syncytial virus, rhinovirus, coxsackie virus, adenovirus, herpes simplex virus type 1, hepatitis C virus, hantaan virus, chikungunya virus, ebola virus, tacaribe arenavirus, and human herpesvirus 8 [9–15]. ARB's broad-spectrum antiviral activity may be due to the mechanisms that it can interact with both virus and cellular proteins [16].

Studies have also shown that ARB can stimulate the humoral and cellular immunity, including restoring balance of pro-inflammatory and anti-inflammatory mediator expressions *in vivo*, as well as inducing serum interferon in cell cultures and in animals and humans [16–18]. Liu Q et al. reported ARB had antiviral activity through decreasing the mortality, alleviating virus-induced lung lesions and viral titers in mice model infection with A/FM/1/47 (H1N1) [11], but these results were contrary to Brooks. M J who reported that ARB can not cause a reduction in lung titers after challenge with A/Aichi/2/68 [19], the reason may be the difference of virus strains. Furthermore, ARB can also inhibited the levels of IL-1β,IL-6,IL-12,TNF-α, and elevated the level of IL-10, in the bronchoalveolar lavage fluids and lung tissues in mice model, but had no influence on the levels of IFN-α and IFN-γ [11], which was consistent with the Brooks. MJ results [19].

Although there are numerous reports about ARB against different virus, the anti-inflammatory activity of ARB against influenza virus *in vivo* is limited, especially in ferret model.

In this study, the ARB against different influenza virus strains and anti-inflammatory *in vitro* and *in mice* were evaluated. Furthermore, to our knowledge, the ferret model has not been used to study efficacy of ARB against influenza virus. Ferrets are susceptible to infection with unadapted human influenza viruses and similar in manifestations of clinical signs akin to human influenza, including nasal discharge, anorexia, watery eyes, otologic symptoms and fever [20]. So, except mice model, ferret model was further used to confirm that ARB could reduce the over-expression of the immune response in the early stage of viral infection, thus relieving the symptoms related with influenza infection.

2. Materials and methods

2.1. Chemicals and reagents

ARB was provided by Shijiazhuang No. 4 Pharmaceutical Co., Ltd, China and Jiangsu Wuzhong Pharmaceutical Co., Ltd, China.

Oseltamivir phosphate (Ose) is the inhibitor of neuraminidase (NA) of influenza virus which is a positive control drug to treat influenza virus infection, it was purchased from Roche. Chemicals were dissolved in distilled water and stored at −20 °C prior to use.

2.2. Viruses and cells

Influenza virus A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), B/Lee/1940 (FluB) and A/HK/Y280/97 (H9N2) were purchased from American Type Culture Collection (ATCC); A/Guangdong/GIRD07/09 (H1N1) was isolated from infected patients; A/China/24/96 (H7N3) was stored in our lab. Viruses were grown in the allantoic cavity of embryonated chicken eggs for 48 h at 35 °C, followed by 12 h at 4 °C, after which the harvested viruses were preserved at −80 °C until further use.

For the mice experiments, mouse lung-adapted variants were generated by repeated infection of mice. The 50% lethal dose (LD_{50}) in mice was determined. Madin-Darby Canine Kidney (MDCK) and human alveolar epithelial carcinoma (A549) cells were grown in 75 cm^2 cell culture flasks and transferred into 96, 24 and 12 well cell culture plates for experiments. Cells were cultured in a monolayer in Minimum Essential Medium (MEM) and Ham's F12 medium respectively, both medium were supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 μg/mL), and the cells were incubated at 37 °C, 5% CO_2.

2.3. Virus titration and virus yield reduction assay

ARB and Ose were dissolved in MEM with 1 μg/mL trypsin and incubated with MDCK cells for 1 h at 37 °C. The cell cultures were then washed twice with phosphate-buffered saline (PBS) (pH = 7.4) and incubated with appropriate viruses (multiplicity of infection, MOI = 0.01) for 1 h. The monolayers were washed twice with PBS, and the same drug-containing medium was added. The plates were incubated for 48 h at 34 °C in the presence of 5% CO_2. The viral titre in the supernatant was further determined by the 50% tissue culture infectious dose ($TCID_{50}$) assay using Methyl Thiazolyl Tetrazolium (MTT) after cultivating virus in MDCK cells for 48 h at 34 °C, 5% CO_2. Each concentration of the chemicals was tested in triplicate. The 50% inhibitory concentrations (IC_{50}), TC_{50}, 50% toxic concentration (TC_{50}) and selection index ($SI = TC_{50}/IC_{50}$) of the drugs were calculated from the obtained data separately [21,22].

2.4. Haemagglutination inhibition (HI) test

The lowest number of virus particles required to agglutinate the chicken erythrocytes was determined to investigate the inhibitory effect of ARB on the haemagglutinating activity. Chicken erythrocytes supplemented with 1.6% sodium citrate (Sigma) in sterile water were separated by centrifugation and washed three times with sterile PBS. 25 μL of ARB of various concentrations was mixed with same volume of virus stock, then 50 μL of chicken erythrocytes (0.5%) was added into the plate. The plate was observed for hemagglutination after incubation for 45 min at 4° C.

2.5. Haemolysis inhibition assay

ARB was gradient diluted by half with the initial concentration of 62.5 μg/mL, the same volume (100 μL) of PR8 virus solution (HI titer >256) was then added and incubated for 1 h. 200 μL of 2%