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14-Deoxy-11,12-didehydroandrographolide attenuates excessive inflammatory responses and protects mice lethally challenged with highly pathogenic A(H5N1) influenza viruses

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ABSTRACT

Traditional Chinese medicine (TCM) has been an excellent treasury for centuries' accumulation of clinical experiences, which deserves to be tapped for potential drugs and improved using modern scientific methods. 14-Deoxy-11,12-didehydroandrographolide (DAP), a major component of an important TCM named *Andrographis paniculata*, with non-toxic concentration of 1000 mg/kg/day, effectively reduced the mortality and weight loss of mice lethally challenged with A/chicken/Hubei/327/2004 (H5N1) or A/PR/8/34 (H1N1) influenza A viruses (IAV) when initiated at 4 h before infection, or A/duck/Hubei/XN/2007 (H5N1) when initiated at 4 h or 48 h before infection, or 4 h post-infection (pi). DAP (1000 or 500 mg/kg/day) also significantly diminished lung virus titres of infected mice when initiated at 4 h or 48 h before infection, or 4 h pi. In the infection of A/duck/Hubei/XN/2007 (H5N1), DAP (1000 mg/kg/day) treatment initiated at 48 h before infection gained the best efficacy that virus titres in lungs of mice in $\log_{10}TCID_{50}$/mL reduced from 2.61 ± 0.14 on 3 days post-infection (dpi), 2.98 ± 0.17 on 5 dpi, 3.54 ± 0.19 on 7 dpi to 1.46 ± 0.14 on 3 dpi, 1.86 ± 0.18 on 5 dpi, 2.03 ± 0.21 on 7 dpi. Moreover, DAP obviously alleviated lung histopathology and also strongly inhibited proinflammatory cytokines/chemokines expression. The mRNA levels of TNF-α, IL-1β, IL-6, CCL-2/MCP-1, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β in lungs of A/duck/Hubei/XN/2007 (H5N1)-infected mice and serum protein expression of TNF-α, IL-1β, IL-6, CCL-2/MCP-1 and CXCL-10/IP-10 in mice infected with all the three strains of IAV were all significantly reduced by DAP. Results demonstrated that DAP could restrain both the host intense inflammatory responses and high viral load, which were considered to contribute to the pathogenesis of H5N1 virus and should be controlled together in a clinical setting. Considering the anti-inflammatory and anti-IAV activities of DAP, DAP may be a promising active component obtained from *A. paniculata*, which can be further investigated as a useful constitute of curative strategies in the future against IAV, the H5N1 strains in particular.

1. Introduction

Influenza A virus (IAV), which belongs to the *Orthomyxoviridae* family, is a highly contagious pathogen that can cause severe epidemics of respiratory illness in humans and many other animal species, such as waterfowl, birds, poultry, swine and ferrets (Beigel et al., 2005; Munster et al., 2009; Olsen et al., 2006). This virus has led to devastating damage to public health, as well as large economic and social developmental losses.

A highly pathogenic avian influenza virus (HPAIV) of H5N1 subtype entered the public view in 1997, and it has gradually attracted significant global attention because of its high morbidity and mortality, continuing evolution, wide circulation and severe transmission to humans (Watanabe et al., 2012; Webster and Govorkova, 2006). The infection of H5N1 HPAIV is characterised by high virus load and excessive immune responses, which may

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contribute to viral pathogenesis (Beigel et al., 2005; Teijaro, 2015). H5N1 infection in animals is always accompanied with acute lung injury, lung pathology and inflammatory infiltration, which may be closely associated with the intense release of proinflammatory cytokines/chemokines and attribute to its mortality (Marsolais et al., 2009; Peiris et al., 2009). Effective methods to counteract pandemic H5N1 HPAIV are urgently needed.

Although vaccination is the primary strategy for the prevention of influenza infection, many problems such as slow vaccine production, rapid antigenic shift and existing side effects may occur (Cinatl et al., 2007), thereby showing the inadequate ability of vaccination. Alternatively, effective antiviral drugs are of utmost importance. The current approved antivirals consist of inhibitors of the M2 ion channel (e.g. amantadine and rimantadine) and those of neuraminidase (NA) (e.g. oseltamivir, zanamivir, laninamivir and peramivir). The drug-resistant virus variants accompanied by the use of amantadine and rimantadine have rapidly appeared, and therefore these drugs are no longer recommended for the treatment of influenza (Ison, 2011). The application of NA inhibitors (NAI) also raises the risk of generation and spread of NAI resistant influenza viruses (McKimm-Breschkin, 2013), such as most seasonal A/H1N1 isolates prior to the 2009 pandemic (the novel pandemic A/H1N1 viruses are still oseltamivir-sensitivive subtypes). However, the existing toxicity and adverse effects of these drugs (Smith et al., 2011) have restricted their use. Therefore, novel anti-IAV drugs, particularly those for anti-H5N1, with new mechanisms of actions should be developed.

Traditional Chinese medicine (TCM) is an excellent treasury to develop novel anti-influenza agents. A large number of TCM herbs, such as *Cinnamomi cortex* (Hayashi et al., 2007), *Ferula assafoetida* (Lee et al., 2009) and *Mosla scabra* (Yu et al., 2010), and their active components (e.g. polyphenols, flavonoids, saponins, glucosides and alkaloids) have shown potential anti-influenza activities (Ge et al., 2010; Grienke et al., 2012; Peng et al., 2014; Sithisarn et al., 2013). *Andrographis paniculata* (Burm. F.) Nees, which belongs to the Acanthaceae family, is an important TCM herb that has been used for centuries in many Asian countries. This herb exhibits multiple pharmacological properties, such as anti-inflammatory (Bao et al., 2009; Guan et al., 2011), antipyretic (Suebsasana et al., 2009), analgesic (Suebsasana et al., 2009), anticancer (Kumar et al., 2004), antiviral (Lee et al., 2014; Wintachai et al., 2015), antibacterial (Singha et al., 2003), anti-diabetic (Reyes et al., 2006), hepatoprotective (Maiti et al., 2010) and immunomodulatory (Kumar et al., 2004) activities. Despite its many pharmacological effects, *A. paniculata* has been commonly used for the treatment of cold, flu and upper respiratory tract infections (Coon and Ernst, 2004). However, to date, the mechanism of actions for its usage remains obscure. Only few studies (Chen et al., 2009) about its anti-influenza activity using modern scientific methods are available. In our previous work, we reported that 14-deoxy-11,12-didehydroandrographolide (14-deoxy-11,12-dehydroandrographolide, DAP, Fig. 1A), exerts potent antiviral activity mainly against HPAIV H5N1 and significantly reduces the upregulated expression of proinflammatory cytokines/chemokines *in vitro* (Cai et al., 2015).

In the present study, we further tested the *in vivo* (a mouse model) efficacy of DAP against lethal challenge with HPAIV A/duck/Hubei/XN/2007 (H5N1), A/chicken/Hubei/327/2004 (H5N1) and A/PR/8/34 (H1N1).

2. Materials and methods

2.1. Drugs and reagents

Sodium carboxymethyl cellulose (CMC–Na) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), dissolved in sterile deionised water and prepared to 0.5% CMC–Na. DAP (98.00%) was purchased from Bioyeargene Biosciences Co., Ltd. (Wuhan, China) and dispersed in 0.5% CMC–Na. Oseltamivir phosphate (oseltamivir) was purchased from Roche Pharmaceuticals Ltd. (Shanghai, China) and also dispersed in 0.5% CMC–Na.

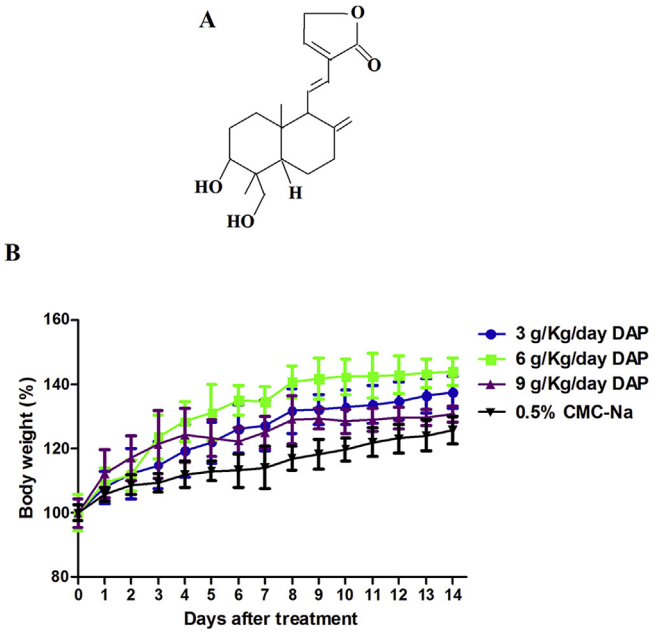

Fig. 1. Toxicity of DAP in mice. (A) Chemical structure of DAP. (B) Female BALB/c mice (n = 6 per group) were treated with DAP (3, 6 or 9 g/kg/day) or 0.5% CMC–Na via intragastric administration. Body weights in each group were monitored daily for 14 days post-treatment. Body weight (%) was calculated according to following formula: body weight (%) = average body weight of mice daily/average body weight of mice before treatment × 100%. Data shown are the mean ± SD for three independent experiments.

2.2. Viruses

Influenza virus strains A/duck/Hubei/XN/2007 (H5N1) [H5N1 (XN), Clade 2.3.2], A/chicken/Hubei/327/2004 (H5N1) [H5N1 (DW), Clade 7] (Zhou et al., 2006) and A/PR/8/34 (H1N1) (PR8) were conserved by the State Key Laboratory of Agricultural Microbiology of China, and propagated in 10-day-old embryonated eggs for 2 (for H5N1) or 3 (for PR8) days at 37 °C before use. Virus titres were determined by calculating the $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL in Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA, USA) using the method developed by Reed and Muench (1938). All experiments with the H5N1 virus were performed in a biosafety level 3 laboratory.

2.3. Mice

BALB/c female mice (five to six weeks old) were purchased from the Animal Experimental Centre of Wuhan University, Hubei Province, China.

2.4. Ethics statement

All animal experiments were performed according to the protocols approved by the Hubei Provincial Animal Care and Use Committee (approval number SYXK 2008-0004) of China.