Short communication

Inhibition of novel reassortant avian influenza H7N9 virus infection *in vitro* with three antiviral drugs, oseltamivir, peramivir and favipiravir

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Background: A novel reassortant avian-origin influenza A (H7N9) virus was isolated from respiratory specimens obtained from three patients and was identified as H7N9 in China. Antiviral agents are required to treat patients with avian influenza H7N9 virus infection.
Methods: In this study, we assessed the antiviral potential of oseltamivir, peramivir, favipiravir (T-705), amantadine and rimantadine against novel reassortant avian-origin influenza H7N9 virus *in vitro*.
Results: All three avian influenza H7N9 virus strains were sensitive to oseltamivir, peramivir and favipiravir (T-705), but resistant to amantadine and rimantadine.
Conclusions: Our data show a pattern of antiviral sensitivity for this novel H7N9 strain of influenza that suggests the compounds oseltamivir, peramivir and favipiravir should be useful for therapy.

Introduction

In February and March of 2013, three patients had been hospitalized with severe lower respiratory tract infection of unknown origin [1]. A novel reassortant avian-origin influenza A (H7N9) virus was isolated from respiratory specimens obtained from these three patients and was identified as H7N9 [1]. On 29 March 2013 the Chinese Center for Disease Control and Prevention (CDC) completed laboratory confirmation of three human infections with an avian influenza A(H7N9) virus. This novel virus had not been previously reported in humans [2]. These infections were reported to the World Health Organization (WHO) on 31 March 2013. Following the detection of the first cases, the Chinese CDC rapidly made specific polymerase chain reaction test kits for the new A(H7N9) viruses available to provincial and local laboratories across China to ensure timely testing of suspected cases [3,4]. Subsequently, the cases were confirmed in nine contiguous provinces (Anhui, Fujian, Henan, Hunan, Jiangsu, Jiangxi, Shandong, Zhejiang and Taiwan) and two cities (Beijing and Shanghai) in eastern China. By 30 May 2013 China had reported 132 confirmed H7N9 infections. Among them, 37 (28%) H7N9 virus-related deaths were reported in China [5]. The WHO then issued a global alert about novel reassortant avian influenza (H7N9) virus infection.

Although vaccination is the primary strategy for the prevention of influenza, vaccines are ineffective against rapidly emerging mutant viral antigens. There are four anti-influenza drugs approved for use in the United States, the M2 ion channel inhibitors amantadine and rimantadine, and the influenza virus neuraminidase inhibitors oseltamivir and zanamivir. Oseltamivir (Tamiflu) is the first effective treatment for all strains of influenza. Another promising drug, favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide, T-705) has been shown to inhibit a variety of influenza viruses [6,7]. Favipiravir might function as a specific inhibitor of influenza virus RNA polymerase [8]. Based on these observations, a total of five drugs (paramivir, oseltamivir, favipiravir, amamtadine and rimantadine) approved for clinical use or in clinical trials in the treatment of influenza virus infections were tested in Madin–Darby canine kidney (MDCK) cells in this study.

Materials and methods

Influenza A/Anhui/1/2013 (H7N9), A/Shanghai/1/2013 (H7N9) and A/Shanghai/2/2013 (H7N9) viruses were provided by the National Influenza Center, Chinese CDC (Beijing, China). These three H7N9 strains were isolated from the throat-swab specimens obtained from all three patients in China. The MDCK cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's minimal essential medium (MEM; Gibco® Life Technologies, Beijing, China) containing 10% fetal bovine serum (Gibco® Life Technologies), 100 U of penicillin G per ml and 100 μg of streptomycin per ml. Viral propagation was carried out in MDCK cells.

Oseltamivir (oseltamivir carboxylate, MW: 284.3) was synthesized from Yichang Yangtze River Pharmaceutical Co., Ltd (Yichang, Hubei, China). Peramivir (peramivir trihydrate, MW: 382.4) and favipiravir (T-705, MW: 157.1) were synthesized by the Beijing Institute of Pharmacology and Toxicology (Beijing, China). Amantadine (MW: 151.2) and rimantadine (MW: 215.7) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Test drugs were dissolved in dimethyl sulfoxide for *in vitro* studies and diluted in MEM for efficacy testing.

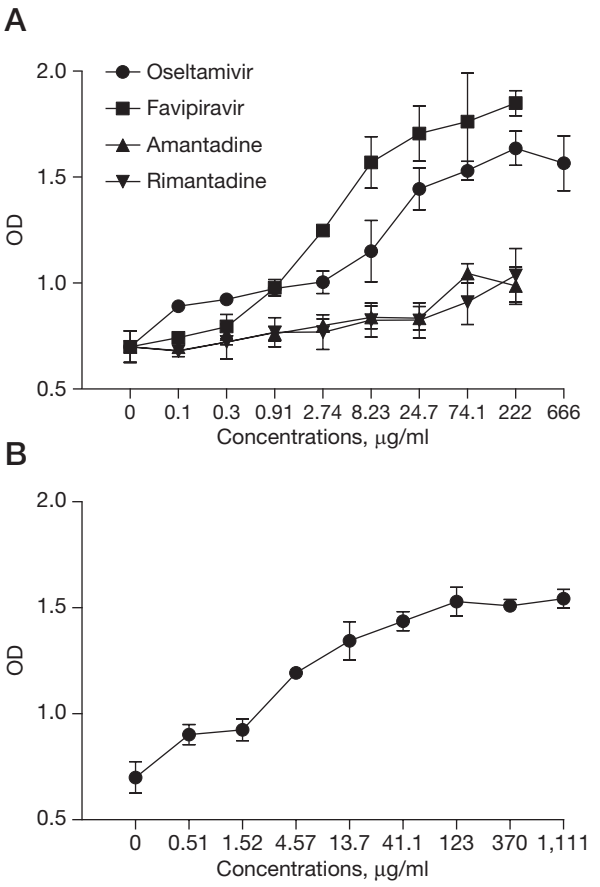
Antiviral activity was determined *in vitro* by the inhibition of virus-induced cytopathic effect (CPE) assay. These methods have been described previously [9]. MDCK cells were incubated for 24 h prior to drug addition. On the next day, eight concentrations of the test drugs, each of which varied by one-half $\log_{10}$ from the next concentration, were evaluated. Standard placebo-treated virus controls, toxicity controls and normal-medium controls were included. Morphological changes resulting from cytotoxicity of test drug or virus CPE were graded on a scale of 0–5, with 5 defined as the appearance of complete cytotoxicity or CPE involving the entire monolayer as observed by light microscopy. The values obtained were then converted to percentages of untreated, uninfected controls. CPE inhibition data were expressed as the 50% inhibitory (viral CPE-inhibitory) concentration (IC_{50}), the 50% cytotoxic (cell-inhibitory) concentration (CC_{50}) and the selectivity index (SI), which was determined as CC_{50}/IC_{50}.

The cell viability assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega Corporation, Madison, WI, USA; G3582) according to the manufacturer's instructions. The CellTiter 96® AQueous One Solution Cell Proliferation Assay kit was purchased from Promega Corporation. Briefly, MDCK cells were cultured and infected with H7N9 virus after addition of test drugs. At 3 h before each of the desired time points, 10 μl of MTS reagent was added into each well and cells were incubated at 37°C for 3 h. The optical density values were determined by a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

Results

We first examined the effect of oseltamivir at different concentrations for antiviral activity against novel reassortant avian influenza H7N9 virus infections in MDCK cells. Oseltamivir was cytotoxic to MDCK cells at 2,000 μg/ml. Oseltamivir inhibited A/Anhui/1/2013 H7N9 virus infection in a dose-dependent manner (Figure 1A), was shown to have an IC_{50} of 2.30 ±1.35 μg/ml determined by visual assay and to have an IC_{50} of 7.93 ±2.66 μg/ml determined by MTS assay. Oseltamivir also inhibited A/Anhui/1/2013 H7N9 isolate with SI values ranging from >90 to >363 (Table 1). The inhibitory effect of peramivir was further tested

Figure 1. Effect of the different test drugs on H7N9 virus infection



(A) Madin–Darby canine kidney (MDCK) cells were treated with oseltamivir, favipiravir, amantadine or rimantadine, respectively, and then infected with H7N9 virus. **(B)** MDCK cells were treated with peramivir and then infected with H7N9 virus. OD, optical density.

Table 1. Antiviral activities of oseltamivir, peramivir, favipiravir (T-705), amantadine and rimantadine against the novel reassortant avian influenza H7N9 virus *in vitro*

Test drugs	Visual assay			MTS assay		
	IC_{50}	CC_{50}	SI	IC_{50}	CC_{50}	SI
Oseltamivir, μg/ml	2.30 ±1.35	>670 ±0.0	>363 ±190	7.93 ±2.66	>670 ±0.0	>90 ±25
Peramivir, μg/ml	0.69 ±0.13	>1,100 ±0.0	>1,600 ±264	6.87 ±4.54	>1,100 ±0.0	>207 ±114
Favipiravir, μg/ml	0.56 ±0.18	>220 ±0.0	>420 ±130	2.40 ±1.05	>220 ±0.0	>106 ±49
Amantadine, μg/ml	>220 ±0.0	>220 ±0.0	0.0 ±0.0	>220 ±0.0	>220 ±0.0	0.0 ±0.0
Rimantadine, μg/ml	>220 ±0.0	>220 ±0.0	0.0 ±0.0	>220 ±0.0	>220 ±0.0	0.0 ±0.0

Values are mean ±SD. CC_{50}, 50% cell cytotoxic concentration; IC_{50}, 50% virus inhibitory concentration; SI, visual assay and MTS assay =CC_{50}/IC_{50}.

on A/Anhui/1/2013 virus infection. Peramivir was not cytotoxic to MDCK cells at the concentrations up to 10,000 μg/ml and provided a complete protection at the higher doses and a linear dose response at the lower part of the concentration curve (Figure 1B). IC_{50} was 0.69 ±0.13 μg/ml determined by visual assay and 6.87 ±4.54 μg/ml determined by MTS assay. Peramivir inhibited A/Anhui/1/2013 H7N9 isolate with SI values ranging from >207 to >1,600 (Table 1).

We next examined the effect of favipiravir (T-705) at the different concentrations for the antiviral activity against avian influenza H7N9 virus infection in MDCK cells. Favipiravir was not cytotoxic to MDCK cells at 222 μg/ml. Favipiravir was efficacious against A/Anhui/1/2013 virus evaluated with IC_{50} values, determined by visual assay and MTS assay, ranging from 0.56 ±0.18 to 2.40 ±1.05 μg/ml (Table 1). This efficacy was generally stronger than that observed with oseltamivir (Figure 1A) but not with peramivir. We also examined whether amantadine and rimantadine have antiviral activity against the avian influenza H7N9 virus *in vitro*. No cytotoxicity was observed for amantadine and rimantadine at 666 μg/ml. However, neither drug inhibited avian influenza virus infection (Table 1).

The susceptibilities of the other two avian influenza H7N9 viruses, A/Shanghai/1/2013 and A/Shanghai/2/2013, to oseltamivir, piramivir and favipiravir were evaluated in visual assay and MTS assay. Similar data were obtained when the MDCK cells were treated with oseltamivir, peramivir or favipiravir and then infected with A/Shanghai/1/2013 or A/Shanghai/2/2013, respectively (data not shown).

Discussion

We noticed that peramivir, oseltamivir and favipiravir (T-705) but not amantadine and rimantadine, were shown to have the potent antiviral activities against the novel reassortant avian influenza H7N9 virus *in vitro*. Gubareva *et al*. [10] reported that oseltamivir, zanamivir and peramivir are potent inhibitors of influenza A (H1N1 and H3N2) and B virus neuraminidase in the low nanomolar range *in vitro*. Hayden *et al*. [11,12] first demonstrated that prophylaxis and early treatment with oral oseltamivir were associated with significant antiviral and clinical effects in experimental human influenza A and B virus infections. Early treatment with oseltamivir is recommended for avian influenza H5N1 virus infection [13]. Gao *et al*. [1] also reported that the three patients with H7N9 virus infection received late treatment with oseltamivir starting on day 7 or 8. The sequences of the NA, M2 and Pol genes for A/Anhui/1/2013 are consistent with the observed drug susceptibilities [1]. In this report, this strain appears to be less sensitive, or at least partially resistant, to oseltamivir. In addition, Barroso *et al*. [14] determined the tolerability and antiviral efficacy of oral peramivir for treatment and prophylaxis of experimental human influenza A and B and concluded that early treatment with peramivir was associated with significant antiviral effects in experimentally induced influenza in humans. Furthermore, Sidwell *et al*. [7] demonstrated that favipiravir was inhibitory to four strains of avian H5N1 influenza virus in MDCK cells. Experiments with mice lethally infected with influenza A/Duck/MN/1525/81 (H5N1) virus showed that favipiravir significantly prevented mortality. Kiso *et al*. [15] also demonstrated that favipiravir effectively protected mice from lethal infection with oseltamivir-sensitive or -resistant highly pathogenic H5N1 virus. Sleeman *et al*. [16] reported that favipiravir also inhibied *in vitro* replication of a wide range of influenza viruses, including drug-resistant influenza virus, H2N2, H4N2, avian H7N2, avian H5N1 and 2009 A(H1N1) viruses.

Given the demonstrated *in vitro* inhibition of avian influenza virus replication, oseltamivir, paramivir and favipiravir (T-705) might qualify as a potential therapy for treating the novel reassortant avian influenza virus infections. These results suggest that the drugs found to be active should be studied further as potential agents for the novel reassortant avian influenza H7N9 virus therapy. More probably, they could be used as the lead drugs for the development of even more potent, non-toxic inhibitors of the novel reassortant avian influenza H7N9 virus.

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Disclosure statement

The authors declare no competing interests.

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