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Development of anti-influenza A compounds: a pilot study

Key Messages

1. There is no effective anti-H5N1 avian influenza agent.
2. A chemical compound—BFDBSC—can inhibit H5N1 virus infection in cell cultures, and such inhibition might be attributable to its halogenated benzoyl residues.
3. This pilot study assessed anti-H5N1 activity and toxicity of four chemical compounds with halogenated benzoyl residues in cell culture system.
4. Two compounds—FP-BFDBSC and BFB-gallate—showed higher antiviral effects than BFDBSC, whearas the other two—BFB-borneol and BFB-menthol—showed lower antiviral effects. These compounds did not show toxicity.
5. The halogenated benzoyl residues may play a key role in anti-H5N1 effects. However, all these compounds showed poor solubility, which may limit their utility.

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Introduction

A highly pathogenic avian influenza A (H5N1) virus has caused severe disease in humans. The first outbreak was in Hong Kong in 1997 and caused six deaths in 18 infected patients.[1] Subsequently, a re-emergence of human H5N1 disease causing two deaths was reported in a family of five members who visited Fujian, China from Hong Kong in January 2003.[2] Thereafter, human infections with H5N1 viruses continued to be identified in many countries. From January 2004 to October 2006, the virus has afflicted 253 patients and claimed 148 lives, including 18 cases with 13 deaths in China.[3] Most of these cases entailed direct exposure to H5N1 virus-infected poultry; the virus was endemic in poultry in Asia since 2003.[4] The virus has spread along migratory flyways linked to Southeast Asia, Siberia, the Middle East, Africa and Europe.[5] Although genetic analysis of H5N1 virus isolated from humans in 1997, 2004, and 2005 revealed that all genes were of avian origin, limited person-to-person transmission was identified during the 1997 outbreak in Hong Kong, during 2004 in Thailand,[6] during 2005 in northern Vietnam, and during 2006 in Indonesia. Taken together, the risk of a pandemic caused by a reassortant virus with efficient and sustained human-to-human transmissibility has increased.

Although an anti-influenza drug—oseltamivir—has been effective against H5N1 in animals,[7] methylprednisolone and oseltamivir did not show obvious clinical benefits in some H5N1 patients.[8,9] Notably, H5N1 is resistant to M2 inhibitors and may rapidly develop resistance to neuraminidase inhibitors.[10] Considering the high mortality rate (>50%) associated with H5N1 avian influenza, there is an urgent need to develop new drugs to combat this disease.

In collaboration with Dr Ulrike Holzgrabe from University of Wurzburg, Germany, we synthesised and tested two chemical compounds—DBSC and BFDBSC—for their anti-H5N1 effects in cell culture system. DBSC did not show antiviral activity, whereas BFDBSC showed anti-H5N1 effect at a 50% inhibitory concentration (IC_{50}) of 80 μM, with a 50% cytotoxic concentration (CC_{50}) of about 7500 μM in Madin-Darby canine kidney (MDCK) cells and no toxicity in Balb/c mice with dose up to 5 mg/kg. As the non-halogenated mother compound, DBSC contained selenium and did not show anti-H5N1 activity, selenium in BFDBSC was also unlikely to show antiviral activity. Instead, the antiviral activity might be attributable to the compound's halogenated benzoyl residues. We therefore synthesised lipophilic bis-(p-fluorophenacyl) ester of BFDBSC (FP-BFDBSC) and three 4-bromo-2-fluorobenzoyl esters (ie BFB-borneol, BFB-menthol, and BFB-gallate) and tested their antiviral activity and toxicity in cell culture system.

Methods

FP-BFDBSC, BFB-borneol (from (1S)-(-)-borneol), BFB-menthol (from natural (-)-menthol) and BFB-gallate (from gallic acid) were chemically synthesised as described previously.[11]

The MDCK cells were used for culture and titration of H5N1 virus (A/Vietnam/1194/04) as described previously.[1,2,12,13] Briefly, about 90% confluent MDCK cells were infected with the virus at 37ºC for 1 hour, and then the virus solution was replaced by MEM with 1% FBS. After 48 to 72 hours at 37ºC,

the supernatants were collected, pooled, and stored at -80ºC for further study. The titres of virus in the supernatants were determined by $TCID_{50}$ assay based on observation of cytopathic effects after 72 hours of culture and/or a haemagglutination assay using turkey red blood cells.[1,2,12,13]

The compounds were serially two-fold diluted to seven dilutions and individually mixed with 100 $TCID_{50}$ of H5N1 virus at 1:1 ratio. The mixtures were inoculated to 96-well plates of MDCK cell cultures in triplicates. Compounds BFDBSC and DBSC were included as positive and negative controls, respectively. Serials of two-fold dilution of the compounds were also inoculated into the cell cultures as cytotoxicity controls of the compounds. Cytopathic effects in the cultures were monitored daily, and the supernatants were collected 48 hours post infection. The released virus in the supernatants was titrated using $TCID_{50}$,[1,2,12,13] whereas copies of viral RNA in the supernatants were measured by real-time RT-PCR as described previously,[1,2,12-17] Briefly, total RNA in the supernatants was extracted using RNeasy Mini kit (Qiagen, Germany) and reverse transcribed to cDNA using applied SuperScript II Reverse Transcriptase (Invitrogen, USA) and a primer 'Uni12' 5'-AGC AAA AGC AGG-3'. H5N1 viral NP gene was measured by SYBR green Mx3000 Real-Time PCR System (Stratagene, USA), using primers NP-Forward: 5'-GAC CAG GAG TGG AGG AAA CA-3', NP-Reverse: 5'-CGG CCA TAA TGG TCA CTC TT-3'.

The compounds diluted from the highest concentrations were inoculated to MDCK and Vero cells. Their potential toxicity was monitored by methylthiazolyldiphenyltetrazolium bromide and their 50% cytotoxic concentration (CC_{50}) was determined as described previously.[18] Amanitin at 30 μg/ml was used as a toxic control.

Results

Anti-H5N1 virus effects of the synthesised compounds (FP-BFDBSC, BFB-borneol, BFB-menthol, and BFB-gallate) were determined in MDCK cell cultures. BFDBSC and BDSC were included as positive and negative controls, respectively. One hour pre-incubation with different concentrations of the compounds potently inhibited the replication of H5N1 virus in MDCK cell cultures as measured by real-time RT-PCR (Fig a) and back titration using $TCID_{50}$ (Fig b). Two of the synthesised compounds (FP-BFDBSC and BFB-gallate) showed stronger antiviral effects than BFDBSC (positive control), whereas the other two compounds (BFB-borneol and BFB-menthol) showed less antiviral effects (Table 1). Notably, all these compounds were not soluble in water and had to be dissolved in DMSO. When the compounds in DMSO were further diluted in culture medium or PBS, BFB-borneol and BFB-menthol showed lower solubility than FP-BFDBSC and BFB-gallate (Table 2).

All the synthesised compounds at their maximum soluble concentration did not show toxicity in MDCK cell cultures (Table 2). However, the toxicity test was limited by low solubility of the compounds.

Discussion

All four synthesised compounds exhibited antiviral effect against H5N1 infection in MDCK cell cultures. The antiviral activity may be attributable to the halogenated benzoyl residues. Although antiviral effects of FP-BFDBSC and BFB-gallate were about 1-fold higher than that of the positive control (BFDBSC), further studies and applications were limited by their poor solubility, which was about 7- to 10-fold lower than that of the positive control. Thus, new

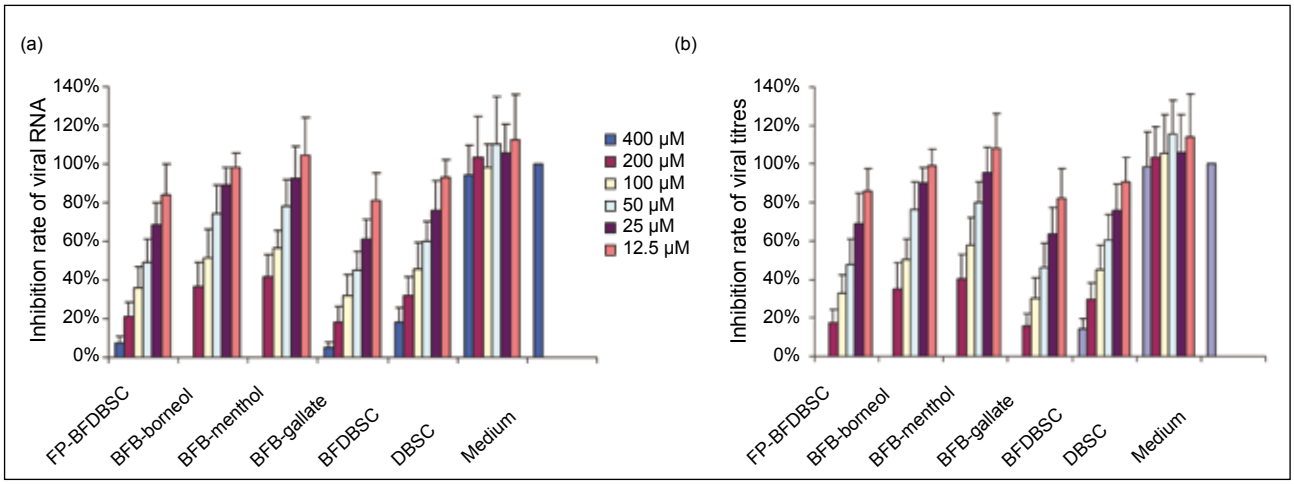

Fig. Inhibition of H5N1 virus infection in Madin-Darby canine kidney (MDCK) cell cultures
Six compounds are mixed with 100 $TCID_{50}$ of H5N1 virus and then inoculated to MDCK cells in triplicates. Antiviral effects of the compounds were determined by measuring viral RNA copies (relative viral RNA copies yielded as compared to untreated controls) using (a) real-time RT-PCR and (b) viral titres (relative infective virus yielded as compared to untreated controls) by titration using $TCID_{50}$ in culture supernatant 48 hours post infection. Three triplicates are tested.

Table 1. 50% inhibitory concentration (IC_{50}) of the compounds against H5N1 viral infection

Compounds	Mean±SD IC_{50} (μM)	
	Real-time RT-PCR	$TCID_{50}$
BFB-borneol	49.2±5.3	48.1±4.8
BFB-menthol	106.8±17.6	102.4±19.3
FP-BFDBSC	172.5±18.8	177.4±17.5
BFB-gallate	45.4±7.1	46.8±5.5
BFDBSC	82.6±8.7	80.8±7.2

Table 2. Solubility and toxicity of the compounds

Compounds	Solubility in 10% DMSO-PBS (μM)	50% cytotoxic concentration (μM)
BFB-borneol	2000	>2000
BFB-menthol	200	>200
FP-BFDBSC	200	>200
BFB-gallate	3000	>3000
BFDBSC	20 000	7478±127

compounds containing the halogenated benzoyl residues with higher solubility should be designed. Furthermore, as over 80% of human influenza infections in Hong Kong appear resistant to the current anti-influenza drug Tamiflu, it is also worth evaluating any potential emergence of drug resistance for the newly designed compounds. This preliminary study has provided the theoretical and practical basis for development of new drugs to combat H5N1 avian influenza.

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