Background: Current vaccination strategies and antiviral drugs only provide limited protection against influenza virus infection. In this study, we investigated the use of a novel antisense oligonucleotide (named IV-AS), which is specific for the 5'-terminal conserved sequence found in all eight viral RNA segments of influenza A virus.

Methods: The activity of IV-AS was monitored both in vitro, in Madin-Darby canine kidney (MDCK) cells, and in vivo using a mouse model. IV-AS was given intranasally to H5N1-infected mice once daily for 6 days starting 6 h after infection. A three-base mismatch of IV-AS was used as a control.

Results: IV-AS inhibited influenza virus A induced cytopathic effects in MDCK cells with the 50% effective concentration (EC50) ranging from 2.2 to 4.4 µM. IV-AS was effective against H5N1 virus in preventing death, lessening weight reduction, inhibiting lung consolidation and reducing lung virus titres. Dosages of 40 and 60 mg/kg/day provided 40% and 60% survival rates and prolonged mean survival days in comparison with the infected control group (P<0.05). The lung index in mice treated with IV-AS, at a dose of 20, 40 or 60 mg/kg/day, had been inhibited on day 4 or 6 (P<0.05 or P<0.01); virus titres in lung had declined to 2.42, 1.51 and 1.54 log10 TCID50/g of lung, respectively, whereas the yields in the infected control mice were 6.00 log10 TCID50/g of lung.

Conclusions: Our results suggest that the 5'-terminal conserved region of influenza A virus RNA segments can be targeted using antisense technology; therefore, IV-AS is a potential drug for prophylaxis and control of influenza virus infections.

Introduction

Influenza is a globally contagious disease that is associated with significant morbidity in the general population and mortality in the elderly and high-risk patients. In addition, influenza presents an ongoing threat of a new pandemic [1,2]. Despite the devastation caused by this respiratory disease, only limited strategies are available for its control. Immunization remains the primary means of prevention of viral infections; however, vaccines against influenza viruses are either unavailable or ineffective due to viral antigenic changes or a poor host immune response. Thus, antiviral drugs constitute a critical treatment option. The M2 channel inhibitors, such as amantadine and rimantadine, are effective against influenza A strains; however, their usage is limited because of the side effects in the central nervous and gastrointestinal systems, emergence of viral resistance, and the lack of effectiveness against influenza B [3,4]. The neuraminidase (NA) inhibitors, such as zanamivir and oseltamivir, are de novo anti-influenza virus drugs that are effective for the prophylaxis and treatment of both influenza A and B [5–7]. Oseltamivir is currently stockpiled by many countries prepared for a major influenza outbreak or a pandemic, but the emergence of resistant variants of influenza A (H5N1) during oseltamivir treatment should not be neglected [8–10].

Antisense oligonucleotides (ASOs) are short, single-stranded, chemically modified DNA molecules that are considered to be complementary to a specific mRNA sequence; they cause the destruction of the targeted mRNAs and, therefore, downregulate respective protein expression [11,12]. Since the US Food and Drug Administration (FDA) approved the first antisense
drug, Vitravene, for the treatment of cytomegalovirus (CMV) that caused retinitis in 1998 [13], a large number of antisense compounds have been studied using in vitro and in vivo models and several have been evaluated in human clinical trials [14,15]. For antiviral therapy, many published studies have shown that ASOs can inhibit various viral pathogens such as human immunodeficiency virus (HIV) [16,17], Epstein–Barr virus (EBV) [18], hepatitis B virus (HBV) [19], hepatitis C virus (HCV) [20], respiratory syncytial virus [21,22], and influenza virus [23–25] by interactions with essential viral genes [26]. However, ASOs specific for the 5′-terminal conserved sequences of all eight influenza A viral RNAs have not been demonstrated so far.

In this study, we designed a 13-mer phosphorothioate ASO (IV-AS) targeting the 5′-terminal conserved region of the eight influenza A viral RNAs, and investigated the antiviral activities of IV-AS both in vitro and in vivo. Our results revealed that IV-AS not only has a broad spectrum of anti-influenza A virus activity without significant cytotoxicity, but also exhibits a therapeutic efficacy in the lethal avian influenza virus infection mouse model.

Methods

Viruses, cells and mice

Influenza viruses, A/Lufang/09/1993(H3N2) and A/Jingfang/01/1986(H1N1), were obtained from the Chinese Centers for Disease Control and Prevention (Beijing, China). Highly pathogenic avian influenza viruses, A/Tiger/Harbin/01/2002(H5N1), A/Tiger/Shanghai/04/2003(H5N1) and A/Tiger/shanxi/02/2002(H5N1), were reserved by Changchun Institute of Veterinary Science. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC; Virginia, USA), and used for virus titration, cytotoxic assay and antiviral assay in vitro. Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal calf serum (FCS) at 5% CO₂. For virus infection, 2% FCS and 2 mg/ml of trypsin were used. Specific-pathogen-free BALB/c mice (weighing 18–20 g) used in this experiment were specific pathogen-free mice. All animals were bought from Animal Centre of Biomedical Institute of China (Beijing, China). Animals were housed in climate-controlled colony rooms (22–26°C, 60% humidity), with a 12 h light/dark cycle and free access to food and water. The experimental protocol was approved by the animal research committee of our institute. Median lethal doses (LD₅₀) were estimated by the Bliss method [27]. The 95% confidence limits (UCL, LCL) of the LD₅₀ values were determined according to Sokal and Rohlf [28]. A series of progressively decreasing doses determined by Drug and Statistics software (DAS version 2.0) [29] (the most commonly used professional software in the field of pharmacology and pharmaceutics in China) from 2.162, 1.726, 1.490, 1.246 to 1.072 g IV-AS/kg body weight, were employed for intravenous injection.

In vitro antiviral assays

Inhibition of virus-induced cytopathic effect (CPE) was used to assay antiviral activity in vitro. Briefly, a serial twofold dilution of the compound started at 0.5 μM for IV-AS and 5 μM for IV-mis. MDCK cells were used for this assay, and virus was inoculated at a 50% tissue culture infective dose (TCID₅₀) of 100/10⁴ cells. After inoculation, cells were washed twice in phosphate buffered saline to remove unabsorbed viruses, and fresh maintenance media in each well was then added with different concentrations of the compounds. Cells were further incubated at 37°C for 2 days. The viabilities of cells were exposed to several concentrations of compounds. Each experiment was performed in triplicate and repeated three times.

In vivo toxicity assays

BALB/c mice (weighing 18–20 g) used in this experiment were specific-pathogen-free mice. All animals were bought from Animal Centre of Biomedical Institute of China (Beijing, China). Animals were housed in climate-controlled colony rooms (22–26°C, 60% humidity), with a 12 h light/dark cycle and free access to food and water. The experimental protocol was approved by the animal research committee of our institute. Median lethal doses (LD₅₀) were estimated by the Bliss method [27]. The 95% confidence limits (UCL, LCL) of the LD₅₀ values were determined according to Sokal and Rohlf [28]. A series of progressively decreasing doses determined by Drug and Statistics software (DAS version 2.0) [29] (the most commonly used professional software in the field of pharmacology and pharmaceutics in China) from 2.162, 1.726, 1.490, 1.246 to 1.072 g IV-AS/kg body weight, were employed for intravenous injection.
expressed as the 50% effective (viral CPE inhibitory) concentration (EC_{50}). Therapeutic index (TI) was determined by the ratio of CC_{50}/EC_{50}. Each experiment was performed in triplicate and repeated three times.

**In vivo antiviral assays**
Mice were anaesthetized with methyl ether and exposed to influenza virus A/Tiger/Harbin/01/2002 (H5N1) (2–3 LD_{50} dose) by intranasal instillation (in the biosafety level 3 laboratory). For each experiment, the mice received one of the following treatments: saline, IV-AS (20, 40, 60 mg/kg/day, respectively), and IV-mis (60 mg/kg/day). A 20 µl aliquot of saline solution containing IV-AS or IV-mis was given by intranasal administration once daily for 6 days, beginning 6 h after infection. Ten infected mice were used for each treatment and control. Parameters for determining treatment effects included prevention of death through 15 days, lessening of weight loss and improvement of mean survival days (MSDs).

**Lung infection parameters**
Mice were infected and treated in the same manner as described above. The lung parameters were assayed on days 2, 4 and 6 post-infection, with six mice from each group killed at each time point. The lungs were observed for colour and consolidation, and were weighed. The lungs were then homogenized in the maintenance media at 1:10 (w/v), and centrifuged at 3,200 × g for 5 min to pellet cell debris. Serial 10-fold dilutions of the lung homogenate supernatant (100 µl) were added to MDCK cell monolayers, as described previously [30]. The monolayers in the wells were observed daily and scored for virus-induced CPE. Infectivity was expressed as the number of TCID_{50} per gram lung tissue. In addition, the lung index was calculated as a parameter of inflammation or consolidation according to the following formula: lung index = lung weight (g)/body weight (g)×100. The lung infection parameters were determined in parallel with these survival experiments.

**Statistics analysis**
Survival percentage was determined by χ^2 analyses. MSDs, virus titres and lung indexes were analysed by the Student’s t-test; *P<0.05 was considered as denoting significance.

**Results**

**Cytotoxicity of IV-AS**
The viability of MDCK cells was determined after 4 days continuous exposure to compounds. IV-AS did not exhibit cytotoxicity on MDCK cells at a concentration up to 500 µM, concentrations of IV-AS >500 µM resulted in cell shrinkage, malformation and shedding over 4 days. As evaluated by using the MTS method, the CC_{50} of IV-AS was 875 µM.

**In vivo toxicity assays**
The (14 day) LD_{50} value for IV-AS on female mice was 1.554 g/kg, the 95% confidence interval (95% CI) of the (14 day) LD_{50} values was 1.695–1.424 g/kg. The (14 day) LD_{50} value for IV-AS on male mice was 1.533 g/kg, the 95% CI of the (14 day) LD_{50} values was 1.679–1.4 g/kg.

**Antiviral activity of IV-AS in vitro**
The anti-influenza virus activity of IV-AS was determined with respect to the inhibition of CPE in MDCK cells by using the MTS method. In order to exclude the nonspecific effects caused by oligonucleotides, we used the mismatched oligonucleotide (IV-mis) as a negative control. The EC_{50} values of IV-AS ranged from 2.22 to 4.44 µM (Table 1); however, antiviral activity of IV-mis was not observed.

**Antiviral activity of IV-AS in vivo**
On the basis of the in vitro studies, we attempted to investigate the antiviral activity of IV-AS against highly pathogenic influenza A (H5N1) virus in our mouse model. Mice that received IV-AS, in a dose-dependent manner, showed less clinical signs of disease and death after virus challenge, as compared with infected control mice or the control mice that received IV-mis treatment (*P<0.05). Complete protection was not observed for all treatments. All the infected control mice died before the treated mice (Figure 1). Of ten mice, four survived when they were administered IV-AS at doses of 40 and 60 mg/kg/day (Table 2). In addition, lowering the dosage to 20 mg/kg/day in the same model prevented lethality in two of the ten mice. No signs of drug-related toxicity were observed when IV-AS was administered intranasally for 5 days at a dose of 120 mg/kg/day. A dose-response relationship exhibited by IV-AS was observed when weight

<table>
<thead>
<tr>
<th>Virus</th>
<th>IV-AS EC_{50} µM*</th>
<th>TI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Jingfang/01/1986 (H1N1)</td>
<td>2.42 ±0.11</td>
<td>361</td>
</tr>
<tr>
<td>A/Lufang/09/1993 (H3N2)</td>
<td>2.22 ±0.09</td>
<td>394</td>
</tr>
<tr>
<td>A/Tiger/Harbin/01/2002 (H5N1)</td>
<td>4.44 ±0.24</td>
<td>197</td>
</tr>
<tr>
<td>A/Tiger/Shanghai/04/2003 (H5N1)</td>
<td>4.02 ±0.16</td>
<td>318</td>
</tr>
<tr>
<td>A/Tiger/Shaxi/02/2005 (H5N1)</td>
<td>3.95 ±0.22</td>
<td>222</td>
</tr>
</tbody>
</table>

*Mean ±SD of EC_{50} values (50% virus inhibitory [effective] concentration) obtained from three independent experiments using the MTS method. †TI, therapeutic index (CC_{50}/EC_{50}).
loss in the infected mice was monitored over time (Figure 2). Lower doses generally resulted in greater weight loss than higher doses.

Lung infection parameters
The effects on lung weights and virus titres on days 2, 4 and 6 are shown in Table 2. Lung consolidation and lung weights increased over time up to day 6 in the infected controls. Reductions in lung consolidation/weight at the 40 and 60 mg/kg/day doses were statistically significant (P<0.05 or P<0.01) in the IV-AS-treated animals compared with the infected controls. A dose-dependent decrease in virus production was observed in the lungs of the IV-AS-treated mice. In the groups treated with IV-AS at a dose of 20, 40 and 60 mg/kg/day, the mean virus yields were reduced to 2.42 (P<0.01), 1.51 (P<0.01) and 1.54 (P<0.01) log_{10} TCID_{50}/g of lung on day 6, respectively, whereas the yields in the infected control mice were as high as 6.00 log_{10} TCID_{50}/g of lung.

Discussion
In this study, we demonstrated that the effect of IV-AS against influenza virus on MDCK cells was sequence-specific, and the CPE caused by the virus was significantly inhibited in IV-AS-treated cells as compared with untreated infected cells. Furthermore, we observed that IV-AS was effective in inhibiting infection of influenza virus in experimentally infected mice.

The influenza virus genome consists of eight single-stranded negative-sense RNA molecules that are present in the virus particle in nearly equivalent molar ratios. Each of the six largest RNA segments encodes a single polypeptide present in both the infected cell and the virion. The two smallest segments (7 and 8) encode two polypeptides each, that is, matrix protein and M2, and NS1 and NS2, respectively [31]. A number of published reports have shown that antisense technology can be employed to inhibit the influenza virus by interfering with viral gene expression in a sequence-specific manner; this technology targets the antisense stands that are specific for genes encoding the nucleocapsid protein (NP) or an RNA transcriptase component (PA, PB1 and PB2) of the influenza virus containing the AUG initiation codon and loop-forming sequences [24–26,32,33]. Antisense technology can abolish the accumulation of not only the corresponding mRNAs, but also the viral

![Figure 1. Effects of intranasal treatment of IV-AS on survival in H5N1 virus-infected mice](image)

The number of mice in each group was 10. IV-AS, a 13-mer phosphorothioate antisense oligonucleotide; IV-mis, a three-base mismatch control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosage, mg/kg/day</th>
<th>No. of survivors/total no.</th>
<th>Lung index, %</th>
<th>Virus titre, log_{10} TCID_{50}/g lung ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>IV-AS</td>
<td>20</td>
<td>2/10</td>
<td>0.9 ±0.33</td>
<td>0.79 ±0.10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4/10‡</td>
<td>11.0 ±3.56‡</td>
<td>0.77 ±0.13</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4/10‡</td>
<td>11.4 ±3.53‡</td>
<td>0.80 ±0.06</td>
</tr>
<tr>
<td>IV-mis</td>
<td>20</td>
<td>6/10</td>
<td>7.9 ±0.08</td>
<td>0.76 ±0.04</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6/10</td>
<td>6.8 ±1.32</td>
<td>0.77 ±0.02</td>
</tr>
<tr>
<td>Infected control</td>
<td>Saline</td>
<td>0/10</td>
<td>0.74 ±0.02</td>
<td>0.77 ±0.01</td>
</tr>
<tr>
<td>Uninfected control</td>
<td>Saline control</td>
<td>10/10</td>
<td>NA</td>
<td>0.74 ±0.02</td>
</tr>
</tbody>
</table>

*Once a day for 6 days beginning 6 h after virus exposure. †A/Tiger/Harbin/01/2002(H5N1). Significant differences from infected control: *P<0.05 and ‡P<0.01. MSD, mean survival days; NA, not applicable; TCID_{50}, 50% tissue culture infective dose.
RNAs in the virion and complementary RNAs in cells. However, the highly variable influenza genome limits inhibition by the antisense targets [34,35]. Interestingly, the 5'- and 3'-terminal sequences of the influenza A virus RNA segments are conserved among the segments and the different subtypes. Additionally, some signals necessary for genome replication and packaging apparently reside in these terminal sequences [31,36]. In this study, we employed a 13-mer ASO specific for the 5'-terminal sequences. The results reveal that IV-AS has a broad spectrum of anti-influenza A virus activity and high therapeutic efficacy in a lethal highly pathogenic avian influenza virus infection mouse model. Therefore, the 5'-terminal sequences of the influenza virus RNA segments are stable and effective targets for antisense technology.

Regarding the lung infection parameters, IV-AS efficiently inhibited the virus yield in the lung and led to a reduction in the lung index. The lung infection parameters were determined in parallel with these survival experiments. Therefore, inconsistencies between the virus titres and the outcome of infection are probably due to the pathogenesis of the highly pathogenic H5N1 virus. The possibility of late viral rebound is not ruled out, although it is less likely as the mice treated with IV-AS and surviving infection were completely recovered after 1 month. The highly pathogenic avian influenza viruses are more potent inducers of proinflammatory cytokines (for example, tumor necrosis factor-α) and chemokines (for example, IP-10), which suggests that cytokine dysregulation might play a role in the pathogenesis of the H5N1 disease. This differential hyper-induction of cytokines and chemokines has been shown to contribute to the extraordinary severity of the human H5N1 disease [37,38]. In addition, it has been demonstrated that highly pathogenic avian influenza viruses cause systemic infections in experimentally infected mice, ferrets and humans [2,39,40]. In this study, influenza viruses could be isolated from the lung, brain, liver and kidney of the mice (data not shown). Clinically, a majority of hospitalized patients are typically started on both broad-spectrum antibiotics and antiviral agents [2]. Although corticosteroids are widely used, they have not shown clear clinical benefits [2]. Thus, it is still worthwhile to investigate whether the use of IV-AS with other supplementary treatments is beneficial in controlling highly pathogenic influenza virus infections in our mice model.

In conclusion, our results demonstrated that IV-AS, which is targeted to the 5'-terminal conserved sequence of eight segmented viral RNAs of influenza A virus, could specifically inhibit influenza A virus both in vitro and in vivo in a dose-dependent manner. This is the first study to demonstrate that the 5'-terminal conserved sequences of influenza A virus RNA segments are stable and effective targets for antisense technology. Our findings show that IV-AS is able to provide an effective strategy for the prophylaxis and control of influenza virus infections.

Acknowledgements

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Disclosure statement

The authors declare no conflict of interest.
References


