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Antisense oligonucleotides targeting the RNA binding region of the NP gene inhibit replication of highly pathogenic avian influenza virus H5N1

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ABSTRACT

The H5N1 avian influenza virus (AIV) causes widespread infections in bird and human respiratory tracts, and vaccines and drug therapy are limited in their effectiveness. Recent studies of AIV structures have been published and provide new targets for designing antiviral drugs such as antisense oligonucleotides (AS ODNs), which effectively inhibit gene replication. In this study, we designed and synthesized three AS ODNs (NP267, NP628, NP749) that were specific for the RNA binding region of nucleoprotein (NP) based on AIV structure. Results showed that all three AS ODNs could inhibit viral replication in MDCK cells. The NP628 showed the best antiviral effect of all through viral titers, quantitative RT-PCR and indirect immunofluorescence (IFA) assays. In addition, the liposome mediated NP628 could partially protect the mice from a lethal H5N1 influenza virus challenge. Moreover, the NP628 group had a lower viral titer and lung index in the infected mice when compared with the viral control. Our results showed that AS ODN targeting of the AIV NP gene could potently inhibit AIV H5N1 reproduction, thus, formulating a candidate for an emergent therapeutic drug for the pathogenic H5N1 influenza virus infection.

1. Introduction

The H5N1 avian influenza virus (AIV) is a highly contagious disease associated with significant morbidity in the general population and mortality in high-risk patients. Avian H5N1 viruses have now spread from Asia to Europe and Africa, and the human mortality rate is about 60% [1–3]. Currently, the most common and efficient way of controlling influenza is by vaccination, however, it takes time to produce an antigenically appropriate and immunogenic vaccine and to deliver it to an entire population. In addition, the constant antigenic drift demands an ongoing development of new vaccines for each season [4], therefore, M2 ion channel blockers, amantadine and rimantadine, and the neuraminidase inhibitors, oseltamivir and zanamivir, have been licensed as anti-influenza drugs. They are beneficial for uncomplicated seasonal influenza, but the therapeutic effects of these agents have been limited because of side effects in the central nervous and gastrointestinal systems and the rapid emergence of drug-resistant viruses [5].

The structural and functional studies of the viral nucleoprotein (NP) of influenza A virus proteins indicate that the conserved NP protein sequence is an excellent drug target for all influenza A virus subtypes [6–10]. Furthermore, some amino acid changes of target sites in these conserved structures are deleterious to viruses; therefore, a virus might not easily develop the resistance to compounds that target these sites. Nucleic acid-based antiviral drugs, including AS ODNs and siRNAs, have many advantages such as a simple design, high specificity, easy preparation and they express low side effects. Moreover, nucleic acid drugs can specifically target the conserved gene and knockdown the selected gene, but have no impact on host gene expression [11,12]. Antisense oligonucleotides are chemically synthesized DNA and RNA molecules with single strands of about 20 nucleotides and base-pair homology against a specific mRNA sequence. They are potentially useful therapeutic agents for the treatment of viral diseases [13]. AS ODNs are effective against the influenza virus in vitro and in vivo by targeting the PA, PB2, NS1 and PB1 genes; these target sites are mainly focused on the conserved AUG initiation codon of the influenza virus [14–17]. There are no current studies of AS ODNs on the NP gene of H5N1 avian influenza.

We designed three phosphorothioate-modified AS ODNs that target the NP gene of the conserved RNA binding region among different avian influenza virus subtypes and strains. Then we evaluated the abilities of the three AS ODNs to inhibit replication of the highly pathogenic avian influenza virus (HPAIV) subtype H5N1 both in vitro and in vivo.

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2. Materials and methods

2.1. Virus, cells and animals

Highly pathogenic AIV (A/Tiger/Harbin/01/2002) subtype H5N1 was obtained from the Changchun Institute of Veterinary Science (Changchun, China). The AIV was propagated in the allantoic cavity of ten-day-old embryonated specific pathogen-free eggs. Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC; Virginia, USA), and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA) with 10% fetal calf serum (Hyclone, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Hyclone, USA) at 37 °C with 5% CO_2. Six to eight-week-old, pathogen-free, female BALB/c mice (weighing 20–25 g) were used to assay antiviral activity in vivo. All experiments with the H5N1 virus were conducted in a biosafety level three laboratory facility at Changchun Institute of Veterinary Science.

2.2. Oligonucleotides

Each of the AS-ODNs were synthesized at the Takara Biotechnology Co. (Dalian, China) and purified with oligonucleotide purification columns. We employed the following strategy to design AS ODNs: (1) all AS-ODNs were phosphorothioate-modified to increase their in vivo stability; (2) AS-ODNs were meant to target the conserved, RNA binding region of the NP gene of AIV; (3) and the Gibbs Free energy value and T_m associated with the AS ODNs in reference to the NP gene were determined. Finally, selected AS-ODNs were submitted to a BLAST search against the human genome sequences to ensure that the human genome was not targeted. Three AS-ODN screenings were based on these strategies, and a scrambled oligo as control. The sequences of the NP-targeted AS-ODNs are shown in Fig. 1.

2.3. MDCK cell transfection and virus infection

AS-ODNs transfection with liposomes were performed according to instructions accompanying Lipofectamine™ 2000 (Invitrogen, USA), a lipid-based carrier. Non-transfected MDCK cells were used as a blank control. Briefly, MDCK cells were added to 24-well culture plates and cultured overnight at 37 °C, 5% CO_2; AS-ODNs were diluted with 50 μl DMEM (final concentration of the AS-ODNs was 4 μM). After 30 min of incubation at room temperature, the transfection complex (Lipofectamine™ 2000 and AS ODNs) was added to each well. At 6 h post-transfection, the MDCK cells were infected with 50 plaque-forming units (PFU) of the H5N1 virus. During 36 h post-transfection, the supernatants were harvested from the cultures and were ready for assay. The transfection efficiency was determined using a FITC-labeled AS ODNs and was analyzed using a flow cytometry assay on a BD FACSCalibur (BD, USA).

2.4. Viral titer assay

Viral titer was measured using 50% tissue culture infective dose ($TCID_{50}$) assays. The serial 10-fold dilutions of treated and viral samples were added onto a monolayer of MDCK cells in 96-well culture plates for 2 to 3 days. The cytopathic effect (CPE) was examined under a light microscope and the viral titers of the samples were calculated by the Reed–Muench method [18].

2.5. Real-time quantitative PCR assay

MDCK cells that had been transfected with AS-ODNs were harvested and total RNA was extracted using Trizol reagent (GIBCO, USA). The RNA was reversely transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA) and poly-T oligonucleotide primer (5′-TTTTTTTTTTTTTTT-3′, Takara, Japan). The levels of NP mRNA transcripts were determined by quantitative real-time PCR using the SYBR Premix Ex Taq Kit (Takara). The PCR reactions (20 μl) were made in duplicate, performed at 95 °C for 30 s, and subjected to 40 cycles of 95 °C for 5 s and 60 °C for 20 s on a Mx 3000P (Stratagene, USA). The sequences of special primers were forward 5′-TTCATCAGAGGGACAAGAGTGG-3′ and reverse 5′-TCAGTTCAAGAGTGTTGGAGTC-3′ for NP (109 bp), and forward 5′-ATGTATCAGTTGTGGATCTGACCTG-3′ and reverse 5′-ATGCCTGCTTCACTA- CCTTCTTG-3′ for GAPDH (86 bp). The relative levels of NP mRNA transcripts for the control of GAPDH were analyzed with MxPro QPCR software and calculated by the double standard curve method.

2.6. Indirect immunofluorescence assay (IFA)

Either non-infected (negative control) or infected (with H5N1 virus) MDCK cells were transfected with liposome supernatants were removed, and cells were fixed with 80% cold acetone for 20 min at 30 h post-infection. The cells were then washed three times with PBS, incubated with mouse anti-NP monoclonal antibody as the primary antibody for 1 h at 37 °C, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) as the secondary antibody for 1 h at 37 °C. Results were observed by fluorescence microscopy (Olympus, Japan).

2.7. Viral challenge in mice

Mice (eight for each group) were anesthetized with methyl ether and infected with the H5N1 influenza virus (10 LD_{50}) by intranasal instillation. After 8 h, the mice received a saline, liposome-mediated NP628 treatment. One hundred microliters of DMEM containing 30 nmol NP628 and 10 μl of liposome were given by intranasal administration. At 48 h post-infection, the mice were treated again as previously described to improve therapeutic effect. Body weight and mortality were monitored until all of the animals had either succumbed

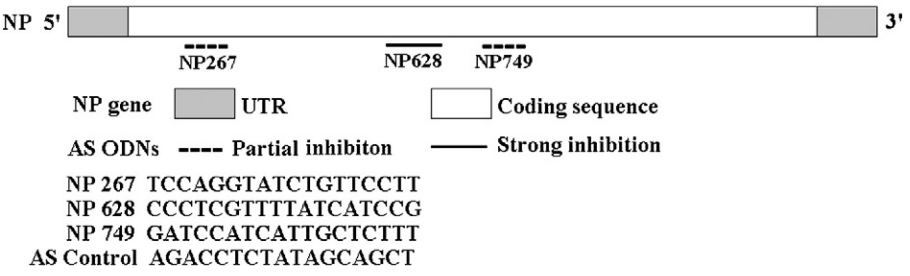

Fig. 1. Schematic diagram showing the location of each AS-ODN in the NP gene and its relative potency in inhibiting influenza virus replication in MDCK cells (based on data in Fig. 2).