5'-Triphosphate-Short Interfering RNA: Potent Inhibition of Influenza A Virus Infection by Gene Silencing and RIG-I Activation

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Limited protection of current vaccines and antiviral drugs against influenza A virus infection underscores the urgent need for development of novel anti-influenza virus interventions. While short interfering RNA (siRNA) has been shown to be able to inhibit influenza virus infection in a gene-specific manner, activation of the retinoic acid-inducible gene I protein (RIG-I) pathway has an antiviral effect in a non-gene-specific mode. In this study, we designed and tested the anti-influenza virus effect of a short double-stranded RNA, designated 3p-mNP1496-siRNA, that possesses dual functions: an siRNA-targeting influenza NP gene and an agonist for RIG-I activation. This double-stranded siRNA possesses a triphosphate group at the 5' end of the sense strand and is blunt ended. Our study showed that 3p-mNP1496-siRNA could potentially inhibit influenza A virus infection both in cell culture and in mice. The strong inhibition effect was attributed to its siRNA function as well as its ability to activate the RIG-I pathway. To the best of our knowledge, this is the first report that the combination of siRNA and RIG-I pathway activation can synergistically inhibit influenza A virus infection. The development of such dual functional RNA molecules will greatly contribute to the arsenal of tools to combat not only influenza viruses but also other important viral pathogens.

Influenza viruses cause annual epidemics and occasional pandemics that have severe consequences for human health and the global economy. An average of 200,000 hospitalizations occur each year in the United States due to respiratory and cardiac illness associated with influenza virus infections (28). Most human influenza infections are caused by influenza A viruses (IAV) of the orthomyxovirus family, with a single-stranded, negative-sense, segmented RNA genome (18). In order to evade the immune response and antiviral interventions, these viruses continue to evolve through genetic mutations caused by the error-prone RNA-dependent RNA polymerase and reassortment of gene segments between viruses. Vaccination and antivirals are the major interventions for prophylaxis and treatment of influenza. However, there are limitations to both measures. Annual vaccine programs can provide protection to most members of the population, but they are less effective for vulnerable groups such as the very young, the elderly, and immunocompromised individuals. From the therapeutic perspective, antivirals are available to treat influenza infection based on M2 or NA inhibition. Unfortunately, the emergence of antiviral-resistant influenza strains continues to be on the rise, limiting their efficacy in the long term (10). The rapid global spread of the 2009 pandemic H1N1 virus and the continued threat of avian influenza virus to humans underscore the urgent need to develop novel therapeutic strategies to treat influenza.

Short interfering RNAs (siRNAs) are found in many eukaryotes. They are short double-stranded (ds) RNAs usually 21 or 22 nucleotides (nt) long with a 2-nt overhang at the 3' end (4). Within cells, each siRNA unwinds into two single-stranded (ss) RNAs: the sense strand and the guide strand (antisense strand). The guide strand is then incorporated into the RNA-induced silencing complex (RISC), which degrades the target mRNA, and the sense strand is degraded (13, 19). Transfection of synthetic 21-nt siRNAs into mammalian cells can activate the siRNA pro-

cess and degrade targeting mRNA. Several studies have shown that siRNAs hold great potential as medical applications against the important human viral pathogens, such as influenza virus (5, 6, 29), human immunodeficiency virus (2, 11, 17), hepatitis B virus (7), hepatitis C virus (20), and dengue virus (1).

Within the host, the innate immune system is an important defense against viral infections. One of the major mechanisms of innate immune responses is to activate intracellular retinoic acid-inducible gene I protein (RIG-I) and its downstream pathways. This leads to type I interferon (IFN) production and activation of host antiviral activity. As a member of the DExD/H helicase protein group, RIG-I contains a helicase domain at its C terminus and two tandem caspase recruitment domains (CARDs) at the N terminus. Binding of dsRNA to the C-terminal RNA helicase domain of RIG-I induces a conformational change that exposes the N-terminal CARD domains to recruit mitochondrial antiviral signaling protein (MAVS), resulting in the activation of host innate immune responses (3, 27).

The exact structures of RNA agonists for RIG-I activation have been controversial (14). Recently, using fully chemical synthetic 5'-triphosphate RNAs, two groups independently identified the exact molecular features of RNA that are required for RIG-I recognition (22, 23). These results demonstrated that for RNA to act as an agonist the following three structures must be in place: (i) a triphosphate group (3p-) at the 5' end of the sense strand of the dsRNA; (ii) a dsRNA of more than 22 nucleotides; and (iii) a blunt 5' triphosphate end

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of the dsRNA (22, 23). Based on these findings, we rationalized that a combination of these two antiviral approaches, namely, suppression of influenza virus replication by siRNA targeting a viral gene and triggering of the host innate immune response by RIG-I activation, should lead to a more effective inhibition of influenza virus infection. In this study, we designed and generated a 3p-siRNA that simultaneously silences the influenza NP gene and activates the RIG-I-mediated interferon pathway. We report its potent inhibition effect on IAV infection.

MATERIALS AND METHODS

Cells and viruses. A549 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, Rockford, IL) containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA). Madin-Darby canine kidney (MDCK) cells were cultivated in minimal essential medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS. Influenza A/Puerto Rico/8/34 H1N1 (PR8), A/Texas/36/91 H1N1 (Tx91), and A/Halifax/210/2009 H1N1 (Halifax210) viruses were propagated in 11-day-old embryonated chicken eggs as previously described (24).

Antibodies. Rabbit polyclonal NS1 and NP antibodies were generated in our laboratory as previously described (25, 26). The other antibodies were purchased from the indicated companies: rabbit polyclonal anti-human RIG-I antibody (Enzo Life Sciences, Farmingdale, NY), monoclonal anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA), and IRDye 800-conjugated donkey polyclonal anti-mouse IgG and IRDye 680-conjugated goat anti-rabbit polyclonal IgG (LI-COR Biosciences, Lincoln, NE).

RNA synthesis. Based on the previous reports (5, 6, 22, 23), the following three single-stranded RNA oligonucleotides were chemically synthesized by Eurogentec (Liege, Belgium): mNP1496-AS-RNA (5'-GUCUCCGAAGAAAUAAGAUCUU-3'), mNP1496-S-RNA (5'-AAGGAUCUUAUUUCUUCGAGACUU-3'), and 3p-mNP1496-S-RNA (5'-ppp-AAGGAUCUUAUUUCUUCGAGACUU-3'). The above-described mNP1496-AS-RNA represents the antisense RNA strand (AS-RNA) or guide RNA strand, and mNP1496-S-RNA and 3p-mNP1496-S-RNA are the sense RNA strands (S-RNA). Notably, 3p-mNP1496-S-RNA containing triphosphate at the 5' end was synthesized from mNP1496-S-RNA using standard phosphoramidite solid-phase synthesis as described previously (23). NP1496-siRNA (sense strand, 5'-GGAUCUUAUUUCUUCGAGAGTdT-3'; guide strand, 5'-CUCCGAAGAAAUAAGAUCdT-3') was purchased from Qiagen (Valencia, CA), and poly(I:C) was obtained from Sigma-Aldrich (St. Louis, MO).

ds-siRNA annealing. Double-stranded siRNAs (ds-siRNAs) were annealed as previously described (6) with minor modifications. Briefly, all chemically synthesized ssRNAs were dissolved in RNase-free water to make a final solution of 10 μ g/ μ l. The mNP1496-S-RNA or 3p-mNP1496-S-RNA was mixed with the same amount of mNP1496-AS-RNA. The mixture was incubated in a beaker containing one liter of 95°C water and was allowed to cool down gradually to 25°C followed by a further incubation at 25°C for 3 h. The annealed dsRNAs were subjected to 16% TBE-acrylamide gel electrophoresis at 100 V in 0.5 $\times$ TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 4°C. RNA bands were visualized by staining with ethidium bromide. The annealed siRNAs were aliquoted and stored at -80°C until use.

siRNA transfection and infection. A549 cells or Vero cells (7 $\times$ 10⁴/well of a 24-well plate) were transfected with siRNAs using X-tremeGene siRNA transfection reagent (Roche, Basel, Switzerland) as described previously (12). Briefly, various amounts of siRNAs and X-tremeGene siRNA transfection reagents were diluted in Opti-MEM (Gibco, Carlsbad, CA) in two separate vials. The diluents were mixed immediately, and the mixture was further incubated at room temperature for 20 min before addition to cells at 30 to 40% confluence. The medium was changed to Opti-MEM 6 h posttransfection, and 24 h posttransfection, cells were infected with IAV

TABLE 1 Assignment of mice in each group^a

Group (n = 8)	Treatment (i.v.)	Infection (i.n.)
1	3p-mNP1496-siRNA	PR8 (5,000 PFU)
2	mNP1496-siRNA	PR8 (5,000 PFU)
3	Off-target siRNA	PR8 (5,000 PFU)
4	PBS	PR8 (5,000 PFU)
5	Off-target siRNA	PBS

^a i.v., intravenous; i.n., intranasal.

at a multiplicity of infection (MOI) of 1. Then, 24 h postinfection, supernatants and cell lysates were harvested and subjected to various assays.

siRNA transfection and RIG-I expression. A549 cells at 30 to 40% confluence in a 24-well plate were transfected with 10 pmol of each siRNA using X-tremeGene siRNA transfection reagent as described above. Total cell lysates were harvested at the indicated time points, and RIG-I protein expression was detected with rabbit anti-human RIG-I-specific antibody.

IFN- β luciferase assay. To examine whether 3p-mNP1496-siRNA would trigger beta interferon (IFN- β) activity, 293T cells (5 $\times$ 10⁵) were transfected with 0.5 μ g of pLuc125 plasmid, which encodes the luciferase gene under the control of the IFN- β promoter, 0.1 μ g of pTK-rLuc, which encodes renilla luciferase, and 10 pmol of siRNAs or 500 ng of poly(I:C) using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) as per the manufacturer's instruction. At 24 hours posttransfection, luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI). Relative luciferase activities were calculated as the ratio of firefly to renilla luciferase light unit. Each luciferase activity value is the average of three independent experiments.

In vitro RIG-I RNA binding and ATPase activity assay. RIG-I ATPase activity assay was performed as described previously (31) with minor modifications. Briefly, Flag-tagged human RIG-I protein was purified from 293T cells transfected with pCMV2-3 $\times$ Flag-RIG-I plasmid encoding a Flag-tagged human RIG-I protein gene. Various amount of dsRNAs were incubated with 1 μ g of the Flag-tagged RIG-I protein in 50 μ l of RNA binding buffer (20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 5% glycerol [vol/vol] and 1.5 mM dithiothreitol [DTT]) in a 96-well plate at 37°C for 1 h. Thereafter, fresh ATP was added to each well at 1 mM final concentration, and the plates were further incubated at 37°C for 15 min. Subsequently, 100 μ l of BIOMOL Green reagent (Enzo Life Science, Farmingdale, NY) was added to each well and incubated at room temperature for another 30 min to allow full development of the green color. Meanwhile, serial dilutions of phosphate standard in the binding buffer were also included. Signals were measured at an optical density of 655 nm (OD₆₅₅) using a microplate reader (Bio-Rad, Hercules, CA).

In vivo study. To assess the inhibitory effect of 3p-mNP1496-siRNA, 6- to 7-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were divided into five groups (Table 1). Mice in each group (n = 8) were intravenously injected with 100 μ g of 3p-mNP1496-siRNA, mNP1496-siRNA, off-target siRNA (Invitrogen, Grand Island, NY), or phosphate-buffered saline (PBS) mixed with *in vivo* DNA or RNA delivery reagent *in vivo*-jetPEI (Polyplus-transfection Inc., New York, NY) in a volume of 100 μ l according to the manufacturer's protocol. On day 1 post-siRNA treatment, mice in groups 1 to 4 were infected with 5,000 PFU PR8 intranasally. Mice in group 5 that received off-target siRNA mixed with *in vivo*-jetPEI were mock infected with PBS. On day 3 postinfection, mice were humanely euthanized and their lungs were collected for further analysis.

Histopathology, immunohistochemistry (IHC), and virus isolation from mouse lung. Tissue samples of left lung lobes were collected from all mice on day 3 post-virus infection. These were fixed in 10% neutral phosphate-buffered formalin, routinely processed, and stained with hematoxylin and eosin (H&E) for histopathologic examination. The immunohistochemical staining was conducted at Prairie Diagnostic Services, Saskatoon, Saskatchewan, Canada, using a technique adapted for an automated slide stainer as previously described (8). For these tissues, pro-

FIG 1 Schematic representations and characterization of siRNAs. (A) Sequence and structure of NP1496-siRNA. Both strands are 21 nt in length, forming a 19-bp double strand with two dTdT overhangs at both 3' ends. (B) Sequence and structure of mNP1496-siRNA. The sense strand and the guide strand are 25 nt and 23 nt in length, respectively, forming a 23-bp dsRNA with a UU overhang at the 3' end of the sense strand. The ds-siRNA is blunt ended at the 5' end of the sense strand. (C) Sequence and structure of 3p-mNP1496-siRNA. The sequence and structure are quite similar to those of mNP1496-siRNA except that the sense strand contains a triphosphate at the 5' end. (D) MALDI-TOF analysis of mNP1496-AS-RNA. The major peak at 7,321.8 represents mNP1496-AS-RNA (calculated molecular weight, 7,316). (E) MALDI-TOF analysis of mNP1496-S-RNA; the single peak at 7,922.8 represents mNP1496-S-RNA (expected molecular weight, 7,922). (F) MALDI-TOF analysis of 3p-mNP1496-S-RNA; expected molecular weight, 8,162; the peak at 8,157.1 Da represents 3p-mNP1496-S-RNA. There are several other peaks representing mNP1496-S-RNA without or with mono- or biposphate at its 5' end. The triphosphate mNP1496-S-RNA constitutes about 30% of the final product. (G) Ethidium bromide staining of annealed dsRNAs; the positions of dsRNA and ssRNA are indicated.

tease XIV (Sigma Chemical Co., St. Louis, MO) digestion was used for epitope retrieval and the primary antibody (goat anti-RNP type A influenza, V304-501-157; National Institute of Allergy and Infectious Diseases; Bethesda, MD) was used at dilutions of 1:5,000 and 1:10,000. Binding of the primary antibody was detected using biotinylated rabbit anti-goat

immunoglobulins and an avidin-biotin immunoperoxidase complex reagent with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Electron Microscopy Science, Ft. Washington, PA) for the chromogen.

For virus isolation, lung tissue was weighed and homogenized in MEM supplemented with antibiotic-antimycotic solution as described previ-

ously (15). Virus titer was determined by plaque assay of the supernatant of the homogenized tissue on MDCK cells.

RNA isolation from mouse lung and RT-qPCR. Total RNA from mouse lung tissues was isolated using TRIzol (Invitrogen, Grand Island, NY) per the manufacturer's instructions. The isolated RNA was further treated with RNase-free DNase I to remove trace genomic DNA. Total RNA were further purified with an RNeasy kit (Qiagen, Valencia, CA). The mRNA levels of RIG-I, IFN- β , and GAPDH were measured by reverse transcription qualitative PCR (RT-qPCR) conducted in an iCycler IQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Briefly, 2.5 μ g of each RNA was reverse transcribed using oligo(dT) followed by PCR using specific primers: mouse RIG-I-forward, 5'-CCACC TACATCCTCAGCTACATGA-3'; mouse RIG-I-reverse, 5'-TGGGCCC TTGTTGTTCTTCT-3'; mouse IFN- β -forward, 5'-GGAGATGACGGA GAAGATGC-3'; mouse IFN- β -reverse, 5'-CCCAGTGCTGGAGAAATT GT-3'; mouse GAPDH-forward, 5'-AACTTTGGCATTGTGGAAGG-3'; mouse GAPDH-reverse, 5'-ACACATTGGGGGTAGGAACA-3'. RIG-I and IFN- β expression was normalized to GAPDH expression in the same sample. Data are presented as relative gene expression to that of untreated cells using the formula $2^{(\Delta CT \text{ of gene} - \Delta CT \text{ of GAPDH})}$ (9), where CT is the threshold cycle. All RNA determinations have been assayed in triplicate and repeated three times.

RESULTS

Design of dual functional siRNAs. It has been reported that chemically synthesized siRNAs specific for conserved regions of the viral genome can potentially inhibit IAV infection in both cell lines and mice (5, 6, 29). Among these siRNAs, NP1496-siRNA that targets the NP gene showed the most profound effect on inhibition of virus replication (6). Our design of dual functional siRNA was therefore based on NP1496-siRNA sequence. Although NP1496-siRNA is an optimized siRNA, it does not have any features to serve as an RIG-I agonist (Fig. 1A). Indeed, Ge et al. have demonstrated that the inhibition of viral RNA accumulation by siRNA is not because of a cellular interferon response (6). To generate a dual functional siRNA, we chemically synthesized three ssRNAs. mNP1496-S-RNA is based on the NP1496-siRNA sense strand with 2 nt (AA) added to the 5' end and a 2-nt (AC) extension at the 3' end prior to the UU sequence of the NP1496-siRNA sense strand (Fig. 1B). mNP1496-AS-RNA is modified from the NP1496-siRNA guide strand with 2 nt (GU) added to the 5' end of the NP1496-siRNA guide strand (Fig. 1B). 3p-mNP1496-S-RNA has the same nucleotide sequence as mNP1496-S-RNA, except it contains triphosphate at the 5' end (Fig. 1C). Annealing of mNP1496-S-RNA and mNP1496-AS-RNA will generate a 23-bp ds-siRNA (Fig. 1B). Annealing of 3p-mNP1496-S-RNA and mNP1496-AS-RNA will generate a 23-bp ds-siRNA with triphosphate and a blunt end at its 5' end, in regard to RNA sense orientation (Fig. 1C); this molecule, designated 3p-mNP1496-siRNA, should fulfill the dual function of siRNA and RIG-I agonist.

All products were purified by high-performance liquid chromatography (HPLC) and verified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Fig. 1D, E, and F). It is worth noting that the yield efficiency of 3p-mNP1496-S-RNA was about 30% of the total input of mNP1496-S-RNA. The synthesized ssRNA and annealed dsRNA were subjected to 16% TBE-acrylamide gel electrophoresis and were visualized by ethidium bromide staining. Figure 1G showed that the annealed ds-siRNA migrated at a different position than ssRNA. Since the binding ability of ethidium bromide to dsRNA is much stronger than that to ssRNA, the dsRNA bands are much brighter than the

FIG 2 Inhibition of IAV infection by mNP1496-siRNA and 3p-mNP1496-siRNA. A549 cells were transfected with mNP1496-siRNA and NP1496-siRNA at concentrations of 0, 5, 10, 15, or 20 pmol in a 24-well plate. Twenty-four hours later, cells were infected with PR8 virus at an MOI of 1. At 24 h postinfection, cells were harvested and the expression of viral NP and NS1 proteins was assayed by Western blotting. Meanwhile, the levels of β -actin were assessed as internal loading controls. NP and NS1 protein levels were first normalized to the level of β -actin in each sample and then compared to that in the sample without any siRNA treatment and expressed as percentages of changes.

ssRNA bands. It is also clear that almost all ssRNAs have been converted to dsRNAs (Fig. 1G).

Modified mNP1496-siRNA executed inhibition on influenza A virus infection. To make mNP1496-siRNA, we have modified NP1496-siRNA as follows: on the basis of the conserved region of the NP RNA segment, we extended the length of dsRNA from 19 nt to 23 nt and generated a blunt end at the 5' end of the sense strand (Fig. 1A and B). We then asked whether the modified siRNA had any inhibition effects on IAV infection. A549 cells were transfected with 0, 5, 10, 15, or 20 pmol of each siRNA, and at 24 h posttransfection, the treated cells were infected with PR8 at an MOI of 1. At 24 h postinfection, cell lysates were prepared and subjected to Western blotting with antibodies specific for NP and NS1 proteins. Levels of β -actin were monitored as a loading control. As shown in Fig. 2, significant amounts of NP and NS1 proteins were detected in the siRNA-untreated and virus-infected cells (lane 1); these levels were normalized to that of the β -actin in the same sample and were set as references (100%). Transfection of NP1496-siRNA and mNP1496-siRNA led to a dose-dependent inhibition of viral protein synthesis, where at 20 pmol, the inhibition effect is the most profound (lanes 5 and 9). The modified mNP1496-siRNA showed inhibition capability similar to that of optimized NP1496-siRNA. Virus titers in the supernatants of 5- and 10-pmol treated samples were evaluated by plaque assay. As shown in Fig. 3B, the virus titers in NP1496-siRNA- and mNP1496-siRNA-treated cells were similar (5.3×10^6 versus 4.6×10^6 PFU/ml at 5 pmol and 8.0×10^5 versus 7.0×10^5 PFU/ml at 10 pmol). These titers are significantly lower than that in untreated samples (1.1×10^7 PFU/ml). These results demonstrated that the extension of dsRNA and blunt-end modifications did not change the inhibition efficiency of the mNP1496-siRNAs.

3p-mNP1496-siRNA showed a stronger inhibition effect on IAV infection than that of mNP1496-siRNA in A549 cells. We have shown that mNP1496-siRNA had inhibition efficiency sim-

FIG 3 Inhibition of IAV infection by 3p-mNP1496-siRNA is more profound than that of mNP1496-siRNA. (A) A549 cells were transfected with 3p-mNP1496-siRNA or mNP1496-siRNAs at concentrations of 0, 0.6, 1.2, 5, or 10 pmol in a 24-well plate. Twenty-four hours later, cells were infected with PR8 virus at an MOI of 1. At 24 h postinfection, cells were harvested and the levels of NP, NS1, and β -actin were evaluated by Western blotting. The percentage of protein level changes was calculated as described in legend to Fig. 2. (B) Virus titers in the supernatant harvested in the above-described experiments were determined by plaque assay. Error bars represent the standard deviations of results from three independent experiments. (C) A549 cells were transfected with 5 pmol off-target siRNA or 3p-mNP1496 siRNA in a 24-well plate. At 24 hours posttransfection, cells were infected with TX91 or Hallifax at an MOI of 1. At 24 hours postinfection, NP, NS1, and β -actin levels were evaluated by Western blotting using specific antibodies.

ilar to that of optimized NP1496-siRNA. Motivated by these results, we synthesized 3p-mNP1496-siRNA which was further modified from mNP1496-siRNA by the addition of triphosphates at the 5' end of the sense strand. Since the 3p-mNP1496-siRNA meets all the basic criteria for full RIG-I binding and activation, we speculated that 3p-mNP1496-siRNA should exert dual functions of siRNA and RIG-I agonist and thus would inhibit IAV infection more potently. We therefore tested the efficiency of 3p-mNP1496-siRNA in inhibiting IAV infection.

A549 cells were transfected with various amounts of 3p-mNP1496-siRNA or mNP1496-siRNA. At 24 hours posttransfection, cells were infected with PR8 at an MOI of 1. At 24 h postinfection, cell lysates were subjected to Western blotting with polyclonal antibodies specific for NP or NS1. Virus titers in the supernatant were determined by plaque assay. As seen in Fig. 3A, treatment of cells with 0.6 and 1.2 pmol of mNP1496-siRNA did not significantly inhibit viral protein synthesis (lanes 2 and 3). With 5 and 10 pmol of mNP1496-siRNA treat-

FIG 4 Activation of RIG-I and IFN- β pathway by 3p-mNP1496-siRNA. A549 cells were transfected with 5 pmol of 3p-mNP1496-siRNA (A) or mNP1496-siRNA (B) and harvested at the indicated times. Total cell lysates were subject to Western blotting with antibodies specific for RIG-I and β -actin. (C) RIG-I ATPase activities after incubating with 3p-mNP1496-siRNAs or mNP1496-siRNA were measured by BIOMOL Green reagent. Three independent experiments were performed, and the mean values are plotted, with the error bars representing standard deviations. (D) 293T cells were transfected with plasmids pLuc125 and pTK-rLuc together with 10 pmol of siRNAs or 500 ng of poly(I:C). Twenty-four hours later, luciferase activity was measured using the dual-luciferase reporter assay system. Relative luciferase activities were calculated as the ratio of firefly to renilla luciferase light units. Fold induction was calculated as the ratio of relative luciferase activity in each sample to that in siRNA untransfected cells.

ment, viral protein synthesis was reduced to about 55% and 20% (lanes 4 and 5), respectively, compared to that in untreated cells (lane 1). In contrast, while treatment of cells with 0.6 pmol of 3p-mNP1496-siRNA led to a 40% reduction of viral protein synthesis, 1.2 pmol of 3p-mNP1496-siRNA could significantly inhibit viral protein synthesis to about 20% (lane 7). Furthermore, viral proteins were barely detectable in the samples that were treated with 5 or 10 pmol of 3p-mNP1496-siRNA (lanes 8 and 9). Consistent with these results, Fig. 3B showed that virus titers in 3p-mNP1496-siRNA-treated samples are dramatically decreased compared to those in mNP1496-siRNA-treated samples (7.3×10^5 versus 9.4×10^6 PFU/ml at 1.2 pmol; 1.9×10^5 versus 4.6×10^6 PFU/ml at 5 pmol, and 1.1×10^5 versus 7.0×10^5 PFU/ml at 10 pmol).

In order to test whether 3p-mNP1496-siRNA would inhibit other strains of IAV infection, A549 cells were treated with 5 pmol of 3p-mNP1496-siRNA and were infected with Halifax 210 and Tx91 at an MOI of 1, and viral protein synthesis was determined by Western blot analysis. As seen in Fig. 3C, in agreement with the results obtained by using PR8 virus, transfection of 3p-mNP1496-siRNA resulted in a significant reduction in viral NP protein and NS1 protein synthesis. Cellular β -actin levels were not altered by siRNA transfection and virus infection.

3p-mNP1496-siRNA induces RIG-I activation and IFN- β transcription. Since 3p-mNP1496-siRNA and mNP1496-siRNA contain the same guide strand (Fig. 1B and C) and this is incorporated into RISC to degrade target mRNA, they should have the same siRNA effects. However, we have shown that the inhibition activity of 3p-mNP1496-siRNA was much stronger than that of mNP1496-siRNA. In order to investigate if this effect on inhibition was due to the activation of RIG-I and IFN- β , two experi-

ments were performed to assess RIG-I activation. First, A549 cells were transfected with 5 pmol of 3p-mNP1496-siRNAs and harvested at the indicated times. Total cell lysates were subject to Western blotting with antibodies specific for RIG-I and β -actin. As seen in Fig. 4A, RIG-I protein was detectable at 4 h post-transfection, and the elevated level of RIG-I was sustained until 60 h posttransfection. In contrast, both off-target siRNA and mNP1496-siRNA were unable to induce RIG-I expression (Fig. 4B). In the second experiment, we evaluated RIG-I ATPase activity, which is critical for antiviral responses (16). Binding of dsRNA to RIG-I activates its helicase ATPase, which will convert ATP to ADP. The free phosphates released from the ATPase hydrolysis was measured with the BIOMOL Green reagent. As seen in Fig. 4C, incubation of 3p-mNP1496-siRNA with RIG-I led to an increased activity of ATPase in a dose-dependent manner. In contrast, binding of mNP1496-siRNA did not induce any RIG-I ATPase activity.

We further investigated whether the IFN- β pathway was activated. To this end, 293T cells were transfected with pLuc125 plasmid and pTK-rLuc, together with different siRNAs. Poly(I:C) is known to be an inducer of IFN- β (31) and thus was included as a positive control. Luciferase activity was determined at 24 h post-transfection. Fold induction of the promoter activity was obtained by normalizing to that in the mock siRNA-treated cells. As shown in Fig. 4D, poly(I:C) and 3p-mNP1496-siRNA transfection led to a significant induction of IFN- β promoter activity. Specifically, 7.3- and 13.3-fold induction were obtained in poly(I:C)- and 3p-mNP1496-siRNA-transfected cells, respectively. In contrast, transfection of off-target siRNA and NP1496-siRNA did not induce IFN- β promoter activity. These data suggest that the stronger inhibition effect of 3p-mNP1496-siRNA can be attributed to RIG-I and IFN- β activation.

FIG 5 Viral inhibition effect of 3p-mNP1496-siRNA in Vero cells. Vero cells were transfected with different amounts of mNP1496-siRNA or 3p-mNP1496-siRNA for 24 h. Cells were then infected by PR8 virus for another 24 h. (A) NP, NS1, and β -actin levels in the cells were determined by Western blotting. NP and NS1 protein levels were measured as described in the legend to Fig. 2. (B) Virus titers in the supernatant were determined by plaque assay. Error bars represent the standard deviations of results from three independent experiments.

3p-mNP1496-siRNA showed an inhibition effect on IAV replication similar to that of mNP-1496-siRNA in Vero cells. We have shown that 3p-mNP1496-siRNA had stronger inhibition of IAV infection than mNP1496-siRNA on A549 cells, and it is very likely correlated with the RIG-I-mediated IFN- β secretion pathway. To further support this finding, Vero cells which are defective in IFN- β expression were used. These Vero cells were transfected with different amounts either of mNP1496-siRNA or 3p-mNP1496-siRNA; 24 h later, cells were infected with PR8 virus at an MOI of 1. At 24 h postinfection, the supernatant was harvested for virus titration and cell lysates were subjected to Western blotting using antibodies specific for NP or NS1 protein. As seen in Fig. 5A, at the concentration of 10 pmol, both 3p-mNP1496-siRNA and mNP1496-siRNA reduced viral protein synthesis to about 40% (lanes 3 versus 6), and at 20 pmol, both siRNAs could completely inhibit viral protein synthesis (lanes 4 versus 7). Figure 5B showed that virus titers are similar in mNP1496-siRNA- and 3p-mNP1496-siRNA-treated Vero cells (5.13×10^5 versus 5.17×10^5 PFU/ml at 5 pmol and 2.47×10^5 versus 2.53×10^5 PFU/ml at 10 pmol).

3p-mNP1496-siRNA treatment also showed potent inhibition of IAV replication in mice. In order to test whether 3p-mNP1496-siRNA strongly inhibited IAV infection *in vivo*, mice were intravenously injected with 100 μ g of 3p-mNP1496-siRNA,

mNP1496-siRNA, off-target siRNA, or PBS mixed with *in vivo*-jetPEI. At 24 hours post-siRNA treatment, mice in groups 1 to 4 were intranasally infected with PR8 (Table 1). Three days post-virus infection, mouse lungs were collected and subjected to the assays to detect virus titer, viral protein expression, RNA extraction, and pathology. During the course of the experiment, mice in group 5 that received only off-target siRNA did not show any side effects in terms of daily activity and weight loss (data not shown). Mice that received mNP1496-siRNA had a lung virus titer that is about 4-fold lower than those in PBS-treated and off-target siRNA-treated groups (1.45×10^8 PFU/ml/g versus 6.33×10^8 PFU/ml/g and 6.21×10^8 PFU/ml/g; Fig. 6A). Mice that received 3p-mNP1496-siRNA treatment had a 10-fold decrease in lung titer compared to those in the PBS and off-target treatment groups (5.13×10^7 PFU/ml/g versus 6.33×10^8 PFU/ml/g and 6.21×10^8 PFU/ml/g; Fig. 6A). In agreement with these results, Western blotting with lung homogenates showed that NP and NS1 protein levels were reduced in both mNP1496-siRNA- and 3p-mNP1496-siRNA-treated groups. However, 3p-mNP1496-siRNA has a more profound inhibition effect (Fig. 6B). The body weight of mice after virus infection was monitored. Figure 6C showed that PR8 virus caused rapid weight loss in mice that received pretreatment of PBS or off-target siRNA. In contrast, pretreatment of mice with mNP1496-siRNA and 3p-mNP1496-siRNA protected mice from

FIG 6 Inhibition of influenza A virus infection by siRNAs in mice. Mice were intravenously administered respective siRNAs and then intranasally infected by PR8 virus. On day 3 postinfection, lung tissue was collected and homogenized. (A) Virus titers were determined on MDCK cells by plaque assay. Mean titer from each group ($n = 8$) were plotted, with the error bars representing standard deviations. Comparison of mouse lung virus titer between control group and testing groups was performed by one-way analysis of variance (ANOVA) in Prism. ***, $P < 0.001$. (B) NP, NS1, and β -actin levels were determined by Western blotting. (C) Changes of body weight after virus infection. Error bars represent the standard deviations of results for 8 mice in one group.

dramatic weight loss and 3p-mNP1496-siRNA had more profound protection.

Inhibition of IAV infection by 3p-mNP1496-siRNA was also evaluated by histopathology. Representative results are shown in Fig. 7. Mice in the PBS-, off-target siRNA-treated, and virus-infected groups developed characteristic influenza lesions, including severe necrotizing bronchiolitis and interstitial pneumonia

(Fig. 7A; PBS-treated sample shown). Comparatively, mice in the mNP1496-siRNA-treated group developed moderate necrotizing bronchiolitis and interstitial pneumonia (Fig. 7B). Mice from the 3p-NP1496-siRNA-treated group and the negative-control group (off-target siRNA-treated and noninfected group) had no inflammation and minimal changes to the bronchiolar epithelial cells, including apoptosis and sloughing (Fig. 7C, 3p-NP1496-siRNA-treated group shown). The lung sections were also evaluated for the presence of IAV-specific antigen using IHC staining. There was strong bronchiolar epithelial and interstitial immunoreactivity in lung for the untreated and off-target siRNA-treated mice (Fig. 7D) and moderate bronchiolar epithelial and interstitial immunoreactivity in lungs from mNP1496-siRNA-treated mice (Fig. 7E). There was rare bronchiolar epithelial immunoreactivity in lungs of 3p-NP1496-siRNA-treated mice (Fig. 7F) and no immunoreactivity in the lung of the negative control (group 5; data not shown).

3p-mNP1496-siRNA treatment also induced RIG-I and IFN- β expression in mice. Total RNA was isolated from mouse lung. Induction of RIG-I and IFN- β mRNA was measured by real-time PCR. As seen in Fig. 8, injection of 3p-mNP1496-siRNA into mice induced a 23.8-fold increase of RIG-I mRNA (Fig. 8A) and 54.2-fold of IFN- β mRNA expression (Fig. 8B). Injection of off-target siRNA as well as mNP1496-siRNA did not induce any RIG-I and IFN- β mRNA expression. Thus, *in vivo* experiments demonstrated that 3p-mNP1496-siRNA induced RIG-I and IFN- β expression and has a more potent inhibition effect on IAV replication.

Therapeutic effect of 3p-mNP1496-siRNA on ongoing influenza virus infection. To investigate the therapeutic effect of 3p-mNP1496-siRNA on virus infection, A549 cells were first infected by PR8 at an MOI of 1. At different hours postinfection, cells were transfected with 5 pmol of 3p-mNP1496-siRNA. At 24 h postinfection, supernatant was harvested for virus titration and cells were lysed for Western blotting. As seen in Fig. 9, when 3p-mNP1496-siRNA was administered at 2 h postinfection, viral protein synthesis (Fig. 9A, lane 2) and virus yield (Fig. 9B) were significantly inhibited compared to those in nontreated, infected cells. When 3p-mNP1496-siRNA was applied at 4 and 6 h postinfection, the inhibition effects were reduced in a time-dependent manner. No inhibitory effect was observed at 8 h postinfection. Figure 9A also showed that compared to the RIG-I expression level in the 2-h postinfection sample, the level decreased to 71% and 21% in samples from 6 and 8 h postinfection, respectively.

DISCUSSION

Human influenza virus infections result in an estimated 3 to 5 million cases of severe illness and between 250,000 and 500,000 deaths every year around the world. Although current vaccination programs and antiviral drugs could provide some protection against influenza virus infection, development of new prophylactic and therapeutic tools is still needed. siRNA is a powerful tool that can specifically inhibit gene expression through degradation of target mRNA. Ge et al. designed and tested a total of 20 siRNAs targeting different influenza genes and found that those that target NP are especially effective (6). Further *in vivo* studies showed that siRNA targeting NP nt 1496 to 1514 (NP1496-siRNA) could inhibit various strains of influenza virus infection in mice (5, 29). The inhibition was sequence and virus specific, indicating that the

FIG 7 Microscopic lung lesions at 3 days postinfection. (A) Lung section from a mouse in the PBS-treated and virus-infected group, with severe necrotizing bronchiolitis and interstitial pneumonia. (B) Lung section from a mouse in the mNP1496-siRNA-treated and virus-infected group, with moderate necrotizing bronchiolitis and interstitial pneumonia. (C) Lung section from a mouse in the 3p-mNP1496-siRNA-treated and virus-infected group; no severe pathological changes were observed. (D) Serial lung section of a PBS-treated mouse with strong bronchiolar epithelial and interstitial immunoreactivity in lung. (E) Serial section of an mNP1496-siRNA-treated mouse with moderate bronchiolar epithelial and interstitial immunoreactivity in lung. (F) Serial section of a 3p-mNP1496-siRNA-treated mouse with rare bronchiolar immunoreactivity (arrowhead). In panels A, B, and C, micrographs are of H&E-stained sections, whereas in panels D, E, and F the sections are IHC stained using anti-influenza antibodies. Scale bars, 200 μ m.

antiviral effects of the siRNA are not mediated by IFN- β . Recently, using T7 RNA polymerase synthesized partial double-strand 5'ppp-RNA, Ranjan et al. have shown that the 5'ppp-RNA can inhibit drug-resistant avian H5N1, 1918, and 2009 pandemic influenza viruses in a RIG-I- and type I IFN-dependent manner in cells and in mice (21). However, the inhibition effect was not in an siRNA-dependent manner, since the sequences of the 5'ppp-RNA are not complementary to any influenza virus mRNAs.

Here, we are interested in developing a special siRNA which can play dual antiviral roles: viral gene-specific silencing and non-gene-specific RIG-I activation. To achieve this goal, we chemically synthesized three short RNA molecules; after annealing, they formed two dsRNA molecules, namely, mNP1496-siRNA and 3p-mNP1496-siRNA. The only difference between these two dsRNA molecules is that mNP1496-siRNA bears a hydroxyl group at the 5' end of the sense strand, whereas 3p-mNP1496-siRNA possesses a triphosphate at the 5' end of the sense strand.

We first tested whether the modified mNP1496-siRNA still retained the typical siRNA function. By using NP1496-siRNA (29) as a positive control, dose titration experiments showed that while mNP1496-siRNA had an inhibition effect similar to that of NP1496-siRNA (Fig. 2), 3p-mNP1496-siRNA exerted the most profound inhibition activity (Fig. 3). These results suggested that the aforementioned modifications of mNP1496-siRNA did not change its siRNA inhibition efficiency and 3p-mNP1496-siRNA might exert dual antiviral functions. To further demonstrate that 3p-mNP1496-siRNA could activate the RIG-I-mediated IFN- β pathway, we assessed RIG-I protein levels after transfection of siRNAs; RIG-I ATPase activity after binding to siRNA, and IFN- β promoter activity after stimulating with siRNA. Our results demonstrated that 3p-mNP1496-siRNA, but not mNP1496-siRNA,

could induce RIG-I and IFN- β activities, which contribute to the efficient inhibition of influenza virus infection (Fig. 4). The results that both mNP1496-siRNA and 3p-mNP1496-siRNA exerted similar inhibition effects of IAV on Vero cells (Fig. 5) further confirmed this notion.

In animal studies, we have demonstrated the pretreatment of mice with dual functional 3p-mNP1496NP-siRNA could significantly reduce virus load and virus-induced pathogenesis (Fig. 6 and 7). Again, the *in vivo* experiment was in line with the *in vitro* results that dual functional siRNA exerted a more profound effect than the monofunctional siRNA. It is notable that when chemically adding the triphosphate group into the 5' end of an RNA molecule, the efficiency is only about 30%, i.e., the yield of 3p-mNP1496-S-RNA was only about 30% of the total input of mNP1496-S-RNA. This is mainly due to the inherent low efficiency of the chemical modification. Taking this into consideration, it is conceivable that a more pure formulation of 3p-siRNA would have a more potent antiviral effect.

We also investigated the therapeutic effect of 3p-mNP1496-siRNA on inhibition of influenza virus infection. The inhibitory effect was achieved when 3p-mNP1496-siRNA was given at 2, 4, and 6 h post-virus infection. When 3p-mNP1496-siRNA was given at 8 h post-virus infection, no viral inhibition was achieved. Concomitantly, RIG-I expression was reduced in this sample; this might contribute to the no-inhibition effect. In addition, at 8 h postinfection, virus has completed one life cycle, and therefore the increased amount of mRNA may have made the siRNA function of 3p-mNP1496-siRNA less efficient.

One challenge in the use of the siRNA for the treatment of IAV infection is how to deliver the siRNA to the target tissues efficiently. Administration of 3p-siRNA would result in an induction

FIG 8 Expression of RIG-I and IFN- β mRNAs in the lung. Total RNA was extracted from mouse lung. RIG-I (A) and IFN- β mRNA (B) levels were measured by quantitative real-time RT-PCR and were normalized to GAPDH expression in the same sample. Fold induction was achieved by setting the PBS-treated and virus-infected group as the reference.

of innate immune responses of the host; therefore, even if less 3p-siRNA is delivered to the virus replication site, it will still exert antiviral activity through the RIG-I activation. Of course, improvement of RNA delivery will greatly enhance the antiviral effect of dual functional siRNA. Transfection reagents and specific vectors are two major media for delivering siRNA into animals. The relatively higher price limited the use of cationic polymer-based transfection reagents in animals. It has been reported that lentivirus vector-based short hairpin RNAs (shRNAs) showed good delivery of NP1496-siRNA into mice (5, 29); therefore, how to establish a vector system in which 3p-mNP1496-siRNA can be highly expressed and delivered needs to be investigated in the future. The other challenge in the use of 3p-siRNA is the cost of chemical synthesis of 3p-RNA. Although T7 polymerase could synthesize RNA bearing triphosphate at the 5' end, the self-coded 3'-extension runoff transcription of T7 RNA polymerase (30) makes it difficult to control the 3' end and get homogenous structure for our studies. Currently, chemical synthesis and modification are still the best ways to obtain homogenous 3p-mNP1496-siRNA.

FIG 9 Therapeutic effect of 3p-mNP1496-siRNA. (A) A549 cells were infected with PR8 at an MOI of 1. At indicated times postinfection cells were transfected with 5 pmol of 3p-mNP1496-siRNA. At 24 h postinfection, cells were lysed and subjected to Western blotting using antibodies specific for NP, NS1, RIG-I, and β -actin. (B) Virus titers in the supernatant were determined on MDCK cells by plaque assay. Error bars represent the standard deviations of results from three independent experiments.

It is worth noting that even though we developed a 3p-mNP1496-siRNA which is specific for anti-IAV infection, the strategy can be implemented with other important viral pathogens.

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