

## RNA Interference-Mediated Silencing of the Respiratory Syncytial Virus Nucleocapsid Defines a Potent Antiviral Strategy<sup>∇</sup>

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**We describe the design and characterization of a potent human respiratory syncytial virus (RSV) nucleocapsid gene-specific small interfering RNA (siRNA), ALN-RSV01. In in vitro RSV plaque assays, ALN-RSV01 showed a 50% inhibitory concentration of 0.7 nM. Sequence analysis of primary isolates of RSV showed that the siRNA target site was absolutely conserved in 89/95 isolates, and ALN-RSV01 demonstrated activity against all isolates, including those with single-mismatch mutations. In vivo, intranasal dosing of ALN-RSV01 in a BALB/c mouse model resulted in potent antiviral efficacy, with 2.5- to 3.0-log-unit reductions in RSV lung concentrations being achieved when ALN-RSV01 was administered prophylactically or therapeutically in both single-dose and multidose regimens. The specificity of ALN-RSV01 was demonstrated in vivo by using mismatch controls; and the absence of an immune stimulatory mechanism was demonstrated by showing that nonspecific siRNAs that induce alpha interferon and tumor necrosis factor alpha lack antiviral efficacy, while a chemically modified form of ALN-RSV01 lacking measurable immunostimulatory capacity retained full activity in vivo. Furthermore, an RNA interference mechanism of action was demonstrated by the capture of the site-specific cleavage product of the RSV mRNA via rapid amplification of cDNA ends both in vitro and in vivo. These studies lay a solid foundation for the further investigation of ALN-RSV01 as a novel therapeutic antiviral agent for clinical use by humans.**

Human respiratory syncytial virus (RSV) is an ubiquitous virus and the most common cause of serious lower respiratory tract infections in infants and young children worldwide, as well as an important pathogen in elderly individuals and immunocompromised patients (5, 10, 11, 18–21, 62, 64). The worldwide disease burden associated with RSV infection is considerable. RSV is the leading cause of hospitalization for infants (44), with infection rates approaching 70% in the first year of life (25). Approximately 30% of RSV-infected children develop lower respiratory tract infections. RSV results in the hospitalization of approximately 3% of previously healthy infants within their first year of life and a substantially greater percentage of infants and children with underlying diseases (8). RSV is a common cause of childhood bronchiolitis and has been implicated in the development and exacerbation of asthma and reactive airway disease in childhood (39, 50, 51, 54).

Despite nearly four decades of research, no RSV vaccine approach has been successful at conferring protection at a level that exceeds the incomplete protection afforded by natural infection. Currently, the only antiviral approved for use for the treatment of RSV infection is ribavirin; but due to its terato-

genicity, limited efficacy, and poorly understood mechanism of action, it has very limited use (43, 73). Prophylactic therapies include the use of the approved humanized monoclonal antibody palivizumab (Synagis), which targets the fusion protein of RSV (2, 27, 36). While this antibody is effective, it is used only for the treatment of high-risk patient populations, including premature infants (3, 48, 66), and as an inhibitor of viral fusion, it may be of limited benefit for the treatment of an established RSV infection. Thus, there is a clear need for an alternative approach to the development of a novel anti-RSV therapeutic agent.

RNA interference (RNAi) is a posttranscriptional mechanism of gene silencing first described as an innate response to viral infections in plants and subsequently in all higher-order eukaryotes (7, 30). RNAi involves the target-specific degradation of RNA transcripts following the incorporation of small double-stranded RNA into the RNA-induced silencing complex. A major advance in the field of RNAi was the demonstration that synthetic double-stranded, small interfering RNAs (siRNAs) were functionally active against target mRNA transcripts in mammalian cells (17). These findings have led to the emergence of a new field of drug discovery with RNAi therapeutics that target a wide variety of human diseases, ranging from cancer to metabolic diseases and viral infections (13). Recent studies have demonstrated the efficacy of siRNAs in inhibiting several viruses, in vitro and in vivo, including hepa-

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titis C virus (9, 59, 75), hepatitis B virus (4, 24, 69), West Nile virus (38, 47, 65), the severe acute respiratory syndrome-associated coronavirus (31, 76, 77, 81), influenza virus (23, 70), and RSV (6, 82), among others. For RSV, Bitko et al. (6) and Zhang et al. (82) have demonstrated the *in vitro* and *in vivo* inhibition of RSV by targeting the phosphoprotein (P protein) and nonstructural (NS1) protein siRNAs, respectively, confirming the feasibility of using a strategy that targets siRNA to achieve activity against this virus. However, the P protein siRNA is limited by its specificity to one particular strain of RSV, while the inhibition of the NS1 protein siRNA of RSV may be attributed to immune modulation, which results in the more robust clearance of the virus by the host rather than the direct targeting of the viral RNA. Furthermore, in both cases, definitive proof of an RNAi-mediated mechanism of antiviral activity remains to be established. Here we describe the pharmacology of a highly potent and specific human RSV siRNA with a broad spectrum of activity that demonstrates antiviral activity *in vitro* and *in vivo* via an RNAi mechanism of action.

#### MATERIALS AND METHODS

**Animals.** Six- to 8-week old, pathogen-free female BALB/c mice were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). The mice were housed in microisolator cages and were fed sterilized water and food *ad libitum*.

**Virus preparation, cell lines, and viral titer determination.** Vero E6 cells were maintained in tissue culture medium consisting of Dulbecco's modified Eagle medium (DMEM; Gibco Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). RSV A2 and RSV B1 were prepared in Vero E6 cells. Briefly, confluent Vero E6 cells (American Type Culture Collection, Manassas, VA) in serum-free DMEM were infected with RSV at a multiplicity of infection of 0.1. The virus was allowed to adsorb for 1 h at 37°C, after which tissue culture medium was added. Infected cells were incubated for 72 to 96 h at 37°C until a cytopathic effect of >90% was observed by light microscopy. Infected cells were harvested by removal of the medium and replacement with a minimal volume of serum-free DMEM, followed by three freeze-thaw cycles at -70°C and 4°C. The contents were collected and centrifuged at 4,000 × g for 20 min at 4°C to remove the cell debris, and the titer was determined by an immunostaining plaque assay, as described previously (72). Briefly, Vero E6 cells were infected with serial dilutions of stock RSV, the virus was allowed to adsorb for 1 h at 37°C, and then the cells were overlaid with 2% methylcellulose medium (DMEM supplemented with 2% fetal bovine serum, 1% antibiotic-antimycotic solution, and 2% methylcellulose). After 5 days at 37°C in 5% CO<sub>2</sub>, the plates were removed and the cells were fixed with ice-cold acetone-methanol (60:40). The cells were blocked with a universal blocking reagent (Powerblock; Biogenix, San Ramon, CA) and incubated with anti-RSV fusion (F)-protein monoclonal antibody (clone 131-2A; Millipore-Chemicon, Temecula, CA) diluted 1:200, followed by the addition of goat anti-mouse immunoglobulin G whole-molecule alkaline phosphatase secondary antibody. The reaction was developed with an alkaline phosphatase substrate kit (Vector Black; Vector Laboratories, Burlingame, CA), and plaques were visualized and counted under a light microscope. For RSV primary isolate cultures, samples were obtained from John DeVincenzo from the University of Tennessee, Memphis. RSV isolates were obtained from RSV-infected children diagnosed either by a conventional direct fluorescent-antibody method or by a rapid antigen detection method in the Le Bonheur Children's Medical Center Virology Laboratory in Memphis, TN. Nasal secretions were collected by aspiration, and isolates from the secretions were grown and passaged in HEP-2 cells and harvested when the cytopathic effect was 90%. Individual aliquots of the supernatant containing RSV were then subjected to nucleic acid extraction with a QIAmp viral RNA minikit, according to the manufacturer's protocol (Qiagen, Valencia, CA). RSV isolates were also obtained from Mark Van Ranst from the University of Leuven, Leuven, Belgium, and Larry Anderson from the Centers for Disease Control and Prevention, Atlanta, GA.

**RSV-specific siRNA selection.** An analysis of the sequences in one of the National Center for Biotechnology Information databases was performed with the Basic Local Alignment Search Tool (BLAST). In that analysis, a sequence comparison algorithm was used to search the sequence databases for optimal

local alignments to a query (1). In this case, the query was the 19-nucleotide (nt) sequence comprising the sense or antisense strand of ALN-RSV01, excluding the dTdT overhang. The Reference Sequence (RefSeq) database provides a comprehensive, integrated, nonredundant set of sequences, including genomic DNA, transcript (RNA), and protein products, and is updated weekly (53). Only siRNAs that showed no significant homology with any sequence from the RefSeq database were selected for synthesis and further study.

***In vitro* RSV inhibition assay.** Vero cells were grown in 24-well plates in a 5% CO<sub>2</sub> humidified incubator at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin (BioChrom, Cambridge, United Kingdom) to 80% confluence. siRNAs were diluted to the indicated concentrations in 50 μl Opti-MEM reduced serum medium (Invitrogen). Separately, 3 μl Lipofectamine 2000 (Invitrogen) was diluted in 50 μl Opti-MEM, and the components were mixed and incubated for 5 min at room temperature. The siRNA and Lipofectamine mixtures were combined, incubated for 20 to 25 min at room temperature, and then added to the cells; and the cells were incubated at 37°C overnight. The mixture was then removed from the cells, and 200 to 400 PFU of RSV A2 (ATCC CC-81) was incubated with the cells for 1 h at 37°C. The infected cells were covered with methylcellulose medium and incubated for 5 days at 37°C, and plaques were visualized by the immunostaining plaque assay in Vero cells, as described previously.

***In vivo* screening of RSV-specific siRNAs.** For the prophylaxis model, BALB/c mice were anesthetized by the intraperitoneal administration of 2,2,2-tribromoethanol (Avertin), and siRNA in a total volume of 50 μl of phosphate-buffered saline (PBS) was instilled intranasally (*i.n.*). At 4 h after siRNA instillation, the mice were anesthetized and infected *i.n.* with 10<sup>6</sup> PFU of RSV A2 in 50 μl. Prior to removal of the lungs at day 4 postinfection, the anesthetized mice were exsanguinated by severing the right caudal artery. Lung tissue was collected in 1 ml ice-cold PBS (Gibco Invitrogen). The RSV titers in the lung tissue were measured by an immunostaining plaque assay. The lungs were homogenized with a handheld Tissuisher homogenizer (Fisher Scientific, Pittsburgh, PA), and the lung tissue homogenates were placed on ice for 5 to 10 min to allow the debris to settle. The clarified lung tissue lysates were serially diluted 10-fold in serum-free DMEM and added to 95% confluent Vero E6 cells cultured in DMEM in 24-well plates (Falcon; Becton Dickinson, San Jose, CA), and plaque assays were performed as described above. For the treatment model, BALB/c mice were anesthetized as described above, and 10<sup>6</sup> PFU of RSV A2 in 50 μl was instilled *i.n.* At 1, 2, 3, or 4 days after viral infection, the mice were reanesthetized, siRNA in 50 μl was instilled *i.n.*, and then the viral concentrations in the lungs were measured on day 5 postinfection, as described above.

**siRNA generation.** RNA oligonucleotides were synthesized by standard solid-phase oligonucleotide synthesis protocols with commercially available 5'-O-(4,4'-dimethoxytrityl)-3'-O-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite monomers of uridine, 4-N-benzyloxytyridine, 6-N-benzoyladenine, and 2-N-isobutyrylguanosine with 2'-O-*tert*-butyldimethylsilyl-protected phosphoramidites. After cleavage and deprotection, the RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography and characterized by electrospray ionization mass spectrometry. To generate siRNAs from RNA single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed, and the siRNAs were further characterized by capillary gel electrophoresis.

**PBMC assay.** To examine the ability of siRNAs to stimulate alpha interferon (IFN-α) or tumor necrosis factor alpha (TNF-α), human peripheral blood mononuclear cells (PBMCs) were isolated from concentrated fractions of leukocytes (buffy coats) obtained from the Blood Bank Suhl, Institute for Transfusion Medicine, Germany. The buffy coats were diluted 1:1 in PBS and added to a tube of Histopaque (Sigma, St. Louis, MO), and the tubes were centrifuged at 2,200 rpm for 20 min to allow fractionation. White blood cells were collected, washed in PBS, and then centrifuged. The cells (concentration, 1 × 10<sup>6</sup> cells/ml) were resuspended in RPMI 1640 culture medium (Invitrogen) supplemented with 10% fetal calf serum, interleukin-3 (10 ng/ml; Sigma), and phytohemagglutinin-P (5 μg/ml; Sigma) for the IFN-α assay or with no additive for the TNF-α assay and seeded onto 96-well plates and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere. The control oligonucleotides were siRNA AL-DP-5048 duplex (5'-GUCAUCACACUGAAUACCAAU-3' and 3'-CACAGUAGUGACUUAUGGUUA-5'), siRNA AL-DP-7296 duplex (5'-CUACACAAAUCAGCGAUUUCCAUGU-3' and 3'-GAUGUUUUAGUCGCUAAAGGUACA-5'), siRNA AL-DP-1730 duplex (5'-CGAUUUUAUUACAGGAUGAdTsdT-3' and 3'-dTsdTGCUAAUAAAUGUCCUACU-5'), and siRNA AL-DP-2153 duplex (5'-GGCUCUAA GCUAAUGAAGdTdT-3' and 3'-dTdTCCGAGAUUCGAUUGACUUC-5'). Cells in culture were combined with either 500 nM oligonucleotide prediluted in Opti-MEM (Invitrogen) or 133 nM oligonucleotide prediluted in Opti-MEM and the Geneporter (GP2) transfection reagent (Genlantis, San

Diego, CA) for the IFN- $\alpha$  assay or were combined with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (Roche, Switzerland) for the TNF- $\alpha$  assay. The cells were then incubated at 37°C for 24 h. IFN- $\alpha$  and TNF- $\alpha$  levels were measured with a Bender MedSystems (Vienna, Austria) instant enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instruction.

**In vitro and in vivo rapid amplification of cDNA ends (RACE).** Total RNA was purified either from in vitro-transfected Vero E6 cells or from lungs harvested at day 5 postinfection, as described above, by using Trizol (Invitrogen), followed by DNase treatment and final processing with RNeasy, according to the manufacturer's instructions (Qiagen). Five to 10  $\mu$ l of the RNA preparation from pooled samples was ligated to a GeneRacer adaptor (5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3') without prior treatment. The ligated RNA was reverse transcribed by use of a gene-specific primer (cDNA primer [5'-CTCAAAGCTTACATGATATC-3']). To detect RNAi-specific cleavage products, two rounds of consecutive PCR were performed with primers complementary to the RNA adaptor and RSV A2 nucleocapsid (N) gene mRNA (primers GR 5' [5'-CGACTGGAGCACGAGGACACTGA-3'] and Rev Primer [5'-CCACTCCATTGCTTTACATGATATCC-3']) for the first round, followed by a second round of nested PCR with primers GRN (5'-GGA CACTGACATGGACTGAAGGAGTA-3') and Rev N Primer (5'-GCTTTTA CATGATATCCCGCATCTCTGAG-3'). The amplified products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Specific cleavage products migrating at the correct size were excised, cloned into a sequencing vector, and sequenced by standard methods.

**Sequence analysis of clinical isolates for ALN-RSV01 target site conservation.** Amplification of the RSV N-gene fragment containing the ALN-RSV01 recognition site was performed by a two-step reverse transcription-PCR. Briefly, RNA was reverse transcribed with random hexamers and Superscript III reverse transcriptase (Invitrogen) at 42°C for 1 h to generate a cDNA library. A 1,200-nt gene-specific fragment was amplified by PCR with Platinum *Taq* polymerase (Invitrogen) and the RSV N forward primer (5'-AGAAAACCTTGATGAAAG ACA-3') and the RSV N reverse primer (5'-ACCATAGGCATTCATAAA-3') for 35 cycles at 55°C for 30 s, followed by 68°C for 1 min. The PCR products were analyzed by 1% agarose gel electrophoresis. As a control, a laboratory strain of RSV A Long was subjected to identical procedures for analysis. The PCR products were purified with a QIAquick PCR purification kit (Qiagen), according to the manufacturer's protocol, and sequenced by standard protocols (Agencourt Bioscience, Beverly, MA). For each amplicon, the forward and the reverse sequences were obtained. The sequences were analyzed and aligned by use of the Clustal W program and ContigExpress with Vector NTI software (Invitrogen).

**RSV genotyping.** The genotyping of all 21 isolates from Belgium was performed as described previously (85). Genotyping of the remaining 78 isolates (which were of U.S. origin) was performed by Jeffrey Kahn's laboratory, Department of Pediatrics, Yale University, New Haven, CT. Analysis of the RSV attachment (G) protein gene was performed by first generating cDNA with random hexamers and Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) at 37°C for 1 h. This was followed by PCR amplification with HotStar *Taq* DNA polymerase (Qiagen) and G-protein-gene-specific primer GTmF (5'-CCGCGGGTCTGGCAATGATAATCTCAAC-3') and subgroup-specific G-protein-gene-specific primer RSV A-GAR2 (5'-GCCG CGTGATAATTCATAAACCTTGGTAG-3') or RSV B-GBR (5'-GGGGCC CCGCGCCGCGCATTAAATAGCAAGAGTTAGGAAG-3') by denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min and then a single 10-min extension at 72°C. The PCR products were analyzed by 2% agarose gel electrophoresis. If a PCR product of the appropriate size was identified (for RSV serotype A, 1,200 nt; for RSV serotype B, 900 nt), the product was purified by using a QIAquick extraction kit (Qiagen), according to the manufacturer's protocol. The purified PCR products were analyzed by agarose gel electrophoresis and were sequenced on a 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA).

The nucleotide sequences were aligned manually, and the alignment was confirmed by using the Clustal W program. RSV serotype A and RSV serotype B isolates were distinguished by comparing the G-gene nucleotide sequences and laboratory standards. Phylogenetic analysis of the RSV serotype A isolates was performed with an aligned 417-nt segment of the G-protein gene corresponding to nucleotide positions 5010 to 5426 (GenBank accession no. M74568). Phylogenetic analysis of RSV serotype B isolates was performed with an aligned 288-nt segment of the G-protein gene corresponding to nucleotide positions 5036 to 5323 (GenBank accession no. AF013254). Data sets containing 1,000 aligned permuted nucleotide sequence sets for bootstrap analysis were produced by using the SEQBOOT program (PHYLIP package, version 3.65). Maximum-

likelihood phylogenetic trees were obtained by using the DNAML program (PHYLIP package, version 3.65) with default settings and the data sets used for bootstrap analysis. The CONSENSE program (PHYLIP package, version 3.65) was used to produce an extended-majority-rule consensus tree, and the trees were produced with the MEGA (version 4) program. Bootstrap values, isolate clustering, and previous genotype assignments (45, 85, 86) were used to determine the RSV genotypes.

## RESULTS

**Bioinformatic analysis of RSV genome and selection of ALN-RSV01.** RSV is a member of the *Pneumovirinae* subfamily in the *Pneumovirus* genus. There are two major groups of human RSV, serotypes A and B, and both serotypes cocirculate in the community. RSV has a negative single-strand RNA genome containing two nonstructural genes (NS2, NS1), followed by eight other genes, namely, the genes for the N, P, matrix (M), small hydrophobic (SH), G, F, second matrix (M2), and RNA-dependent RNA polymerase (L) proteins, in the order 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'. The N, P, and L proteins are contained within the nucleocapsid of the virion and are required for various steps within the replication cycle. Consistent with their absence from the outer virus surface, the RNAs encoding the N, P, and L proteins are among the most highly conserved regions of the RSV genome (67, 68). We therefore reasoned that the screening of siRNAs targeting these mRNAs would result in the selection of the most potent and broad-spectrum inhibitors of viral replication.

To select appropriate siRNAs, sequences with GenBank accession numbers AF035006 (RSV A2), AF013255 (RSV B1), AY911262 (RSV A Long), and D00736 (RSV 18537) were aligned by using the Clustal W algorithm to identify conserved 19-mers among all RSV sequences analyzed. To determine the uniqueness of each 19-mer across the human genome, an analysis of the sequences with those in the RefSeq database was performed with BLAST. Only siRNAs with homology to any gene in the human genome of 16 nt or fewer were selected for further analysis.

Seventy siRNAs targeting the RSV N, P, and L genes were analyzed in an in vitro RSV plaque inhibition assay; and 19 inhibited plaque formation by >80% compared with that achieved with a PBS control at siRNA concentrations of 20 nM (data not shown). Of these 19 siRNAs, the siRNA designated ALN-RSV01 (Fig. 1A), which targets the N-protein gene, consistently demonstrated the highest antiviral activity. Indeed, ALN-RSV01 showed a 50% inhibitory concentration (IC<sub>50</sub>) of 0.7 nM in the RSV plaque inhibition assay (Fig. 1B). The in vitro activity of ALN-RSV01 was further evaluated with human lung epithelial cells (A549 cells), with which it demonstrated >97% inhibition and an IC<sub>50</sub> of 0.6 nM (data not shown).

**ALN-RSV01 inhibition of RSV primary isolates.** In order to evaluate the ability of ALN-RSV01 to provide broad-spectrum activity across RSV strains, the ALN-RSV01 recognition elements from a series of primary low-passages isolates (genotype analysis; Fig. 2A and B) taken from the nasal washes of children with confirmed RSV disease were sequenced. Of the RSV primary isolates sequenced, 94% (89/95) showed absolute sequence conservation (19/19 nt) across the ALN-RSV01 target site. The six isolates that were not 100% conserved each had a single-base alteration within the ALN-RSV01 target site. Four





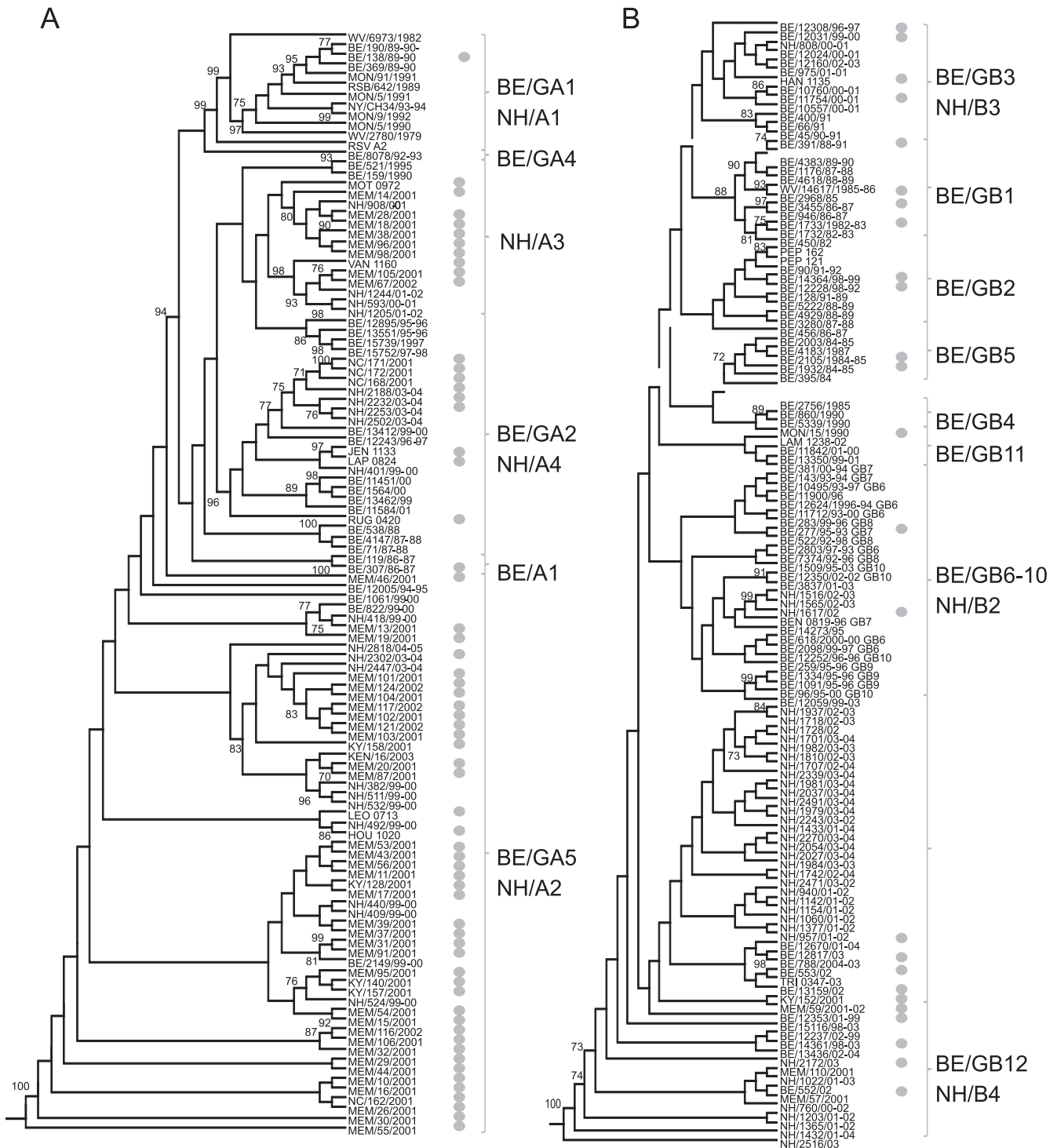


FIG. 2. Genotype analysis of RSV primary isolates. Maximum-likelihood phylogenetic trees for RSV serotype A (A) and RSV serotype B (B) strains on the basis of analysis of the RSV G-protein gene. Bootstrap values of  $\geq 70\%$  (1,000 replicates) are shown at the corresponding nodes. Circles, isolates whose ALN-RSV01 target sites were analyzed.

siRNAs, have been shown to stimulate innate immune responses through a variety of RNA binding receptors (58, 60). This stimulation can be monitored in vitro in a PBMC assay (32, 37, 63). While the immunostimulatory property of an siRNA could act in combination with an RNAi-mediated

mechanism to produce a more robust anti-RSV effect, such a feature might also confound the interpretation of the results related to an siRNA treatment strategy. Accordingly, ALN-RSV01 was evaluated for its ability to stimulate IFN- $\alpha$  and TNF- $\alpha$  in vitro by incubating ALN-RSV01 with freshly purified

TABLE 1. ALN-RSV01 target site within the RSV N gene of primary clinical isolates and the RSV A2 laboratory strain

Isolate name	Sequence at target site <sup>a</sup>	Subtype	IC <sub>50</sub> (nM)
RSV A2	GGCTCTTAGCAAAGTCAAG	A	1
LAP 0824	GGCTCTTAGCAAAGTCAAG	A	4.9
HAN 1135	GGCTCTTAGCAAAGTCAAG	B	4.7
LAM 1238	GGCTCTTAGCAAAGTCAAG	B	1.2
LEO 0713	GGCTCTTAGCAAAGTCAAG	A	1
RUG 0420	GGCTCTTAGCAAAGTCAAG	A	4.8
MOT 0972	GGCTCTTAGCAAAGTCAAG	A	4.9
BEN 0819	GGCTCTTAGCAAAGTCAAG	B	1.2
JEN 1133	GGCTCTTAGCAAAGTCAAG	A	1.1
VA 37C	GGCTCTTAGCAAAGTCAAG	A	4.8
VA-38C	GGCTCTTAGCAAAGTCAAG	A	1
VA-54C	GGCTCTTAGCAAAGTCAAG	A	1.2
RSV 32	GGCTCTTAGCAAAGTCAAG	A	1

<sup>a</sup> Nucleotides not conserved in the target region are indicated in boldface.

PBMCs, as described above. High concentrations of ALN-RSV01 (133 nM) that exceeded the IC<sub>50</sub> for an antiviral effect by over 100-fold were used in these assays. After 24 h, only modest levels of both IFN- $\alpha$  and TNF- $\alpha$  were detected by ELISA, with an average of approximately 147 pg/ml of IFN- $\alpha$  (Fig. 5A) and 1,500 pg/ml of TNF- $\alpha$  (Fig. 5B) being induced, whereas no IFN- $\alpha$  or TNF- $\alpha$  was induced in the controls treated with medium alone.

To verify that the antiviral activity of ALN-RSV01 was not influenced by this modest induction of cytokines, two non-RSV-specific siRNAs shown to induce either IFN- $\alpha$  or TNF- $\alpha$  more significantly than ALN-RSV01 were assayed in the in vivo BALB/c mouse model. Neither AL-DP-1730, a TNF- $\alpha$  inducer (Fig. 6A), nor AL-DP-2153, an IFN- $\alpha$  inducer (Fig. 6B), inhibited RSV A2 when administered to the mice i.n. (100  $\mu$ g), whereas strong inhibition was observed when ALN-RSV01 was administered (Fig. 6C). Importantly, even 10-fold higher doses of AL-DP-1730 had no effect on the viral load when it was delivered prophylactically 4 h prior to infection (data not shown).

To further evaluate the role of immune activation on the

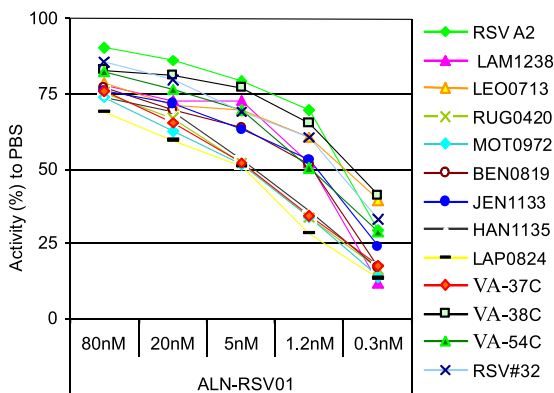


FIG. 3. In vitro inhibition of primary RSV isolates by ALN-RSV01. Vero cells in 24-well plates were transfected with decreasing concentrations of ALN-RSV01, followed by infection with 200 to 300 PFU of RSV primary isolates. At day 5 postinfection, the cells were fixed, immunostained, and counted. The activity of ALN-RSV01 was plotted as a percentage of that of PBS.

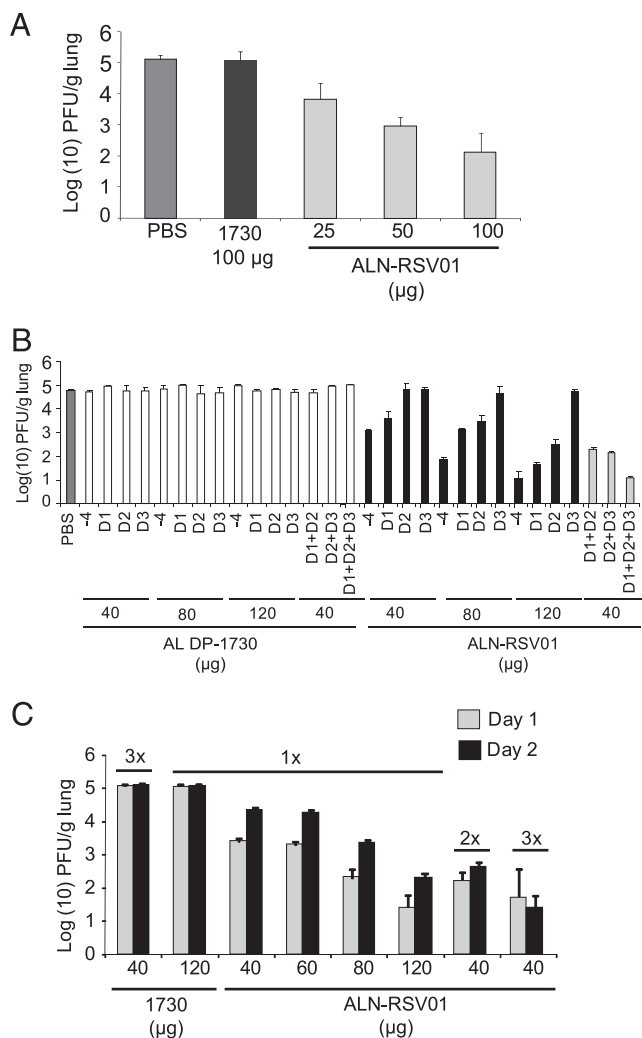


FIG. 4. In vivo activity of ALN-RSV01 following administration of single and multiple doses in BALB/c mice. In each panel, each group represents five animals, error bars represent standard deviations, and the threshold of detection of the assay is 1.7 log<sub>10</sub> PFU/g. (A) ALN-RSV01 in vivo dose-response curve. BALB/c mice were treated i.n. with ALN-RSV01 at increasing concentrations (25  $\mu$ g, 50  $\mu$ g, or 100  $\mu$ g), negative control siRNA AL-DP-1730 (1730; 100  $\mu$ g), or PBS 4 h prior to infection with  $1 \times 10^6$  PFU of RSV A2. The lungs were harvested, and virus was quantified by a standard immunostaining plaque assay and plotted as log<sub>10</sub> PFU/g lung. (B) ALN-RSV01 multi-dose study. BALB/c mice were treated i.n. with ALN-RSV01 or mismatch siRNA (AL-DP-1730) at either 40  $\mu$ g, 80  $\mu$ g, or 120  $\mu$ g (single-dose treatment) or 40  $\mu$ g (multiple-dose, daily treatment). The lungs were harvested, and virus was quantified by an immunostaining plaque assay on day 5. -4, 4 h prior to infection; D1, day 1 postinfection; D2, day 2 postinfection; D3, day 3 postinfection. (C) ALN-RSV01 same-day multidose study. BALB/c mice were treated i.n. with ALN-RSV01 or mismatch siRNA (1730) at either 40  $\mu$ g, 60  $\mu$ g, 80  $\mu$ g, or 120  $\mu$ g for the single-dose groups at day 1 or 2 after RSV infection or 40  $\mu$ g two or three times daily for the multidose groups at day 1 or 2 after RSV infection. The lungs were harvested, and virus was quantified by an immunostaining plaque assay.

antiviral efficacy of ALN-RSV01, an immune-silent form of this siRNA, AL-DP-16570, containing 2'-methoxy modifications, was synthesized. The sense strand is 5'-GGu uCU UAG cAa AGu cAa GdTdT-HP-3', and the antisense strand is

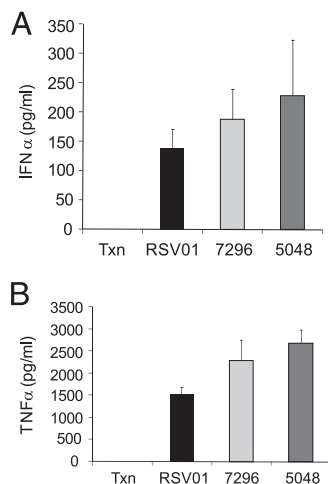


FIG. 5. ALN-RSV01 is a modest stimulator of IFN- $\alpha$  and TNF- $\alpha$  in vitro. Transfection reagent alone (Txn) or siRNAs (ALN-RSV01 [RSV01] or mismatch-positive controls siRNA AL-DP-7296 [7296] and siRNA AL-DP-5048 [5048]) were transfected into PBMCs and assayed by ELISA for cytokine induction at 24 h posttransfection. (A) IFN- $\alpha$  induction; (B) TNF- $\alpha$  induction.

5'-Cuu GAC UUU GCu AAG AGC cdTdT-HP-3'. The lowercase letters indicate the 2'-methoxy modifications, and HP indicates hydroxyproline. AL-DP 16570 showed potent antiviral activity in vitro and had an  $IC_{50}$  of  $\sim 1$  nM (data not shown), which is comparable to that measured for ALN-RSV01. Furthermore, in PBMC assays, the use of high concentrations of AL-DP-16570 (133 nM) resulted in no significant induction of either IFN- $\alpha$  or TNF- $\alpha$  (Fig. 7A and B, respectively). AL-DP-16570 was then tested for its in vivo antiviral efficacy in the mouse model. Compared with the activity of a nonspecific siRNA control, AL-DP-16570 showed potent antiviral activity that was comparable to that of the parent sequence ALN-RSV01 (Fig. 7C). These data support the conclusion that the antiviral effects of ALN-RSV01 are mediated by an RNAi mechanism and not via the induction of innate immunity.

**In vitro and in vivo RACE analysis of ALN-RSV01 cleavage product.** The RNA-induced silencing complex-mediated cleavage of a specific mRNA transcript occurs exactly 10 nt from the 5' end of the siRNA antisense strand. To definitively confirm an RNAi-mediated mechanism of action for ALN-RSV01, a 5' RACE assay was used. This assay allows the capture and sequence analysis of the specific RNAi cleavage product mRNA intermediate following ALN-RSV01 treatment both in vitro and in vivo. Following siRNA transfection into Vero cells and subsequent infection with RSV A2, a specific cleavage fragment could be detected only in the samples treated with ALN-RSV01 and not in samples treated with either PBS or a mismatch control siRNA (AL-DP-2153) (data not shown). In these experiments, 92% of the sequenced clones resulted from site-specific cleavage (between positions 26 and 27 of RSV A2 N mRNA) (data not shown). When they were analyzed in vivo, 60 to 82% of the clones isolated from the lung tissue of ALN-RSV01 treated, RSV-infected mice demonstrated site-specific cleavage of the N-gene transcript between positions 26 and 27 (Fig. 8). Only animals treated with ALN-RSV01, in contradistinction to those treated with PBS or the mismatch control

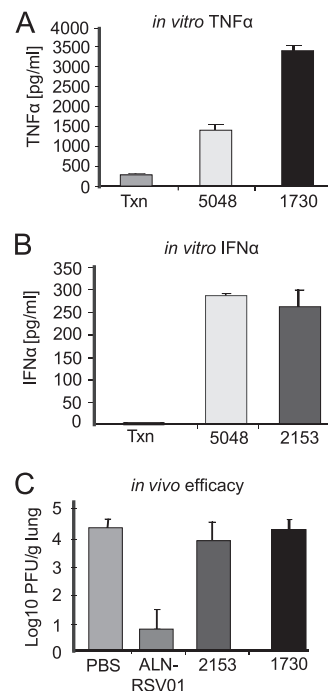


FIG. 6. TNF- $\alpha$ - and IFN- $\alpha$ -stimulatory mismatched siRNAs do not modulate RSV in vivo. (A) Transfection reagent alone (Txn) or siRNAs (AL-DP-5048 [5048] and AL-DP-1730 [1730]) transfected into PBMCs were assayed for TNF- $\alpha$  stimulation at 24 h posttransfection. (B) siRNAs (AL-DP-5048 [5048] and AL-DP-2153 [2153]) transfected into PBMCs were assayed for IFN- $\alpha$  stimulation at 24 h posttransfection. (C) Lung virus concentrations from mice dosed i.n. with RSV at day 0 and with RSV-specific siRNAs (ALN-RSV01) or mismatch control immunostimulatory siRNAs (AL-DP-2153 [2153] and AL-DP-1730 [1730]) at 4 h before inoculation. Lung RSV concentrations were measured by an immunostaining plaque assay at day 5 postinfection.

siRNA, yielded significant numbers of clones whose sequence was confirmed to be the result of cleavage at the predicted site (Fig. 8).

## DISCUSSION

While a large number of anti-RSV therapeutic strategies have been evaluated, including strategies that use antisense oligonucleotides (12, 52, 71), deoxyribozymes (79, 83, 84), small-molecule inhibitors (15, 16, 55), fusion protein inhibitors (22, 26, 56), and morpholino compounds (42), no approach has led to any new positive clinical data or FDA-approved products. At the same time, the level of unmet need for safe and effective therapies remains high because of the more than 125,000 pediatric hospitalizations (61) and 170,000 adult hospitalizations (19) in the United States annually. In this report, we describe the generation of a novel RNAi therapeutic agent for the treatment of RSV infection. This pan-RSV siRNA, ALN-RSV01, demonstrates antiviral activity both in vitro and in vivo in a number of dosing paradigms relevant to clinical use and shows an RNAi-specific antiviral mechanism of action.

ALN-RSV01 is a double-stranded siRNA duplex with 19 paired nucleotides complementary to a highly conserved region of the RSV N-protein gene and 2-nt dT overhangs at the 3' end of each strand; this structure represents the canonical



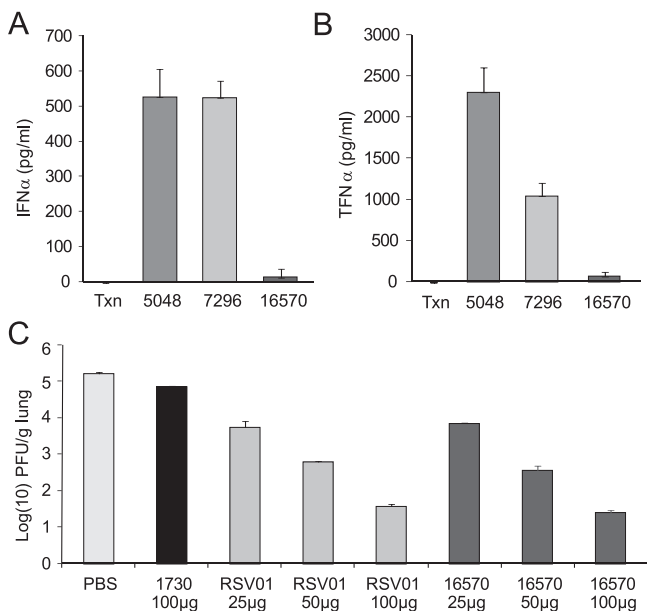


FIG. 7. Chemically modified ALN-RSV01 (AL-DP-16571) is immunologically silent, while it maintains antiviral activity. (A) Transfection reagent alone (Txn), AL-DP-16570 (16570), or positive control siRNAs AL-DP-5048 (5048) and AL-DP-7296 (7296) were transfected into PBMCs and assayed for IFN- $\alpha$  stimulation at 24 h posttransfection. (B) AL-DP-16570 or positive control siRNAs AL-DP-5048 and AL-DP-7296 were transfected into PBMCs and assayed for TNF- $\alpha$  stimulation at 24 h postinfection. (C) Lung virus concentrations from mice dosed i.n. with RSV at day 0 and with ALN-RSV01 (RSV01), AL-DP-16570 (16570), or a mismatched control at the indicated concentrations at 4 h before inoculation. Lung RSV concentrations were measured by an immunostaining plaque assay at day 5 postinfection.

Tuschl design structure that is most validated in the literature (17). The molecule was purposefully designed to be stable as an agent for local delivery to the lung and unstable in systemic circulation to minimize any potential systemic exposure. Indeed, in serum, ALN-RSV01 has a half-life of ~30 min, while in respiratory secretions from RSV-positive children, the half life is >5 h (data not shown). Thus, ALN-RSV01 would be rapidly degraded after distribution from the lung, preventing any prolonged systemic exposure.

A major consideration when an RSV therapeutic is developed is the requirement that the molecule be broadly active across the spectrum of viral genotypes. RSV can be classified into two subgroups, serotypes A and B, and the sequences of all 11 genes identified differ between the two serotypes. Genotypes can vary from year to year, with certain serotypes dominating in some years, but the cocirculation of multiple RSV strains is commonly observed (28, 41). Although RSV serotype A may produce greater viral loads in infants (29, 46, 49, 74) and RSV serotype A may produce disease slightly more severe than that produced by RSV serotype B, both RSV serotype A and RSV serotype B produce a similar and clinically indistinguishable disease spectrum. In the case of an RNAi therapeutic, broad-spectrum activity is achieved by selecting an siRNA molecule whose corresponding mRNA target site is conserved across the various circulating RSV isolates. This is achieved for ALN-RSV01 by targeting the relatively conserved viral N protein, which is less prone to mutation than

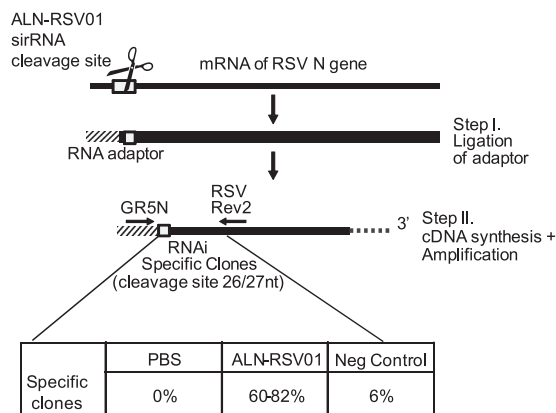


FIG. 8. ALN-RSV01 viral inhibition is mediated by RNAi in vivo. Shown is a schematic representation of the 5' RACE assay used to demonstrate the generation of the site-specific cleavage product. Boxed are the results of sequence analysis of individual clones from PCR amplification of cDNAs generated from linker-adapted RSV N-gene mRNA isolated from an in vivo viral inhibition assay in which mice were inoculated with RSV at day 0 and treated with ALN-RSV01, AL-DP-2153 (Neg Control), or PBS at day 3, followed by lung homogenization and evaluation by RACE at day 5 postinfection.

surface viral proteins, such as the G or the F protein. Indeed, within RSV, the N-protein gene is among the most conserved, with approximately 86% identity at the nucleic acid level and 96% identity at the amino acid level. Sequence differences within the target site were observed at a low frequency in clinical specimens and largely created a G-U wobble between the antisense strand of the siRNA and the target RSV mRNA. As expected, all clinical isolates tested were effectively inhibited in vitro by ALN-RSV01 (Fig. 4). Although the IC<sub>50</sub> for the two isolates with G-U wobbles was 5 nM (the top of the range), it should be noted that other isolates, namely, isolates RUG 0420 and MOT 0472, both of which show 100% sequence conservation at the ALN-RSV01 target site, also exhibited IC<sub>50</sub>s of 5 nM (Table 1). This suggests that the target site divergence in the isolates with nonconserved sequences does not account for the slightly higher IC<sub>50</sub>. It is likely that the growth and replication properties of these isolates account for their slightly higher IC<sub>50</sub>s. These primary isolates spanned genotypes A and B, indicating that ALN-RSV01 is likely active against all currently circulating strains.

The mechanism of RNAi is such that the specificity is determined by sequence complementarity, and thus, ALN-RSV01 would not be effective against other viruses, even closely related viruses within the subfamily *Pneumoviridae* (i.e., human metapneumovirus). In fact, the ALN-RSV01 target region within the N-protein gene of human metapneumovirus diverges from ALN-RSV01 target region within the N-protein gene of RSV almost completely, and we therefore predict that ALN-RSV01 would not inhibit human metapneumovirus. In addition, Bitko et al. (6) have also demonstrated the specificity of RNAi-mediated inhibition of RSV by showing that their RSV-specific siRNA did not inhibit parainfluenza virus type 3. Although we have not attempted to generate recombinant viruses via reverse genetics to identify the positions which would make viruses resistant to ALN-RSV01, our large genetic data set of circulating primary RSV isolates shows a high



degree of conservation of the sequence within this target region in both subtype A and subtype B viruses (Fig. 2A and B). This conservation likely predicts an important function for this region and suggests an evolutionary bias against sequence changes there.

The importance of target sequence conservation was also explored in a study evaluating an siRNA targeting the RSV P gene (data not shown). Specifically, this siRNA was identical to the sequence in laboratory strain RSVA2 but had a single-base variation compared with the sequence of laboratory strain RSV A Long. Interestingly, while complete inhibition of RSV A2 was achieved, no inhibition of RSV A Long was observed *in vitro*. This result was expected on the basis of the specific position of the mismatch (position 7 with respect to the antisense strand of the siRNA), as this position in the siRNA seed is known to be important for target recognition (34).

In a large series of *in vivo* studies, ALN-RSV01 was found to be a potent antiviral in both prophylaxis and treatment paradigms, achieving up to 3-log-unit viral load reductions compared to the reductions achieved with either PBS or nonspecific siRNA controls. Bitko et al. (6) and Zhang et al. (82) have also previously demonstrated siRNA inhibition of RSV in BALB/c mice. They targeted either the P-protein mRNA (6) or the NS1-protein mRNA (82) and achieved reductions similar to those described here. These RNAi-mediated antiviral activities are similar to those previously reported for ribavirin and palivizumab (33, 35, 78). While these other siRNAs were effective inhibitors of viral replication *in vivo*, neither is ideally suited for RNAi therapeutic development. The P-protein gene targeting the siRNA of Bitko et al. (6) is directed against a region of the virus that is not conserved across RSV serotypes A and B. Furthermore, the NS1 protein-targeting siRNA of Zhang et al. (82) is believed to function (at least in part) as an immune modulator by inhibiting the production of the RSV protein that inhibits the induction of IFN.

Applications of ALN-RSV01 in clinical settings will require optimization of the dosing strategy in terms of dose level and frequency. In the current study, we evaluated the effects of multidosing by comparing a single-dose regimen with the administration of either multiple daily doses or multiple doses on only a single day. Multidosing with low doses of siRNA was equivalent or slightly more effective than the equivalent total dose delivered in a single administration, suggesting that multidosing may be the optimal strategy for the delivery of ALN-RSV01.

Recent studies have suggested that some siRNAs may function not by the intended mechanism of RNAi but by nonspecifically activating the innate immune system (37, 40, 57, 80). Such a nonspecific effect might confound the interpretation of a direct antiviral mechanism, as immune stimulation could itself provide an antiviral response. Numerous experiments were performed to confirm that the mechanism of viral inhibition by ALN-RSV01 is due to RNAi. First, as a control for the antiviral efficacy of ALN-RSV01, nonspecific siRNAs were used. Second, non-RSV-targeting siRNAs with robust IFN- $\alpha$  (AL-DP-2153) or TNF- $\alpha$  (AL-DP-1730) stimulatory activity were found to be ineffective in our *in vivo* model. Third, a derivative of ALN-RSV01 (AL-DP-16570) that was chemically modified to reduce immune stimulatory activity maintained essentially indistinguishable antiviral activity *in vitro* and *in vivo*.

AL-DP-16570 is one of a series of molecules currently under evaluation in our *in vivo* RSV model. Fourth, and most importantly, 5' RACE was employed to capture the specific cleavage products from both the *in vitro* and the *in vivo* degradation of the target N-gene mRNA. By this technique, isolated clones from ALN-RSV01-treated mice demonstrated site-specific cleavage of the N-gene mRNA at a position corresponding to that exactly 10 nt from the 5' end of the siRNA antisense strand (Fig. 8). The small percentage of clones resulting from nonspecific cleavage at other sites in the RSV N gene reflects the inherent instability of the 5' end of the RSV N gene mRNA. In aggregate, these data demonstrate that the inhibition of RSV replication by ALN-RSV01 is via an RNAi mechanism and not via immune modulation.

RNAi represents a promising strategy for the discovery and development of antiviral medicines. Our studies of ALN-RSV01 thus provide a road map for considerations in the advancement of this approach in general, including the selection of conserved target sequences, *in vivo* pharmacology, and proof of the mechanism of action. Furthermore, these studies lay a foundation for the further study of ALN-RSV01 and other virus-targeting siRNAs in human clinical trials (14).

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