RNA Interference-Mediated Silencing of the Respiratory Syncytial Virus Nucleocapsid Defines a Potent Antiviral Strategy

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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 is available; but due to its teratogenicity, 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-
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 washes with DMEM. The infected cells were harvested by removal of the medium and re-plated with a minimal volume of serum-free DMEM, followed by three washes with DMEM. The infected cells were incubated for 5 days at 37°C, and plaques were visualized by the immunostaining plaque assay, as described previously (72).

siRNA generation. RNA oligonucleotides were synthesized by standard solid-phase oligonucleotide synthesis protocols with commercially available 5′-O-(2′-deoxymethylinyl) 3′-O-(2′-cyanoethyl-N,N-diisopropyl) phosphoramidite monomers of uridine, 4-N-benzoylcytidine, 6-N-benzyladenosine, 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. siRNA oligonucleotides were then annealed with RNA:RNA complementary strands to form RNA duplex oligonucleotides that were used for in vivo or in vitro studies.

siRNA specific siRNA selection. An analysis of the sequences in one of the 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 subjected for synthesis and further study.

In vitro RSV inhibition assay. Vero cells were grown in 24-well plates in a 5% CO2 humidified incubator at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (BioChem, 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) were 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 methylenechloride 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-trichloroethanol (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, infected i.n. with 105 PFU of RSV A2 in 50 µl. Prior to removal of the lungs at day 4 postinfection, the anesthetized mice were randomly selected by their ear tag numbers, and the lungs were harvested, placed on ice for 5 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 105 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 loads in the lungs were measured on day 5 postinfection, as described above.

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 2,000 rpm for 20 min to allow fractionation. White blood cells were collected, washed in PBS, and then centrifuged. The cells (concentration, 1 × 106 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% CO2 atmosphere. The control oligonucleotides were siRNA AL-0P-5048 duplex (5′-GUCUACUACCGUGAUAAUCACAAU-3′ and 3′-CUAGUAUUGUGACUAUUAGGUUA-5′), siRNA AL-0P-7296 duplex (5′-CCUACACGGAUAUAACUUCCGUU-3′ and 3′-GGAGUGUUGUUUGCUACAAU-5′), siRNA AL-0P-1730 duplex (5′-GCAUGUAUAACUGAUUGAUAA-3′ and 3′-UAUCCUGUAUAUGUGUCUCAU-5′), and siRNA AL-0P-2153 duplex (5′-GCCUCUAUGUGAGAGAAUUAU-3′ and 3′-GACAUUGUGAGAGAAUUAU-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-α assay or were combined with N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (Roche, Switzerland) for the TNF-α assay. The cells were then incubated at 37°C for 24 h. IFN-α and TNF-α 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 µl of the RNA preparation from pooled samples was ligated to a GeneRacer adapter (5′-CGACUGGAGCACGGAGGACACUGCAUCGGAGGAAGAGA-3′) without prior treatment. The ligated RNA was reverse transcribed by use of a gene-specific primer (cDNA primer 5′-CTCAAAGGCTCTACATCATATCC-3′). To detect RNAspecific cleavage products, two rounds of consecutive PCR were performed with primers complementary to the RNA adaptor and RSV A2 nucleosapid (N) gene mRNA (primers GR 5′-5′-CGACTGGAGCACGGAGGAAGTGCTA-3′ and Rev Primer 5′-CCATCTGTGTTTTTATGATATCC-3′) for the first round, followed by a second round of nested PCR with primers GRN (5′-GGAACTGCCTGAGCCTGGGATGAC-3′) and Rev N Primer (5′-GCCGTTAGTATACCCCGATCTGTTAG-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 followed by a two-step reverse transcription-Polymerase chain reaction (RT-PCR). Briefly, RNA was reverse transcribed with random hexamers and Superscript III reverse transcriptase (Invitrogen) for 42°C for 1 h to generate a cDNA library. A 1,200-nt sequencing vector, and sequenced by standard methods.

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 (IC₅₀) 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 IC₅₀ 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-passage 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
had C-to-U mutations at position 4 with respect to the 5' end of the antisense strand of RSV01, one had an A-to-G mutation at position 7, and one had a G-to-A mutation at position 1. A subset of these 95 isolates was tested by the in vitro viral inhibition assay, including one isolate with a mismatch at position 4 and another with a mismatch at position 7 (Table 1). Of these, 12/12 (100%) exhibited 70% inhibition with 80 nM ALN-RSV01 compared to the level of inhibition achieved with the PBS control, and all had similar dose-response curves for inhibition by ALN-RSV01 (Fig. 3).

**In vivo studies of ALN-RSV01.** The BALB/c mouse is a well-established model for RSV infection and was thus chosen as the in vivo system to be used for evaluation of the antiviral effect of ALN-RSV01. Studies were initially performed with a prophylaxis model; siRNA was administered i.n. to mice 4 h prior to infection with 10^6 PFU of RSV A2. There was a dose-dependent inhibition of RSV A2 replication in the lungs of mice, with a 100-μg dose of ALN-RSV01 reducing the titers by between 2.5 and 3.0 log_{10} PFU/g lung compared to the titers for mice treated with either PBS or a nonspecific siRNA (Fig. 4A). Fifty- and 25-μg doses yielded reductions of approximately 2.0 and 1.25 log_{10} PFU/g, respectively (Fig. 4A).

To evaluate the efficiency of viral inhibition in a treatment paradigm, ALN-RSV01 was delivered i.n. in single or multiple daily doses at 1, 2, and/or 3 days postinfection; and the results were compared to those achieved with prophylactic dosing. When ALN-RSV01 was delivered as a single dose, the most efficacious antiviral effect was seen when it was given as a prophylactic (~4 h) dose, and this occurred in a dose-dependent fashion. Compared to the mismatch control (AL-DP-1730), the administration of 120 μg of ALN-RSV01 as a single prophylactic dose resulted in maximal viral inhibition, attenuating the lung concentrations of RSV to background levels in this assay. When ALN-RSV01 was administered in a treatment regimen as a single dose following viral inoculation, the dose-dependent antiviral efficacy was found to decrease as a function of the time of dosing after viral infection (Fig. 4B). Indeed, by day 3 postinfection, single doses as high as 120 μg did not yield any significant viral inhibition. However, when multiple 40-μg doses of ALN-RSV01 were delivered daily on days 1, 2, and 3, potent antiviral activity was maintained and the viral titers were again reduced to background levels (Fig. 4B).

To further explore alternative dosing paradigms that could be employed in future clinical studies, additional multidose regimens were evaluated. To this end, RSV-infected mice were treated with ALN-RSV01 either two times per day or three times per day. Interestingly, the multiple-daily-dose regimen of the RSV-specific siRNA (40 μg three times a day) was found to be as efficacious as a single 120-μg dose (Fig. 4C). In the aggregate, these data show that an ALN-RSV01 multidose treatment regimen can provide a maximal antiviral effect in a fashion readily applicable to human clinical studies.

**ALN-RSV01 and cytokine induction.** Many nucleic acids, including double-stranded RNAs, single-stranded RNAs, and

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**FIG. 1.** Structure of ALN-RSV01 duplex and IC<sub>50</sub> of ALN-RSV01 in vitro. (A) ALN-RSV01 is a synthetic double-stranded RNA oligonucleotide formed by hybridization of two partially complementary single-stranded RNAs in which the 3' ends are capped with two thymidine units. (B) Vero cells in 24-well plates were transfected with decreasing concentrations of ALN-RSV01, followed by infection with 200 to 300 PFU of RSV A2. At 5 days postinfection, the cells were fixed, immunostained, and counted. The activity of ALN-RSV01 was plotted as a percentage of that of PBS, and the IC<sub>50</sub> was measured with XLFit software. Error bars represent standard deviations.
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-β/H9251 and TNF-α/H9251 in vitro by incubating ALN-RSV01 with freshly purified PBMCs. Cytokine production was measured by ELISA.

**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 ≥70% (1,000 replicates) are shown at the corresponding nodes. Circles, isolates whose ALN-RSV01 target sites were analyzed.
PBMCs, as described above. High concentrations of ALN-RSV01 (133 nM) that exceeded the IC₅₀ for an antiviral effect by over 100-fold were used in these assays. After 24 h, only modest levels of both IFN-α/H9251 and TNF-α/H9251 were detected by ELISA, with an average of approximately 147 pg/ml of IFN-α/H9251 (Fig. 5A) and 1,500 pg/ml of TNF-α/H9251 (Fig. 5B) being induced, whereas no IFN-α or TNF-α 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-α/H9251 or TNF-α/H9251 more significantly than ALN-RSV01 were assayed in the in vivo BALB/c mouse model. Neither AL-DP-1730, a TNF-α inducer (Fig. 6A), nor AL-DP-2153, an IFN-α inducer (Fig. 6B), inhibited RSV A2 when administered to the mice i.n. (100 μg), 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 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

<table>
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<th>Isolate name</th>
<th>Sequence at target site</th>
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<th>IC₅₀ (nM)</th>
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<td>1</td>
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* Nucleotides not conserved in the target region are indicated in boldface.
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 IC50 of 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-α or TNF-α (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 contrast to those treated with PBS or the mismatch control 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
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. Ge
tonotypes 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, 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 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 IC50 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 IC50s 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 IC50. It is likely that the growth and replication properties of these isolates account for their slightly higher IC50s. 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 RSV A2 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 vitro 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 vitro, 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 innate 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-α (AL-DP-2153) or TNF-α (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).

REFERENCES


