

Original article

Inhibition of human metapneumovirus replication by small interfering RNA

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Background: Human metapneumovirus (hMPV) is a major respiratory viral pathogen in young children, elderly individuals and immunocompromised patients. Despite its major effects related to bronchiolitis, pneumonia and its potential role in recurrent wheezing episodes, there is still no commercial treatment or vaccine available against this paramyxovirus.

Methods: We tested a therapeutic strategy for hMPV that was based on RNA interference.

Results: An hMPV genome-wide search for small interfering RNAs (siRNAs) by computational analysis revealed 200 potentially effective 21-mer siRNAs. Initial screening with a luciferase assay identified 57 siRNAs of interest. Further evaluation of their inhibitory potential against the four hMPV subgroups by quantitative real-time reverse transcriptase PCR and plaque immunoassay identified two

highly potent siRNAs with 50% inhibitory concentration (IC_{50}) values in the subnanomolar range. siRNA45 targets the nucleoprotein messenger RNA (mRNA) and had IC_{50} values <0.078 nM against representative strains from the four hMPV subgroups, whereas siRNA60, which targets the phosphoprotein mRNA, had IC_{50} values between 0.090 – <0.078 nM against the same panel of hMPV strains. Longer 25/27-mer siRNAs known as Dicer substrates designed from the top two siRNA candidates were also evaluated and were at least as effective as their corresponding 21-mer siRNAs. Interestingly, the presence of one or two nucleotide mismatches in the target mRNA sequence of some hMPV subgroups did not always affect hMPV inhibition *in vitro*. **Conclusions:** We successfully identified two highly efficient siRNAs against hMPV targeting essential components of the hMPV replication complex.

Introduction

Human metapneumovirus (hMPV) was first identified in the Netherlands in 2001 [1] and has been reported worldwide since then [2,3]. hMPV is mainly associated with pneumonia and bronchiolitis or bronchitis in young children, elderly individuals and immunocompromised patients, whereas immunocompetent adults present mostly flu-like illnesses or cold symptoms [2,4]. hMPV infections account for about 5–10% of hospitalizations of young infants suffering from acute respiratory tract infections [2]. Moreover, hMPV outbreaks have been reported in long-term care facilities [5,6]. Based on findings from a mouse model [7] and a recent study in young infants [8], hMPV infections could be associated with long-term effects such as recurring wheezing episodes and asthma. There are two major lineages of hMPV (A and B) that often cocirculate during the same winter–spring

period in temperate countries. These two groups can be further divided into additional subgroups (A1, A2, B1 and B2) based on genomic analysis [2,3,9–12].

hMPV contains a non-segmented single-stranded negative-sense RNA genome of 13.3 kb and belongs to the *Metapneumovirus* genus within the *Paramyxoviridae* family. Its genome contains eight genes coding for nine proteins in the order 3'-N-P-M-F-M2.1-M2.2-SH-G-L-5' [13]. Based on previous paramyxovirus studies [14–16], hMPV gene expression should follow a transcriptional gradient, that is, the nucleoprotein (N) gene is transcribed first and in larger amounts than the polymerase (L) gene, which is expressed last and in very small amounts. The N, phosphoprotein (P) and L proteins form the functional polymerase complex required for hMPV transcription and replication [15,16]. These

proteins are considered suitable targets for antiviral drug development because they are essential for hMPV replication and less subject to immunological pressure than the surface proteins, that is, fusion (F), attachment (G) and small hydrophobic (SH) proteins [17–19].

Because no vaccines are currently available to prevent hMPV infections, there is an important need for developing efficient antiviral agents. One promising technology involves RNA interference (RNAi) [16]. RNAi is a mechanism of post-transcriptional gene silencing found in almost all eukaryotes and is known to confer natural antiviral protection in plants [20,21]. RNAi is triggered by endogenous small non-coding micro RNAs (miRNAs) or small interfering RNAs (siRNAs) exogenously introduced in cells. Whereas miRNAs mostly induce repression of messenger RNA (mRNA) translation, siRNAs instead lead to cleavage of the target mRNA. Sequence-specific double-stranded siRNA molecules of 19–21 nucleotides can efficiently elicit degradation of the cognate mRNAs through the RNA-induced silencing complex (RISC) located in the cytoplasm, and thus suppress the expression of the corresponding proteins [16]. In the endogenous pathway, long primary miRNAs are processed in the nucleus into pre-miRNAs by a ribonuclease (RNase) III enzyme called Drosha, and then exported to the cytoplasm where Dicer, another RNase III enzyme, further shortens the pre-miRNAs into miRNAs. Similarly, exogenously-introduced long double-stranded RNA can be processed by Dicer into siRNA [22]. The siRNA duplex consists of a passenger strand (sense strand) and a guide strand (antisense strand). When the latter is integrated into RISC, it allows for mRNA pairing and subsequent degradation. The 5' region of the siRNA guide strand, mainly nucleotides 2–8 (named the seed region) is responsible for recognition and pairing to the target mRNA, whereas positions 10 and 11 are crucial for the cleavage of the mRNA by RISC [23–25]. In the context of a viral infection, delivery of siRNA to infected cells could inhibit viral mRNA translation to proteins, and thus virus replication. Reports on human respiratory syncytial virus (hRSV), avian metapneumovirus (aMPV) and influenza virus inhibition by siRNA have demonstrated a great potential for this novel and specific nucleic acid-based therapy for respiratory viral infections [26–35]. In this study, we aimed to identify highly efficient siRNAs that inhibit hMPV replication from a pool of siRNAs covering most of the coding sequences within the hMPV genome.

Methods

Cell lines and viruses

LLC-MK2 (rhesus monkey kidney) and HEP-2 (human laryngeal carcinoma) cells were grown in minimum essential medium (MEM; Invitrogen, Burlington, ON,

Canada) supplemented with 10% fetal bovine serum (FBS; Wisent, St-Bruno, QC, Canada). A549 (human lung carcinoma) cells were grown in Ham's F12K medium (Invitrogen) containing 10% FBS. The hMPV clinical strains C-85473 (group A1), CAN97-83 (group A2), CAN97-82 (group B1) and CAN98-75 (group B2) were used in this study.

Virus quantification

LLC-MK2 cells were infected with hMPV using Opti-MEM (Invitrogen) containing 2 µg/ml of trypsin (Sigma, Oakville, ON, Canada) until maximal cytopathic effects were noted. Supernatant was collected and virus titre was reported as 50% tissue culture infectious dose (TCID₅₀) per ml or plaque forming units (PFU) per ml by immunostaining [36].

siRNA design and synthesis

Alignment of hMPV sequences using CAN98-75 (GenBank AY297748.1) as the base sequence along with two other hMPV full length genome sequences available in GenBank (AF371337 and AY297749) and one from our laboratory (C-85473) was carried out using ClustalW2 to find conserved regions among viral strains. Algorithms available in the public domain [37,38] were used to identify 200 siRNA sequences. The criteria for selecting sequences were as follows: A to G and C to U mismatches (sense strand) were allowed in any position and G to A or C to A mismatches (sense strand) were not allowed in any position other than 1, 18 and 19. Positions 10–17 (sense strand) should have no or one mismatch and positions 1–9 on the sense strand were allowed two or three mismatches. No GGGG, CCCC, AAAA, TTTT or SSSS (G or C) stretches, or stretches longer than four nucleotides, were allowed in any position. siRNAs were synthesized (IDT Inc., Coralville, IA, USA) at a 10 nM scale and purified by RNase-free HPLC so that the full length 21-mer siRNA products constituted >85% of the total yield and the remaining impurities were smaller fragments, which are inefficient in RISC-associated RNAi. All siRNAs were double-stranded and contained 19 RNA bases and two chimeric DNA base overhangs at the 3' terminus for each strand. None of the siRNAs were chemically modified. For testing, siRNAs were resuspended at 100 µM and 5 µM concentrations in a RNase-free Duplex Buffer (Dharmacon Inc., Lafayette, CO, USA) containing 100 mM potassium acetate and 30 mM HEPES at pH 7.5. An siRNA sequence with no known target within the mammalian genome (sense 5'-UUCUCCGAACGUGUCACGUDtDt-3'; synthesized by Dharmacon Inc.) or a Dicer substrate scrambled siRNA (sense 5'-CUUCCUCUCUUUCUCUCCCUUGUDGdA-3'; synthesized by IDT Inc.)

served as negative controls in all experiments. Longer 25/27-mer Dicer substrate siRNAs were designed and synthesized (MDRNA Inc., Bothell, WA, USA) based on the most potent 21-mer (RISC substrate) siRNAs. Dicer substrate siRNAs contained 23 RNA bases and two chimeric DNA bases at the 3' terminus for their sense strand, whereas their antisense strand contained 27 RNA bases. No chemical modification of these Dicer substrate siRNAs was made.

Luciferase assay

hMPV gene fragments (N, P, F, M2 and L), corresponding with each target region, were cloned in psiCHECK-2 Vector (Promega, Madison, WI, USA) and the accuracy was verified by sequencing. Vectors (100 ng) and corresponding siRNAs (10 nM) were cotransfected (in triplicate) in 85% confluent A549 cells in 96-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. At 24 h post transfection, Dual-Glo Luciferase Reagent (Promega) was added to the well and incubated for 10 min before quantification of firefly luciferase activity using a Microplate Luminescence Microtiter (Thermo Labsystems, Milford, MA, USA). Dual-Glo Stop & Glo Reagent (Promega) was then added to the well and *Renilla* luciferase activity was quantified after 10 min. Ratios of dual luciferase activity were determined to normalize *Renilla* luciferase values, and siRNAs showing >40% reduction in *Renilla* luciferase activity were selected for further evaluation.

Quantitative reverse transcriptase PCR assay for hMPV inhibition

LLC-MK2 cells in 24-well plates that were 95% confluent were transfected with different siRNAs in 300 µl/well of Opti-MEM using 1.5 µl/well of Lipofectamine 2000. Each siRNA was transfected in triplicate. The medium was removed after 5 h and 500 µl of infection medium containing 10^7 TCID₅₀ of hMPV in Opti-MEM with 2 µg/ml of trypsin were added to the cells for 1.5 h. The medium was then replaced with fresh medium containing no virus, and cells were incubated for 3 days. Supernatants were collected and stored at -80°C until viral RNA extraction and quantification using a real-time reverse transcriptase (RT)-PCR assay for the hMPV N gene as previously described [36].

Immunostaining assay for hMPV inhibition

LLC-MK2 cells that were 95% confluent were transfected with siRNA using Lipofectamine 2000 as described above. After 5 h, cells were infected with 30–50 PFU of hMPV for 1.5 h and the medium was replaced with 1 ml/well of methylcellulose overlay. Cells were incubated for 3 days then immunostained as

previously described [36]. These inhibition assays were done between two and four times.

Viral yield assay

LLC-MK2 cells were transfected with siRNA and infected, as described above, with the overlay containing only Opti-MEM and trypsin. Cells were incubated for 3 days, then supernatants were collected and titrated by plaque assay and immunostaining [36].

Cytotoxicity assay

Cytotoxicity of control and viral siRNAs was evaluated with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in LLC-MK2 cells. In brief, serial dilutions of each siRNA were transfected in triplicate using Lipofectamine 2000 as described above. After a 3-day incubation period, 100 µl of 1 mg/ml of MTT was added to each well for 2 h at 37°C. The dye was solubilized with 100 µl/well of acidic isopropanol and absorbance was read at a wavelength of 570 nm with reference at 650 nm. An absorbance value that was significantly lower than that of the control cells indicated cytotoxicity.

Statistical analyses

Analyses were performed with GraphPad Prism 5 software using one-way analysis of variance and the Tukey multiple comparisons test.

Results

Selection and pre-screening of siRNAs in the absence of virus

Complete hMPV genome sequences that were available in GenBank at the time of the design, that is, AY297748 CAN98-75 (group B2), AF371337 NL-001 (group A1) and AY297749 CAN97-83 (group A2), in addition to that of clinical strain C-85473 (group A1) used in our laboratory, were aligned (using CAN98-75 as consensus sequence) and analysed using ClustalW2. The most conserved hMPV genes (N, P, M, F, M2-1, M2-2 and L) were targeted for siRNA selection. We identified 200 siRNA sequences based on specific requirements such as sequence stability at the 5' end of the antisense strand relative to the 3' terminus and insertion of chimeric DNA base overhangs at the 3' terminus of each strand.

These siRNAs were pre-screened in the absence of virus using a luciferase assay. Partial sequences from hMPV genome were cloned downstream of the *Renilla* translational stop codon in the psiCHECK-2 Vector. RNAi-based cleavage and subsequent degradation of the fusion mRNA could be easily monitored. The vector also contains the firefly luciferase gene, which allows for normalization of gene expression and transfection efficiency in every well. Each siRNA was

cotransfected with the vector in A549 cells and the expression of both luciferase genes was measured to determine activity ratios, that is, normalized luciferase values. *Renilla* luciferase inhibition by the siRNAs varied between -58–97%; there were siRNAs showing good inhibition of *Renilla* luciferase expression in all target genes, as shown in Figure 1. All 57 siRNAs associated with inhibition scores >40% were selected for further inhibition studies against hMPV. None of the siRNAs unable to inhibit luciferase expression were selected for further testing against hMPV; therefore, the value of the luciferase test as a screening assay could not be determined.

Screening and evaluation of siRNA activity against hMPV

A quantitative real-time RT-PCR assay was used to determine siRNA activity against hMPV strains. The 57 siRNAs identified by dual luciferase were evaluated at two concentrations (10 and 100 nM) against the hMPV group A1 clinical strain (C-85473). Viral RNA in the supernatants of siRNA-transfected cell cultures was quantified and compared with that of control siRNA. The control siRNA had no viral or mammalian target sequence and did not decrease or increase hMPV replication in LLC-MK2 cells at all tested concentrations. The target sequences of these 57 siRNAs and their inhibitory activity against hMPV A1 are shown in Figure 2. Not all siRNAs predicted by the luciferase assay showed good inhibition activity against hMPV when tested by quantitative RT-PCR. At 10 nM, 20 (35%) of the 57 siRNAs inhibited hMPV replication by >50% and only 10 (17.5%) had inhibition activity >80% against hMPV A1. Also, some

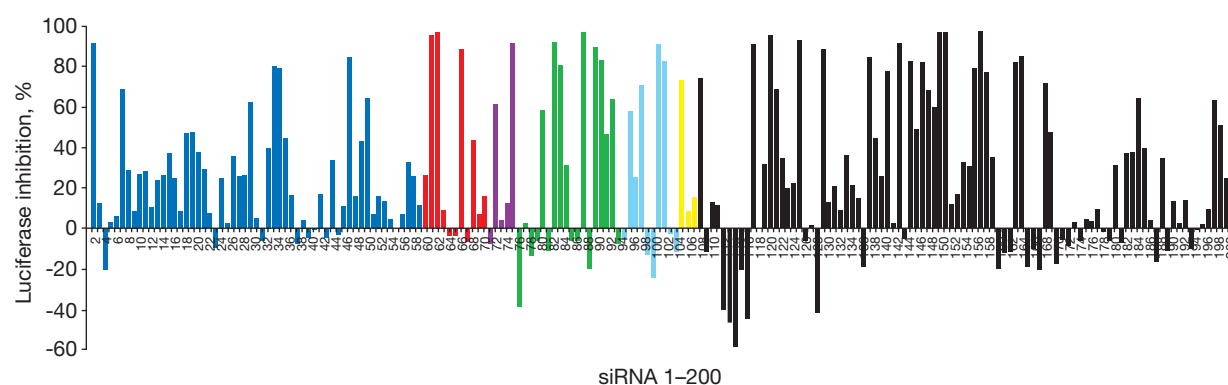
siRNAs showed reverse dose-response activity against hMPV when evaluated with the RT-PCR assay, which could be related to the variability seen in this assay.

50% Inhibitory concentration of the most active siRNAs against hMPV

The 10 lead siRNAs showing >80% inhibitory activity at a 10 nM concentration were selected for determination of 50% inhibitory concentration (IC_{50}) values using the quantitative RT-PCR assay. The same hMPV group A1 strain was initially tested against a more extensive array of siRNA concentrations, that is, twofold dilutions from 10 nM to 0.078 nM. Table 1 presents the IC_{50} values for the 10 most active siRNAs. Three siRNAs (siRNA45, siRNA60 and siRNA118) showed potent inhibition with IC_{50} values <0.078 nM against hMPV A1, whereas all others had intermediate activity. Four siRNAs were tested twice to assess the reproducibility of this assay: the three most active siRNAs (siRNA45, siRNA60 and siRNA118) and one siRNA with intermediate activity (siRNA1). Divergent values between both replicates were noted for siRNA118 (<0.078 and 0.141 nM). Such differences could be attributed to the transfection efficiency or the prolonged period of time (months) between initial siRNA resuspension and testing, which might have caused siRNA degradation.

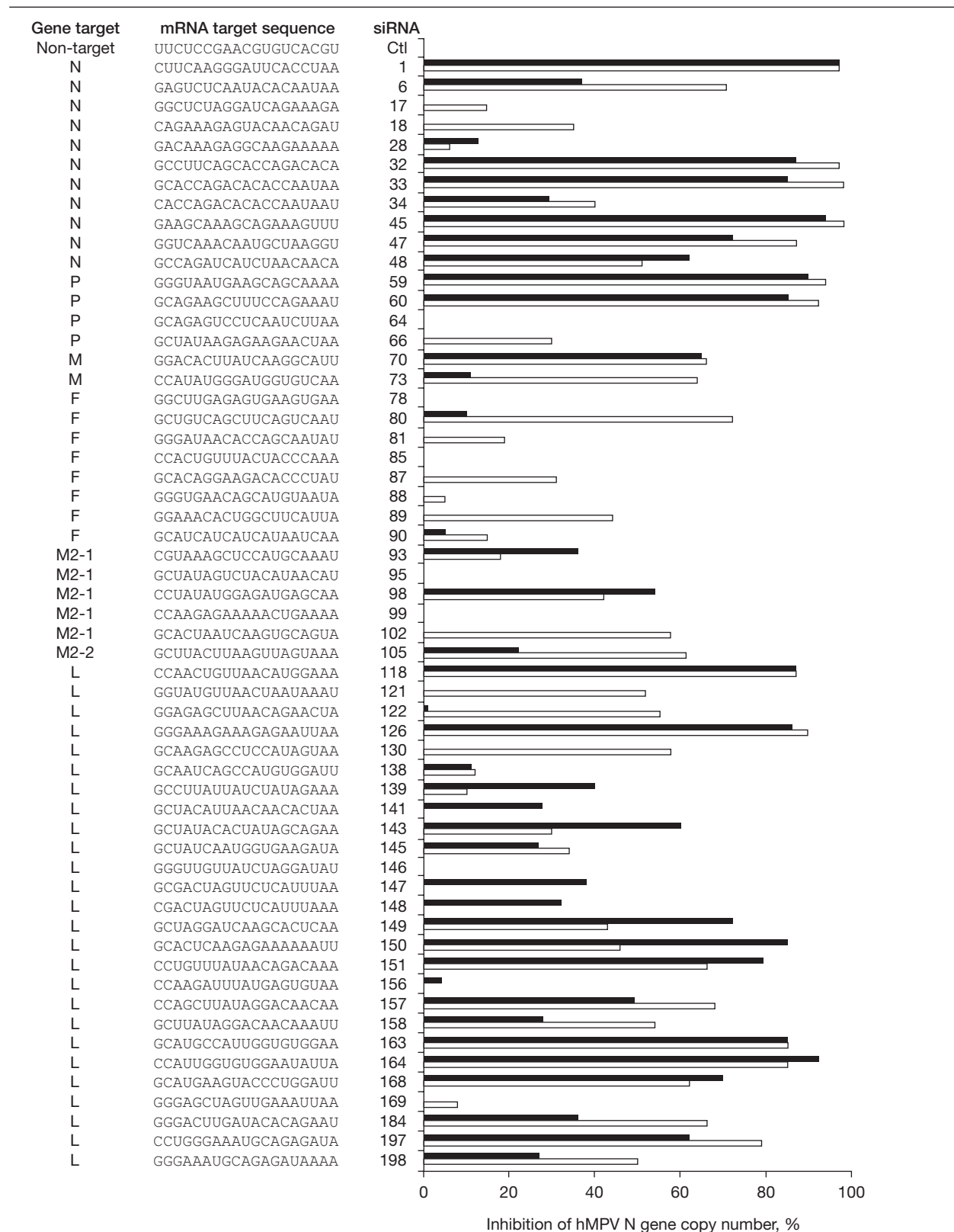
The inhibitory activity of these four siRNAs was also evaluated against hMPV group B2 by quantitative RT-PCR. siRNA1 had a lower IC_{50} value against the B2 strain compared with the A1 strain, whereas siRNA118 showed no inhibition against the B2 strain (>10 nM). siRNA45 and siRNA60 had IC_{50} values <0.078 nM against both hMPV A1 and B2 strains (Table 1).

Figure 1. Activity of 200 siRNAs targeting hMPV genome determined by the percentage inhibition of luciferase expression



Percent inhibition was calculated as follows: a small interfering RNA (siRNA) can inhibit *Renilla* luciferase expression, but not firefly luciferase expression, thus the percentage of luciferase inhibition corresponds to the ratio of *Renilla* luciferase expression to firefly luciferase expression in the presence of the siRNA. In the absence of siRNA, the ratio of *Renilla* luciferase expression to firefly luciferase expression is one, that is, 0% inhibition. Columns represent siRNAs targeting the nucleoprotein gene (blue), the phosphoprotein gene (red), the M gene (purple), the F gene (green), the M2-1 gene (light blue), the M2-2 gene (yellow) and the polymerase gene (black). hMPV, human metapneumovirus.

Figure 2. Screening of 57 siRNAs at two concentrations (10 and 100 nM) using a real-time RT-PCR assay



Cells were first transfected with small interfering RNAs (siRNAs) then infected with human metapneumovirus (hMPV). After 3 days, viral RNA was extracted, reverse transcribed and quantified by PCR. Target genes, sequences and siRNA numbers are indicated. Black bars represent the percentage of hMPV inhibition by siRNAs using a 10 nM concentration; white bars represent the percentage of hMPV inhibition by siRNAs using a 100 nM concentration. Ctl, control siRNA; L, polymerase; mRNA, messenger RNA; N, nucleoprotein; P, phosphoprotein; RT, reverse transcriptase.

Table 1. Inhibition data obtained for 10 siRNAs against hMPV groups A1 and B2 with the quantitative real-time RT-PCR and against hMPV group A1 with the plaque assay followed by immunostaining

siRNA	Target gene	siRNA sequence*	IC ₅₀ value against hMPV A1, nM			IC ₅₀ value against hMPV B2, nM	
			RT-PCR (first experiment)	RT-PCR (second experiment)	Plaque assay†	RT-PCR (first experiment)	RT-PCR (second experiment)
1	N	5'-CUUCAAGGGAUUCACCUAAAdTdT-3' 3'-dTdTGAAGUCCCUAAGUGGAUU-5'	0.215	0.099	>10	<0.078	<0.078
32	N	5'-GCCUUCAGCACCAGACACAdTdT-3' 3'-dTdTTCGGAAGUCGUGGUCUGUGU-5'	0.459	ND	>10	ND	ND
33	N	5'-GCACCAGACACACCAUAAAdTdT-3' 3'-dTdTTCGUGGUCUGUGUGGUAAUU-5'	0.082	ND	0.473 ±0.272	ND	ND
45	N	5'-GAAGCAAAGCAGAAAGUUUdTdT-3' 3'-dTdTTCUUCGUUUCGUCUUUCAA-5'	<0.078	<0.078	0.046 ±0.027	<0.078	<0.078
59	P	5'-GGGUAAUGAAGCAGCAAAAdTdT-3' 3'-dTdTCCCAUUCUUCGUCGUUUU-5'	0.084	ND	0.377 ±0.149	ND	ND
60	P	5'-GCAGAAGCUUUCAGAAAUdTdT-3' 3'-dTdTTCGUCUUCGAAAGGUCUUUA-5'	<0.078	<0.078	0.075 ±0.068	<0.078	<0.078
118	L	5'-CCAACUGUUAACAUGGAAAdTdT-3' 3'-dTdTGGUUGACAAUUGUACCUUU-5'	<0.078	0.141	>10	>10	>10
126	L	5'-GGGAAAGAAAGAGAAUUAAdTdT-3' 3'-dTdTCCCUUUCUUUCUCUUAUU-5'	0.130	ND	>10	ND	ND
163	L	5'-GCAUGCCAUUGGUGUGGAAdTdT-3' 3'-dTdTTCGUACGGUAACCACACCUU-5'	0.127	ND	>10	ND	ND
164	L	5'-CCAUUGGUGUGGAAUUAUAdTdT-3' 3'-dTdTGGUAACCACACCUUAUAU-5'	0.087	ND	>10	ND	ND

*Sense strand 5'-3', antisense strand 3'-5'. †Results are means (±SD) from two to four independent experiments. hMPV, human metapneumovirus; IC₅₀, 50% inhibitory concentration; L, polymerase; N, nucleoprotein; ND, not done; P, phosphoprotein; RT, reverse transcriptase; siRNA, small interfering RNA.

The inhibitory activity of the 10 siRNAs was also evaluated using a plaque assay followed by immunostaining. Of note, six of the 10 siRNAs were unable to inhibit replication of hMPV A1, even at the highest tested concentration of 10 nM (Table 1). Two siRNAs remained very effective against hMPV A1 with mean ±SD IC₅₀ values of 0.046 ±0.027 nM for siRNA45 and 0.075 ±0.068 nM for siRNA60, whereas two had intermediate activity with mean ±SD IC₅₀ values of 0.473 ±0.272 nM for siRNA33 and 0.377 ±0.149 nM for siRNA59 (Table 1).

An infectious viral yield reduction assay was also performed for siRNA60 against hMPV A1 (in duplicate) to confirm its inhibitory activity. The progeny viral titre was determined by titration of supernatant of transfected cells and results showed potent activity with mean IC₅₀ values <0.019 nM.

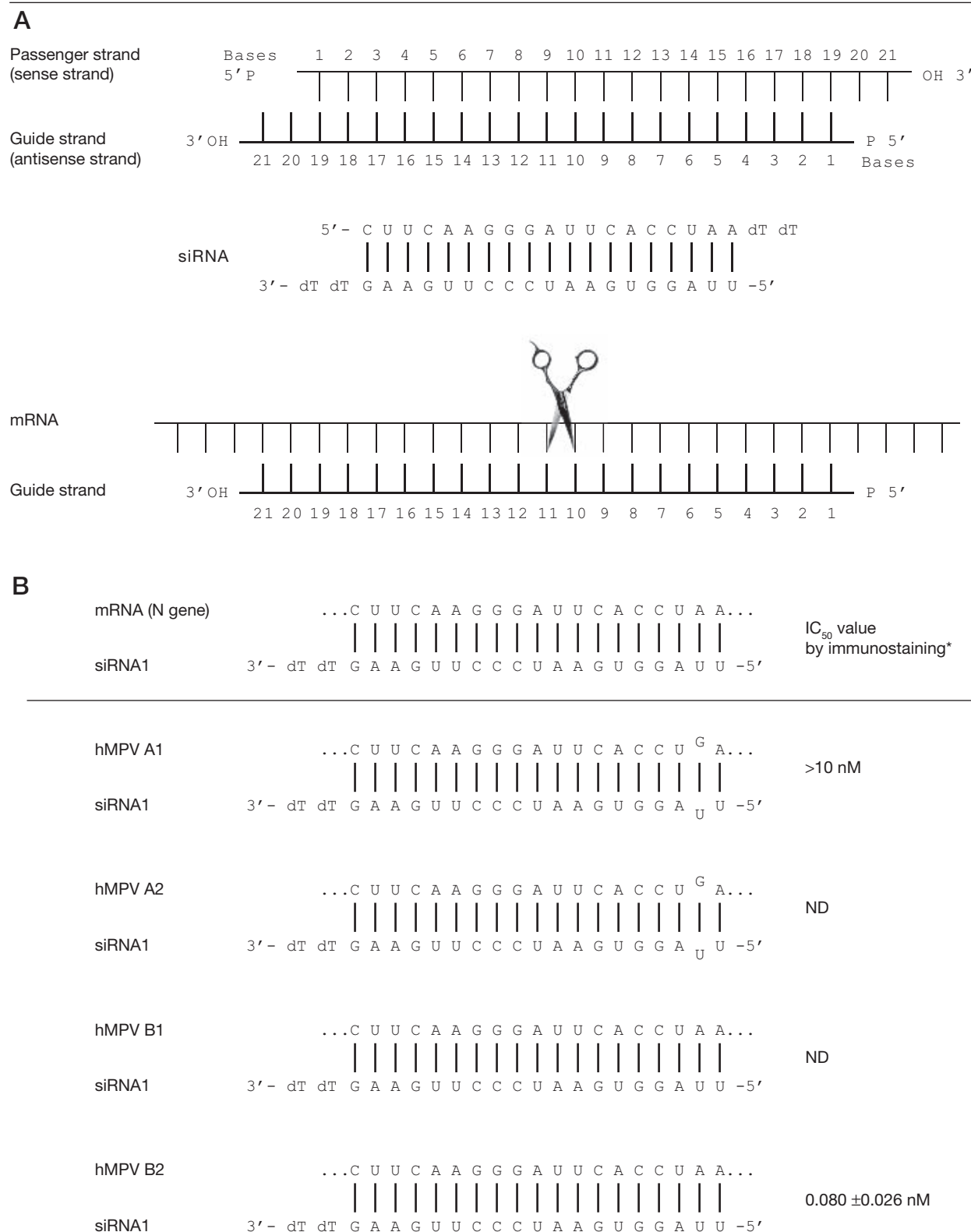
Effects of mismatches between the siRNA and its target
In the present study, the designed siRNAs were not 100% complementary to the various hMPV strains tested because siRNA sequences were selected using CAN98-75 as the consensus sequence. Sequence analysis based on the four hMPV genotypes showed that five different mismatches were found between siRNA1, siRNA45, siRNA60 and their respective targets. Three

were located in the 5' region of the siRNAs and two in the 3' region (Figure 3).

siRNA1 was 100% complementary with hMPV B2 and was indeed very effective against this strain (IC₅₀ value 0.080 ±0.026 nM), whereas there was a mismatch with the A1 strain at position 2 (A to G) resulting in an IC₅₀ value >10 nM against this strain (Figure 3B).

In contrast, two mismatches were found when siRNA45 was compared with the hMPV A1 strain, one at position 2 (U to C) and the other at position 18 (A to C; Figure 3C). Surprisingly, siRNA45 remained fully active against this strain (IC₅₀ value 0.046 ±0.027 nM). The same mismatch at position 18 was found between hMPV A2 and siRNA45 without any loss of activity (IC₅₀ value <0.078 nM). hMPV B1 and B2 strains were 100% complementary to siRNA45 and, as expected, excellent inhibitory activity (IC₅₀ value <0.078 nM) was observed.

Similar to siRNA45, siRNA60 was very effective against the four hMPV strains tested (IC₅₀ values 0.075 ±0.068 nM against hMPV A1, 0.090 ±0.051 nM against hMPV A2 and <0.078 nM against hMPV B1 and B2) despite a single mismatch for two strains (Figure 3D). The hMPV A2 strain had a mismatch in the target region corresponding to position 5 of the siRNA (G to A), whereas the mismatch in hMPV B1 strain corresponded to position 17 (A to C).

Figure 3. Nucleotide mismatches between siRNA1, siRNA45 and siRNA60 with their respective hMPV targets

*Values represent the mean of two to four independent experiments with errors expressed as $\pm$ sd. (A) Illustration of a typical 21-mer small interfering RNA (siRNA) duplex composed of a passenger strand (sense strand) and a guide strand (antisense strand), followed by pairing with its target messenger RNA (mRNA) and cleavage of mRNA between base positions 10 and 11. (B) Sequence of siRNA1 with location of mismatches for target mRNAs and corresponding 50% inhibitory concentration (IC₅₀) values. (C) Sequence of siRNA45 with location of mismatches for target mRNAs and corresponding IC₅₀ values. (D) Sequence of siRNA60 with location of mismatches for target mRNAs and corresponding IC₅₀ values. hMPV, human metapneumovirus; N, nucleoprotein; ND, not done; P, phosphoprotein.

Figure 3. Continued

C

mRNA (N gene)	...G A A G C A A A G C A G A A A G U U U...	IC ₅₀ value by immunostaining*
siRNA45	3' - dT dT C U U C G U U U C G U C U U U C A A A -5'	

hMPV A1	...G ^C A G C A A A G C A G A A A G U ^C U...	0.046 ±0.027 nM
siRNA45	3' - dT dT C _U U C G U U U C G U C U U U C A _A A -5'	

hMPV A2	...G ^C A G C A A A G C A G A A A G U U U...	<0.078 nM
siRNA45	3' - dT dT C _U U C G U U U C G U C U U U C A A A -5'	

hMPV B1	...G A A G C A A A G C A G A A A G U U U...	<0.078 nM
siRNA45	3' - dT dT C U U C G U U U C G U C U U U C A A A -5'	

hMPV B2	...G A A G C A A A G C A G A A A G U U U...	<0.078 nM
siRNA45	3' - dT dT C U U C G U U U C G U C U U U C A A A -5'	

D

mRNA (P gene)	...G C A G A A G C U U U C C A G A A A U...	IC ₅₀ value by immunostaining*
siRNA60	3' - dT dT C G U C U U C G A A A G G U C U U U A -5'	

hMPV A1	...G C A G A A G C U U U C C A ^A A A A U...	0.075 ±0.068 nM
siRNA60	3' - dT dT C G U C U U C G A A A G G _C U U U A -5'	

hMPV A2	...G C A G A A G C U U U C C A G A A A U...	0.090 ±0.051 nM
siRNA60	3' - dT dT C G U C U U C G A A A G G U C U U U A -5'	

hMPV B1	...G C ^C G A A G C U U U C C A G A A A U...	<0.078 nM
siRNA60	3' - dT dT C G _U C U U C G A A A G G U C U U U A -5'	

hMPV B2	...G C A G A A G C U U U C C A G A A A U...	<0.078 nM
siRNA60	3' - dT dT C G U C U U C G A A A G G U C U U U A -5'	

Evaluation of siRNA cocktail efficiency

Simultaneous transfection of the two most active siRNAs (siRNA45 and siRNA60) was evaluated by

plaque assay followed by immunostaining. Equimolar concentrations of each siRNA (from 0.039 nM to 100 nM) were tested simultaneously. This siRNA

cocktail was as potent as individual siRNAs with an IC_{50} value against hMPV A1 of 0.039 ± 0.028 nM compared with 0.046 ± 0.027 nM for siRNA45 alone and 0.075 ± 0.068 nM for siRNA60 alone (Table 2).

Longer Dicer substrates versus shorter siRNAs

Longer siRNAs containing 25/27 bases, known as Dicer substrate siRNAs, were also evaluated against hMPV A1 using plaque assay followed by immunostaining. Because siRNA45 contains two mismatches compared with the reference hMPV A1 strain, we designed two different Dicer substrate siRNAs, that is, one with the mismatch at position 18 but not at position 2 compared with the original siRNA45 sequence (siRNA45-Dicer), and another one that was fully complementary to the A1 strain (siRNA45-mut-Dicer). These two Dicer substrate siRNAs showed slightly better hMPV inhibition than the original 21-mer siRNA45 with IC_{50} values of 0.025 ± 0.023 nM, 0.018 ± 0.011 nM and 0.046 ± 0.027 nM for siRNA45-mut-Dicer, siRNA45-Dicer and siRNA45, respectively (Table 2). Another 19-mer siRNA was also designed from the siRNA45 target sequence to correct the mismatches against hMPV A1, that is, the mismatch at position 18 in siRNA45 was corrected and this shorter siRNA version (siRNA45-mut) did not contain the other mismatch (at position 2). This siRNA had decreased activity against hMPV A1 with an IC_{50} value of 0.476 ± 0.326 nM compared with 0.046 ± 0.027 nM for the original 21-mer siRNA (Table 2). Finally, another 25/27-mer siRNA was designed based on the siRNA60 sequence (siRNA60-Dicer), and its

activity against hMPV A1 was also slightly better than the corresponding 21-mer siRNA60 with IC_{50} values of 0.022 ± 0.000 nM for siRNA60-Dicer and 0.075 ± 0.068 nM for siRNA60 (Table 2).

Cytotoxicity associated with siRNA transfection

We used Lipofectamine 2000 to achieve siRNA transfection in cells and noticed that, in some experiments, Lipofectamine 2000 alone showed some toxicity compared with untransfected cells (data not shown). We also evaluated the cytotoxicity of the cocktail and that of nine siRNAs (control siRNA, siRNA1, siRNA45, siRNA60, siRNA118, siRNA45-mut, siRNA-mut-Dicer, siRNA45-Dicer and siRNA60-Dicer). All but one siRNA had no effect on cell viability when compared with Lipofectamine 2000 alone at the highest tested concentration (100 nM). siRNA118 was cytotoxic at the two highest concentrations (10 and 100 nM).

Discussion

In this study, we demonstrated that hMPV can be successfully inhibited *in vitro* by specific siRNAs. From a starting pool of 200 siRNAs identified by computational analysis of the hMPV genome, we first selected 57 siRNAs using a luciferase assay which did not require infectious particles. We next screened the selected siRNAs at two concentrations (10 and 100 nM) against an hMPV group A1 strain by real-time quantitative RT-PCR. The top 10 siRNAs were then evaluated against two to four hMPV subgroups by RT-PCR and

Table 2. IC_{50} values for a cocktail of two siRNAs (siRNA45 and siRNA60), individual siRNAs and Dicer substrate siRNAs against an hMPV group A1 strain by plaque assay

siRNA	Length	Target gene	siRNA sequence ^{a†}	IC_{50} , nM [‡]
Cocktail of siRNA45 + siRNA60	21-mer	N + P	5'-GAAGCAAAGCAGAAAGUUUdTdT-3' 3'-dTdTTCUUCGUUUCGUCUUUCAA-5' + 5'-GCAGAAGCUUCCAGAAAUdTdT-3' 3'-dTdTTCGUCUUCGAAAGGUCUUUA-5'	$0.039 \pm 0.028^§$
siRNA45	21-mer	N	5'-GAAGCAAAGCAGAAAGUUUdTdT-3' 3'-dTdTTCUUCGUUUCGUCUUUCAA-5'	0.046 ± 0.027
siRNA45-mut	19-mer	N	5'-GCAGCAAAGCAGAAAGUdTdT-3' 3'-dTdTTCGUCGUUUCGUCUUUCA-5'	0.476 ± 0.326
siRNA45-mut-Dicer	25/27-mer	N	5'-GCAGCAAAGCAGAAAGUCU AUUUdGdT -3' 3'-UCCGUCGUUUCGUCUUUCAG AUAAACA -5'	0.025 ± 0.023
siRNA45-Dicer	25/27-mer	N	5'-GAAGCAAAGCAGAAAGUCU AUUUdGdT -3' 3'-UCCUUCGUUUCGUCUUUCAG AUAAACA -5'	0.018 ± 0.011
siRNA60	21-mer	P	5'-GCAGAAGCUUCCAGAAAUdTdT-3' 3'-dTdTTCGUCUUCGAAAGGUCUUUA-5'	0.075 ± 0.068
siRNA60-Dicer	25/27-mer	P	5'-GCAGAAGCUUCCAGAAAUCAUUAAdA-3' 3'-AUCGUCUUCGAAAGGUCUUAGUAAUU-5'	0.022 ± 0.000

^aSense strand 5'-3', antisense strand 3'-5'. [†]Bold, underlined nucleotides represent change in small interfering RNA (siRNA) sequence compared with the original siRNA45. [‡]Means ($\pm$ SD) of two to four independent experiments. [§]Equal amounts of each siRNA (siRNA45 and siRNA60) were used in the cocktail. hMPV, human metapneumovirus; IC_{50} , 50% inhibitory concentration; N, nucleoprotein; P, phosphoprotein.

plaque assay followed by immunostaining. We finally identified two highly efficient siRNAs (siRNA45 and siRNA60) that were similarly active against all four hMPV subgroups.

siRNA activity against other paramyxoviruses such as hRSV, human parainfluenza virus (hPIV) and aMPV has also been reported. siRNAs targeting aMPV P and N mRNAs, which are the closest genetic homologues to hMPV, have been successfully identified *in vitro* [33,34]. Also, *in vivo* experiments have shown that hRSV and hPIV replication can be efficiently inhibited by siRNAs targeting their P mRNA, and that a cocktail of both siRNAs can inhibit a dual viral infection in mice [28]. The hRSV NS1 gene (which is absent in hMPV) has also been successfully inhibited by siRNA *in vitro* and *in vivo* [35]. Moreover, Phase I and II trials of ALN-RSV01 (an siRNA targeting the hRSV N mRNA delivered via nasal spray and inhalation) have been recently completed in adults [39].

In our study, widely different inhibitory activities were sometimes obtained for siRNAs depending on the evaluation method. For instance, some siRNAs showed good hMPV inhibition when evaluated by quantitative RT-PCR performed on cell culture supernatant, but were not active ($IC_{50} > 10$ nM) when evaluated by plaque assay followed by immunostaining (Table 1). It is likely that PCR analyses are less representative of viable viral progeny production compared with plaque assays, which directly estimate the amount of infectious particles present in a sample. Incomplete viral genomes or viral mRNAs (for example, N or P mRNAs) could be present in cell culture supernatant and might have led to an overestimation of siRNA activity when evaluated by PCR. Consequently, plaque assay followed by immunostaining appears to be more accurate for assessing siRNA potency in inhibiting hMPV replication, although additional *in vitro* and *in vivo* evaluations are required. Also, transfection efficiency and other reasons besides intrinsic on-target siRNA activity can be responsible for the differences observed between evaluation methods and between replicates of the same experiment.

We cannot completely rule out the possibility that hMPV inhibition might be due to immune stimulation of LLC-MK2 cells following siRNA transfection. However, the absence of antiviral activity associated with the control siRNA and control Dicer substrate would suggest that interferon induction was not involved in hMPV inhibition.

siRNA sequences were chosen from conserved regions of different hMPV genomes, but the final sequences were selected based on hMPV B2, the consensus sequence. This approach resulted in a few mismatches between siRNAs and some of the strains used in subsequent evaluation. Among our top siRNA candidates, we found some mismatches with viral targets that were not always

associated with a loss of activity. Most reports assessing the influence of target mismatches on siRNA activity showed that a single nucleotide change can reduce or completely abolish the siRNA silencing effect [27,40]. According to studies on miRNA efficacy and tolerance to mismatches, the 5' region is crucial for recognition and pairing to the target mRNA and does not usually accept major changes [25,41]. The base at position 1 of the guide strand does not seem to pair with the mRNA target sequence, but rather interacts with proteins from RISC. However, nucleotides 2–8, which are responsible for recognition of mRNA target, do not easily tolerate mismatches. On the other hand, the 3' region of miRNA allows for more base substitutions without drastically affecting its efficacy. It is believed that siRNA molecules follow the same rules as miRNA [42] but, according to our findings, some changes could be tolerated, even in the 5' region of siRNAs. Figure 3A illustrates the position of the nucleotides forming the siRNA and the cleavage of target mRNA by RISC between positions 10 and 11 of the siRNA guide strand. Here, we report on two siRNAs (siRNA45 and siRNA60) for which mismatches in the 5' region (positions 2 and 5, respectively) did not abrogate their activity, whereas a mismatch at position 2 of siRNA1 was indeed associated with loss of function (Figure 3). As expected, mismatches located in the 3' region of siRNAs (position 18 of siRNA45 and position 17 of siRNA60) did not affect their anti-hMPV potency. Furthermore, siRNA45, which simultaneously presented two mismatches when compared with the hMPV A1 target sequence, remained highly effective against this strain. The position of mismatch is important for siRNA activity but the type of substitution could also be a key element in siRNA tolerance to mismatches. Substitutions of U to C (at position 2), of G to A (at position 5), of A to C (at position 17) and of A to C (at position 18) were well-tolerated, whereas a substitution of A to G (at position 2) was deleterious for siRNA activity. This suggests that some mismatches can be tolerated in the siRNA sequence, but more detailed studies must be conducted to identify essential base-pairing positions and types of substitutions allowed for retaining siRNA activity.

We further demonstrated that a cocktail of two siRNAs (siRNA45 and siRNA60) can be successfully used to inhibit hMPV replication, at least as efficiently as individual siRNAs, without inducing cytotoxicity. The use of a cocktail of siRNAs might theoretically be helpful to prevent treatment failure if deleterious mutations do happen in the target mRNA. This is a possibility considering the number of variations reported in so-called conserved viral genes.

Our two best siRNA candidates target mRNA of essential structural proteins. siRNA45 targets the N mRNA and siRNA60 targets the P mRNA. These proteins form the replication-transcription complex along

with the L protein and are essential for viral replication [3]. Thus, silencing of these components leads to a nearly total loss of all RNA synthesis [16]. Moreover, *de novo* synthesis of N and P proteins following infection is essential for efficient viral replication of another paramyxovirus, such as the Sendai virus [43]. The N and P proteins are good targets because they are generally well conserved between all hMPV strains and less prone to immunological selective pressure than surface proteins [17–19]. Even though the N and P mRNAs are the two most frequent mRNAs produced in hMPV-infected cells [15], their cleavage induced by siRNA45 and siRNA60 transfection is highly effective in blocking replication, even at very low concentrations, that is, in the subnanomolar range against all hMPV subgroups (Table 1 and Figure 3). Because these experiments involved transfection of cells in order to observe inhibition of hMPV replication, we cannot compare our siRNA IC₅₀ values to those of other antiviral compounds such as ribavirin [44].

In this study, we initially used 21-mer siRNAs and identified two highly potent siRNAs against hMPV. Because some reports demonstrated better activity associated with longer siRNAs known as Dicer substrates [45–49], we designed and evaluated 25/27-mer Dicer substrates corresponding to our top 21-mer siRNA candidates, that is, siRNA45 and siRNA60. Two Dicer substrates derived from siRNA45 and one from siRNA60 were tested against hMPV A1 strain. siRNA45-mut-Dicer had two RNA bases modified from the original siRNA45 sequence to obtain perfect complementarity with the hMPV A1 strain, whereas siRNA45-Dicer still contained the mismatch corresponding to position 18 in siRNA45. siRNA60-Dicer had no modification compared with the original RNA bases and was fully complementary to the hMPV A1 strain. When evaluated by plaque assay followed by immunostaining, these three Dicer substrate siRNAs were at least as effective (and possibly slightly more active) against hMPV A1 than the 21-mer siRNAs (Table 2), which suggests that longer siRNAs can be an equal or even better option. Moreover, a gain in stability could be obtained with these longer siRNAs. A revised and shorter (19-mer) version of siRNA45 that contained no mismatches compared with hMPV A1 was also evaluated, but showed decreased activity against this strain compared with the original 21-mer siRNA. Thus, both the length and sequence of siRNAs are important prerequisites for effective viral inhibition.

As these siRNAs are highly potent in inhibiting hMPV replication *in vitro* and are not associated with cytotoxicity in LLC-MK2 cells, subsequent evaluation of their activity against hMPV in a small animal model in the presence or absence of delivery vehicles is extremely desirable. This will be further supplemented

with the cytokine profiling from the bronchoalveolar lavage fluid of siRNA-treated mice. Considering that lung epithelial cells can be targeted by RNAi therapeutics following intranasal challenge with naked siRNAs or with the use of a carrier [28,45,50,51], topical antiviral therapy based on RNAi appears a promising avenue for hMPV and other respiratory viral infections [26,29].

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Disclosure statement

KC, QG, SS, MVT, SCQ and PHJ are employees or former employees of MDRNA Inc. All other authors declare no competing interests.

References

1. van den Hoogen BG, de Jong JC, Groen J, *et al.* A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 2001; 7:719–724.
2. Deffrasnes C, Hamelin ME, Boivin G. Human metapneumovirus. *Semin Respir Crit Care Med* 2007; 28:213–221.
3. Broor S, Bharaj P. Avian and human metapneumovirus. *Ann N Y Acad Sci* 2007; 1102:66–85.
4. Boivin G, Abed Y, Pelletier G, *et al.* Virological features and clinical manifestations associated with human metapneumovirus: a new paramyxovirus responsible for acute respiratory-tract infections in all age groups. *J Infect Dis* 2002; 186:1330–1334.
5. Boivin G, De Serres G, Hamelin ME, *et al.* An outbreak of severe respiratory tract infection due to human metapneumovirus in a long-term care facility. *Clin Infect Dis* 2007; 44:1152–1158.
6. Louie JK, Schnurr DP, Pan CY, *et al.* A summer outbreak of human metapneumovirus infection in a long-term-care facility. *J Infect Dis* 2007; 196:705–708.
7. Hamelin ME, Prince GA, Gomez AM, Kinkad R, Boivin G. Human metapneumovirus infection induces long-term pulmonary inflammation associated with airway obstruction and hyperresponsiveness in mice. *J Infect Dis* 2006; 193:1634–1642.
8. Garcia-Garcia ML, Calvo C, Casas I, *et al.* Human metapneumovirus bronchiolitis in infancy is an important risk factor for asthma at age 5. *Pediatr Pulmonol* 2007; 42:458–464.
9. Boivin G, Mackay I, Sloots TP, *et al.* Global genetic diversity of human metapneumovirus fusion gene. *Emerg Infect Dis* 2004; 10:1154–1157.
10. Mackay IM, Bialasiewicz S, Jacob KC, *et al.* Genetic diversity of human metapneumovirus over 4 consecutive years in Australia. *J Infect Dis* 2006; 193:1630–1633.

11. Mackay IM, Bialasiewicz S, Waliuzzaman Z, et al. Use of the P gene to genotype human metapneumovirus identifies 4 viral subtypes. *J Infect Dis* 2004; 190:1913–1918.
12. Manoha C, Espinosa S, Aho SL, Huet F, Pothier P. Epidemiological and clinical features of hMPV, RSV and RVs infections in young children. *J Clin Virol* 2007; 38:221–226.
13. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. Analysis of the genomic sequence of a human metapneumovirus. *Virology* 2002; 295:119–132.
14. Cowton VM, McGivern DR, Fearn R. Unravelling the complexities of respiratory syncytial virus RNA synthesis. *J Gen Virol* 2006; 87:1805–1821.
15. Easton AJ, Domachowske JB, Rosenberg HF. Animal pneumoviruses: molecular genetics and pathogenesis. *Clin Microbiol Rev* 2004; 17:390–412.
16. Barik S. Control of nonsegmented negative-strand RNA virus replication by siRNA. *Virus Res* 2004; 102:27–35.
17. Skiadopoulos MH, Biacchesi S, Buchholz UJ, et al. Individual contributions of the human metapneumovirus F, G, and SH surface glycoproteins to the induction of neutralizing antibodies and protective immunity. *Virology* 2006; 345:492–501.
18. Ulbrandt ND, Ji H, Patel NK, et al. Isolation and characterization of monoclonal antibodies which neutralize human metapneumovirus *in vitro* and *in vivo*. *J Virol* 2006; 80:7799–7806.
19. Padhi A, Verghese B. Positive natural selection in the evolution of human metapneumovirus attachment glycoprotein. *Virus Res* 2008; 131:121–131.
20. Baulcombe D. RNA silencing in plants. *Nature* 2004; 431:356–363.
21. Wang MB, Metzclaff M. RNA silencing and antiviral defense in plants. *Curr Opin Plant Biol* 2005; 8:216–222.
22. de Fougères A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* 2007; 6:443–453.
23. Anderson EM, Birmingham A, Baskerville S, et al. Experimental validation of the importance of seed complement frequency to siRNA specificity. *RNA* 2008; 14:853–861.
24. Ui-Tei K, Naito Y, Zenno S, et al. Functional dissection of siRNA sequence by systematic DNA substitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. *Nucleic Acids Res* 2008; 36:2136–2151.
25. Yuan YR, Pei Y, Ma JB, et al. Crystal structure of A. aeolicus argonaute, a site-specific DNA-guided endoribonuclease, provides insights into RISC-mediated mRNA cleavage. *Mol Cell* 2005; 19:405–419.
26. Barik S, Bitko V. Prospects of RNA interference therapy in respiratory viral diseases: update 2006. *Expert Opin Biol Ther* 2006; 6:1151–1160.
27. Bitko V, Barik S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. *BMC Microbiol* 2001; 1:34.
28. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat Med* 2005; 11:50–55.
29. Ge Q, Eisen HN, Chen J. Use of siRNAs to prevent and treat influenza virus infection. *Virus Res* 2004; 102:37–42.
30. Ge Q, Filip L, Bai A, et al. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc Natl Acad Sci U S A* 2004; 101:8676–8681.
31. Ge Q, McManus MT, Nguyen T, et al. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc Natl Acad Sci U S A* 2003; 100:2718–2723.
32. Zhang W, Yang H, Kong X, et al. Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene. *Nat Med* 2005; 11:56–62.
33. Munir S, Kaur K, Kapur V. Avian metapneumovirus phosphoprotein targeted RNA interference silences the expression of viral proteins and inhibits virus replication. *Antiviral Res* 2006; 69:46–51.
34. Ferreira HL, Spilki FR, de Almeida RS, Santos MM, Arns CW. Inhibition of avian metapneumovirus (AMPV) replication by RNA interference targeting nucleoprotein gene (N) in cultured cells. *Antiviral Res* 2007; 74:77–81.
35. Kong X, Zhang W, Lockey RE, et al. Respiratory syncytial virus infection in Fischer 344 rats is attenuated by short interfering RNA against the RSV-NS1 gene. *Genet Vaccines Ther* 2007; 5:4.
36. Deffrasnes C, Hamelin ME, Prince GA, Boivin G. Identification and evaluation of a highly effective fusion inhibitor for human metapneumovirus. *Antimicrob Agents Chemother* 2008; 52:279–287.
37. Thermo Fisher Scientific Inc. Dharmacon RNAi Technologies, siDesign® Centre. (Accessed 26 August 2008.) Available from <http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx>
38. Whitehead Institute for Biomedical Research. WI siRNA selection program. (Accessed 26 August 2008.) Available from <http://jura.wi.mit.edu/bioc/siRNAext>
39. DeVincenzo J, Cehelsky JE, Alvarez R, et al. Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). *Antiviral Res* 2008; 77:225–231.
40. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature* 2002; 418:435–438.
41. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev* 2004; 18:504–511.
42. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *Embo J* 2001; 20:6877–6888.
43. Wiegand MA, Bossow S, Schlecht S, Neubert WJ. *De novo* synthesis of N and P proteins as a key step in Sendai virus gene expression. *J Virol* 2007; 81:13835–13844.
44. Wyde PR, Chetty SN, Jewell AM, Boivin G, Piedra PA. Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by ribavirin and immune serum globulin *in vitro*. *Antiviral Res* 2003; 60:51–59.
45. Akinc A, Zumbuehl A, Goldberg M, et al. A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat Biotechnol* 2008; 26:561–569.
46. Kim DH, Behlke MA, Rose SD, et al. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* 2005; 23:222–226.
47. Siolas D, Lerner C, Burchard J, et al. Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* 2005; 23:227–231.
48. Amarzguioui M, Lundberg P, Cantin E, et al. Rational design and *in vitro* and *in vivo* delivery of Dicer substrate siRNA. *Nat Protoc* 2006; 1:508–517.
49. Kubo T, Zhelev Z, Ohba H, Bakalova R. Modified 27-nt dsRNAs with dramatically enhanced stability in serum and long-term RNAi activity. *Oligonucleotides* 2007; 17:445–464.
50. de Fougères AR. Delivery vehicles for small interfering RNA *in vivo*. *Hum Gene Ther* 2008; 19:125–132.
51. Novobrantseva TI, Akinc A, Borodovsky A, de Fougères AR. Delivering silence: advancements in developing siRNA therapeutics. *Curr Opin Drug Discov Devel* 2008; 11:217–224.