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RNA interference *in vitro* and *in vivo* using DsiRNA targeting the nucleocapsid N mRNA of human metapneumovirus ☆

Magali Darniot [a,1], Verena Schildgen [b,1,2], Oliver Schildgen [b,1,2], Brian Sproat [c,1,3], Michael Kleines [d,1,4], Vanessa Ditt [b,1,2], Cécile Pitoiset [a,1], Pierre Pothier [a,1], Catherine Manoha [a,*,1]

[a] *Laboratoire de Virologie, CHU Dijon, 2 Rue Angélique Ducoudray, 21070 Dijon cedex, France*
[b] *Institute of Virology, University of Bonn Medical Centre, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany*
[c] *Integrated DNA Technologies, BVBA, Interleuvenlaan 12A, 3001 Leuven, Belgium*
[d] *Division of Virology, MUI Innsbruck, Fritz-Pregl-Straße 3, Innsbruck 6020, Austria*

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ABSTRACT

Human metapneumovirus causes respiratory diseases with outcomes that can be severe in children, the immunocompromised, and the elderly. Synthetic small interfering RNAs (siRNAs) that silence targeted genes can be used as therapeutic agents. Currently, there is no specific therapy for hMPV. In this study, we designed Dicer-substrate siRNAs (DsiRNAs) that target metapneumovirus sequences on the mRNAs of the N, P, and L genes. *In vitro*, six DsiRNAs were shown to inhibit virus replication using cell proliferation tests. Of those, the DsiRNA that targets the most conserved mRNA sequence was then resynthesized in Evader™ format with heavy 2′-*O*-methyl modification of the guide strand. In a murine model, the prophylactic administration of this Evader™ DsiRNA was effective at partially inhibiting viral replication of hMPV (13×10^3 vs. 29×10^3 PFU/g of lung; $p < 0.01$), which was not the case for the control, a mismatched DsiRNA. Inhibition was achieved without inducing cytokines or off-target effects. Moreover, the specificity of the siRNA mechanism of action was demonstrated *in vitro* and *in vivo* using 5′-RACE methodology. This *in vivo* approach of using a DsiRNA against hMPV is an important step in the development of synthetic siRNA as a therapeutic agent for this virus.

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1. Introduction

hMPV is a major pathogen that causes acute respiratory illness (ARI) worldwide in individuals of all ages (Bastien et al., 2003; Boivin et al., 2002; Stockton et al., 2002; van den Hoogen et al., 2001). Higher morbidity is observed in young children, the elderly, and immunocompromised adults. The clinical characteristics of hMPV infection range from asymptomatic or mild upper respiratory tract infection to severe respiratory failure. hMPV is an important cause of hospitalization and critical illness, particularly among high-risk patients. The main clinical symptoms include rhinorrhea, cough, dyspnea, wheezing and exacerbation of asthma (Manoha et al., 2007; Peiris et al., 2003; Walsh et al., 2008; Williams et al., 2004). There is no vaccine for hMPV or specific antiviral therapy approved for the treatment of hMPV. Therapy consists mainly of symptomatic treatment. Up to now, only ribavirin, a guanosine analogue used in some cases for patients with severe respiratory syncytial virus (RSV) infection, and immunoglobulin have been used in some cases of severe hMPV infections (Bonney et al., 2009; Raza et al., 2007), even though potential adverse effects of ribavirin have been reported.

RNA interference (RNAi) is a natural major mechanism of post-transcriptional gene silencing that occurs in eukaryotic cells (Hannon, 2002; Sharp, 1999). This natural cellular process has led to the development of synthetic siRNA to silence targeted gene expression. RNAi can be used to analyse gene function, or be developed as therapeutic agents (Dorsett and Tuschl, 2004; Elbashir

Abbreviations: hMPV, human metapneumovirus; N, nucleoprotein; RNAi, RNA interference; D, dicer; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; TLR, Toll-like receptor; IFN-α, interferon-α; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; i.n., intranasal(ly); BAL, bronchoalveolar lavage; AO, airway obstruction; HRP, horseradish peroxidase; AEC, 3-amino-9-ethyl-carbazole; CCID, tissue culture infectious dose.

☆ DsiRNA design was done in Leuven, Belgium. *XTT* screening was done in Bonn, Germany. Imaging was done in Aachen, Germany. *In vivo* work was done in Dijon, France. Race analysis was done in Dijon, France.

* Corresponding author. Tel.: +33 3 80 29 50 02; fax: +33 3 80 29 36 04.
E-mail addresses: schildgeno@kliniken-koeln.de (O. Schildgen), fa205401@skynet.be (B. Sproat), michael.kleines@i-med.ac.at (M. Kleines), catherine.manoha@chu-dijon.fr (C. Manoha).
[1] All authors contributed equally.
[2] Current address: Kliniken der Stadt Köln gGmbH, Krankenhaus Merheim, Klinikum der Privaten Universität Witten/Herdecke, Institut für Pathologie, Ostmerheimer Str. 200, D-51109 Köln, Germany. Tel.: +49 (0) 221 8907 13467; fax: +49 (0) 221 8907 3542.
[3] Current address: Chemconsilium GCV, Jaarmarktstraat 48, 2221 Booischot, Belgium. Tel.: +32 15 245667.
[4] Current address: Division of Virology, MUI Innsbruck, Fritz-Pregl-Straße 3, 6020 Innsbruck, Austria. Tel.: +43 512 9003 71712; fax: +42 512 9003 73701.

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et al., 2001) to target disease-associated genes. RNAi is mediated through the RNA-induced silencing complex (RISC) and leads to the sequence-specific degradation of mRNA by the complementary antisense strand of a double-stranded, small interfering RNA (siRNA) (Elbashir et al., 2001). The expression of the protein related to the targeted gene is suppressed in a highly selective and specific way. The Dicer endonuclease is involved in loading the siRNA into RISC and assembly (Lee et al., 2004; Rose et al., 2005; Sontheimer, 2005). Dicer-substrate RNAs (DsiRNAs) are longer duplex RNAs compared to traditional 21-mer siRNAs and usually have increased potency in RNAi because they are optimally processed by Dicer (Kim et al., 2005).

One drawback encountered with siRNAs is the possible activation of the innate immune system. Innate immune response activation results in the release of inflammatory cytokines and interferons such as IFN-α, triggered by TLR ligands (Hornung et al., 2005; Judge et al., 2005). This inflammatory response can be severe. Moreover, to avoid nuclease degradation *in vivo*, chemical modifications to the 2′-OH group in the ribose residues have to be included during siRNA synthesis. Small interfering RNAs must be stabilized and chemically modified without impairing the gene-silencing activity. In certain cases, however, these chemical modifications may have a negative impact on RNAi activity (Behlke, 2008; Bramsen et al., 2010; Collingwood et al., 2008; Prakash et al., 2005). Small interfering RNAs against respiratory viruses, such as influenza and RSV (DeVincenzo et al., 2010; Saravolac et al., 2010) have already been developed. The siRNA ALN-RSV01 directed against a highly conserved region of the mRNA encoding the nucleocapsid (N) protein of RSV (DeVincenzo et al., 2010) has already undergone early clinical trials. In addition, three siRNAs have previously been shown to inhibit hMPV replication *in vitro* (Deffrasnes et al., 2008). In this study, we report on the *in vitro* as well as *in vivo* silencing of the viral N mRNA by the prophylactic intranasal administration of a DsiRNA. An RNAi-mediated silencing mechanism was confirmed and corroborated the decrease of viral load; moreover, this was achieved in the absence of immune stimulation.

2. Materials and methods

2.1. Cells and virus

Human hepatoma HepG2 cells (ATCC HB-8065) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) (Schildgen et al., 2010). LLC-MK2 cells were maintained in supplemented Eagles' minimal essential medium with 5% FCS (Invitrogen/Gibco, Life Technologies). The hMPV strain (C4-CJP05), belonging to the A2 genotype, was a clinical strain, isolated in our laboratory that was cultured with a low number of passages through LLC-MK2 in EMEM containing 0.3% BSA and 0.025% trypsin (infection medium) (Darniot et al., 2005).

2.2. Mice

Six- to 8-week-old, pathogen-free female BALB/c mice were purchased from Janvier (Le Genest saint Isle, France). Throughout the study, they were allowed *ad libitum* access to food and water. This work was approved by the local ethics committee of University of Burgundy, France.

2.3. siRNA

The PubMed nucleotide sequence database contained 6 complete genomes when the siRNA design was performed: CAN97-83 (accession code NC_004148), BJ1816 (accession code DQ843658), BJ1887 (accession code DQ843659), NL/1/99 (accession code AY525843), CAN98-75 (accession code AY297748) and 00-1 (accession code AF371337). Based upon the RSV work we expected that the N, P and L genes would be good candidates for targeting by RNAi. Thus, a standard sequence comparison algorithm was used to search for optimal local alignments in the N, P and L genes, however alignment of these sequences gave no regions with 100% conservation for longer than about 20 nucleotides. Since the DsiRNA is longer than 20 nucleotides it proved impossible to design potent DsiRNAs capable of hitting N, P or L mRNAs for all genotypes. In order to find a compromise two strategies were chosen: (i), the best predicted DsiRNAs for each genotype were designed, giving 27 unique N designs, 27 unique P designs and 25 unique L designs; (ii), the best common sites with site variants to target each genotype were chosen, resulting in 23 unique N designs, 23 unique P designs and 18 unique L designs. This analysis led to 128 different DsiRNAs (there were a few sequence overlaps) that were chemically synthesized on a small scale as unmodified DsiRNAs by Integrated DNA Technologies, Inc. (Coralville, Iowa) and purified by reversed phase HPLC (C18 column) for *in vitro* screening.

DsiRNA sequences targeting hMPV were then selected using cell viability tests for the determination of their viral inhibitory activity (Schildgen et al., 2010). For *in vivo* experiments, 2′-*O*-Me modifications were then added to the unique selected siRNA that was called Evader™ DsiRNA. A mutated version of Evader™ DsiRNA, called DsiRNActrl II, was designed by Mark Behlke from Integrated DNA Technologies. The mutation pattern kept the same identical base composition but switched key residues at all possible seed region sites as well as at the base 10,11 positions in the mature 21-mer siRNA where Ago2 cleavage occurs during RNAi (Behlke, 2008). Large scale DsiRNA syntheses were performed by Integrated DNA Technologies BVBA (Leuven, Belgium); guide and passenger strands were purified by preparative anion-exchange HPLC under sterile conditions, annealed, desalted and the DsiRNA was lyophilized. Analysis was done by analytical HPLC, mass spectroscopy and endotoxin testing was performed to ensure suitability for *in vivo* use.

2.4. In vitro screening

The 128 DsiRNAs were tested for their ability to inhibit HMPV replication *in vitro* in HepG2 cells. Lyophilized DsiRNAs were reconstituted in 20 μl annealing buffer (IDT, Coralville, Iowa) and heated to 95 °C for 2 min. The solutions were cooled to room temperature, the final concentrations were 100 μM. Two nmol DsiRNA were transfected into the HepG2 cells with Lipofectamine™ RNAiMAX (Invitrogen, Karlsruhe, Germany) using the reverse transfection procedure as recommended by the Manufacturer for high-throughput transfection. Fifty microliters of HepG2 cell culture suspension containing 5×10^4 cells were added to each well of a 96-well cell culture plate containing 9.5×10^4 PFU of hMPV and incubated overnight at 33 °C. The infection medium was discarded and cells were washed twice with DMEM (PAA, Pasching, Austria). Cells were incubated with DsiRNAs diluted with DMEM to a final concentration of 1 μM for an additional 24 h at 37 °C before transfection medium was replaced by 150 μl fresh DMEM. Cells were incubated for 4 days at 33 °C and checked for their viability by the XTT assay as previously described. (Lüsebrink et al., 2010; Schildgen et al., 2010). The assays were performed in quadruplicate. The approach is reciprocal, i.e. the cells are protected from cytopathic effects and lysis by the siRNA and their viability can be measured by colorimetric XTT processing. Thus, a stronger antiviral effect leads to higher cell viability, i.e. a higher absorption in the XTT assay as more cells survive that in turn can metabolize the XTT reagent. Correspondence between optical density (OD) values and viral load was established using serially diluted titrated virus.

A regression analysis of the curve enabled the conversion of OD values into viral load. Viral inhibition was then calculated by taking the "virus + transfectant" value as reference.

2.5. In vitro siRNA transfection prior plaque assay or Rapid amplification of cDNA ends assay

LLC-MK2 cells were transfected with Evader™ DsiRNA C11 or DsiRNActrl II using Lipofectamine™ RNAiMAX and forward transfection procedure (Invitrogen, Life Technologies, Cergy Pontoise, France). LLC-MK2 cells were seeded into 24-well plates to ·90–95% confluence. Transfection with Lipofectamine™ RNAiMAX was done as follows: 1.5 μl of Lipofectamine™ RNAiMAX (Invitrogen, Life Technologies, Cergy Pontoise, France) was diluted in 50 μl Opti-MEM. The DsiRNA (0.09 μM or serial dilutions of DsiRNA for subsequent RACE or plaque assay, respectively) and Lipofectamine™ RNAiMAX mixtures were mixed (vol/vol) and incubated for 25 min at room temperature. At the end of the incubation, LLC-MK2 cells were washed before adding 200 or 500 μL of optiMEM/well and the 100 μl of mixtures (siRNA/RNAiMAX) for 5 h at 37 °C. Thus, the final concentration of DsiRNA was 15 nM and from 50 nM to 0.05 nM, respectively. Then, the cells were washed with PBS and infected with 500 or 60 PFU, respectively, of the C4-CJP05 viral strain in infection medium for 2 h at 37 °C. The inoculum was subsequently removed. For RACE assay, the cells were washed once and incubated with infection medium for 24 h at 37 °C. Total RNA was then recovered as described in Section 2.12. For plaque assay, the cells were covered with infection medium containing 0.5% agarose and incubated for 3 days at 37 °C. Detection of hMPV cytopathic plaques by immunostaining was conducted as follows: the agarose was removed and the cells were fixed in cold methanol (between each step, the cells were washed with PBS). A human anti-hMPV serum diluted to 1:100 in PBS was added to the cells, and the plates were incubated at 37 °C for 30 min. The cells were then incubated with an HRP-labeled anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) at 37 °C for 30 min before the addition of the AEC substrate for peroxidase (Vector Laboratories, Burlingame, CA). They were then left for 10 min at room temperature. The plates were finally rinsed with water. Cytopathic plaques were counted after AEC staining. The inhibition assay was done in triplicate.

2.6. In vivo imaging

In order to enable *in vivo* biodistribution studies of the active Evader™ DsiRNA C11 targeting hMPV, 1.76 mg of a highly purified version of this duplex was chemically synthesized at Integrated DNA Technologies BVBA (Leuven, Belgium) with a near-infrared (NIR) dye, CF™ 750 (Biotium Inc., Hayward, CA 94545) attached to the 5′-terminus of the guide strand via a C6-aminolinker. The duplex was analyzed by analytical HPLC and electrospray ionization mass spectroscopy (ESI-MS) which confirmed the correct mass of the CF™ 750-labelled guide strand at 11904.3 Da. This particular dye had not previously been used to label RNA. It is a very sensitive dye that absorbs at 750 nm and emits at 780 nm and has an extinction coefficient of ~250000 M^{-1} cm^{-1}. It has the additional advantage of higher photostability than similar dyes.

The CF750-labelled hMPV-specific DsiRNA was evaluated for intranasal delivery to the lungs using an *in vivo* model. The Visen Medical FMT 2500 imaging system was calibrated for detection of CF750-labelled hMPV-specific DsiRNA with 100 μl of 2.365 μM siRNA. The thorax of BALB/c mice ($n = 6$) was shaved to prevent biasing of the results by autofluorescence of hair. Next, they were nasally inoculated with 4 mg/kg of the CF750-labelled hMPV-specific DsiRNA in a volume of 20 μl. Within 15 min after the inoculation the animals were immobilized with a constant isofluorane anesthesia of 2% which was maintained throughout the procedure. Thoracal fluorescence was detected for a period close to 5 min starting 30 min after the inoculation of the siRNA. After the detection period the animals were allowed to wake up again. They were reanesthetized in advance to further time points of detection (1, 3, 5, 7, and 24 h). The imaging system's software calculated fluorescence images for each mouse. These were analyzed to determine the quantity of fluorescent siRNA molecules in the lung.

2.7. Experimental model of hMPV infection

The mice were anaesthetized with ketamine/xylazine. Infected groups ($n = 125$ in total) were either untreated or DsiRNA-treated ($n = 125$) as follows: The Evader™ DsiRNA C11 or DsiRNActrl II, were instilled intranasally (i.n.) (4 mg/kg) 30 min before hMPV i.n. challenge (1.10^6 PFU/mouse in 25 μl). The mice were then either killed by pentothal injection at day 1 post-challenge for the 5′ rapid amplification of cDNA ends (RACE) analysis on lung samples ($n = 3$/group) or at day 3 post-challenge for analysis of lung virus titers ($n = 7–12$) or monitored for body weight ($n = 12–61$) and airway obstruction ($n = 12–47$) until day 14 as an indicator of disease.

In addition, control groups ($n = 23$) were constituted as follows to evaluate siRNA safety: mice were either untreated or instilled with DsiRNAs but not infected. They were either killed at day 1 post-challenge to determine and analyze cell infiltration in bronchoalveolar lavages (BAL) ($n = 4$) and to measure the quantity of cytokines in BAL, or monitored for body weight and airway obstruction until day 14 ($n = 4–8$) as an indicator of tolerance to treatment.

2.8. hMPV quantification in lungs

To quantify viral replication, the weighed lungs were individually homogenized with 1 mm glass beads in a Mini-BeadBeater homogenizer (Biospec products, Bartlesville, OK). The suspension was centrifuged at 10,000*g* for 1 min at 4 °C and clarified. LLC-MK2 cells were seeded into 24-well plates 24 h prior to titration. Immediately before titration, the medium was aspirated from the cells which were then washed with PBS before inoculation with serial 10-fold dilutions of the virus in the infection medium. The plates were incubated at 37 °C for 2 h. The inoculum was subsequently removed and the cells were covered with infection medium containing 0.5% agarose. The virus infection was left to propagate for 3 days. Detection of hMPV cytopathic plaques by immunostaining was conducted as described in Section 2.5. Virus titer was expressed as PFU/g of lung tissue.

2.9. Bronchoalveolar lavage (BAL)

To test DsiRNA safety, BALs were collected 24 h after initial DsiRNA administration. Lavage of the airways was performed twice via a tracheal cannula with 1 ml of PBS. The resulting fluid was centrifuged (at 500*g* for 5 min). The supernatants were removed and stored at −80 °C for cytokine quantification by ELISA. IFN-α (PBL Biomedical, Piscataway, NJ), IL-6, and TNF-α (DuoSet ELISA Development System, RnD Systems, Minneapolis, MN) were detected by sandwich ELISA according to the Manufacturer's instructions. The lower limits of detection were 12.5, 15 and 60 pg/ml, respectively. The concentrations of the cytokines were calculated from a standard curve by using recombinant mouse protein as the standard. Pellets of harvested BAL cells were resuspended in 500 μl of RPMI medium and layered onto glass slides (Cytospin, ThermoShandon, Pittsburgh, PA) by cytocentrifugation (400*g*, 4 min, low speed). Specific cell populations were distinguished using EMB (RAL555 kit, RAL reagents, Martillac, France).

2.10. Body weight and airway obstruction

Body weight was measured daily until day 14 to monitor disease progression. Weight loss was calculated as a percentage of the starting weight (100%). Whole-body, unrestrained plethysmography (Buxco Electronics Inc; Sharon, CT) was used to monitor and express the respiratory dynamics of the mice in a quantitative manner. The enhanced pause (Penh) as measured by plethysmography, has already been validated in animal models of infection-associated airway obstruction (AO) (Hamelmann et al., 1997). The mice were allowed to acclimatize to the plethysmograph chamber, and then baseline readings were recorded to determine AO. Airway function was evaluated daily until day 14 post-infection.

2.11. In vitro and in vivo rapid amplification of cDNA ends (RACE)

To demonstrate that our chemically modified DsiRNA C11 functions via an RNAi mechanism, we used the RACE method to detect RNA-induced silencing complex (RISC) mediated cleavage of the hMPV N-mRNA (Fromont-Racine et al., 1993; Volloch et al., 1994). The RACE method was previously used to demonstrate *in vivo* that siRNA-mediated cleavage occurs precisely between the 10th and 11th positions when measured from the 5′-end of the guide strand (antisense) (Soutschek et al., 2004).

Total RNA was purified either from *in vitro*-transfected LLC-MK2 cells or from lungs that were harvested at day 1 p.i. and stored in RNAlater (Ambion, Life Technologies, Cergy Pontoise, France). RNA was purified by using Trizol (Invitrogen, Life Technologies, Cergy Pontoise, France), then Nucleospin RNAII according to modified Manufacturer's instructions (Macherey Nagel, Hoerdt, France). RNAs were precipitated using ethanol and RNA quality was confirmed by 2% agarose gel electrophoresis. 5′-RNA ligase-mediated RACE (5′-RLM RACE) was performed according to the Invitrogen GeneRacer manual with modifications. Total RNA was mixed with the GeneRacer RNA adaptor (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′), heated to 65 °C for 5 min, and snap-cooled on ice prior to ligation. RNA ligation was performed at 37 °C for 1 h according to the Manufacturer's instructions (Gene racer Kit, Invitrogen, Life Technologies, Cergy Pontoise, France). Samples were then purified by diafiltration using Amicon Ultra-0.5 ml 100 K filters (Millipore, Molsheim, France). 10 μl of the purified RNA ligation product was reverse transcribed using SuperScript III (Invitrogen, Life Technologies, Cergy Pontoise, France) and an hMPV-specific primer (hMPVcDNA: 5′-GCTTCTTT-TTCTTCA TCTGTGAGTCC 3′) designed to hybridize to a target site 3′ to the predicted hMPV siRNA–mediated mRNA cut site. Specific hMPV primers were designed using Primer express software (Applied Biosystems, Life Technologies, Cergy Pontoise, France). Reverse transcription was carried out at 53 °C for 45 min followed by inactivation at 70 °C for 15 min and snap-cooling on ice. To detect RNAi-specific cleavage products, 5′-RLM RACE-PCR was performed using forward GR5 (5′-CGACTGGAGCACGAGGACACTGA-3′) and hMPV revspec primer (5′- TGTTTGGCACTCTCCCTCGATACA-3′). These primers were chosen because they span the predicted hMPV N-mRNA cut site. Amplification was performed in an Eppendorf Mastercycler using Platinum Taq DNA polymerase High Fidelity (Invitrogen, Life Technologies, Cergy Pontoise, France) and touchdown PCR conditions of 94 °C for 2 min (1 cycle), 94 °C for 30 s and 68.1 °C for 1 min then 10 cycles with a decrease of 1 °C per cycle, 94 °C for 30 s and 58.1 °C for 30 s, 68 °C for 1 min (25 cycles), and 68 °C for 10 min (1 cycle). The PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The amplified product of 359 bp obtained in *in vitro* experiments was gel purified, cloned into pGEMt easy vector (pGEMt easy, Promega, Charbonnières les Bains, France), and sequenced.

For *in vivo* experiments, the identity of the PCR product was further confirmed by nested PCR amplification using primers (GR5′nested: 5′-GGACACTGACATGGACTGAAGGAGTA-3′) and (hMPVpcr2spec: 5′-GGCACTCTCCCTCGATACATACCTATTATG-3′). Amplification conditions for PCR2 were 94 °C for 2 min (1 cycle), 94 °C for 30 s and 65.5 °C for 1 min then 10 cycles with a decrease of 1 °C per cycle, 94 °C for 30 s and 55.5 °C for 30 s, 68 °C for 1 min (25 cycles), and 68 °C for 10 min (1 cycle). A strip of gel corresponding to between 320 and 350 bp was excised and the DNA fragment was cloned into pGEMt easy vector before sequencing. Similar assay conditions and primer design were employed to amplify cleavage products using DsiRNActrl II.

2.12. Statistical analysis

Data are presented as means ± SEM. Mean values were compared by one-way ANOVA and multiple comparison tests (Scheffe) in order to determine significant differences between the groups at the same time point. Bartlett's test was used to assess the homogeneity of variance. A non-parametric test (Kruskal–Wallis) was performed when the data were not normally distributed, when the variance was heterogeneous or when the sample size was too small. $p < 0.05$ was considered significant. The initial threshold was increased to take into account the number of tests performed. All analyses were performed with STATA software (version 8).

3. Results

3.1. In vitro DsiRNA testing

The XTT assay was previously shown to be a reciprocal read out system for virus infections (Schildgen et al., 2010; Lüsebrink et al., 2010). *In vitro* testing was performed with 2 nmol/well of each unmodified DsiRNA using cell viability assays. OD values obtained for each siRNA relative to OD values obtained for "virus + transfectant" were converted into viral inhibition percentages as described in Section 2.4. In total, 6 out of 128 DsiRNAs displayed an antiviral effect, two sequences targeted the N gene and 4 the L gene (Fig. 1a, b). Antiviral activity is also shown for three randomly selected non-potent siRNAs targeting the N gene (Fig. 1a). Of the 6 DsiRNAs the DsiRNA targeting the gene region mostly conserved among the so far known hMPV strains, namely C11, was used for further *in vivo* analyses after synthesis scale-up.

3.2. DsiRNA for in vivo experiments

The most potent Dicer-substrate siRNA, C11, targeting the mRNA coding for the N protein of hMPV homologous to A2 strains (represented by strains CAN97–83 (Canada), NL/00/17 (The Netherlands) and C4-CJP05 (France)) was chemically synthesized with suitable chemical modifications in Evader™ DsiRNA format to prevent immune stimulation and to provide additional nuclease resistance for *in vivo* use. Likewise, an inactive negative control DsiRNA, DsiRNActrl II, was synthesized with chemical modifications and mismatched nucleotides (Table 1).

3.3. Inhibitory activity of the Evader™ DsiRNA candidate C11 evaluated by plaque assay

In vitro antiviral activity of chemically modified C11, i.e. Evader™ DsiRNA was then confirmed by plaque assay. The inhibitory response was dose-related with a 50% reduction plaque on C4-CJP05 strain at a concentration of 0.65 nM.

3.4. In vivo distribution of hMPV specific DsiRNA in the lung 30 min to 24 h post inoculation

BALB/c mice were intranasally inoculated with the dye-DsiRNA (4 mg/kg). The fluorescence images showed that siRNA could be detected in the lungs as early as 30 min after inoculation.

At this time point, fluorescence signals were still slightly visible on the nose, the site of inoculation. The intensity of fluorescence, which corresponds to the amount of siRNA molecules in the lungs, increased after 1 h and stayed relatively constant until 5 h post-inoculation. At 7 h post-inoculation a decrease in fluorescence was observed leading to complete disappearance of lung-associated fluorescence after 24 h. The quantification results support the qualitative estimation based on the fluorescence images. A median of 115.3 pmol DsiRNA could be detected in the lungs 30 min post-inoculation. A maximal accumulation occurred after 1 h with 227.6 pmol. No dramatic change was observed until 5 h post-inoculation. After 7 h, the amount of DsiRNA present in the lungs was reduced to 130.5 pmol. At 24 h post-inoculation the DsiRNA was completely cleared from the lungs (Fig. 2).

3.5. No immune stimulation following siRNA administration in vivo: analysis of BAL fluid

There was no increase in the cell number in the BAL of DsiRNA-treated mice that could be induced by undesired immune stimulation, either by Evader™ DsiRNA C11 or by DsiRNActrl II (mean in both groups of treated mice $<10^6$ cells, that is similar to that of untreated mice) (Fig. 3a). BAL cells were then evaluated on day 1 for cellular infiltration of the airways. The cellular infiltrate comprised mostly monocytes/macrophages, similar to untreated mice (Fig. 3b).

We then assessed the production of IFN-α cytokine, proinflammatory cytokine IL-6 as well as TNF-α, a cytokine associated with inflammation in the BALs of mice (Fig. 4). At day 1 post infection, IFN-α, IL-6 and TNF-α in the BALs of mice in all groups were at levels close to the limits of detection of the assays and there was no statistical difference between Evader™ DsiRNA C11-treated and untreated groups (mean of IL-6 in groups of mice: Evader™ DsiRNA C11-treated, DsiRNActrl II-treated, untreated: 37, 27 and 30 pg/ml, respectively; mean of IFN-α: 4, 65 and 2 pg/ml, respectively; mean of TNF-α: 66, 47 and 72 pg/ml, respectively). Our findings revealed that our modified DsiRNAs including selective incorporation of 2′-*O*-Me-modified nucleotides prevented stimulation of the immune response.

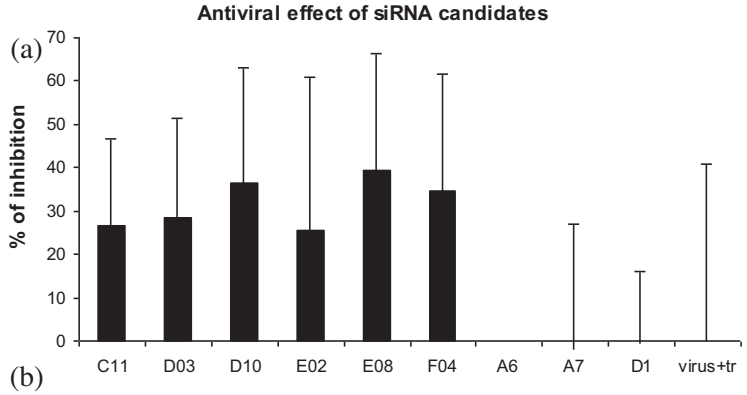
3.6. Viral replication in vivo

To assess whether RNAi could inhibit hMPV virus replication *in vivo*, we used an established murine model of hMPV infection. BALB/c mice were treated with hMPV-specific or control DsiRNAs. Preliminary assays on a small number of mice (data not shown) using different administration times led us to choose to administer a single dose intranasally (4 mg/kg) 30 min before infection. At day 3 post-challenge, the lungs were removed and lung homogenates were assayed for the virus. Virus titers were significantly lower in animals given the Evader™ DsiRNA C11 than in those given DsiRNActrl II or untreated infected animals (mean virus titer in Evader™ DsiRNA C11-treated mice: $13 \times 10^3 \pm 1.8 \times 10^3$ PFU/g; mean virus titer in hMPV-infected mice $29 \times 10^3 \pm 4.1 \times 10^3$; mean virus titer in DsiRNActrl II -treated mice: $30 \times 10^3 \pm 3 \times 10^3$, $p = 0.004$, KW) (Fig. 5).

3.7. Kinetics of body weight and airway obstruction after DsiRNA administration

Treatment with 2′-*O*-Me-modified DsiRNA was well tolerated. The administration of Evader™ DsiRNA C11 alone, without subsequent infection, did not lead to weight loss, AO or induction of a cytokine response. We therefore considered that this DsiRNA had no *in vivo* toxicity.

During the course of infection, hMPV induced a considerable weight loss and airway obstruction (Darniot et al., 2005, 2009),



(b)

Targeted gene	Sense Sequence	Antisense Sequence	Short Name
N	\5Phos\rGrCrArGrCrArArArGrCrArGrArArArGrUrUrUrArUrUrCGT	rArCrGrArArUrArArArCrUrUrUrCrUrGrCrUrUrUrGrCrUrGrCrCrU	C11
N	\5Phos\rArGrArUrCrArUrCrCrArArCrArArCrArUrArArArUrGrCrUAG	rCrUrArGrCrArUrUrArUrGrUrUrGrUrUrGrGrArUrGrArUrCrUrGrG	D03
L	\5Phos\rCrCrArArUrCrArArCrCrArArGrCrArUrUrArArGrUrGrAGA	rUrCrUrCrArCrUrUrArArUrGrCrUrUrGrGrUrUrGrArArUrUrGrGrArC	D10
L	\5Phos\rCrCrArCrArArCrArCrArArUrUrArGrArUrUrArCrUrUrCCC	rGrGrGrArArGrUrArArUrCrUrArArUrUrGrUrGrUrUrGrGrA	E02
L	\5Phos\rArGrCrUrArGrUrArGrArUrArArGrArUrArArCrUrUrCrUGA	rUrCrArGrArArGrUrUrArUrCrUrUrArUrCrUrArCrUrArGrCrUrUrA	E08
L	\5Phos\rArCrUrUrGrArUrArCrArCrArGrArArUrArArGrUrArArAGA	rUrCrUrUrUrArCrUrUrArUrUrCrUrGrUrGrUrArUrCrArArGrUrCrC	F04

Fig. 1. *In vitro* screening. (a) Antiviral effect of DsiRNAs. The cell viability was measured as a reciprocal parameter for the cytopathic effect. The higher the OD the lower the cytopathic effect, i.e. the higher the antiviral potency of a given siRNA. The ODs were converted into viral load and then expressed as viral inhibition percentages by comparison with the viral load value obtained for "virus + transfectant". C11, D03, D10, E02, E08 and F04 are six potent inhibitor candidates and A6, A7, D1, three randomly selected non-potent siRNAs. (b) Sequences of the six DsiRNAs that are potent inhibitors of hMPV replication cells.

Table 1
DsiRNA sequences.

Evader™ DsiRNA C11	
5′- pGCAGCAAAGCAGAAAGUUUAUUCgt-3′	passenger strand
3′- **UCC**G**U**C**G**U**U**U**C**G**U**C**U**U**U**C**A**AAUAAGCA -5′ (11 10)	guide strand
DsiRNActrl II	
5′- pGCAGCAAA**C**G**A**GAAA**U**G**U**UAU**C**U**C**gt -3′	
3′- **UCC**G**U**C**G**U**U**U**G**C**U**C**U**U**U**A**C**A**AUA**G**A**C**A -5′	(grey = base changes)

Upper case letters represent ribonucleotides, lower case letters represent deoxyribonucleotides, bold underlined capital letters represent 2′-*O*-methylribonucleotides and p is a phosphate residue. Grey letters represent base changes. Positions 10 and 11 on the guide strand where theoretical cleavage occurs are labelled. The 2′-*O*-Me modification pattern prevents TLR7/8 interaction and minimizes interaction with Rig-I.

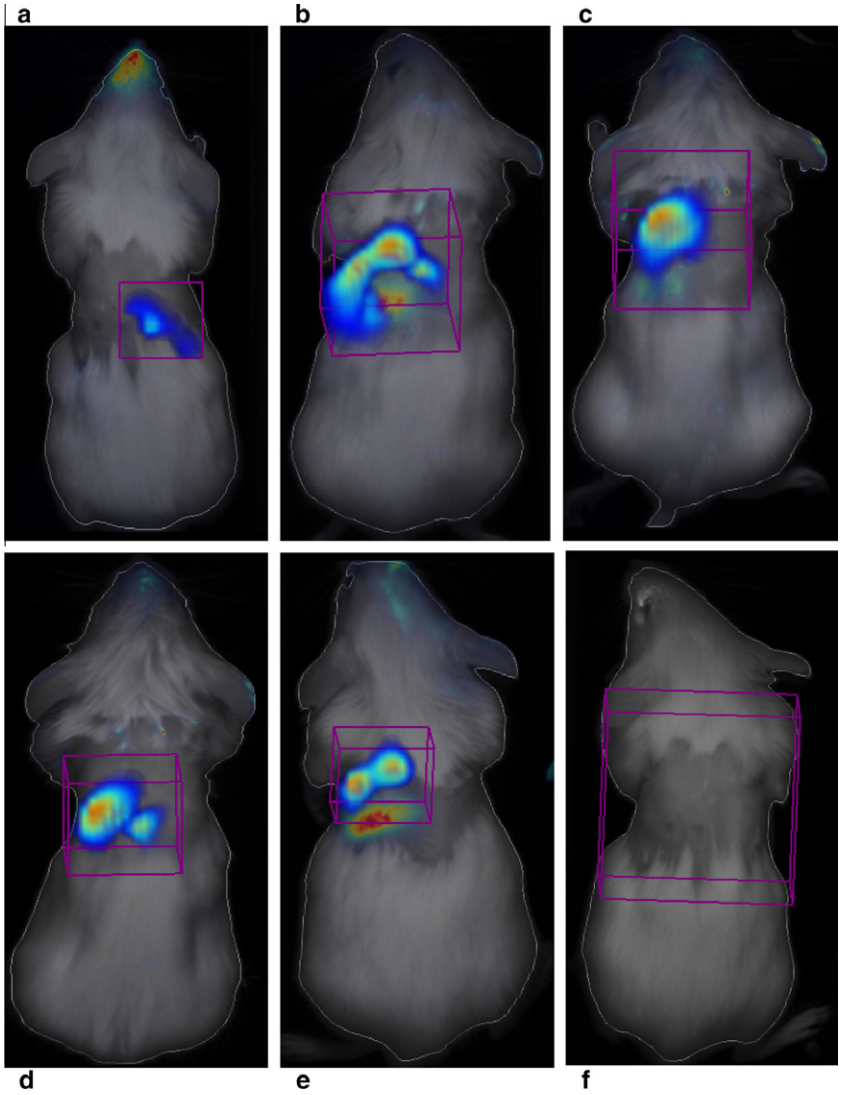


Fig. 2. Evader™ DsiRNA C11 lung delivery. Distribution of hMPV-specific NIR dye labelled Evader™ DsiRNA C11 in representative BALB/c mice 30 min (a), 1 h (b), 3 h (c), 5 h (d), 7 h (e), and 24 h (f) post inoculation ($n = 6$).

as shown in Fig. 6. Treatment with Evader™ DsiRNA C11 gave no significant clinical improvement, based on weight loss and AO (Fig. 6). There was no significant difference between groups in survival rates. We performed histopathology at day 6 post infection. Untreated and DsiRNA-treated lungs of infected mice presented histopathological abnormalities similar in nature and intensity. As previously shown (Darniot et al., 2009), alveolitis and parenchymal pneumonia were observed as well as perivascular lymphocytic infiltrate (data not shown).

3.8. RNA interference by rapid amplification of cDNA ends (RACE)

With the primers that we used, the expected size of the amplified product was 359 bp for the first PCR round and 344 bp for the second PCR round. *In vitro*, RACE was performed with total RNA from LLC-MK2 cells. As shown by gel electrophoresis, following siRNA transfection into LLC-MK2 cells and subsequent infection with hMPV, a cleavage fragment could be detected in the Evader™ DsiRNA C11 transfected cells. No PCR product of 359 bp was

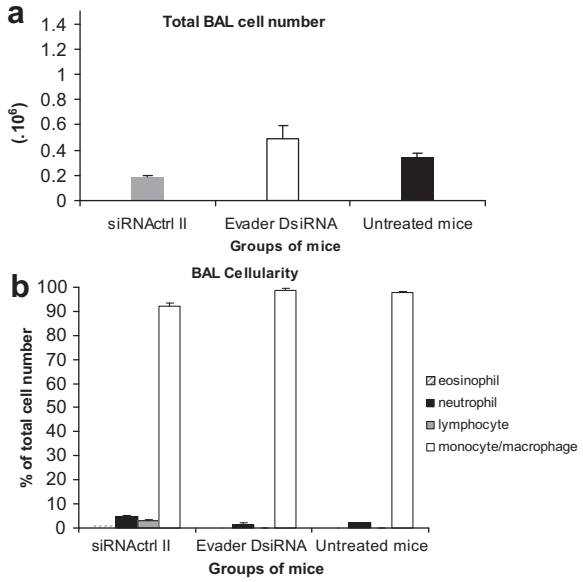


Fig. 3. Total and differential cell counts in the BAL population. (a) BALs were evaluated on day 1 post-administration for numbers of cells. There was no significant increase in cell numbers between the following groups: mice treated with DsiRNActrl II or Evader™ DsiRNA C11, or untreated mice. (b) At day 1, monocyte/macrophage represented the main population in BAL cells in both mice groups, as for untreated mice. Values are means ± SEM (n = 4/group).

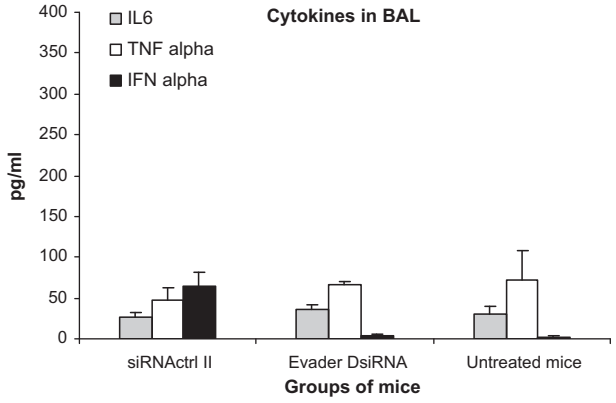


Fig. 4. DsiRNA C11 does not mediate induction of interferon alpha, TNF alpha or IL-6 *in vivo*. IFN-α or inflammatory cytokines such as TNF-α and IL-6 were assessed in BALs by ELISA at 24 h. There was no significant difference in cytokines between the following groups: mice treated with DsiRNActrl II or Evader™ DsiRNA C11, or untreated mice. Values are means ± SEM (n = 4–8/group).

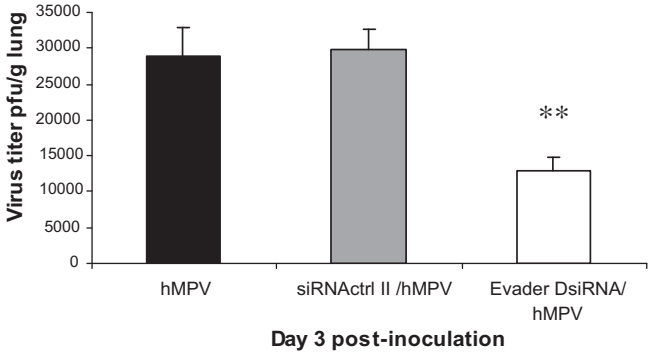


Fig. 5. Viral load in lung tissue. Viral replication in the lung assessed at day 3 post-infection in the Evader™ DsiRNA C11-treated mice was significantly lower than in the hMPV-infected mice (P = 0.004). No significant difference was observed between the DsiRNActrl II-treated mice and the hMPV-infected mice. Values are expressed in PFU/g of lung tissue as means ± SEM (n = 7–12 mice/group).

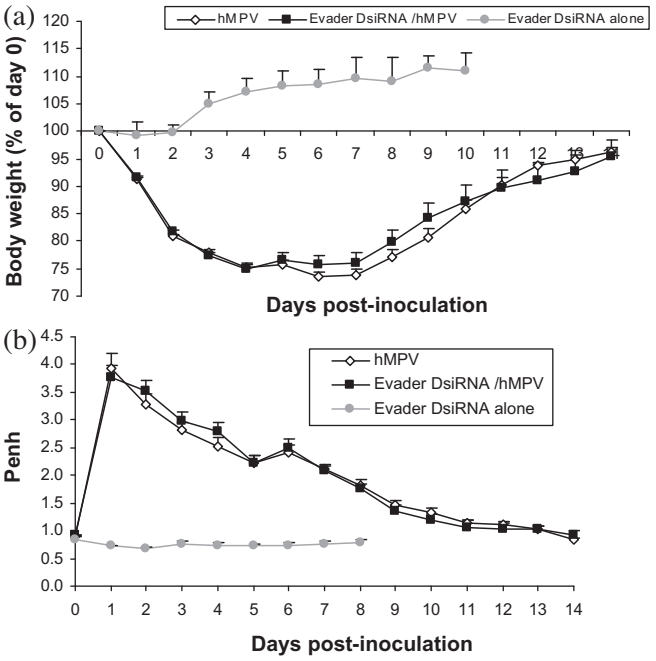


Fig. 6. Clinical manifestations in mice kinetics of weight loss (a) and airway obstruction (b) in Evader™ DsiRNA C11-treated uninfected mice, Evader™ DsiRNA C11-treated infected mice and untreated infected mice. (a) Weight was calculated as a percentage of the starting weight (100%) (n = 12–61); (b) the airway obstruction, reported as enhanced pause (Penh) values, was determined using whole-body unrestrained plethysmography (n = 12–47).

obtained in cells transfected with the mismatched control, DsiRNActrl II (Fig. 7a). All PCR products were then subcloned in pGEMt and sequenced to confirm the correct junction between the adaptor sequence and the predicted cleavage site of the siRNA. *In vivo*, RACE analysis of total lung RNA led to the amplification of the expected product in the Evader™ DsiRNA C11-treated mice, but not in DsiRNActrl II-treated mice (Fig. 7b). A 344-bp PCR product (obtained after the second round of PCR), was observed in Evader™ DsiRNA C11-treated mice, and at a lower intensity in the mismatch control siRNA, DsiRNActrl II.

3.9. Sequence analysis of cloned PCR products

A RACE assay followed by sequencing was used to confirm the existence of an RNAi-mediated mechanism of action for Evader™ DsiRNA C11. The sequence of the cloned 359-bp PCR product included the predicted junction between the adaptor sequence (underlined) and the cleavage site of hMPV N-mRNA (…GTAGAAACAGAAA….). *In vitro* RACE also detected the predicted mRNA cleavage product in Evader DsiRNA C11 treated cells. Sequencing revealed the specific predicted cleavage site between nucleotide positions 718 and 719.

In vivo, sequencing of the cloned PCR2 products showed the predicted junction between the adaptor sequence and the cleavage site of hMPV N-mRNA in Evader™ DsiRNA C11 treated mice, but not in DsiRNActrl II-treated mice.

This RACE assay and the sequence analysis made it possible to visualise and identify the specific RNAi cleavage product of the N-gene mRNA following Evader™ DsiRNA C11 treatment both *in vitro* and *in vivo*. These results demonstrate that Evader™ DsiRNA C11 works through an RNAi pathway to mediate specific RNA

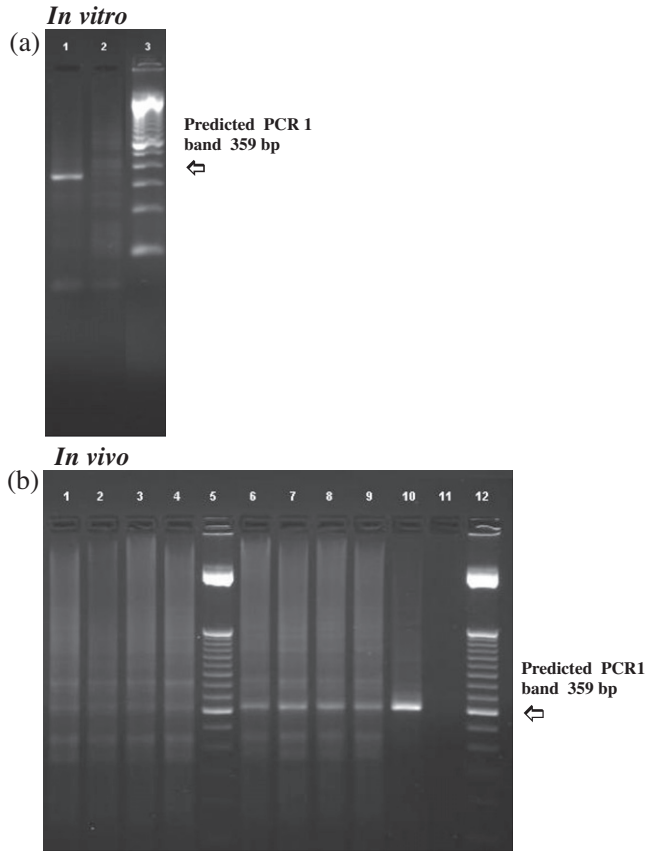


Fig. 7. Identification of cleavage products within the hMPV RNA genome by 5′ rapid amplification of cDNA ends (RACE). (a) LLC-MK2 cells were transfected with the DsiRNA using RNAimax as transfectant; lane 1: Evader™ DsiRNA C11 targeting hMPV N-mRNA; lane 2: control DsiRNA, DsiRNActrl II; lane 3: 100 bp mass ladder ; (b) BALB/c animals were injected prophylactically (4 mg/kg i.n.) with the DsiRNA targeting hMPV N-mRNA or with the control siRNA. Analysis of lung RNA isolated from Evader™ DsiRNA C11 or control siRNA-treated mice. Lanes 1–4: PCR product in pooled lung tissue of mice ($n = 3$) treated with DsiRNActrl II; lanes 6–9: PCR product in lung tissue of mice treated with Evader™ DsiRNA C11 targeting hMPV N-mRNA; lane 10: LLC-MK2 cells transfected with Evader DsiRNA/RNAi max as positive control; lanes 5 and 12: 50 bp mass ladder; lane 11: PCR1 with no template.

cleavage. The impact of Evader™ DsiRNA C11 observed on viral load is consistent with cleavage of the nucleoprotein (N) mRNA via a targeted RNAi-specific mechanism.

4. Discussion

RNA interference (RNAi) is an effective means to suppress virus replication. Here we demonstrated that a small interfering RNA (siRNA) specific for a conserved region of the nucleoprotein mRNA inhibited virus replication *in vitro*. Moreover, treatment with this siRNA significantly reduced lung virus titers in infected mice *in vivo* even in the absence of a transfectant. This inhibition was specific and not mediated by an antiviral IFN-α response. These results indicate that RNAi is a promising strategy for the control of hMPV infection.

RNAi is an emerging technology that specifically inhibits gene expression. Small interfering RNAs (siRNAs), mediators of RNAi, are short (21–27 nt), RNA duplexes that inhibit gene expression by inducing sequence-specific degradation of mRNA. Numerous studies have reported gene expression silencing by siRNA when delivered into mammalian cells *in vitro*. Among these, *in vitro* inhibition of replication by RNAi of respiratory RNA viruses has been shown for influenza virus (Ge et al., 2003), RSV (Alvarez et al., 2009), the related avian metapneumovirus (aMPV) (Ferreira et al., 2007) and recently, hMPV (Deffrasnes et al., 2008). Moreover, *in vivo* silencing of viral genes has been demonstrated (Alvarez et al., 2009; Bitko et al., 2005; Zhang et al., 2005; Zhou et al., 2007).

In this study, we first demonstrated that *in vitro* transfection of human HepG2 cells with DsiRNAs specific for the hMPV nucleoprotein mRNA could inhibit replication and protect infected cells from cytopathic cell lysis induced by hMPV. We then confirmed the antiviral activity of Evader™ DsiRNA C11 *in vitro* by plaque assay and demonstrated its silencing specificity *in vitro* by RACE assay using the Lipofectamine™ RNAiMAX. Lipofectamine™ RNAiMAX was chosen as it is very efficient for siRNA delivery into cells; however, it is not recommended for *in vivo* intranasal administration. Two potent siRNAs, called siRNA45 and siRNA60, targeting the hMPV nucleoprotein and the hMPV phosphoprotein mRNAs, respectively, have been identified *in vitro* (Deffrasnes et al., 2008). The siRNA45 was similar in sequence to Evader™ DsiRNA C11. This matching finding obtained using different methods for screening underlines the importance of this sequence targeting the nucleoprotein mRNA.

We then investigated the inhibitory activity of these DsiRNAs against virus infection and disease in our animal model. To use siRNA *in vivo*, it must be delivered to the appropriate tissue efficiently. Utilizing NIR imaging we demonstrated that DsiRNA administered intranasally can reach the lung. To be suitable for *in vivo* treatment, selective 2′-*O*-Me modifications were added to the DsiRNAs. Selective 2′-*O*-Me modification presents a robust approach to overcome the problem of immune activation by siRNA, whilst reducing the risk of negatively affecting RNAi activity and also results in increased stability *in vivo* (Behlke, 2008). Behlke proposed a pattern of modification that was shown to be applicable to many siRNA sequences that have an inherent capacity to stimulate the innate immune response. In our experience, we were successful in generating non-inflammatory DsiRNAs as proved by the absence of inflammatory cytokines and negligible infiltration in BALs induced by both Evader™ DsiRNA C11 and DsiRNActrl II. The induction of interferons by siRNAs has been implicated in mediating off-target gene effects (Kim et al., 2004), but both DsiRNAs used in this study were shown to be nonstimulatory.

We showed that administration of an hMPV-specific DsiRNA can decrease lung virus titers in mice. The inhibition of virus replication was specific, requiring homology between the siRNA and the gene target, and was not the result of IFN-α induction by double-stranded RNA. Most importantly, we confirmed the RNAi mechanism of action of Evader™ siRNA C11 *in vivo*. The specific detection of mRNA cleavage by RACE is the only method to confirm the knockdown of mRNA by RNA interference, but has rarely been reported for *in vivo* studies (Alvarez et al., 2009; Frank-Kamenetsky et al., 2008; Judge et al., 2009; Saberi et al., 2009; Soutschek et al., 2004). The RACE method was an essential tool to detect mRNA cleavage and to corroborate the decrease in viral replication following treatment with Evader™ siRNA C11 in mice.

The administration of Evader™ siRNA C11 in our experiments led to a significant decrease in viral load but had no significant effect on clinical disease. These results suggest either that the inhibition of viral replication was too limited to be critical for clinical improvement or that an aberrant immune response, not related to the viral burden, contributes to disease severity in mice. Available data are limited to assess the impact of hMPV viral load on severity of illness. A significant association between viral load in children and several, but not all, markers of disease severity was reported (Martin et al., 2008). Our data showed that the half-log decrease in viral load does not result in improvement in clinical disease, represented by weight loss and disease airway obstruction. It does not preclude other markers of severity. As for respiratory

Table 2
Sequences in the target region.

Evader DsiRNA C11		GCAGCAAAGCAGAAAGTTTATTCGT	
Strains	Genotype	Sequence in the target region	Accession No.
C4-CJP05	**A2**	GCAGCAAAGCAGAAAGTTTATTCGT	
NL/00/17	**A2**	GCAGCAAAGCAGAAAGTTTATTCGT	FJ168779.1
00-1	**A1**	GCAGCAAAGCAGAAAGT**C**TATT**T**GT	AF371337.2
NL/1/94	**B2**	G**A**AGCAAAGCAGAAAGTTT**G**TT**T**GT	FJ168788.1
NL/1/99	**B1**	G**A**AGCAAAGCAGAAAGTTT**G**TT**T**GT	AY525843.1
CAN97-83	**A2**	GCAGCAAAGCAGAAAGTTTATTCGT	AY145278.1
CAN98-75	**B2**	G**A**AGCAAAGCAGAAAGTTT**G**TT**T**GT	AY297748
CAN97-82	**B1**	G**A**AGCAAAGCAGAAAGTTT**G**TT**T**GT	AY145277.1

Sequences from isolates representing each genotype: strains from the Netherlands (NL/00/17; 00-1; NL/1/94 and NL/1/99) and Canada (CAN97-83, CAN98-75, and CAN97-82) and C4-CJP05. Nucleotides not conserved are in bold and underlined.

syncytial virus (RSV), hMPV disease severity is probably due to both host immunopathology and direct viral cytopathic effects driven by high-level viral replication. As for RSV (El Saleeby et al., 2011) partial but significant reduction of hMPV replication may reduce direct cytopathic effects and thus improve subsequent morbidity.

The decrease of the viral titer, although significant, was moderate compared with that observed with ALN-RSV01, an siRNA directed against RSV that is in clinical development (Alvarez et al., 2009). On intranasal delivery before RSV inoculation, lung viral replication was decreased by 2.5 to 3 logs. The targeted mRNA sequence is also located on a part encoding the nucleoprotein. For hMPV, several ways should be explored in the future to obtain a higher anti-hMPV effect. The imaging revealed disappearance of the DsiRNA from the lung after 24 h. In the lung department, epithelial cells have a slow turn over (Bowden, 1983) and thus here, cell division is probably not the major factor of siRNA disappearance. Moreover, chemical modifications improve the half-life of siRNAs (Behlke, 2008) and the Evader™ DsiRNA is highly modified. The major reasons for this disappearance may be rapid absorption in the bloodstream and renal elimination (Moschos et al., 2011) or possibly phagocytosis by lung macrophages (Merkel and Kissel, in press). To prolong the RNAi effect, different strategies can be followed. Taking in account its relatively rapid clearance, it will be necessary to establish an appropriate posology in order to improve DsiRNA efficiency; additional administrations of siRNA could lead to prolonged and more effective hMPV gene silencing. New siRNA conjugation strategies and delivery vehicles could also lead to improvements in the RNAi effect. (Bartlett and Davis, 2006; Raemdonck et al., 2008). Indeed, naked siRNA molecules enter cells but significantly more siRNA can be delivered if carrier vehicles are used. Formulation strategies could facilitate intracellular uptake of Evader™ DsiRNA and improve its pharmacokinetics. Combination with other siRNAs may also improve the effectiveness. Studies are underway to test alternative delivery vehicles and new posology schedules.

The N protein is essential for viral replication (Herfst et al., 2004) and as it is not exposed, undergoes little immunological pressure, offering a potential ideal target for RNAi. RNAi needs homology between the siRNAs and the viral gene targets. The Evader™ siRNA C11 sequence is in a reasonably conserved region of the N protein (A2) which suggests that it should have antiviral activity against a broad spectrum of hMPV strains, including both genotypes. As shown in Table 2, two positions on the guide strand are affected by mismatching, positions 2 and 18, for A1 and B strains, respectively. The tolerance to mismatch varies from one position to another and a single mismatch may prevent siRNA action (Bitko and Barik, 2001; Elbashir et al., 2001). Perfect matching is not essential and Huang et al. reported a variable gene silencing tolerance on nucleotide mismatched targets in terms of position and identity (Huang et al., 2009) and showed that positions 2 and 18 are tolerant.

The siRNA ALN-RSV01 displays significant anti-RSV activity in healthy volunteers whilst being safe (DeVincenzo et al., 2010), (Phase IIa of clinical trial), taking us one step closer to siRNA becoming a reality for RNAi therapy against a respiratory virus. In this study, we showed that Evader™ DsiRNA C11 is a potent *in vivo* inhibitor of hMPV. It is a first important and promising step towards using DsiRNA in therapeutic applications against hMPV.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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