RNAi Targeting of Human Metapneumovirus *P* and *N* Genes Inhibits Viral Growth

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Abstract

Background/Aims: Human metapneumovirus (hMPV) is an important human respiratory pathogen and is implicated in an array of respiratory illnesses, ranging from asymptomatic infection to severe bronchiolitis. Currently, there is no reliable vaccine or specific antiviral therapy for hMPV infection and treatment is supportive. The use of ribonucleic acid interference has the potential to change that with the targeting of essential viral genes via small interfering RNAs (siRNAs) offering the ability to directly and rapidly treat viral infections. **Method:** The human lung carcinoma epithelial cell line, A549, was transfected with siRNAs targeting the *N* and *P* genes before infecting with hMPV A2 CAN97-83. Viral growth inhibition was then measured by the viral plaque assay and nucleoprotein (*N*) and phosphoprotein (*P*) gene knockdown was determined by real-time PCR. **Results:** In vitro prophylactic use of siRNAs targeting the 3'-abundantly expressed *N* and *P* genes of hMPV resulted in potent, sequence-specific viral inhibition. The viral inhibition was specific and not mediated by an anti-viral interferon- β response

or cell death. **Conclusion:** The findings presented here confirmed the highly potent, sequence-specific antiviral effect of siRNAs targeting the *N* and *P* gene of hMPV. These results may facilitate the development of a novel therapeutic agent for hMPV control.

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The cells lining the respiratory tract are continuously exposed to the external environment, making the lungs a particularly susceptible site for infection [1]. Respiratory tract infections (RTIs) are a significant cause of morbidity and mortality worldwide [2], and while typically self-limiting in healthy adults, respiratory infections play an integral role in illness and deaths of the elderly, immunocompromised patients, and children [3, 4]. Many pathogens have the capacity to cause RTIs, with viruses responsible for 30–40% of cases [5]. A significant proportion of RTIs with viral aetiology can be attributed to human metapneumovirus (hMPV). The hMPV, although only formally identified in 2001 [6], is one of the principal viral pathogens responsible for acute RTIs [7, 8]. Approximately 5–10% of hospitalisations of infants suffering from acute RTIs can be attributed to hMPV [9], with epidemiological studies demonstrating that hMPV infects

Table 1. Conserved regions between candidate siRNA sequences and selected hMPV isolates

siRNA	hMPV_siN		hMPV_sip	
Sequence	GCAGCAAAGCAGAAAAGTT		GATACTTCATCATTAAGCA	
Strains	Genotype	Sequence in the target region		Accession No.
00-1	A1	GCAGCAAAGCAGAAAAGTCT	GATACTTCATCATTAAGCA	AF371337.2
CAN97-83	A2	GCAGCAAAGCAGAAAAGTTT	GATACTTCATCATTAAGCA	AY145278.1/AY145251.1
NL/00/17	A2	GCAGCAAAGCAGAAAAGTTT	GATACTTCATCATTAAGCA	FJ168779.1
CAN97-82	B1	<u>GA</u> AGCAAAGCAGAAAAGTTT	GATAC <u>AT</u> CATCA <u>CTA</u> AGCA	AY145277.1/AY145250.1
NL/1/99	B1	<u>GA</u> AGCAAAGCAGAAAAGTTT	GATAC <u>AT</u> CATCA <u>CTA</u> AGCA	AY525843.1
CAN98-75	B2	<u>GA</u> AGCAAAGCAGAAAAGTTT	GA <u>CACAT</u> CATCA <u>CTA</u> AGCA	AY297748

Strains are from Canada (CAN97-83, CAN97-82, CAN98-75) and the Netherlands (NL/00/17, 00-1, NL/1/99). Nucleotides that are not conserved are underlined and in bold.

approximately 46% of children within their first year of life, with 70% of children infected by the age of 5 years [8, 10]. Currently, there is no specific antiviral therapy for hMPV infection and so treatment is only supportive. The novel use of small interfering RNAs (siRNAs) against hMPV has the potential to provide a specific therapeutic benefit. Inhibition of hMPV infection has been previously achieved using siRNA molecules that target viral mRNA for degradation [11, 12], with this therapeutic approach having several advantages over traditional pharmaceutical interventions. Here we explore a panel of newly synthesised siRNAs targeting the nucleoprotein (N) and phosphoprotein (P) genes of hMPV to evaluate their effectiveness as potential novel antiviral treatments.

The human lung carcinoma epithelial cell line, A549, and monkey kidney epithelial cell line, Vero E6, were obtained from the American Tissue Culture Collection (ATCC). A549 cells were cultured in Dulbecco's modified Eagle medium nutrient mixture F-12 (DMEM/F12; Gibco-Invitrogen, Waltham, MA, USA), whereas Vero E6 cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco-Invitrogen). All growth media were supplemented with 10% foetal calf serum (Gibco-Invitrogen) and 100 units/mL penicillin G, 100 µg/mL streptomycin sulphate, and 0.29 mg/mL L-glutamine (Gibco-Invitrogen). The hMPV used in this study, hMPV A2 CAN97-83, is a clinical isolate strain obtained from Kristen Spann, University of Queensland. hMPV particles were cultivated in Vero E6 cells. Targeting siRNAs (Sigma-Aldrich, St Louis, MO, USA; Table 1) were transfected using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) for 24 h as per the manufacturer's protocol before infecting with hMPV at a multi-

plicity of infection (MOI) of 0.1. Non-targeting siRNA against the green fluorescence protein (GFP), siGFP (5'-GCACGACUUCUUCAAGUCCUU[dT][dT]-3'; Sigma-Aldrich), was used as a control for any off-target effects. The cells were harvested and RNA extracted at 72 h post-infection. The hMPV N (F 5'-GGATGGA-CATACCAAAAATCGCTA-3', R 5'-AGCATTGTTT-GACCAGCACC-3'; Sigma-Aldrich) and P (F 5'-CCAG-GAAAATACACAAAGTTGGAGA-3', R 5'-CCTGCT-GTAGCAATGTTGAGTG-3'; Sigma-Aldrich) mRNA levels were quantified using the QuantiFast® SYBR® Green PCR Kit (Qiagen) on the Rotor-Gene Q (Qiagen). Shed hMPV particles were quantified using plaque assay under a methylcellulose overlay containing trypsin followed by immunostaining with the mouse anti-hMPV N antibody (catalogue No. MAB80121; Millipore, Burlington, MA, USA) and detected with an anti-mouse IRdye800 antibody (Rockland, Limerick, PA, USA) on an Odyssey Imager (Li-Cor Biosciences, Lincoln, NE, USA). Immunoplaques were counted manually and expressed as plaque-forming units (PFU)/mL. For measuring interferon (IFN)-β expression, A549 cells were transfected with 40 nM of siRNAs, 100 µg/mL of the positive immune-stimulatory control, poly I:C (Sigma-Aldrich) or siGFP. The cells were incubated for 72 h post-infection before harvesting and RNA extraction. IFN-β gene expression was assessed on the Rotor-Gene Q (Qiagen) using the Qiagen QuantiFast® Probe Duplex Assay kit (Qiagen) and QuantiFast® Probe RT-PCR Plus kit (Qiagen).

Previous work has established that in vitro inhibition of the hMPV viral genome can be achieved using siRNA molecules targeting the N and P mRNA of hMPV [11, 13].

Fig. 1. a siRNA specifically knocks down hMPV *N* and *P* gene expression. A549 cells were untransfected (control) or transfected with 40 nM of targeting siRNAs before infecting with hMPV at an MOI of 0.1. siGFP was used as a control for any off-target effects. Cells were harvested and RNA extracted 72 h post-infection. hMPV *N* and *P* mRNA levels were quantified by RT-PCR. Human β -actin was used as the internal housekeeping gene. Statistical significance compared to cells treated only with transfection reagent (control) is shown by unpaired *t* test and the error bars shown are the standard deviation (SD) (***) $p < 0.001$ from 2 independent experiments. **b** siRNA-mediated hMPV *N* and *P* gene knockdown reduced viral growth. A549 cells were either untransfected (UT), treated only with transfection reagent (-), or transfected with 40 nM of the siRNAs listed above before infecting with hMPV at an MOI of 0.1. Viral supernatants were collected 72 h post-infection and the shed hMPV particles were quantified using the plaque assay. Significance compared to cells treated only with transfection

reagent is shown by unpaired *t* test and the error bars shown are the SD (***) $p < 0.001$ from 2 independent experiments. The immunoplaque images are representative of the data shown. Treatment of cells with siRNA targeting hMPV *N* and *P* genes neither induced cell death (**c**) nor a type I IFN response (**d**). **c** A549 cells were either treated only with transfection reagent (-) or with 40 nM of the siRNAs listed above before measuring cell viability by the MTT assay after 72 h. Error bars shown are the SD from 4 independent experiments. **d** For measuring IFN- β expression, A549 cells were transfected with 40 nM of siRNAs, 100 μ g/mL of poly I:C or siGFP. Cells were incubated for 72 h post-infection before harvesting and RNA extraction. IFN- β gene expression was assessed by RT-PCR. Human β -actin was used as the internal housekeeping gene. Data are representative of fold IFN- β expression to cells treated only with transfection reagent (control) and error bars are the SD from 2 independent experiments.

The viral proteins *N* and *P* form part of the hMPV viral replication complex and are critical for viral replication [14], and targeting them via ribonucleic acid interference (RNAi) may be an effective means of viral inhibition [15].

Informed by this, a panel of new siRNAs directed against the key non-structural genes *N* and *P* at different locations along the transcript were synthesised, and their gene silencing capabilities were screened on the human lung

carcinoma epithelial cell line, A549. A549 supports both hMPV replication [11, 12] and RNAi-mediated silencing [12, 15–18], therefore providing an appropriate cell model in this study. siRNA-mediated silencing of the *N* and *P* genes (siN and siP, respectively) resulted in efficient knockdown of the target genes when compared to the control siRNA (Fig. 1a). All the siRNAs designed target the negative sense, 3′–5′ strand RNA of hMPV, and therefore target the genome directly. This approach is unique as other siRNAs designed against hMPV have targeted the sense strand of the virus. The ratio of siRNA seed strand to target genome is higher when targeting the 3′–5′ RNA due to the limited number of viral genomes present upon primary infection [19]. Targeting the initial genome before transcription of viral mRNA and positive sense antigenomic strand (cRNA) takes place may provide increased therapeutic benefit as less siRNA would be required to give an antiviral result. This would also have the added advantage of decreasing the potential for off-target RNAi-induced effects. Next, we tested whether candidate siRNAs had any effect on hMPV viral titres in infected A549 cells. Compared to cells treated only with transfection reagent, pretreatment of A549 cells with siN and siP resulted in a significant decrease in infectious viral titre (Fig. 1b). Similar levels of hMPV suppression were also achieved by Deffrasnes et al. [11] using a comparable siRNA, with an additional study finding that siRNA targeting the *N* gene resulted in the most consistent antiviral effect [13]. Although a previous study also demonstrated hMPV suppression by RNAi of *N* and *P* genes [11], we have employed a uniquely distinct set of *N* and *P* targeting siRNA molecules in our study. Notably, we observed a 10-fold log decrease in hMPV titre (Fig. 1b) with siN, higher than what was previously observed in vitro [13]. hMPV is an obligate intracellular virus that requires live cells to propagate and therefore any decrease in cell viability would have a direct effect on the infectious virus titre. To rule out this possibility, the cell viability of A549 cells was measured post-siRNA transfection. As a positive control for cell death, transfection with siRNA targeting polo-kinase 1 (PLK-1), which is known to induce apoptosis, reduced cell viability (Fig. 1c). However, the viability of cells transfected with siRNA molecules against *N* and *P* genes were comparable to that of cells treated with and without siGFP, suggesting that these molecules are not toxic to A549 cells. siRNA has been shown to be a potent activator of the IFN response [20–22]. To exclude non-specific immune effects as the mediator of antiviral efficacy for the siRNAs, they were tested for their ability to cause siRNA-induced immunostimulation. Analysis

of pro-inflammatory cytokine IFN- β gene induction by siRNA transfection in vitro showed that none of the candidate siRNAs or siGFP significantly changed IFN- β expression when compared to cells treated only with transfection reagent (Fig. 1d), indicating that the viral inhibition seen was caused by RNAi rather than non-specific effects mediated by immune activation. However, the use of in vitro immune stimulation assays to assess siRNA-induced immune activation have been shown to be limited in its ability to measure immunostimulatory potential in vivo [23]. Therefore, analysis of the immune stimulation caused by transfection with the candidate siRNAs needs to be tested further in in vivo animal models. Furthermore, it would be beneficial to investigate if innate immune system activation is altered due to siRNA treatment in the presence of hMPV infection in vitro and in vivo. To be clinically beneficial as an antiviral therapeutic, siRNAs must be efficacious against all lineages of hMPV, as a single nucleotide mismatch between a siRNA and its target site can render it ineffective [24]. This presents a considerable challenge when targeting viruses that display genetic variability. Targeting conserved regions of the viral genome can negate this, with such a strategy also useful in limiting the development of escape mutants during treatment as these areas are more likely to be structurally or functionally constrained. This is expected as all siRNAs in this study were generated based on the hMPV strain A2 (NCBI reference sequence: NC_004148.2), with no nucleotide mismatches present (Table 1). Only single nucleotide mismatches between the guide strand of siN1 and the viral targets were identified, with a mismatch present on position 18 for A1, and position 2 for B1 and B2 hMPV strains. For siP, we identified 2 mismatches at position 6 and 13 on the guide strand for strain B1, with an additional mismatch at position 3 for B2. A single mismatch between the siRNA sequence and target region can render the siRNA ineffective [18, 25]; however, tolerance to mismatches can be position dependent [26]. Therefore, additional analysis of each siRNA against other hMPV strains would be beneficial to ensure they display sufficient antiviral effects against a broad spectrum of strains.

The ultimate goal of this work was the development of an hMPV-specific novel antiviral therapy. While the results presented in this concise study show that in vitro prophylactic treatment of siRNAs targeting the 3′ abundantly expressed *N* and *P* genes of hMPV result in potent sequence-specific viral inhibition, the transition from in vitro to in vivo testing is complex. Currently, there is no specific therapy for hMPV, and while the disease burden associated with hMPV is not yet well defined, the sub-

stantial disease pathogenesis associated with hMPV infections is sufficient enough to warrant the rapid clinical assessment of these potential therapeutics. Previous research has demonstrated that lung epithelial cells can be targeted by RNAi therapeutics through the intranasal delivery of siRNA molecules [17, 27], with phase I and II clinical trials of an siRNA targeting the respiratory syncytial virus (RSV) N mRNA (ALN-RSV01) completed in adults [28, 29], providing some insight into the use of RNAi in the treatment of respiratory viruses. The phase II clinical trial conducted by DeVincenzo et al. [29] showed consistent trends towards decreased viral loads in treated subjects when compared to controls and provided evidence towards the use of the siRNA prophylactically. Importantly, in vivo prophylactic intranasal delivery of dicer-substrate RNAs targeting the *N* gene of hMPV have been attempted, albeit with partial effectiveness at reduc-

ing hMPV replication in an hMPV mouse model [13]. There is potential for the prophylactic use of siRNA-based therapeutics in those most at risk, specifically children, the elderly, and the immunocompromised.

Disclosure Statement

The authors declare that they have no conflicts of interest.

Author Contributions

D.T.C. and N.A.M. conceived the study. K.M.N. performed all experiments and wrote the manuscript. A.I. was involved in the writing and proofreading of the manuscript. All authors read and approved the final manuscript.

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