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Inhibition of Henipavirus infection by RNA interference

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ABSTRACT

Nipah virus (NiV) and Hendra virus (HeV) are recently emerged zoonotic paramyxoviruses exclusively grouped within a new genus, Henipavirus. These viruses cause fatal disease in a wide range of species, including humans. Both NiV and HeV have continued to re-emerge sporadically in Bangladesh and Australia, respectively. There are currently no therapeutics or vaccines available to treat Henipavirus infection and both are classified as BSL4 pathogens.

RNA interference (RNAi) is a process by which double-stranded RNA directs sequence-specific degradation of messenger RNA in animal and plant cells. Small interfering RNAs (siRNAs) mediate RNAi by inhibiting gene expression of homologous mRNA and our preliminary studies suggest RNAi may be a useful approach to developing novel therapies for these highly lethal pathogens. Eight NiV siRNA molecules (four L and four N gene specific), two HeV N gene specific, and two non-specific control siRNA molecules were designed and tested for their ability to inhibit a henipavirus minigenome replication system (which does not require the use of live virus) in addition to live virus infections in vitro. In the minigenome assay three out of the four siRNAs that targeted the L gene of NiV effectively inhibited replication. In contrast, only NiV N gene siRNAs were effective in reducing live NiV replication, suggesting inhibition of early, abundantly expressed gene transcripts may be more effective than later, less abundant transcripts. Additionally, some of the siRNAs effective against NiV infection were only partially effective inhibitors of HeV infection. An inverse correlation between the number of nucleotide mismatches and the efficacy of siRNA inhibition was observed. The demonstration that RNAi effectively inhibits henipavirus replication in vitro, is a novel approach and may provide an effective therapy for these highly lethal, zoonotic pathogens.

1. Introduction

In the past decade a number of novel viral diseases have emerged, particularly in areas of Australia and Southeast Asia (Mackenzie and Field, 2004). These viral diseases include the highly pathogenic and zoonotic Hendra virus (HeV) and Nipah virus (NiV), both negative-sense single-stranded RNA viruses belonging to the new genus Henipavirus in the Paramyxoviridae family. NiV emerged in peninsula Malaysia in 1998 (Chua et al., 1999) while the closely related HeV (70–85% sequence homology to NiV) emerged in eastern Australia in 1994 (Murray et al., 1995b). Both HeV and NiV have neurological and pneumonic tropisms (Mackenzie and Field, 2004). The only two human HeV fatalities succumbed to either an acute respiratory disease or encephalitis (Murray et al., 1995a,b; O'Sullivan et al., 1997). In horses, HeV infection presents more commonly as an acute respiratory syndrome (Mackenzie and Field, 2004). During NiV infection, the predominant clinical syndrome in humans was encephalitic with a smaller number in respiratory distress. In pigs, NiV infection was characterised by acute fever with respiratory involvement with or without neurological signs (Mackenzie and Field, 2004). Both viruses have continued to re-emerge on an almost annual basis, with NiV responsible for as many as 50 infections each year in Bangladesh and neighbouring India with case fatality rates of up to 90% (Icddr, 2003, 2004a,b, 2005).

The initial NiV outbreak in Malaysia resulted in 265 human cases of encephalitis, including 105 deaths. In an attempt to control the outbreak, 1.1 million pigs were culled. The majority of human cases were males who had direct contact with the pigs or pig products and epidemiological evidence suggested that the primary means of spread between farms and between regions was the movement

Abbreviations: BSL4, biosafety level 4; BHK, baby hamster kidney; CAT, chloramphenol transferase; DAPI, 4,6-diaminidino-2-phenylindole; eGFP, enhanced fluorescent green protein; HDV, hepatitis delta virus ribozyme; HeV, Hendra virus; NiV, Nipah virus; PA, acid polymerase; RNAi, RISC, RNA-induced silencing complex, RNA interference; RSV, respiratory syncytial virus; SARS, sever acute respiratory syndrome; siRNA, small interfering RNA; $TCID_{50}$, 50% tissue culture infective dose.

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of pigs (Mackenzie and Field, 2004). Seroepidemiological studies confirmed the reservoir host for both these viruses to be fruit bats of the genus Pteropus in the suborder Megachiroptera (Yob et al., 2001; Young et al., 1996). It has been proposed that NiV passed from bats to pigs to initiate the outbreak (Daszak et al., 2006; Mackenzie et al., 2003). However, many of the recent outbreaks of NiV in Bangladesh and India have no apparent association with an animal source. It is probable that epidemiological investigations may have been inadequate to demonstrate this (Mackenzie and Field, 2004).

Of particular concern is the recent observation of human-to-human transmission in some of the Bangladesh outbreaks, not before seen with either NiV or HeV (Gurley et al., 2007). Significantly, NiV has been isolated from human respiratory secretions (Chua et al., 2001; Gurley et al., 2007). There are currently no vaccines or post-exposure therapeutics specifically indicated for Henipavirus infection. A limited non-randomised trial of ribavirin during the initial NiV outbreak in Malaysia showed ribavirin therapy reduced the mortality associated with acute NiV encephalitis (Chong et al., 2001). While this study reported no serious side effects, ribavirin has been associated with a range of side effects primarily related to haemolytic anaemia (De Franceschi et al., 2000). Ribavirin has also been shown to be effective against HeV in vitro, where virus yield was reduced more than 50-fold (Wright et al., 2005). A more recent study showed that the 5-ethyl analogue of ribavirin, but not ribavirin, was able to prevent mortality in five of six animals in a hamster model of NiV infection (Georges-Courbot et al., 2006) suggesting that other replication inhibitors may be effective against henipaviruses. While there are a number of therapy options being explored experimentally including active and passive vaccination, fusion inhibition and receptor blockade, there is clearly a need for further advances in this area.

RNA interference (RNAi) is a conserved eukaryotic pathway involved in RNA-guided gene silencing. RNAi consists of several pathways in which sequence-specific mRNA is degraded. The core feature of RNAi detected in all organisms is the production of small interfering RNAs (siRNAs) (Hammond, 2005). Long dsRNA is cleaved by an enzyme called dicer into siRNAs. These siRNAs are then incorporated into a complex called RNA-induced silencing complex (RISC) that mediates gene silencing. A number of studies have demonstrated the inhibition of replication of RNA viruses in vitro by RNAi, including polio virus (Gitlin et al., 2005), HIV (Fanning et al., 2003; Han et al., 2004; Novina et al., 2002), hepatitis C virus (Yokota et al., 2003) and severe acute respiratory syndrome (SARS) virus (Lu et al., 2004; Zheng et al., 2004). There are also a few reports on the in vivo inhibition of viruses by RNAi, including hepatitis B virus (Giladi et al., 2003; McCaffrey et al., 2003) and foot and mouth disease virus (Chen et al., 2004). Data from the first RNAi therapeutic to enter human clinical development for an infectious disease has been promising. ALN-RSV01 an RNAi therapeutic for the treatment of respiratory syncytial virus (RSV) infection, demonstrated the drug was safe and well tolerated when administered intranasally to adult human volunteers in Phase I clinical trials (DeVincenzo et al., 2008). These studies indicate that RNAi has enormous therapeutic potential for a number of serious infectious diseases.

Henipaviruses appear to be good targets for inhibition via RNAi as they are RNA viruses without any DNA intermediates during their entire life cycle. Because the genomic RNA of henipaviruses is negative sense, transcription must occur before viral proteins are expressed. The transcription complex consisting of the polymerase (L) and phosphoprotein (P) proteins initiates transcription at the 3′ terminus of the genomic RNA. Monocistronic capped and polyadenylated mRNAs are synthesized for each viral gene. An intergenic region consisting of a GAA trinucleotide exists between each gene. Immediately upstream of the gene junctions, the transcription complex encounters a stop signal and then either proceeds to initiate transcription of the next gene downstream or disengages from the viral RNA to reinitiate transcription at the 3′ terminus. Therefore, the relative abundance of a viral transcript varies inversely with its distance from the 3′ terminus of the genome. As free nucleoprotein (N) accumulates in the cytoplasm, the polymerase switches from transcription to genome replication and reads through the stop signal and intergenic regions to synthesize full-length plus sense viral RNA that serves as a template for synthesis of negative sense, genomic RNA (Bellini et al., 1998; Lamb et al., 2001). Unlike vaccines that require the recipients to have a normal immune system, siRNA-based treatment does not depend on a functional immune system and hence, may be used in elderly and immunocompromised individuals as well.

In this study, we prepared siRNAs using a commercially available kit, targeting the nucleocapsid (N) and large polymerase (L) genes of NiV and HeV for specific inhibition of virus replication. As indicators of siRNA functionality, we measured the reduction in replication of a henipavirus minigenome (Halpin et al., 2004; Magoffin et al., 2007) and the reduction in viral RNA and protein expression in infected cell cultures.

2. Materials and methods

2.1. Virus and cells

BHK-21 and BHK-T7 cells were grown in Eagle's Minimal Essential Medium containing Earle's salts (EMEM), antibiotics (100 U/ml Penicillin, 100 μg/ml Streptomycin and 500 μg/ml Fungizone), 5% foetal calf serum, 5% tryptose phosphate broth. Additionally BHK-T7 cells were cultured in the presence of 0.4 mg/ml Geneticin (G418). HeV was isolated in Vero cells from the lung of a horse infected in the Brisbane outbreak in October 1994 (Murray et al., 1995b) and was passaged five times in Vero cells followed by triple plaque purification and a further five passages in Vero cells as previously described (Hyatt and Selleck, 1996). NiV was isolated in Vero cells from the brain of a human fatally infected in the 1998–1999 Malaysian outbreak and was passaged three times in Vero cells then double plaque purified and passaged a further three times in Vero cells as previously described (Shiell et al., 2003). HeV and NiV stock titers were adjusted to 1×10^6 $TCID_{50}$/ml.

2.2. siRNA construction

Initially small interfering RNA template oligonucleotide sequences targeting the coding sequence of Nipah virus N and L (GenBank Accession no. AJ564622) were designed using the Dharmacon siDESIGN Centre Algorithm at http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx. The design included a leader sequence of any nucleotide (NN) and a total G/C content of between 30% and 52%, based on the sequence of this transcript and the Ambion siRNA template design tool at http://www.ambion.com/techlib/misc/silencer_siRNA_template.html for the Silencer™ siRNA Construction Kit. The highest-ranking results were selected for construction of siRNAs and a total of four were selected for each individual gene. siRNAs were constructed with the Silencer™ siRNA Construction Kit (Ambion) according to the manufacturer's instructions. Subsequent experiments led us to design an additional two HeV-specific siRNA molecules as described above. SiRNAs were also designed against the enhanced fluorescent green protein (eGFP) gene, to provide a knockdown control for initial minigenome studies while a non-specific target siRNA (influenza

Table 1
Sequences of siRNA oligonucleotide templates

Sequence (5′–3′)	Description[a]
siRNA Nipah L gene (27 mer)	
AATTAGCGTTAGGTAGAGTAATATACGCCTGTCTC	siL3619
AAACCTTCTTGTAACATCTTATATGATCCTGTCTC	siL4877
AAAGAAGTACTATCAGATTGACCAACCCCTGTCTC	siL6200
AAACATGGACTATAGCAACTATCCCCTCCTGTCTC	siL2145
siRNA Nipah N gene (21 mer)	
AAAGAGAGTCAATCCGTTCTTCCTGTCTC	siN600
AATATCAATCGTGGTTATCTTCCTGTCTC	siN1045
AAATAATCTCAGACATCGGAACCTGTCTC	siN749
AATGTTCAGGCTAGAGAGGCACCTGTCTC	siN1219
siRNA Hendra N gene (21 mer)	
AACATGCAGGCAAGAGAAGCCCCTGTCTC	siN1219(HeV)
AAAGAGGGTCAATCCATTCTTCCTGTCTC	siN600(HeV)
siRNA controls (21 mer)	
AAGCAATTGAGGAGTGCCTGACCTGTCTC	siPA
AAGCTGACCCTGAAGTTCATCCCTGTCTC	sieGFP

[a] The description of the siRNA refers to the nucleotide position of the AA dinucleotide start of each siRNA on the matching (L or N) gene.

PR8 acid polymerase (PA) gene) was also constructed as above. All oligonucleotide templates used to synthesize the siRNAs used in this study (Geneworks) are listed in Table 1.

2.3. Minigenome replication assay

Our collaborative partners initially constructed the minigenome to encode a NiV RNA analogue in which the coding regions for all viral genes were replaced with a negative-sense copy of the chloramphenol transferase (CAT) reporter gene (Halpin et al., 2004). This system has been subsequently enhanced with these modified reporter systems involved replacing the CAT reporter with an eGFP reporter gene (Magoffin et al., 2007) or utilising a HeV minigenome with a Renilla luciferase (LUC) reporter gene (gift from Linfa Wang). Briefly for these constructs, or construction of the eGFP reporter minigenome a DNA fragment containing the NiV L 3′ UTR, NiV trailer region and T7 promoter sequence was amplified by PCR. A second DNA fragment containing the NiV leader region, NiV N 5′ UTR, hepatitis delta virus ribozyme (HDV) sequence and two T7 terminator sequences was also amplified by PCR. The CAT reporter gene was removed from a plasmid by digestion and all DNA parts were then ligated, generating the pNiV-GFP or pHeV-LUC minigenomes. The precise initiation site of the T7 promoter, and the self-cleavage of the RNA at the 3′ end by the HDV ribozyme, ensured that the minigenome had identical genome termini to the complete viral genomic RNA (Halpin et al., 2004; Magoffin et al., 2007). The rule of six was maintained in both minigenomes. For paramyxoviruses the N, P and L genes are all required for genome replication (Neumann et al., 2002). The reporter gene can only be detected when plasmids encoding the minigenome, NiV N gene, NiV P gene and NiV L gene were transfected into mammalian cells (Halpin et al., 2004; Magoffin et al., 2007).

The NiV support plasmids consisted of the NiV N, P and L genes, cloned individually into the pTM1 expression vector (pTM1-NiV-N, pTM1-NiV-P and pTM1-NiV-L, respectively) and wee used interchangeably with the HEV minigenome (Halpin et al., 2004). When the minigenome is transcribed by T7 polymerase an RNA molecule containing exact copies of the 3′ and 5′ non-coding regions of the genomic RNA of NiV is generated. The minigenome system and support plasmids were transfected into baby hamster kidney (BHK) cells which provide the T7 polymerase activity. A recombinant cell line (BHK-T7) stably expressing T7 RNA polymerase (Ito et al., 2003) was used for all minigenome experiments (gift from Biao He).

2.4. Transfection of BHK-T7 cells with minigenome and support plasmids

BHK-T7 cell monolayers were seeded in 96-well plates at 2×10^4 cells/well in 200 μl EMEM and incubated overnight at 37 °C. The optimum ratio of plasmid concentrations in the minigenome assay was pre-determined to be a mixture of 0.2 μg minigenome plasmid, 0.15 μg N plasmid, 0.1 μg P plasmid and 0.05 μg L plasmid. For each minigenome assay, Lipofectamine 2000 (Invitrogen) was added to the plasmid DNA in a 3:1 ratio (3 μl lipofectamine:1 μg DNA) and then serum free EMEM was added to bring the final sample volume to 50 μl. After incubation for 30 min at room temperature, a further 150 μl of serum free EMEM was then added to the sample. This transfection mix was added to the cells and allowed to incubate at 37 °C with 5% CO_2 for 6 h, after which time the transfection mix was removed and replaced with growth media. Each minigenome assay was performed in 4–8 replicates per 96-well plate and each experiment was carried out at least three times. LUC reporter minigenome experiments were incubated for 24 h then 100 μl of chemiluminescent substrate (Steady-Glo, Promega) was added, incubated for 15 min and plates read in a Luminoskan Ascent luminometer (Thermo Fisher Scientific, Waltham, USA) using 100 ms integration per well.

2.5. siRNA transfections

siRNA constructed as described above was diluted to a working stock solution of 5 μM. A transfection mix containing siRNA and lipofectamine was prepared; 1 μl (0.07 μg) of siRNA was mixed with 0.21 μg lipofectamine 2000 (ratio 3:1), followed by addition of serum free EMEM to bring the final sample volume to 50 μl. After incubation for 30 min at room temperature, a further 50 μl of serum free EMEM was added to the sample, bringing the concentration of siRNA/well to 50 nM. To simplify siRNA screening experiments, a single dose of 50 nM was chosen after a review of the in vitro siRNA transfection literature indicated this was a typically effective dose for viral inhibition. This transfection mix was added to the cells and allowed to incubate at 37 °C with 5% CO_2 for 6 h, after which time the transfection mix was removed and replaced with growth media as described above.

2.6. Live virus siRNA antiviral assays

BHK-21 cells were transfected with test or irrelevant siRNA, followed by infection with NiV or HeV. BHK-21 monolayers were seeded in 96-well plates at 2×10^4/200 μl per well and incubated overnight at 37 °C. After 24 h, cells were transfected with 50 nM siRNA and incubated for 6 h. 96-well plates were then taken into a BSL4 laboratory medium removed and 100 μl of virus solution was added, followed by incubation of 30 min at 37 °C for 18 h. For both viruses stock solutions were diluted to a final concentration of 1500 $TCID_{50}$/100 μl for NiV and 2000 $TCID_{50}$/100 μl for HeV. After 30 min, inoculum was removed and 200 μl growth media was added to each well, followed by overnight incubation at 37 °C. Unprotected cells developed syncytia after overnight culture, detectable by immunofluorescence for viral protein. The following day media was removed and cells were fixed with ice-cold methanol for 10 min, removed from the BSL4 laboratory and air dried for 1–2 h prior to immunofluorescent labeling.

2.7. Immunofluorescence

The fixed and dried 96-well plates were washed with phosphate-buffered saline (10 mM PBS pH 7.6) containing 0.05%

(v/v) Tween 20 (PBS-T). A blocking solution of 1% BSA in PBS-T was added to fixed cells and incubated at 37 °C for 15 min. After blocking, cells were again washed three times with PBS-T followed by incubation with 50 μl/well of an anti-NiV/HeV N protein antibody (1:1000) for 30 min, at 37 °C. After this incubation cells were washed three times with PBS-T, followed by incubation with a goat anti-rabbit Alexa-Fluor 488 conjugate (Invitrogen, 1:1000) at 37 °C for 30 min in the dark. For nuclear staining, 1 μg/ml of 4,6-diaminidino-2-phenylindole (DAPI, ICN Biomedicals, Costa Mesa, USA) was added to the secondary antibody solution. After incubation the immunolabeled and stained cells were washed three times in PBS-T, and then covered with 100 μl PBS to prevent cells and the fluorophores from drying out. Syncytia numbers were then quantified using image analysis microscopy as previously described (Porotto et al., 2007). Briefly, cells were visualized using an IX71 inverted fluorescence microscope (Olympus), images were captured using a DP70 camera (Olympus) and image analysis was performed using AnalySIS® image analysis software (Soft Imaging System GmbH, Munster, Germany).

2.8. Isolation of viral RNA and Taqman PCR

After overnight virus infection viral media was removed from cells and 150 μl cell lysis buffer (RLT, Qiagen, containing 0.1% β-mercaptoethanol) was added directly to wells in 96-well plates. The cell lysate was aspirated into PCR tubes and removed from the BSL4 laboratory. RNA was extracted using the Qiagen RNeasy Mini kit as per the manufacturer's instructions. RNA was eluted in a final volume of 50 μl RNase free water. Samples were stored at −20 °C prior to Taqman PCR analyses.

The primers and Taqman probe to detect NiV or HeV genome sequences were designed from the N gene sequence of NiV (AF212302) and HeV (AF017149), respectively, using criteria specified in the Primer Express Software (version 1.5; Applied Biosystems, Foster City, CA). The specific NiV Taqman primers, probes and reaction conditions were used as previously reported (Mungall et al., 2006). The HeV-specific oligonucleotide primers were Hendra-N1433F and Hendra-N1572R, the fluorogenic 5′ nuclease (Taqman) probe was Hendra-1642comp-FAM-MGBFNQ. 18S rRNA was used to normalize for variations in RNA extraction. The 18S oligonucleotide primers were 18S rRNAF and 18S rRNAR. The fluorogenic 5′ nuclease (Taqman) probe for 18S rRNA was 18S rRNA-VIC. All Taqman PCR oligonucleotide primer and probe sequences used in this study are available on request.

Assays were performed in triplicate using a one-step protocol consisting of an initial reverse transcription reaction followed immediately by cDNA amplification. All Taqman reagents were purchased from Applied Biosystems except the primers, which were obtained from Geneworks. RNA (2 ml) was added to 23 μl of PCR mix in each well of a MicroAmp optical reaction plate containing 12.5 μl of Taqman One-Step PCR Mastermix, 0.625 μl of 40× Multiscribe/RNase inhibitor, 5.75 μl of distilled water, 1.25 μl each of 18 μM NiV or HeV forward and reverse primers, 1.25 μl of 5 μM HeV or NiV FAM-labeled probe, 0.125 μl each of 10 μM 18S rRNAF and 18S rRNAR, and 0.125 μl of 40 μM 18S rRNA-VIC-labeled probe. The samples were amplified in a GeneAmp 7500 sequence detection system (Applied Biosystems) using the following program: 48 °C for 30 min, 1 cycle; 95 °C for 10 min, 1 cycle; and 95 °C for 15 s and 60 °C for 60 s, 45 cycles. Linear regression analysis was used to quantify the NiV or HeV RNA based on cDNA standards. To correct for sample variation, Ct values for viral genome in samples were normalized against 18S rRNA expression, and viral genome was expressed relative to a standard cDNA dilution (3.6 pg of cDNA).

3. Results

3.1. siRNA production

As gene specific silencing was being evaluated in both a minigenome assay and in vitro against live virus infection, siRNAs were only designed to targets likely to suppress henipavirus replication in both systems (e.g. N and L gene). In total 12 siRNAs were designed and produced (Table 1); eight sequence-specific siRNAs targeting NiV (four targeting the L gene of NiV and four for the N gene of NiV), two sequence-specific siRNAs targeting HeV N gene, one irrelevant/non-specific siRNA (negative control) and one siRNA targeted for GFP (positive control). Previous studies have indicated that siRNAs with 3′ overhanging UU dinucleotides are the most effective, consequently all RNA transcripts used for chemical synthesis begin with an AA dinucleotide (Elbashir et al., 2001). Additionally, as chemical synthesis makes use of T7 RNA polymerase, an 8 nt sequence complementary to the T7 promoter sequence was also added to the 3′ of each designed oligonucleotide, 5′-CCTGTCTC-3′. Kim and colleagues have previously suggested that 27 bp siRNAs are more effective than 21 mers (Kim et al., 2004). In order to evaluate this hypothesis, all N gene specific siRNAs utilised in the current study were 21 nucleotides in length while all L gene specific siRNAs were 27 mers. Following synthesis, the concentration of all end products was determined using the Nanodrop® Nb-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the products were analyzed by gel electrophoresis. Concentrations of the siRNAs were in the range of 200–400 ng/μl and all samples showed a band of the expected size on an agarose gel (data not shown).

3.2. Minigenome assays

Initial experiments evaluating optimum plasmid ratios and siRNA efficacy were performed using BHK-T7 cells transfected with the NiV minigenome encoding eGFP as a reporter (data not shown). However, quantitation of GFP expression in monolayers requires intensive digital microscopy and image analysis prompting us to modify this system to one more amenable to automated screening. Subsequent experiments were all performed using a HeV minigenome construct containing a firefly luciferase (LUC) reporter. Halpin and colleagues (Halpin et al., 2004) showed that the support plasmids for the HeV and NiV minigenomes are interchangeable enabling simple substitution without extensive reoptimisation. A luciferase-based assay is not only more sensitive than GFP-based assays, the time to measure expression of the reporter gene was reduced from 72 to 24 h, vastly improving cell viability due to lipofectamine toxicity. Preliminary studies evaluating the optimum transfection efficiency with minimal lipofectamine-induced toxicity indicated that all transfections be carried out for 6 h only, followed by removal of transfection media and replacement with maintenance media. Utilising this LUC minigenome assay, all the siRNAs that targeted the N gene of NiV partially inhibited HeV minigenome replication (30–50% inhibition), while only two of the four L gene specific siRNA effectively inhibited (50% and 80%) replication (Fig. 1). Minigenome lacking one of the support plasmids (−L) was included as a negative control demonstrating minimal replication as evidenced by reduced luciferase activity (Fig. 1).

3.3. Live virus assays

Transfection of siRNAs (50 nM) prior to virus infection of BHK-21 cells showed that three of the four siRNAs that targeted the N gene of NiV were >60% effective in inhibiting viral replication of NiV as indicated by a reduction in the number of syncytia (Fig. 2). In

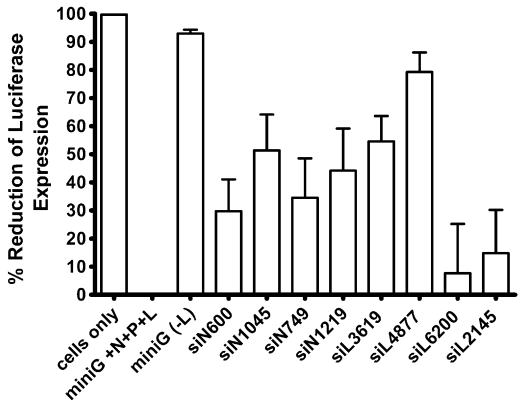

Fig. 1. siRNA inhibition of luciferase expression using a HeV minigenome. BHK-T7 cells were transfected with the HeV minigenome (encoding luciferase as a reporter gene) and its support plasmids (miniG + N + P + L), together with the different siRNAs (50 nM) to be tested. Values are expressed as % of control wells (miniG + N + P + L only) and are the mean ± S.E. of six biological replicates. Wells missing the L gene plasmid (miniG (−L)) served as negative controls. MiniG, minigenome; N, nucleocapsid; P, phosphoprotein; L, polymerase.

contrast, none of the siRNAs that targeted the L gene of NiV showed effective inhibition (<20%) of viral replication (Fig. 2). Interestingly, of these three N targeted siRNAs, only one (siN1045) was equally effective against HeV (>70% inhibition) while the other two (siN600 and siN1219) were significantly less effective against HeV (10–30% inhibition). In order to investigate this effect, the sequence of each siRNA was aligned (multiple alignment, Clustal W) against both NiV and HeV to determine the homology (Table 2).

The alignment shows that siN1045, the siRNA that proved to be most effective in inhibiting both HeV and NiV, contains only 1 nt mismatch compared with the HeV nucleotide sequence. Furthermore siN600 and si1219 contained 2 and 5 nt mismatches resulting in <30% and <15% inhibition of HeV infection, respectively. To evaluate whether the decrease in siRNA efficacy in silencing its target sequence (inhibiting viral replication) was related to nt mismatches, HeV-specific siRNAs were designed against HeV sequence at the identical nucleotide position as the siN600 and si1219 of NiV (designated siN600(HeV) and si1219(HeV)). BHK-21 cells were transfected with siRNA and infected with virus as described above.

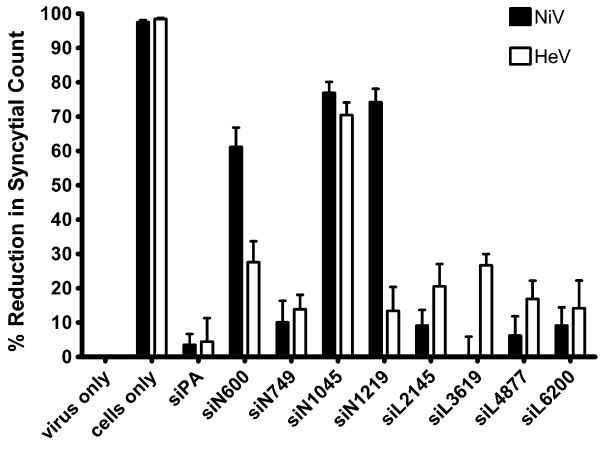

Fig. 2. siRNA inhibition of live NiV and HeV. BHK-21 cells were transfected with the different siRNAs (50 nM) to be tested. 6 h post-transfection, 100 μl of virus was added, cells were incubated for 18 h, then fixed in ice-cold methanol, removed from the BSL4 laboratory and viral protein detected using immunofluorescence. Image analysis of digital images of each well was performed to determine the number of viral syncytium. Values are expressed as % inhibition of syncytial numbers in virus only control wells (untreated) and are the mean ± S.E. of 12 biological replicates.

Table 2
Multiple alignment of HeV, NiV and siN600, si1045 and siN1219

siN600(NiV)	AAAGAGAGTCAATCCGTTCTT
NiV	AAAGAGAGTCAATCCGTTCTT
HeV	AAAGAGGGTCAATCCATTCTT
Mismatches	* *
siN1045	AATATCAATCGTGGTTATCTT
NiV	AATATCAATCGTGGTTATCTT
HeV	AACATCAATCGTGGTTATCTT
Mismatches	*
siN1219(NiV)	AATGTTCAGGCTAGAGAGGCA
NiV	AATGTTCAGGCTAGAGAGGCA
HeV	AACATGCAGGCAAGAGAAGCC
Mismatches	* * * * *

The nucleotide mismatches in the siRNA sequence when aligned to the HeV sequence are indicated (*).

Results show that the HeV-specific siRNAs are now more effective at inhibiting HeV than NiV (Fig. 3) although we observed less overall inhibition than in previous experiments. Despite improved homology, siN1219(HeV) is still not very effective (<25%) against HeV infection in contrast to the equivalent NiV siRNA (>45% inhibition of NiV). These results support the hypothesis that the more mismatches the siRNA contains, the less effective it becomes in silencing its target. One mismatch appears to have only a minor effect in terms of efficacy in silencing the target sequence, but two or more mismatches dramatically reduce the efficacy of the siRNA in silencing its target.

To evaluate possible synergistic effects of multiple siRNA therapy a cocktail of two siRNAs (25 nM each of siN1045 and siN1219(NiV)) were tested to determine if multiple siRNAs were more effective at inhibiting viral replication than using a single siRNA. The cocktail was able to inhibit NiV replication by more than 60%, but did not have any effect on HeV replication, despite the 30% reduction in HeV replication with siN1219 application alone (Fig. 3). Given that the concentration of each siRNA in the cocktail was reduced to half that of all the other siRNA transfections, the inhibition of NiV was considerable, suggesting a cumulative effect of both siRNAs.

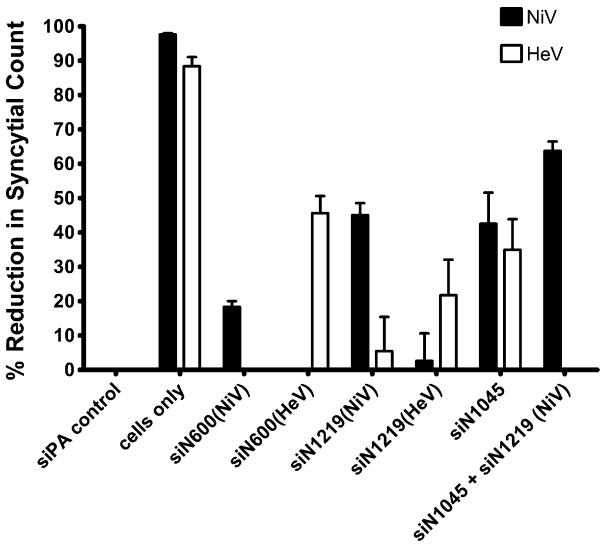

Fig. 3. Differences in the inhibition of virus depending on the specificity of the siRNA. siRNAs (50 nM) specific for NiV are compared to HeV-specific siRNAs. Values are shown as normalized percentage inhibition compared to the siPA control group (siPA), as the mean of three replicates ± standard error.

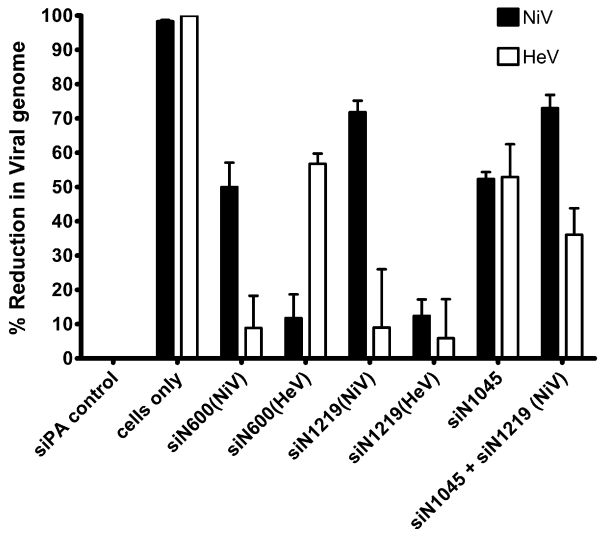

Fig. 4. NiV genome in BHK cells transfected with various siRNAs alone (50 nM), or in combination (25 nM each), detected by Taqman PCR. Values are shown as normalized percentage inhibition compared to the siPA control group (siPA), and are the mean of nine replicates ± standard error.

3.4. Virus quantification using Taqman PCR (RT-PCR)

To confirm the results obtained in the live virus assay (detection based on viral protein expression), virus infection was also quantified using real-time PCR (Taqman PCR) as described previously (Mungall et al., 2006). Results obtained with the Taqman assay corresponded well with the results obtained in the live virus inhibition assays (Fig. 4). All three NiV N gene specific siRNAs able to inhibit live virus NiV replication also reduced viral RNA levels while the HeV-specific siRNA inhibition also corresponded well between these two assays (Fig. 4). BHK cells that were infected with NiV and transfected with 50 nM siN600(NiV), siN1219(NiV) or siN1045 showed a decrease in viral RNA of approximately 50%, 75% and 55%, respectively. BHK cells that were transfected with the siRNA cocktail (25 nM siN1219(NiV) and 25 nM siN1045) also showed a 75% decrease in viral genome, indicating that the usage of multiple siRNAs to inhibit viral replication may provide an enhanced therapeutic approach.

4. Discussion

Given the additional constraints involved with performing antiviral assays against BSL4 agents in vitro, the development of surrogate assays to screen and evaluate antivirals at BSL2 is important. Both NiV (Negrete et al., 2005, 2006) and HeV (Porotto et al., 2007, 2006) pseudotype assays have recently been developed providing excellent surrogate BSL2 assays for the evaluation of virus entry and fusion mechanisms, enabling wider access for potential antiviral evaluation. However, as these pseudotype viruses are either replication deficient, or rely on the replication proteins of the pseudotype virus (VSV), they may not be applicable for specific henipavirus gene knockdown and subsequent siRNA evaluation. In the current study we have compared a minigenome replication assay (Halpin et al., 2004), also requiring no infectious virus, with live virus assays to evaluate candidate siRNA molecules as potential antivirals. Comparison of the results obtained in the live virus assay with the results obtained in the minigenome assay reveals an interesting observation. Using the minigenome, two out of the four siRNAs targeting the L gene of NiV were effective inhibitors of viral replication (inhibiting expression of a luciferase reporter), whereas all of the siRNAs that targeted the N gene of NiV partially inhibited minigenome replication. In contrast, when the siRNAs were tested against live virus infection, none of the siRNAs targeting the L gene were effective inhibitors of viral replication, and only three out of the four siRNAs targeting the N gene were effective. The differences within these two assays between siRNAs targeting different genes might have several explanations. Minigenome replication is dependent on T7-mediated expression of support proteins, a step that does not occur in a naturally infected cell. Thus, the dynamics of siRNA inhibition of plasmid gene expression may be markedly different from in situ virus gene inhibition. Further, the minigenome system is a surrogate assay used to evaluate siRNA inhibition of viral replication in the absence of most of the other viral proteins present when live virus is used. This suggests that results obtained using this surrogate assay may not necessarily correspond to the results obtained in vitro using live viruses. Quite simply, the minigenome assay is a relatively restricted assay system containing only the essential elements for replication, which might explain why the greater efficacy of the siRNAs targeting the L gene (encoding the viral polymerase) of NiV compared to the siRNAs targeting the N gene. Alternatively, the shorter length of the siRNAs targeting the N gene (21 nt) compared to the L gene targets (27 nt) may reduce the strength of binding to the target sequence, reducing their efficacy. Further experiments using equivalent length siRNA molecules are needed to address this. The fact that in the live virus assay the siRNAs targeting the N gene were very effective in inhibiting viral replication while the L gene targets appeared to have little to no effect appears more complex. This may reflect the relative abundance of the N protein compared to the L protein during the early stages of viral replication. The switch of the polymerase from 'transcriptive mode' to 'replicative mode' is thought to be triggered by the intracellular accumulation of viral protein, most probably the N protein necessary for RNA encapsidation (Barik, 2004; Bellini et al., 1998; Lamb et al., 2001).

The current study evaluated only N and L gene targets for siRNA inhibition, selected as the first and last genes expressed by henipaviruses in order to evaluate the effect of transcript quantity on siRNA efficacy. Against live virus, N gene siRNA targets proved much more effective than L gene targets suggesting transcript quantity does influence siRNA efficacy. We have identified several siRNA molecules as effective inhibitors of NiV and HeV infection, however, a number of additional targets remain to be tested. If viral gene transcript abundance is crucial to siRNA efficacy, molecules targeted to the phosphoprotein (P) gene may also be highly effective. While the P protein plays an essential role in the formation and function of the polymerase complex (Eaton et al., 2006), the P gene of henipaviruses is also predicted to encode for an additional three proteins, V, W, and C (Harcourt et al., 2000; Wang et al., 1998). While the target of the C protein is not known, it has been shown that NiV P, V and W proteins all act on the IFN signaling or JAK/STAT pathway by a distinct mechanism involving direct inhibition of STAT1, through a domain in their common N terminus (Rodriguez and Horvath, 2004; Shaw et al., 2004). Due to its conserved role in all three proteins, this region in particular may prove to be an attractive target for siRNA-mediated inhibition. Similarly, the pivotal role of the matrix (M) protein in viral budding indicated by VLP studies (Ciancanelli and Basler, 2006) suggests M gene targets may also inhibit key points in the viral life cycle. While the G and F proteins might appear to be attractive targets due to their central roles in receptor binding and membrane fusion, respectively, their physical exposure to antigenic pressures makes them more susceptible to mutation, facilitating viral escape from antiviral therapy. While escape from siRNA-mediated inhibition is likely to be governed quite differently to antigenic mutation pressures, we anticipate that surface proteins are likely to be

inherently more variable than unexposed proteins (Harcourt et al., 2005).

An important observation in the current study is the apparent cumulative effect of multiple siRNA treatment. This strategy may provide the most effective approach currently available to limiting the development of antiviral resistance. Single siRNA therapy may ultimately be susceptible to viral escape via selective mutations but the use of multiple gene targets makes viral escape much less likely. Additionally, previous studies of influenza escape mutants suggest these are likely to have reduced viral fitness (Carr et al., 2002; Herlocher et al., 2002; Ives et al., 2002; McKimm-Breschkin, 2000). Logically, the combination of several barriers to virus infection (multiple siRNA targets) should reduce the likelihood of viral escape. However, studies have shown that the effective concentration of siRNAs may reach a plateau (Kamath et al., 2001), suggesting that the RNAi-induced silencing complexes of the cell are saturable. Further studies with multiple siRNA targets are necessary to adequately evaluate this effect both in vitro and in vivo.

5. Conclusions

In summary, using a surrogate minigenome assay and a live virus infection assay, we have evaluated a number of potential antiviral candidate siRNA molecules derived from the N and L genes of NiV. We have identified a number of N gene targets effective against live virus infection. Additionally, we observed an inverse correlation between the number of nucleotide mismatches and the efficacy of siRNA inhibition. Given the high homology between HeV and NiV, individual virus specificity may be attained by targeting slightly heterologous regions. Alternatively, broad-spectrum antiviral activity may be achieved by selecting strictly homologous targets. Given the recurrent re-emergence of these viruses and the current lack of therapeutic intervention, the demonstration that RNAi effectively inhibits henipavirus replication in vitro, provides a novel approach and may provide an effective therapy for these highly lethal zoonotic pathogens.

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