Veterinary Microbiology 176 (2015) 10–18

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Combination siRNA therapy against feline coronavirus can delay the emergence of antiviral resistance *in vitro*

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form 8 December 2014
Accepted 11 December 2014

Keywords:
Feline infectious peritonitis virus
Feline infectious peritonitis
RNA interference
Antiviral
siRNA
Viral escape

ABSTRACT

Virulent biotypes of feline coronavirus (FCoV), commonly referred to as feline infectious peritonitis virus (FIPV), can result in the development of feline infectious peritonitis (FIP), a typically fatal immune mediated disease for which there is currently no effective antiviral treatment. We previously reported the successful *in vitro* inhibition of FIPV replication by synthetic siRNA mediated RNA interference (RNAi) in an immortalised cell line (McDonagh et al., 2011). A major challenge facing the development of any antiviral strategy is that of resistance, a problem which is particularly acute for RNAi based therapeutics due to the exquisite sequence specificity of the targeting mechanism. The development of resistance during treatment can be minimised using combination therapy to raise the genetic barrier or using highly potent compounds which result in a more rapid and pronounced reduction in the viral replication rate, thereby reducing the formation of mutant, and potentially resistant viruses. This study investigated the efficacy of combination siRNA therapy and its ability to delay or prevent viral escape. Virus serially passaged through cells treated with a single or dual siRNAs rapidly acquired resistance, with mutations identified in the siRNA target sites. Combination therapy with three siRNA prevented viral escape over the course of five passages. To identify more potent silencing molecules we also compared the efficacy, in terms of potency and duration of action, of canonical *versus* Dicer-substrate siRNAs for two previously identified effective viral motifs. Dicer-substrate siRNAs showed equivalent or better potency than canonical siRNAs for the target sites investigated, and may be a more appropriate molecule for *in vivo* use. Combined, these data inform the potential therapeutic application of antiviral RNAi against FIPV.

1. Introduction

Feline infectious peritonitis (FIP) remains one of the leading causes of death in young cats (Pedersen, 2014). Feline infectious peritonitis virus (FIPV), the aetiological agent, is a virulent feline coronavirus (FCoV) biotype that demonstrates enhanced and sustained replication in cells of the monocyte lineage, and is responsible for triggering and perpetuating the immune mediated pathology seen in FIP (Kipar et al., 2005). Immune modulating drugs have been the mainstay of treatment for affected cats, however such treatments when examined in controlled trials, have provided little if any benefit (Fischer et al., 2011; Ritz et al., 2007). Direct acting antiviral therapy for FIP has received considerably less clinical attention.

The potential of RNAi as an antiviral therapeutic has been demonstrated *in vitro*, and in a few cases *in vivo*, against numerous human and veterinary pathogens

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http://dx.doi.org/10.1016/j.vetmic.2014.12.009
0378-1135/

from diverse viral families (as reviewed by Shah and Schaffer, 2011). We have previously demonstrated effective *in vitro* inhibition of FIPV using synthetic siRNAs targeted against sequences encoding structural and non-structural proteins and the 5′ UTR (McDonagh et al., 2011). One of the major challenges facing the development of antiviral therapeutics is resistance, which may develop during treatment, or be pre-existing in a virus population. Whilst this is a concern with any antiviral compound, it is particularly problematic for RNAi based approaches due to the exquisite sequence specificity of the targeting mechanism. A single nucleotide mismatch within the target site can dramatically reduce the efficacy of RNAi-based viral inhibition by impairing base pairing between the guide strand and its target (Gitlin et al., 2005). This is especially relevant for feline coronaviruses, which are known to exist as a quasispecies (Kiss et al., 1999).

This report extends our previous work on siRNA mediated inhibition of FIPV to investigate and address the challenges of antiviral resistance. Experiments were conducted to determine the efficacy of combination siRNA therapy against FIPV and its ability to delay or prevent the emergence of resistance *in vitro*. For two highly effective siRNAs, the efficacy of Dicer-substrate siRNAs (DsiRNAs), a structural siRNA variant that has been reported in some studies to be more efficacious than sequence matched canonical siRNA (Kim et al., 2005), was investigated in terms of potency and duration of action compared to canonical siRNAs targeting the same motif.

2. Materials and methods

2.1. Cells and viruses

Crandell Rees Feline Kidney (CRFK) cell line was propagated as previously described (McDonagh et al., 2011). FCoV FIPV WSU 79-1146 (FIPV1146) was purchased from American Type Culture Collection (ATCC: VR990; Virginia, USA).

2.2. siRNA/Dicer-substrate siRNA

Anti-FCoV siRNA sequences (Table 1) were designed using previously described criteria (McDonagh et al., 2011). DsiRNAs, based on previously identified highly effective anti-FIPV siRNAs L2 and N1 (McDonagh et al., 2011), in addition to the non-silencing control siRNA (NSC siRNA) (Table 2), were designed as outlined by Amarzguioui et al. (2006). Custom synthesised DsiRNAs and a transfection control DsiRNA duplex conjugated to TYE^{TM} 563 (Integrated DNA Technologies [IDT] Coralville, IA, USA) were resuspended to 5 μM in nuclease-free duplex buffer (IDT) containing 100 mM potassium acetate and 30 mM HEPES. siRNAs and DsiRNAs were stored in single use aliquots at −20 °C until use.

2.3. Nucleic acid transfection

Reverse transfection was conducted as per manufacturer's guidelines using Lipofectamine 2000 (Life Technologies, Mulgrave, VIC, Australia) in 24 well or 96 well plates (Greiner Bio-One, Germany). Pre-prepared complexes were added to wells followed by 8×10^4 cells well^{-1} in 500 μl (24-well plate) or 1×10^4 cells well^{-1} in 100 μl (96-well plate). Plates were gently rocked to evenly distribute cells and incubated at room temperature for 30 min to minimise edge effect, prior to incubation at 37 °C for a further 6 h. Culture media was removed and the cells washed with DMEM prior to infection.

2.4. Virus titration

Titration of extracellular virus was performed using the TCID50 method and by plaque assay as previously reported (McDonagh et al., 2011, 2014).

Table 1
Sequence of siRNAs targeting FCoV and their position in FCoV FIPV1146 genome (accession number DQ010921). Sequence of non-silencing control siRNA (NSC-GFP) targeting GFP is also shown.

siRNA	Sequence	Position in genome
Leader2 (L2)		
Sense	5′-GGACACCAACUCGAACUAAdTdT-3′	79–97
Antisense	5′-UUAGUUCGAGUUGGUGUCCdTdT-3′	
Membrane1 (M1)		
Sense	5′-CCUAGUAGAACCAUCGUUUdTdT-3′	26611–26629
Antisense	5′-AAACGAUGGUUCUACUAGGdTdT-3′	
Nucleocapsid1 (N1)		
Sense	5′-GGAGUCUUCUGGGUUGCAAdTdT-3′	27112–27130
Antisense	5′-UUGCAACCCAGAAGACUCCdTdT-3′	
Nucleocapsid2 (N2)		
Sense	5′-GGCAUACACAGAUGUGUUUdTdT-3′	27885–27837
Antisense	5′-AAACACAUCUGUGUAUGCCdTdT-3′	
Non-silencing control (NSC-GFP)		
Sense	5′-[FITC] CAAGCUGACCCUGAAGUUCdTdT-3′	
Antisense	5′-GAACUUCAGGGUCAGCUUGdTdT-3′	

Table 2
Dicer-substrate siRNA sequences. RNA bases are uppercase, DNA bases are lower case. Previously tested siRNA sequences are shown shaded in grey (the central 19mer duplex). Dicer-substrate siRNAs designed based on the reported sequence of FCoV FIPV1146 (accession number DQ010921).

DsiRNA	Sequence
Leader (DsiRNA-L2)	
Sense	5′-GGACACCAACUCGAACUAAACGAaa-3′
Antisense	3′-AGCCUGUGGUUGAGCUUGAUUUGCUUU-5′
Nucleocapsid (DsiRNA-N1)	
Sense	5′-GGAGUCUUCUGGGUUGCAAGGGAtg-3′
Antisense	3′-UACCUCAGAAGACCCAACGUUCCCUAC-5′
NSC-GFP (DsiRNA-NSC)	
Sense	5′-CAAGCUGACCCUGAAGUUCAUCUgc-3′
Antisense	3′-CCGUUCGACUGGGACUUCAAGUAGACG-5′

2.5. Viral consensus sequencing

2.5.1. RNA extraction

Tissue culture supernatant stored at −80 °C was thawed at 37 °C in a water bath and clarified by centrifugation at 2000 × *g* for 3 min. RNA was extracted using QIAamp Viral RNA mini kit (QIAGEN, Doncaster, VIC, Australia).

2.5.2. RT-PCR

Reverse transcription was performed as described by McDonagh et al. (2011) with the exception that a fixed volume (13 μl) of RNA was used instead of a fixed mass. cDNA was stored at −80 °C until use.

PCR primers were designed using Primer3 software (Rozen and Skaletsky, 2000) to flank the target sites of siRNAs L2 and N1 (Table 3). Conventional PCR was performed in an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf) in 50 μl reactions containing 10 μl 5× GoTaq colourless reaction buffer, 12.5 nmol of each dNTP, 12.5 pmol forward and reverse primers, 125 μmol $MgCl_2$, 1.25 units GoTaq DNA polymerase, 2 μl cDNA, 22.75 μl nuclease-free water. Cycling conditions were denaturation of DNA at 95 °C for 5 min, 35 cycles of 30 s at 95 °C, 45 s at 58 °C, and 60 s at 72 °C, and a final extension at 72 °C for 5 min.

2.5.3. DNA sequencing

PCR products were purified prior to sequencing using the Wizard® SV Gel and PCR Clean-Up System (Promega).

Table 3
Primers for sequencing L2 and N1 target sites. FCoV primers designed based on reported sequence of FCoV FIPV1146 (accession number DQ010921).

Target	Primer sequence	Product size (bp)
Leader2		
Forward	AAAGTGAGTGTAGCGTGGCTAT	452
Reverse	AGGTCACGACGGTATTCAGG	
Nucleocapsid1		
Forward	TCGCTGAGAGGTGGTTCTTT	461
Reverse	CTTGCCTGCAGTTTTCTTCC	

Prior to purification 1 μl of PCR product was electrophoresed on a standard 2% (w/v) agarose horizontal gel slab, containing 1 μl 100 ml^{-1} GelRed (Biotium, Hayward, CA, USA), in TAE buffer and visualised by UV transillumination (Dolphin View, Wealtec, Sparks, NV, USA) to confirm a single band of the appropriate size. The optical density of the purified product was measured at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to assess sample concentration and purity. Purified PCR products resuspended to 50 ng μl^{-1} in nuclease-free water were submitted to Macrogen Inc. (Seoul, South Korea) for sequencing in both directions using an ABI *3730xl* DNA Analyser. Resultant electropherograms were aligned and edited using Geneious (Version 4.6 Biomatters Ltd., Auckland, NZ) and Bioedit Sequence Alignment Editor software (Version 7.2.0).

2.6. Immunofluorescence assay

For some experiments cells grown in μClear® 96-well imaging plates (Greiner Bio-One) were fixed and stained *in situ* for FIPV antigen expression. At the end of the infection period cells were fixed in 20% formaldehyde in PBS and permeabilised in ice cold methanol. Viral antigen was detected with a biotinylated anti-feline coronaviruses (FCoV) antibody (CCV2-2; Custom Monoclonals International, Sacramento, CA, USA) and visualised with streptavidin-conjugated Alexafluor 555. To enable accurate segmentation, cells were stained with the whole cell stain HCS Cell Mask Blue (Life Technologies) in addition to DAPI (Life Technologies) to enhance nuclear visualisation. Fluorescent imaging was performed using the BD Pathway 855 Bioimager (BD, Franklin Lakes, NJ, USA). Images of wells were acquired using a 3 × 3 montage with laser autofocus performed for each montage frame. HCS Cell Mask Blue/DAPI images were acquired with Ex 380/10 BP and Em 435 LP filters, and Alexa Fluor 555 images acquired with Ex 548/20 BP and Em 570 LP filters. Image analysis was performed using the free open-source image analysis software CellProfiler (R11710, www.cellprofiler.org) (Carpenter et al., 2006). A CellProfiler pipeline, was constructed to measure whole cell fluorescent intensity and data exported to FCS Express Image Cytometry (De Novo Software, Los Angeles, CA, USA) for analysis. Calculation of the percentage of infected cells was performed by placing a region marker on fluorescence intensity histograms to exclude negative cells as defined by uninfected control samples.

2.7. Combination siRNA treatment

To assess the efficacy of combination treatment, CRFK cells were reverse transfected in 96-well plates (μClear®, Greiner Bio-One) using single or a combination of multiple effective anti-FCoV siRNAs. The total concentration of siRNA per treatment was held constant at 30 nM, meaning for treatment involving two, three, or four siRNAs the concentration or each siRNA was 15 nM, 10 nM, and 7.5 nM respectively. Following transfection, cells were infected with FIPV1146 at MOI 0.2 and incubated at 37 °C in 5% CO_2

in air. At 36 hpi efficacy was assessed *via* titration of extracellular virus using the TCID50 protocol and by *in situ* IFA staining for viral antigen. Each treatment was performed in triplicate and repeated in three independent experiments. Data represent mean ± SE.

2.8. Viral escape from siRNA mediated inhibition

The ability of FIPV1146 to evolve resistance to RNAi mediated inhibition was investigated by serially passaging the virus in the presence of one or a combination of effective siRNAs. CRFK cells were reverse transfected in 24-well plates (Sarstedt), using one, or a combination of two or three siRNAs, with four wells per treatment. Cells transfected with a non-silencing control (NSC) siRNA and untreated cells were included as controls. The total siRNA concentration per treatment was fixed at 30 nM irrespective of the number of different siRNAs used per treatment. For the first passage infection (P1) cells were infected with original stock virus (P0) at MOI 1 and wells were monitored every 8 h (beginning 24 hpi) for the development of CPE. When three or more wells of a particular treatment showed greater than 80% CPE, the culture media from all four wells was harvested, pooled, and stored at −80 °C. For subsequent passages (P2–P5) cells were transfected as described above and infected with 50 μl of culture media from identically treated cells of the previous passage which had been clarified by centrifugation at 1000 × *g* for 3 min. At the end of the experiment all serially passaged viruses were titrated by plaque assay and consensus sequencing performed.

To determine if mutations identified in viruses serially passaged in siRNA treated cells conferred a resistant phenotype, siRNA treated cells were infected with serially passaged viruses. Cells were transfected or mock transfected in 96-well uClear® plates using single or multiple siRNAs, with a total siRNA concentration per treatment of 30 nM. At the end of the transfection period cells were infected at MOI 0.2. For each siRNA treatment (or control) duplicate wells were infected with P0 (original stock virus) and P1–P5 viruses from identically treated cells. Additionally P1–P5 viruses from cells treated with a single siRNA (either siRNA L2 or N1) were also tested in duplicate in cells transfected with the other single siRNA to which the virus had not previously been exposed. Untreated and uninfected control wells were included on each plate. Cells were fixed and stained for viral antigen expression 40 hpi as previously described.

2.9. Comparison of Dicer-substrate and canonical siRNAs

A concentration–response experiment was performed to compare the antiviral effect of canonical and Dicer-substrate siRNAs. Cells were reverse transfected in 96-well plates (Sarstedt) with half-log dilutions from 10 nM to 0.1 nM of single siRNA or DsiRNA. The potency of combination treatment was compared by transfecting cells with 5 nM, 1.5 nM, or 0.5 nM combinations of two canonical or Dicer-substrate siRNAs. Cells transfected with NSC siRNA and DsiRNA, in addition to untreated cells, were included as controls. At the end of the transfection period cells were infected (or mock infected) with FIPV1146 at MOI 0.2. Culture media was harvested at 40 hpi for titration of extracellular virus by TCID50 endpoint assay. Data analysis were conducted in GraphPad Prism (GraphPad Prism V5.03 for Windows, GraphPad Software, San Diego, CA, USA) with 50% inhibitory concentration (IC50) values calculated using the inbuilt non-linear curve fitting functions following $\log_{10}$ transformation of compound concentration. Each treatment was performed in triplicate and repeated in three independent experiments. Data represent mean ± SE.

A further experiment was conducted to assess the duration of antiviral action of canonical *versus* Dicer-substrate siRNAs against FIPV1146 using single and combination treatment. Cells were reverse transfected with single or dual combination siRNAs or DsiRNAs with a total concentration of 5 nM. At the end of the transfection period cells were infected with FIPV1146 at MOI 0.5 and incubated at 37 °C in 5% CO_2 in air. Culture media was harvested at 20, 40, 60, and 80 hpi for titration of extracellular virus by TCID50 endpoint assay. Each treatment was performed in triplicate and repeated in two independent experiments. Data represent mean ± SE.

3. Results

3.1. Efficacy of combination siRNA treatment

Based on extracellular viral titre and intracellular viral antigen expression, pre-treatment with multiple siRNAs was highly effective at inhibiting viral replication, with no evidence of antagonistic effects noted at the concentrations tested. Pre-treatment with single siRNAs at 30 nM using reverse transfection resulted in a reduction of extracellular viral titres of 99.7%, 99.6%, 93.9%, and 91.4% for siRNAs L2, N1, M1, and N2 respectively (Fig. 1a). Although the cell culture and infection conditions were different, the reduction in relative viral titre using the reverse transfection protocol was significantly greater than previously reported for forward transfected cells, particularly for siRNAs M1 and N2 (McDonagh et al., 2011). This suggests that reverse transfection is more efficient in delivering siRNAs in CRFK cells, in agreement with the findings of Reid et al. (2009). Pre-treatment with a combination of two, three, or four siRNA, at a total concentration of 30 nM, reduced the viral titre by greater than 99.5% for all tested combinations. Analysis of viral antigen expression supported these findings.

Combination treatment with multiple siRNAs was highly effective, resulting in a greater reduction in the percentage of infected cells compared to treatment with a single siRNA (Fig. 1b). Interestingly, the percentage of infected cells following single treatment with siRNAs M1 or N2 (92.5% and 66.1% respectively) was significantly higher than would be expected given the degree of inhibition of extracellular virus (93.9% and 91.4% reduction respectively). This large difference was not apparent for cells treated singly with siRNAs L2 and N1, or cells treated with siRNA combinations, where significant inhibition of extracellular viral titre was associated with a similarly large reduction in the percentage of infected cells.

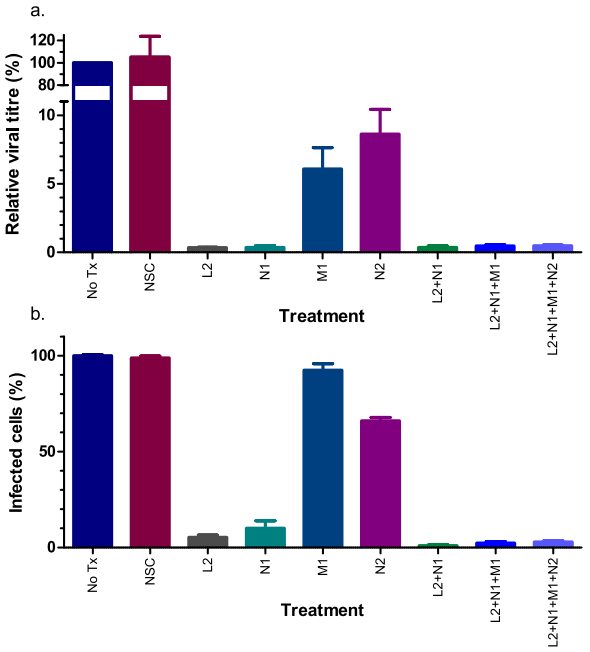

Fig. 1. Efficacy of combination siRNA treatment against FCoV FIPV1146. Efficacy was determined 36 hpi by (a) TCID50 titration of extracellular virus and (b) assessment of intracellular viral antigen expression by IFA.

3.2. Viral escape from siRNA mediated inhibition

Serial passage of FIPV1146 through cells treated with one or multiple siRNAs was associated with the development of a number of single nucleotide polymorphisms within the sequenced siRNA target sites (Fig. 2). For virus passaged through cells treated with a single siRNA, mutations were identified at P1 (for siRNA L2) and P2 (for siRNA N1), while for virus passaged through dual and triple siRNA treated cells, mutations were identified first at P2 and P4 respectively.

Mutations were identified at two separate locations within the L2 target site. The first of these was a C-to-U transition at position ten (in virus passaged through cells treated with siRNA L2 alone and with siRNA combination L2+N1+M1) and the second, a C-to-A transition at position six (in virus passaged through cells treated with siRNA combination L2+N1). As siRNA L2 targets the leader sequence in the 5′ UTR there are no associated amino acid changes as a result of these mutations. The L2 target site is however located within the putative leader-TRS hairpin, thought to be an important *cis*-acting element (Dye and Siddell, 2005), as well as incorporating several nucleotides of the proximal end of the transcriptional regulatory sequence.

For the N1 target site mutations were also identified at two locations. At position nine two polymorphisms were noted: a C-to-A transition (in virus passaged through cells treated with siRNA N1 alone) and a C-to-G transition (in virus passaged through cells treated with siRNA combinations L2+N1 and L2+N1+M1). The second mutation was a G-to-C transition at position seventeen. This mutation was seen only in virus passaged through cells treated with siRNA combination L2+N1+M1. All of the nucleotide substitutions within the N1 target site were associated with amino acid changes. Although position nine of the target site corresponds to the wobble position of the codon for phenylalanine in the original sequence, both identified

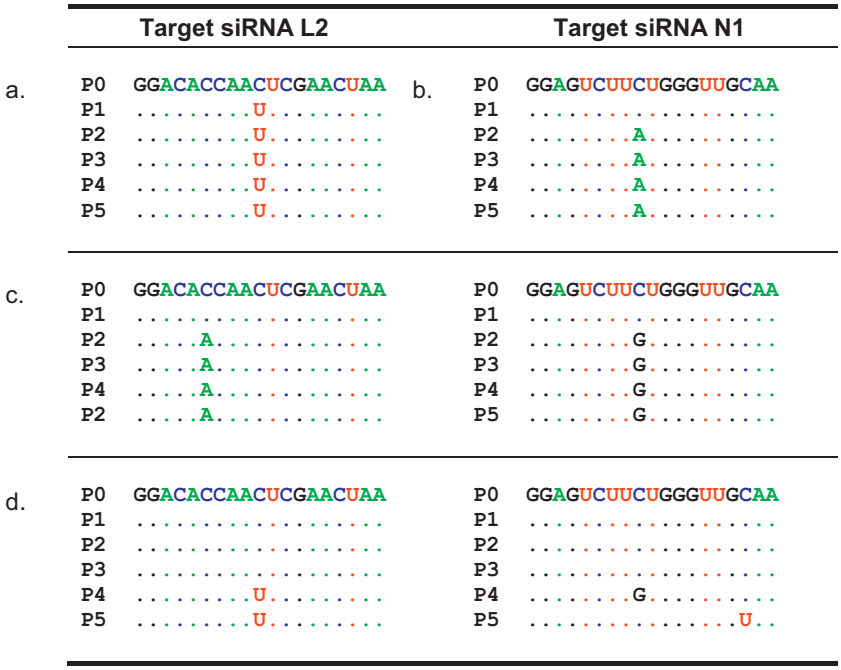

Fig. 2. Mutations identified in siRNA target sites for FCoV FIPV1146 serially passaged in cells treated with (a) siRNA L2, (b) siRNA N1, (c) siRNAs L2+N1, and (d) siRNAs L2+N1+M1. P0 represents original virus stock, P1–P5 represent passages 1–5 respectively.

polymorphisms at this location resulted in an amino acid change to leucine. Position seventeen corresponds to the middle base of the codon for alanine in the original sequence, and the G-to-C transition results in an amino acid substitution to valine.

Based on the inhibition of viral antigen expression, the identified mutations resulted in the acquisition of a fully or partially resistant phenotype (Fig. 3). When treated with a single siRNA the acquisition of a mutation at the target site was associated with an almost complete loss of inhibition. For siRNA N1, although the C-to-A transition was not noted until P2, a highly resistant phenotype was apparent from P1. Analysis of the P1 electropherogram at this site shows a small adenine peak in addition to cytosine suggesting a subpopulation of the P1 viruses had undergone this mutation during the first passage. Given the 40 h infection period used in this study encompasses multiple replication cycles, the presence of even a low-prevalence resistant sub-population would likely result in the appearance of a highly resistant phenotype in this study. The development of resistance to siRNA mediated inhibition was demonstrated to be sequence dependent rather than mechanism dependent as viruses with mutations in the L2 target site remained susceptible to RNAi mediated inhibition by siRNA N1 and *vice versa* (data not shown).

Mutations were not apparent until P2 for virus passaged through dual combination siRNA treated cells. A partially resistant phenotype was noted at P1 with relative viral antigen expression increasing from 3.3% at P0 to 27.7% at P1. By P2 mutations were present in both L2 and N1 target sites, with an accompanying increase in relative viral antigen expression to 91.1%. Interestingly, relative viral antigen expression for P3 and P4 dual treated viruses remained at approximately 90% despite the presence of the apparently fixed mutations in both target sites, with complete resistance not seen until P5.

Virus passaged through triple combination siRNA treated cells remained susceptible to RNAi mediated inhibition for all five passages, although low level resistance was apparent from P1. Despite this early appearance of low level resistance, mutations were not identified in the L2 or N1 target regions until P4. At P4, a C-to-U transition was identified at position ten of the L2 target and a possible C-to-G transition was identified at position nine of the N1 target, although the electropherogram at this latter site showed two almost equal peaks. At P5 the mutation in the L2 target site remained and position nine of the N1 target had reverted to a C, although a small G peak was evident suggesting a subpopulation of viruses retained this mutation. A second novel mutation in the N1 target, a C-to-U transition at position seventeen, was identified at P5. None of the mutations present from P4 onwards appeared to confer a significant increase in resistance compared to earlier passages.

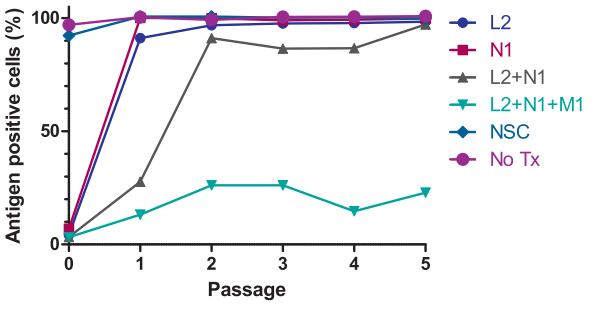

Fig. 3. Effect of target site mutations on the inhibitory effect of antiviral siRNAs against FCoV FIPV1146. The percentage of infected cells was calculated with the value of the no treatment control defined as 100%. Results represent mean of duplicate wells. No Tx, no treatment; NSC, non-silencing control.

3.3. Comparison of Dicer-substrate siRNAs and canonical siRNAs

The relative potency of the RNAi triggers investigated in this study was shown to be target dependent. For the L2 target site DsiRNA was more potent than siRNA, with IC50 values of 0.31 and 1.18 nM respectively (Fig. 4; Table 4). For target N1 the IC50 values for DsiRNA and siRNA were similar at 2.00 and 1.76 nM respectively. Dual combination

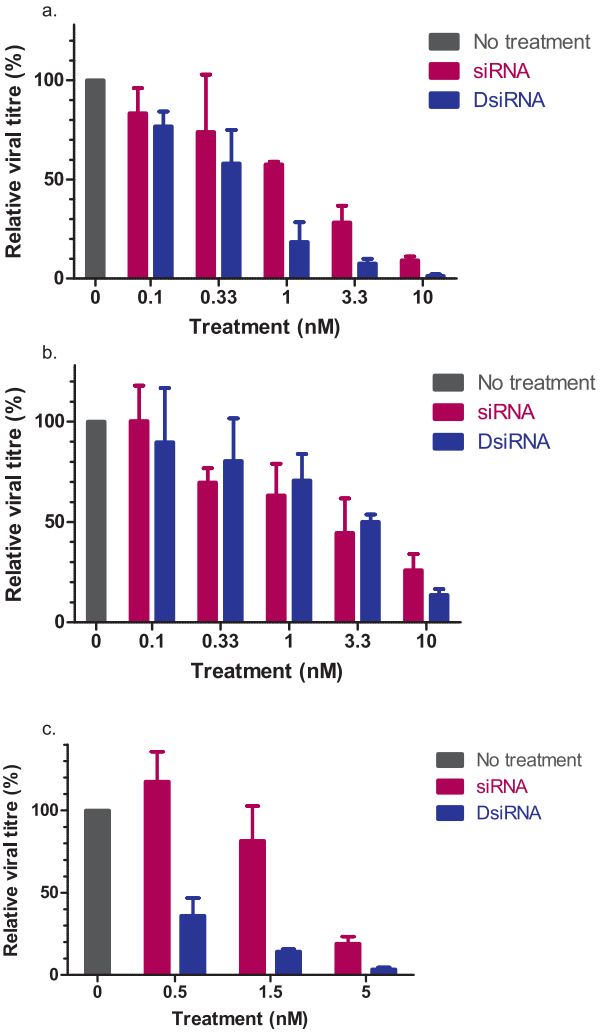

Fig. 4. Comparison of antiviral efficacy of siRNAs and DsiRNAs targeting L2 and N1. (a) siRNA / DsiRNA L2, (b) siRNA / DsiRNA N1, and (c) dual treatment siRNA / DsiRNA L2+N1. Data are expressed as mean ± SE.

Table 4
IC50 values (with 95% confidence intervals) for single and dual combination treatment with siRNAs and DsiRNAs targeting L2 and N1.

Target	siRNA IC50 (nM)	DsiRNA IC50 (nM)
L2	1.18 (0.49–2.55)	0.31 (0.22–0.43)
N1	1.76 (0.96–3.25)	2.00 (1.07–3.87)
L2+N1	2.65 (1.54–4.57)	0.28 (0.13–0.63)

DsiRNAs were considerably more potent than dual combination siRNAs, with an almost 10-fold reduction in IC50 value (0.28 nM compared to 2.65 nM).

Based on the shape of the time–response curves generated, the duration of antiviral response was similar between siRNAs and DsiRNAs designed against the same target (Fig. 5) and overall the duration of antiviral response appeared related to siRNA potency. For target L2, pre-treatment with DsiRNA resulted in almost complete inhibition of viral replication out to 40 hpi, followed by a rapid rise in extracellular viral titre, to reach a maximum titre approximately equal to that of untreated cells at 60 hpi. Pre-treatment with siRNA L2 resulted in a similar response, although in this case the degree of inhibition at 40 hpi was lower than that seen with DsiRNA pre-treatment. By comparison extracellular viral titres of untreated cells, and cells pre-treated with NSC siRNAs and DsiRNAs (data not shown), were similar, rising rapidly at 20 hpi and peaking at 40 hpi, before declining. A similar picture was seen for cells pre-treated with dual combination siRNA or DsiRNA against L2 and N1, although in this case the magnitude of the peak viral titre of treated cells was lower than in untreated cells (or NSC siRNA/DsiRNA treated cells). Molecules targeted at N1, which had previously been demonstrated to be less potent that those against L2, demonstrated a shorter duration of action, with peak viral titre occurring at 40 hpi.

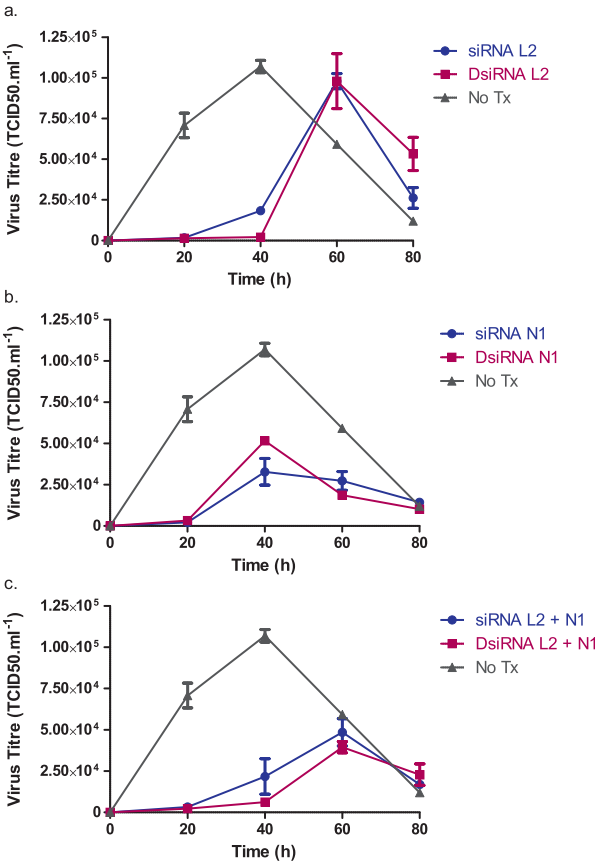

Fig. 5. Duration of antiviral activity of canonical and Dicer-substrate siRNAs. (a) siRNA / DsiRNA L2, (b) siRNA / DsiRNA N1, and (c) dual treatment siRNA / DsiRNA L2+N1. Data represent mean ± SE of duplicate experiments.

4. Discussion

The present study investigated the ability of FCoV FIPV1146 to develop resistance against siRNA mediated inhibition and strategies to minimise this. We have shown the rapid emergence of resistance following siRNA treatment during monotherapy, where a resistant population was identified following a single passage in cells treated with siRNA L2 or N1. Combination therapy with siRNAs can significantly delay the emergence of resistance.

Treatment with a combination of siRNAs may be effective in delaying or preventing the emergence of resistant viruses due to the low probability of multiple simultaneous mutations occurring, or due to the rapid and pronounced reduction in the viral replication rate (Hodge and Field, 2010). Whilst theoretically each additional antiviral added to a treatment regimen will further reduce the likelihood of resistance occurring during treatment, this must be balanced against the potential for increased toxicity or antagonistic effects. For RNAi-based therapeutics, each additional siRNA will compete for access to the RISC machinery, and thus combination therapy with multiple siRNAs may result in highly potent siRNAs being diluted by the presence of less potent molecules (Bitko et al., 2005; Castanotto et al., 2007). Similarly, competition for incorporation into RISC between siRNAs and endogenous microRNAs may increase the risk of off-target effects. The current study supports the notion that for FIPV, combination therapy with siRNAs targeting at least three independent regions is required.

An interesting extension of the combinatorial therapy approach to minimising the emergence of resistance during treatment is to use a combination containing a highly effective siRNA in addition to a number of siRNAs designed to recognise and inhibit the most likely escape sequences for that targeted region, a so-called second generation of siRNAs. In the current study, despite targeting conserved regions, selection pressure due to siRNA treatment resulted in the generation of two unique mutations for L2 and three unique mutations for N1. Further large scale screening for escape sequences would be required to determine whether the mutations identified in the current small scale study represent the most likely route of viral escape, and also whether such an approach is practical for these targets based on the overall number of escape sequences possible. Recent studies using this approach with HIV have shown that although viral escape *via* mutations targeted by the second generation of

siRNAs are inhibited, selection pressure results in the development of resistance *via* alternate routes (Schopman et al., 2010).

The structure of the double-stranded RNA molecule responsible for mediating RNAi is a 21 nucleotide duplex with two nucleotide overhangs at the 3′ end of each strand, often referred to as the "19 + 2" structure (Elbashir et al., 2001). Chemically synthesised siRNAs are traditionally designed to mimic this structure, with a central 19 nucleotide duplex complementary to the target sequence, and with the overhangs commonly deoxythymidines. In the search for improved efficacy and reduced off-target effects, a range of non-classical siRNA structural variants have been investigated, some of which appear to offer advantages over the canonical "19 + 2" structure (Chang et al., 2007; Kim et al., 2005; Rose et al., 2005; Salomon et al., 2010). One of these, a 27 nt duplex referred to as a Dicer-substrate siRNA, was shown by Kim et al. (2005) to be 10–100 times more potent than its sequence matched canonical siRNA, without inducing interferon or protein kinase R responses and having an enhanced duration of action. Further refinement of the originally blunt-ended 27-mer duplex resulted in the DsiRNA structure used in the current study: a 25 nucleotide sense strand in which the two 3′ terminal nucleotides are replaced with DNA, and a 27 nt antisense strand hybridised to give a blunt ended sense 3′ terminus and 2 nt overhang at the sense 5′ terminus (Amarzguioui et al., 2006). Asymmetry and the incorporation of DNA bases into the DsiRNA structure results in the preferential incorporation of the antisense strand into RISC, improved complex formation with proteins known to be important for RNAi, as well as minimising the activation of the innate immune response (Amarzguioui et al., 2006; Snead et al., 2013). Not all published data however supports the contention that Dicer-substrate siRNAs offer any advantage over the canonical siRNAs on which they are based. Foster et al. (2012) compared the performance of DsiRNA and siRNA against a range of targets and found no class effect, with both canonical and Dicer-substrate siRNAs comparable in terms of potency and duration of activity, both *in vitro* and *in vivo*. Dicer-substrate siRNA were also reported to be less tolerant of chemical modifications and more immunostimulatory, however in regards to antiviral therapy, the latter may actually be a desirable feature (Stewart et al., 2011).

In the current study the relative efficacy of canonical and Dicer-substrate siRNAs varied with target site. For monotherapy against target L2, and combination treatment for L2 and N1, DsiRNA were more potent, while there was little difference between siRNA and DsiRNA for monotherapy targeted at N1. Increased potency was greatest for combination therapy, with the IC50 of dual combination DsiRNA almost 10-fold lower than that for dual siRNA therapy. The duration of action appeared related to the potency of the molecule, with increased potency resulting in increased duration of activity. Previous studies investigating the duration of activity of canonical *versus* Dicer-substrate siRNAs have targeted cellular genes. In these cases the cessation of silencing is thought to be related to a reduction of intracellular siRNA due to the activity of ribonucleases or through a dilution effect caused by cell division (Takahashi et al., 2012). In the current study the apparent cessation of antiviral effect may also be due to the emergence of resistant isolates or through a simple overwhelming of the RNAi machinery, as cells are exposed to progressively higher MOI as viral replication proceeds. Both of these hypotheses fit with the experimental data showing that prolonged duration of activity correlated with increased potency.

A potential limitation of this study is the lack of testing against a diverse range of FIPV isolates. The virus used in the current study was a type II FCoV. The prevalence of type II FCoV varies worldwide, however in all reported studies infection with type I viruses is more common (Pedersen, 2009). It is unlikely that the higher prevalence of type I infections would invalidate any therapeutic application of the siRNAs tested in this study, as the primary difference between type I and II FCoVs is the spike protein, a region not targeted by any of the tested siRNAs. Furthermore, siRNAs were selected based on homology to reported strains, including both type I and type II FCoVs, and subsequent alignment of siRNA targets against the most recently published sequences confirmed a high degree of conservation among both types.

The current study and previous work by the authors suggests that siRNA or DsiRNA mediated RNAi, especially combination therapy targeting at least three regions, maybe worthy of further investigation as a therapeutic option for FIP treatment.

Acknowledgements

The authors would like to acknowledge the assistance of Dr. Adrian Smith and Steven Allen of the Centenary Institute of Cancer Medicine and Cell Biology for their assistance with fluorescence imaging.

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