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Inhibition of multiple strains of Venezuelan equine encephalitis virus by a pool of four short interfering RNAs

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Abstract

RNA interference, mediated by short interfering RNAs (siRNAs), has been shown to have activity against a wide range of viruses and is a promising new antiviral therapy. Using multiple siRNAs that target conserved areas of the genome allows for increased chances of antiviral activity against different viral strains and also helps to prevent the emergence of escape mutants. In this study, four siRNAs were designed to target areas of conserved sequence between divergent strains of Venezuelan equine encephalitis virus (VEEV). A pool of these siRNAs inhibited the replication of all six strains of VEEV tested. A single nucleotide mismatch at the extreme 3′ end of one of the siRNA sense strands did not affect antiviral activity but other mutations were not tolerated. Two strains of VEEV were tested for their abilities to overcome the inhibitory effects of RNA interference following 10 consecutive incubations in the presence of siRNAs. One strain remained susceptible throughout the course of the experiment but the other strain became resistant to the activity of siRNAs. Sequence analysis of the siRNA target sites in this strain showed that no mutations had been generated, indicating that the virus may had become resistant in some other manner. In the absence of effective antiviral drugs and vaccines to combat VEEV infection, these siRNAs offer a potential new therapeutic approach but, as with all antimicrobial agents, caution needs to be exercised with respect to the generation of resistance.

Keywords: RNA interference; Short interfering RNA (si RNA); Venezuelan equine encephalitis virus

1. Introduction

Venezuelan equine encephalitis virus (VEEV) is a small, enveloped RNA virus belonging to the Alphavirus genus. The genome is a positive-sense, single-stranded RNA of approximately 11.4 kb, capped at the 5′ end and polyadenylated at the 3′ end. The 5′ two-thirds of the genome encodes for four non-structural proteins (nsp1–nsp4) which are required for RNA replication. The subgenomic (26S) messenger RNA, equivalent to the 3′ one-third of the genome, encodes for three structural proteins (capsid, E1 and E2) and two small cleavage products (E3 and 6K) (Strauss and Strauss, 1994). On infection, the non-structural proteins are translated directly from the genomic RNA, which also acts as a template for the synthesis of the negative-sense strand. The negative-sense strand then provides the template for the synthesis of both genomic and subgenomic RNA (Strauss and Strauss, 1994). The release of progeny virus occurs approximately 6–8 h after entry of the host cell.

VEEV occurs naturally in South America and the southern United States where it is maintained in a cycle between mosquitoes and small mammals (Weaver et al., 2004). Viruses in serogroup I are capable of infecting large mammals, for example the IA/B and IC strains are associated with epizootic spread in equines. Infected equines can act as amplification hosts and replicating virus may be transmitted to humans through mosquitoes, possibly resulting in large outbreaks of febrile illness and neurological disease (Weaver et al., 2004). Viruses in other serogroups do not appear to be equine-virulent and persist in a stable enzootic cycle. Natural transmission of enzootic viruses to humans is rare but may be associated with severe disease (Johnson and Martin, 1974).

In the artificial environment of the laboratory it has been shown that strains of VEEV are readily transmissible via the airborne route and numerous laboratory workers have been accidentally infected in this way (Lennette and Koprowski, 1943; The Subcommittee on Arbovirus Laboratory Safety of the American Committee on Arthropod-borne Viruses, 1980). An

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attenuated, live vaccine (TC-83), derived by repeated passage of a virulent IA/B strain in foetal guinea pig heart cells (Berge et al., 1961), is available for the immunization of equines but it is not currently licensed for use in humans. TC-83 is poorly immunogenic in humans (Pittman et al., 1996) and can cause serious side-effects (Alevizatos et al., 1967; Casamassima et al., 1987; Rayfield et al., 1976). Additionally, there are no effective antiviral drugs presently available for use against VEEV. Work is ongoing to develop new VEEV vaccines suitable for human use (Greenway et al., 1998; Hodgson et al., 1999; Kinney et al., 1988; Phillpotts et al., 2005; Pratt et al., 2003) but in their absence the availability of effective antiviral therapies is extremely desirable.

Short interfering RNAs (siRNAs) are small, double-stranded RNA molecules that direct the degradation of complementary messenger RNAs via a cellular process known as RNA interference. siRNAs are incorporated into a protein complex, known as the RNA-induced silencing complex (RISC), and the antisense strand of the siRNA guides RISC to the target RNA which is then cleaved. RNA interference is an evolutionary conserved mechanism within eukaryotes and its functions include endogenous gene regulation, viral defence and the maintenance of genomic stability (Fountaine et al., 2005). It has yet to be proven that mammals use RNA interference as a viral defence mechanism but in the laboratory, the introduction of siRNAs into mammalian cells can be used to inhibit the expression of viral genes. RNA interference has been shown to be active against many human pathogenic viruses (Geisbert et al., 2006; Reuter et al., 2006; Tan and Yin, 2004), including the alphaviruses Semliki Forest virus (Caplen et al., 2002) and O'nyong-nyong virus (Keene et al., 2004). The efficient and sequence-specific nature of RNA interference has raised the possibility of using siRNAs as an antiviral therapy. Here, the ability of a pool of four siRNAs to specifically inhibit the replication of six human pathogenic strains of VEEV is described.

2. Materials and methods

2.1. Cells and viruses

BHK 21 and Vero cell lines (European Collection of Animal Cell Cultures, UK) were propagated by standard methods using the recommended culture media. For transfection procedures (see Section 2.4.1), cells were maintained in Glasgow Minimum Essential Medium supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 10 mM Hepes and 10% (v/v) tryptose phosphate broth (GMM). For virus infections (see Section 2.4.2) and viral titrations (see Section 2.4.3), cells were maintained in Leibovitz L-15 media supplemented with 2% (v/v) foetal calf serum, 2 mM L-glutamine and 10 mM Hepes (L15MM). Media and supplements were all supplied by Sigma (UK).

Strains of VEEV from serogroups IA/B (Trinidad donkey; TrD), IC (P676), ID (3880), IE (Mena II), II (Fe37c) and III (BeAn8) were kindly supplied by Dr. B. Shope (Yale Arbovirus Research Unit, University of Texas, Austin TX). Virulent virus stocks were prepared and the titer determined as described by Phillpotts et al. (2005). All work with virulent VEEV was carried out under UK Advisory Committee on Dangerous Pathogens Level 3 containment.

Strain A7(74) of Semliki Forest Virus (SFV) was kindly supplied by Dr. P. Bernard (Viragen Scotland Ltd., Edinburgh, UK). Virus was grown and the titer determined as described above.

2.2. Design and synthesis of siRNAs

The genomes of VEEV strains TrD, P676, 3880, Mena II, Fe37c and BeAn8 (GenBank accession nos. J04332, L04653, L00930, AF075252, AF075251 and AF075253, respectively) were aligned using Clone Management Suite software (SciEd Software, USA) and areas of conserved sequence identified. Using SMARTpool® technology, Dharmacon (Lafayette, CO) designed and synthesised a pool of four double-stranded siRNAs from the conserved sequences (Cat. No. M-040016-00). The individual siRNAs contained within this VEEV siRNA pool are shown in Table 1. Analysis confirmed that the selected siRNA sequences were only present once in the genomes of the VEEV strains and that they were absent from the SFV, human and mouse genomes. The VEEV siRNA pool and the four individual siRNAs contained within the pool were supplied by Dharmacon. All siRNAs were reconstituted according to the Manufacturer's instructions before use.

The siRNA designed to the housekeeping gene Lamin A/C (Qiagen, Crawley, UK) and a pool of non-targeting siRNAs (Dharmacon, Cat. No. D-001206-13) were used in the appropriate experiments to ensure the specificity of the observed effects. These negative control siRNAs lack any sequence homology to the VEEV genome.

2.3. Sequencing of siRNA target sites in strains of VEEV

RT-PCR was performed on extracted VEEV genomic RNA (RNeasy Mini Kit, Qiagen, UK) using primers that flanked each of the four siRNA target sites and Ready-To-Go™ RT-PCR Beads (Amersham Biosciences, Buckinghamshire, UK). The amplified products were resolved by agarose gel electrophoresis and visualised by ethidium bromide staining. The sequences of the amplified products were determined by sequencing the excised bands (High Pure PCR Product Purification Kit, Roche, Buckinghamshire, UK).

2.4. Assessing the antiviral activity of siRNAs

Briefly, BHK 21 cells were transfected with siRNAs designed to VEEV or with the appropriate negative control siRNAs. The cells were infected with the VEEV strains 24 h after transfection. The amount of virus in the culture supernatants was ascertained by titration in Vero cells 24 h post-infection (with the exception of one experiment to determine if the VEEV siRNA pool maintained viral inhibition over a longer period of time, where the culture supernatants were titrated 48 h post-infection).

2.4.1. Transfection of BHK 21 cells with siRNAs

Initial experiments had determined the optimal conditions for transfecting siRNAs into BHK 21 cells. Briefly, 9×10^4 cells

Table 1
Sequences and locations of siRNAs in the VEEV siRNA pool

siRNA[a]	Target gene	Nucleotide sequence (sense-strand)	Nucleotide location (bp)[b] in virus strain					
			TrD	P676	3880	Mena II	Fe37c	BeAn8
1	nsp4	5′GGACCGAAUUGGAGAUUUC3′	5,788–5,806	5,788–5,806	5,764–5,782	5,808–5,823	5,742–5,760	5,672–5,690
2	nsp1	5′GGAGAAAGUUCACGUUGAC3′	47–65	47–65	47–65	46–64	46–64	45–63
3	nsp1	5′CAAAGUGACAGACACAUUG3′	989–1,007	989–1,007	989–1,007	988–1,006	988–1,006	987–1,005
4	E1	5′GAGUUCAGGCUCCAAAUAU3′	11,087–11,105	11,090–11,108	11,066–11,084	11,104–11,122	11,041–11,059	10,971–10,989

[a] All siRNA sequences are conserved in VEEV strains TrD, P676, 3880 and Fe37c. Only the sequences of siRNAs 2 and 4 are conserved in strain Mena II and none of the siRNA sequences are conserved in strain BeAn8.
[b] According to the appropriate genome sequence in the GenBank database, accession nos. as described in Section 2.2.

in 900 μl GMM were plated into each well of a 24-well plate (Corning, Acton, MA). The cells were transfected with siRNAs 24 h later using the transfection reagent Lipofectamine™ 2000 (Invitrogen, UK), according to the Manufacturer's guidelines.

2.4.2. Infection of BHK 21 cells with VEEV and SFV

BHK 21 cells were infected at 24 h post-transfection using a multiplicity of infection (m.o.i.) of 0.01. Media was removed from each 24-well and 100 μl of virus diluted in L15MM applied to the monolayer. After an incubation of 30 min at room temperature, 900 μl of L15MM was carefully added. Infected cells were incubated for 24 h (or in one experiment 48 h, see above) after which time the supernatant was carefully removed and stored at −80 °C.

2.4.3. Titration of virus in culture supernatants

The titer of virus in supernatants was determined by plaque formation under a 1.5% (w/v) carboxymethyl cellulose overlay in Vero cells. Briefly, cells were seeded into 24-well plates (1×10^5 cells well^{-1}) and incubated overnight. Media was removed and the cells overlaid with 100 μl of virus diluted in L15MM. After an incubation of 30 min at room-temperature, 1 ml of double-strength L15MM (Invitrogen) diluted in 3% (w/v) carboxymethyl cellulose (Sigma) was carefully added to each well. Cells were incubated for 72 h, fixed with 10% (v/v) formal saline for 4 h and then stained with 0.1% (w/v) crystal violet (Sigma). The plaques were counted and the amount of virus was calculated.

2.5. Statistical methods

Students *t*-tests and one-way ANOVA were performed using Minitab™ statistical software (Minitab™ Inc., www.minitab.com).

3. Results

3.1. The VEEV siRNA pool

A pool of siRNAs was designed to target areas of conserved sequence in the genomes of six strains of VEEV. The sequences and target genes of the four individual siRNAs that are contained within this pool are shown in Table 1. It was found impossible to design siRNAs that were conserved in all six VEEV genomes. Strains TrD (serogroup IA/B), P676 (serogroup IC), 3880 (serogroup ID) and Fe37c (serogroup II) are genetically similar and all four siRNAs are conserved in these genomes. Strains Mena II (serogroup IE) and BeAn8 (serogroup III) are the most genetically diverse from strain TrD, consequently only siRNAs 2 and 4 are conserved in the Mena II genome and none of the siRNAs are conserved in the BeAn8 genome. The sequences (sense-strand) of the siRNA target sites in the Mena II genome are: siRNA 1 5′GGACCGAAUU-**A**GAGAU**C**UC3′, siRNA 3 5′CAA**G**GUGAC**G**GACA-C**G**C**U**U3′. The sequences of the siRNA target sites in the BeAn8 genome are: siRNA 1 5′GGAC**G**GA**G**UUGGAGA**A**U-UC3′, siRNA 2 5′GGAGAAAGUUCACGUUGA**U**3′, siRNA 3

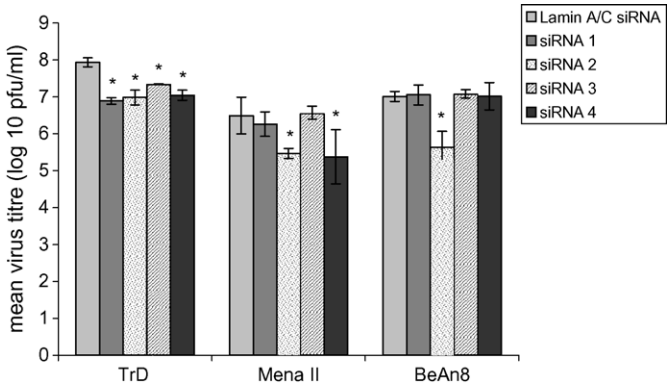

Fig. 1. Inhibition of VEEV replication by individual siRNAs. Titers of TrD, Mena II and BeAn8 strains grown in BHK 21 cells transfected with 100 nM of each siRNA (see text for details). $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.01$.

5′CAA**G**GU**A**ACAGA**U**AC**G**CU**C**3′, siRNA 4 5′GAGU-U**U**A**AA**U**U**G**C**A**G**AU**C**U3′. Differences from the conserved sequence are shown in bold.

3.2. Sequences of siRNA target sites in strains of VEEV

RNA viruses undergo high rates of mutation due to error-prone replication by RNA polymerase. It is therefore possible that the genome sequences of VEEV strains used in this work differ from the published genome sequences. For this reason, the siRNA target sites in the VEEV genomes were sequenced. It was found that all the target site sequences matched the published genome sequences (results not shown).

3.3. Inhibition of VEEV by individual siRNAs

As none of the siRNA sequences are conserved in the BeAn8 genome, the siRNAs in the VEEV siRNA pool would not be expected to have activity against VEEV strain BeAn8 unless mismatches between the siRNA and the target RNA could be tolerated. Similarly, siRNAs 1 and 3 would not be expected to have activity against VEEV strain Mena II, as their sequences are not conserved in the Mena II genome. To confirm the expected activity of the individual siRNAs that make up the VEEV siRNA pool (Table 1), they were tested against VEEV strains TrD, Mena II and BeAn8. In order to ensure that any observed antiviral activity was due to RNA interference rather than the induction of interferon (IFN), BHK 21 cells were used in all experiments. These cells have defects in IFN production (Fournier et al., 2003; Liu et al., 2006).

BHK 21 cells were transfected with 100 nM of each siRNA contained in the VEEV siRNA pool or with 100 nM of a control siRNA designed to Lamin A/C (Bitko and Barik, 2001; Déctor et al., 2002). After 24 h, the transfected cells were infected with the VEEV strains and the amount of virus in the culture supernatants 24 h post-infection was ascertained by titration (Fig. 1). Viral titers were not affected by the Lamin A/C siRNA and viruses replicated to the same extent as in non-transfected cells (results not shown). However, all of the individual siRNAs exerted a significant ($P \leq 0.01$) antiviral effect against VEEV strain TrD. Compared to viral replication in the control cells, siRNAs 1–4 inhibited the replication of TrD by 91%, 89%, 75% and 87% respectively. In contrast, only siRNAs 2 and 4 significantly ($P \leq 0.01$) inhibited the replication of VEEV strain Mena II (by 91% and 92%, respectively, compared to the control) and only siRNA 2 was able to significantly ($P \leq 0.01$) inhibit the replication of VEEV strain BeAn8 (by 96%, compared to the control). The sequences of siRNAs 2 and 4 are conserved in the Mena II genome but siRNA 2 differs from the BeAn8 genome sequence by a single nucleotide at the extreme 3′ end (Section 3.1). The activity of siRNA 2 against this strain indicates that a mutation at this position can be tolerated. However, the position or number of mutations in the remaining siRNAs cannot be tolerated, resulting in a lack of antiviral activity against strains Mena II and BeAn8.

3.4. Varying antiviral activity of individual siRNAs at reduced concentrations

In order to establish if there were any differences between siRNAs 1–4 in their antiviral activity, BHK 21 cells were transfected with 10 or 1 nM of each siRNA. The transfected cells were subsequently infected with VEEV strain TrD and the amount of virus in the culture supernatants 24 h post-infection was determined by titration (Fig. 2). At a concentration of 10 nM, all of the individual siRNAs significantly ($P \leq 0.01$) inhibited the replication of strain TrD (by 83–97%) compared to viral replication in cells transfected with Lamin A/C siRNA. However, at a concentration of 1 nM, only siRNA 2 exerted significant ($P \leq 0.05$) antiviral activity (88% decrease in titer, compared to the control), indicating that this siRNA is the most effective against VEEV.

3.5. The VEEV siRNA pool specifically inhibits multiple strains of VEEV

Experiments with VEEV strain TrD had determined that the VEEV siRNA pool retained its antiviral activity when diluted from 100 to 10 nM but that activity decreased between 5 and 0.1 nM (Fig. 3). To test the activity of the VEEV siRNA pool against multiple strains of VEEV, BHK 21 cells were trans-

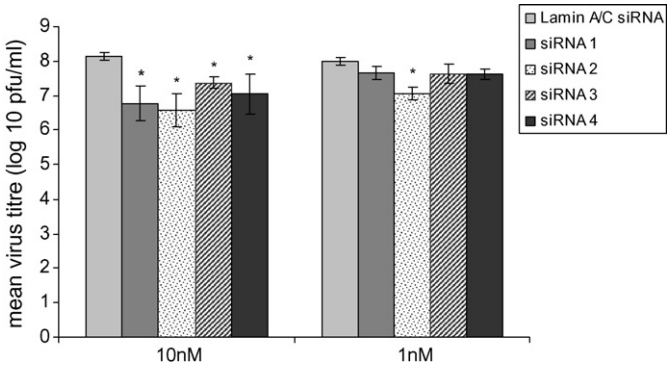

Fig. 2. Antiviral activity of individual siRNAs at reduced concentrations. Titers of strain TrD grown in BHK 21 cells transfected with 10 or 1 nM of each siRNA. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

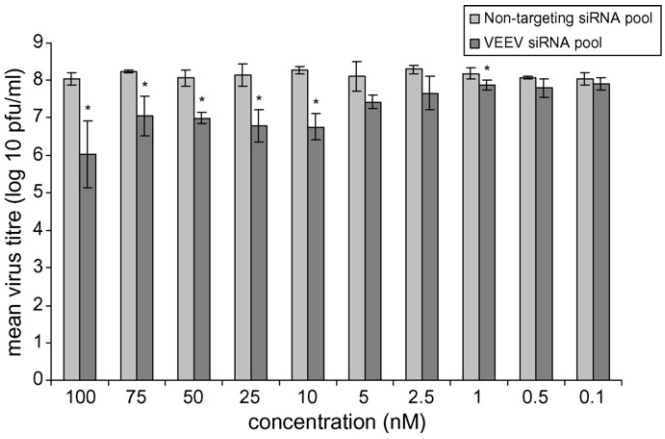

Fig. 3. Effect of dilution of the VEEV siRNA pool on antiviral activity. Titers of strain TrD grown in BHK 21 cells transfected with decreasing concentrations of a non-targeting siRNA pool or VEEV siRNA pool. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

fected with 10 nM of the siRNA pool or with 10 nM of a pool of non-targeting siRNAs. Transfected cells were then infected with six strains of VEEV and one strain of Semliki Forest virus (SFV). None of the siRNA sequences are conserved in the SFV genome. The amount of virus in the culture supernatants 24 h post-infection was determined by titration (Fig. 4). Viruses replicated to the same extent in cells transfected with the pool of non-targeting siRNAs as in non-transfected cells (results not shown). The antiviral activity of the VEEV siRNA pool was statistically significant ($P \leq 0.05$) against all six VEEV strains but not against SFV, demonstrating that the activity of the siRNA pool was specific for VEEV. The VEEV siRNA pool inhibited the replication of VEEV by 96–97%, compared to viral replication in cells transfected with the pool of non-targeting siRNAs.

3.6. The activity of the VEEV siRNA pool against strain BeAn8 is due solely to siRNA 2

Although results obtained with the individual siRNAs and strain BeAn8 (Fig. 1) indicated that only siRNA 2 possessed

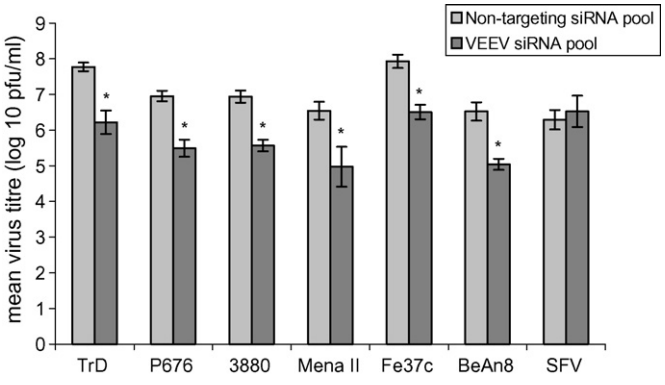

Fig. 4. Specific inhibition of viral replication by a pool of four siRNAs designed against VEEV. Titers of six strains of VEEV and one strain of SFV grown in BHK 21 cells transfected with 10 nM of a non-targeting siRNA pool or VEEV siRNA pool. $n = 6$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

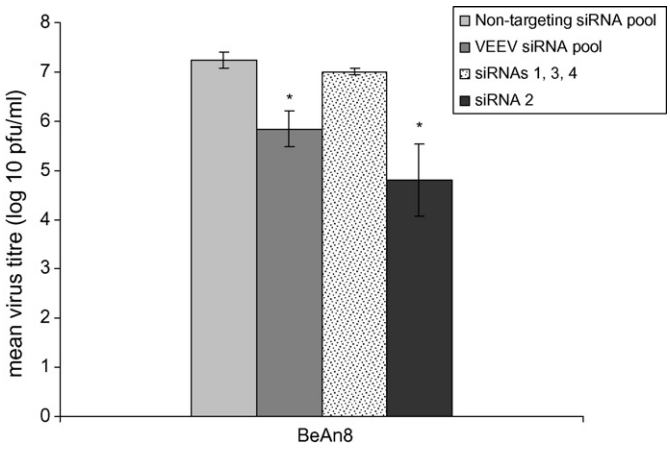

Fig. 5. siRNA 2 is responsible for the inhibition of strain BeAn8 by the VEEV siRNA pool. Titers of strain BeAn8 grown in BHK 21 cells transfected with 10 nM of a non-targeting siRNA pool, VEEV siRNA pool, a combination of siRNAs 1, 3 and 4 or siRNA 2. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

antiviral activity against this strain of VEEV, it was not known if seemingly inactive siRNAs could gain activity when combined in the VEEV siRNA pool. To test this, BHK 21 cells were transfected with a combination of siRNAs 1, 3 and 4 or with siRNA 2 alone (final concentration 10 nM) and infected 24 h later with VEEV strain BeAn8. The culture supernatants were harvested 24 h post-infection and titrated. For comparison, strain BeAn8 was also incubated in the presence of 10 nM of the pool of non-targeting siRNAs or the VEEV siRNA pool (Fig. 5).

siRNA 2 alone and the VEEV siRNA pool exerted significant antiviral activity ($P \leq 0.05$, 99.6% and 96% decrease in titer respectively, compared to the titer in cells transfected with the non-targeting siRNA pool) but the combination of siRNAs 1, 3 and 4 did not inhibit the replication of strain BeAn8. This demonstrates that siRNA 2 is solely responsible for the antiviral activity exhibited by the VEEV siRNA pool against strain BeAn8. On average, the inhibition of strain BeAn8 was greater with siRNA 2 alone than with the VEEV siRNA pool, most likely reflecting the greater concentration of siRNA 2 in cells transfected with siRNA 2 alone than with the VEEV siRNA pool.

3.7. The VEEV siRNA pool is not able to induce long-term viral inhibition

The amount of virus present in culture supernatants was generally determined 24 h post-infection. However, in this experiment the culture supernatants were harvested at 24 and 48 h post-infection to test the ability of the VEEV siRNA pool to inhibit viral replication over longer time periods (non-transfected cell monolayers are completely lysed after 48 h incubation with VEEV). Although significant ($P \leq 0.05$) viral inhibition was observed at 24 h post-infection (96% decrease in titer), this inhibition was not sustained over a 48 h period (Fig. 6). As RNA viruses have high mutation rates, it was possible that VEEV had mutated to overcome the effects of RNA interference. To test this, three samples of virus incubated in

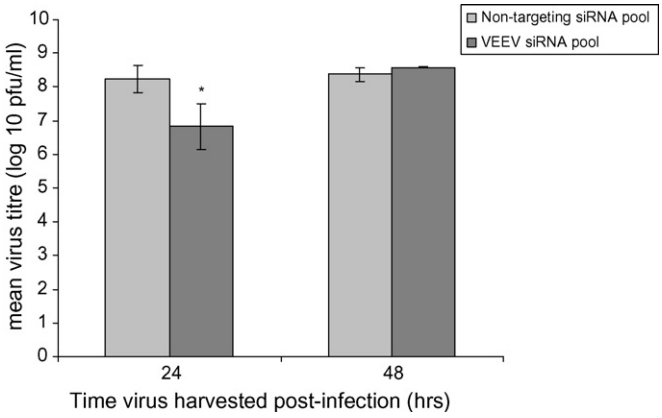

Fig. 6. The VEEV siRNA pool is unable to maintain viral inhibition over an extended period. Titers of strain TrD grown in BHK 21 cells transfected with 10 nM of a non-targeting siRNA pool or VEEV siRNA pool. Culture supernatants were harvested 24 or 48 h post-infection. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

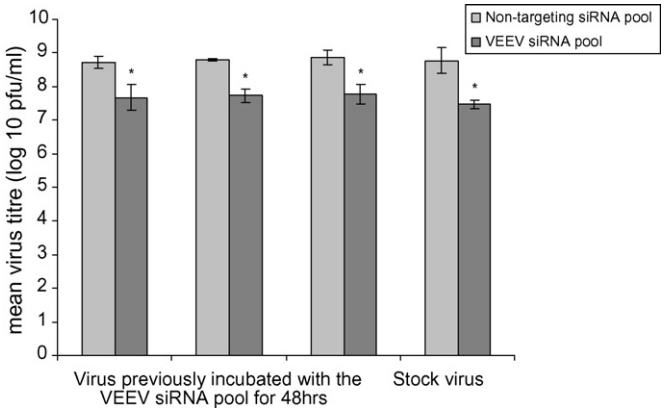

Fig. 7. Virus previously exposed to the VEEV siRNA pool for 48 h remains susceptible to the effects of RNA interference. Titers of strain TrD grown in BHK 21 cells transfected with 10 nM of a non-targeting siRNA pool or VEEV siRNA pool. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

the presence of the siRNA pool for 48 h were re-exposed to the VEEV siRNA pool (Fig. 7). Re-exposed virus was still sensitive to the effects of the VEEV siRNA pool over a 24 h period (91%, 92% and 92% decrease in titer, $P \leq 0.05$) and the level of inhibition was indistinguishable from that observed with stock VEEV. This result indicates that VEEV had not become resistant to RNA interference. Instead, the siRNA pool causes a lag in viral replication, apparent at 24 h as a reduced titer, but at 48 h viral titers have caught up to levels in cells transfected with non-targeting siRNAs.

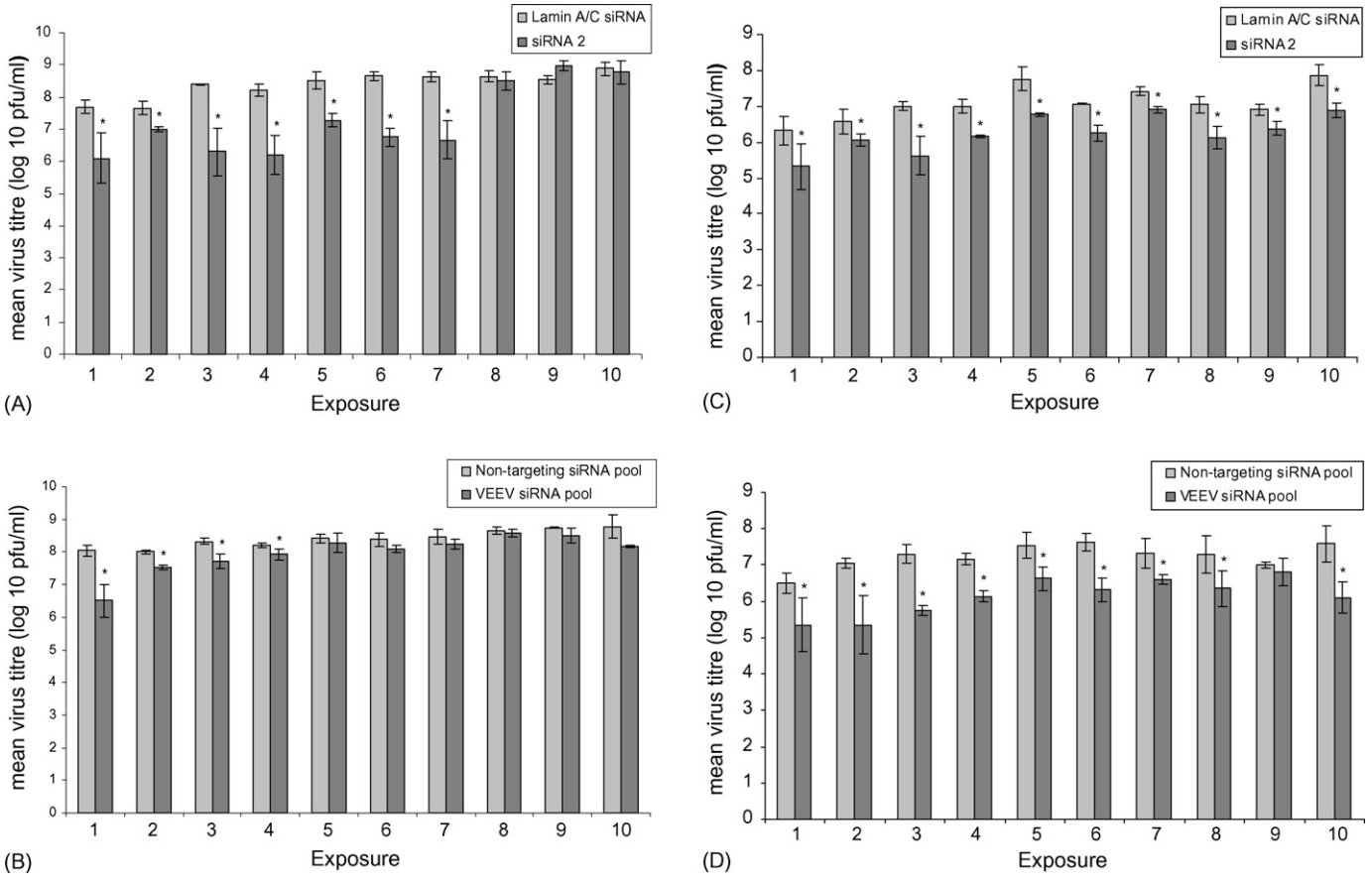

Fig. 8. Resistance to RNA interference during continuous incubation with siRNAs is observed with VEEV strain TrD but not strain BeAn8. Titers of strains TrD (A and B) and BeAn8 (C and D) in the course of 10 consecutive incubations with siRNAs. Virus was grown in BHK 21 cells transfected with 2.5 nM Lamin A/C siRNA or siRNA 2 or with 10 nM of a non-targeting siRNA pool or VEEV siRNA pool. Virus was harvested, titrated and then used to infect cells transfected with the appropriate siRNA. This process was repeated until the virus had undergone 10 exposures to siRNAs. $n = 3$ for all data points, 95% confidence intervals are shown, $^{*}P \leq 0.05$.

3.8. Long-term exposure to siRNAs can result in resistance to RNA interference

It can be envisaged that the use of siRNAs as an antiviral treatment for VEEV infection would involve a multiple-dosing regime, resulting in the virus being exposed to siRNAs over an extended time period. As RNA viruses have high mutation rates, there is a strong probability of VEEV escaping the inhibitory effects of RNA interference by introducing mutations in the siRNA target sites. To determine the ability of VEEV to overcome RNA interference, strains TrD and BeAn8 were subjected to 10 consecutive incubations in the presence of siRNA 2, Lamin A/C siRNA, the pool of non-targeting siRNAs or the VEEV siRNA pool (Fig. 8). Individual siRNAs were used to test whether resistant mutations arose more readily in the presence of single siRNAs compared to pools of siRNAs. However, in the case of VEEV strain TrD, resistance to the siRNA pool was observed after four exposures, whilst resistance to siRNA 2 alone was observed after seven exposures (Fig. 8A and B). In contrast, VEEV strain BeAn8 remained susceptible to either the individual siRNA or the pool of siRNAs throughout the experiment (Fig. 8C and D).

RNA was extracted from virus that had been exposed to 10 consecutive incubations in the presence of siRNA 2 or the VEEV siRNA pool. The sequences of the siRNA target sites were analysed and found to be identical to the conserved sequences (results not shown).

4. Discussion

This is the first study to show the effectiveness of RNA interference against the alphavirus VEEV and it is the first demonstration of siRNAs designed to areas of conserved sequence specifically inhibiting the replication of six strains of virus. As the only siRNA to inhibit strain BeAn8 replication, siRNA 2 is responsible for the activity of the VEEV siRNA pool against this virus. Additional siRNAs could be designed against strain BeAn8 (and Mena II) for inclusion in the siRNA pool. All the individual siRNAs found within the pool inhibit the replication of VEEV strain TrD, although siRNA 3 was on average the least effective. Huesken et al. (2005) have recently described preferences for nucleotides at certain positions in the anti-sense strand of active siRNAs. siRNAs 1 and 2 have four of the preferred bases (at positions 7, 10, 17 and 19 and positions 2, 10, 17 and 19, respectively) and siRNA 4 has three of the preferred nucleotides (at positions 1, 2 and 19). However, in siRNA3, only one of the base preferences is present (at position 17), perhaps explaining it's lack of effectiveness compared to the other siRNAs. Combining the four siRNAs did not cause a large increase in antiviral activity against strain TrD and this is in accordance with the results of others (Schubert et al., 2005; Yuan et al., 2005). It can be envisaged that using multiple siRNAs may lead to a saturation of RISC, thereby reducing the incorporation of individual siRNAs into the complex and decreasing their activity. However, this does not appear to have taken place with the VEEV siRNA pool.

The single base pair mismatch at the 3′ end of siRNA 2 did not affect antiviral activity against VEEV strain BeAn8 but other mismatches did abolish activity against strains Mena II and BeAn8. It has been widely observed that one mismatch between the siRNA and its target can be tolerated but in some cases several mismatches can be tolerated (for a review see Dykxhoorn and Lieberman, 2005; Snøve and Holen, 2004). Du et al. (2005) recently reported that mutations at the two terminal nucleotides at either the 5′ or 3′ end of the target site were well tolerated and that uracil was the best tolerated mismatch base for siRNA anti-sense strand positions with guanine. These results (Du et al., 2005) are in agreement with the finding here that siRNA 2 has antiviral activity against strain BeAn8, despite the base pair mismatch at position 1 of the anti-sense strand of siRNA 2 (a G:U pairing).

It is possible for double stranded RNA to stimulate innate antiviral defence mechanisms which lead to the production of inflammatory cytokines and type I interferons and the non-specific inhibition of gene expression. Receptors involved in RNA recognition include members of the transmembrane Toll-like receptor family (TLR) and cytosolic RNA-binding proteins such as PKR and the helicases RIG-1 and Mda5 (for a review, see Schlee et al., 2006). However, the evidence to date indicates that short synthetic siRNA duplexes can only be detected by TLR7 (located in the endosomal membrane of immune cells such as plasmacytoid dendritic cells) and recognition requires the presence of specific motifs (Hornung et al., 2005; Judge et al., 2005). As the BHK cells used in these experiments are of fibroblast origin and have defects in IFN production (Fournier et al., 2003; Liu et al., 2006) and none of the siRNAs contain the immunostimulatory motifs described by Hornung et al. (2005) and Judge et al. (2005), it seems unlikely that non-specific inhibition of gene expression was taking place. In addition, the replication of SFV was not affected in cells transfected with the VEEV siRNA pool confirming that the siRNAs were specifically inhibiting VEEV replication.

Long-term silencing of viral protein expression by siRNAs has been reported to result in the emergence of viruses resistant to RNA interference (Das et al., 2004; Gitlin et al., 2005), an issue that will need to be addressed if siRNAs are to be used therapeutically. Resistance was caused by the generation of mutations in the siRNA target site. This problem is particularly relevant for RNA viruses that exhibit increased genetic variation due to error-prone replication. To ensure siRNAs remained effective, viral RNA sequences that are conserved, and normally invariant between different strains, have been targeted (Chang et al., 2005; Dave and Pomerantz, 2004) or several viral sequences have been simultaneously targeted (Chang et al., 2005; Geisbert et al., 2006; Gitlin et al., 2005). The work described here utilizes both these approaches and it was successful against VEEV strain BeAn8 but resistance to RNA interference was observed with strain TrD. The mechanism of siRNA resistance in strain TrD is uncertain. Mutations did not occur in the siRNA target sites, indicating that escape mutants do not readily arise when these particular conserved areas of sequence are targeted. Silent mutations, which alter the nucleotide sequence but do not affect the encoded amino acid, have the potential to prevent recog-

nition of the target RNA by the siRNA but even these did not occur. A number of mammalian viruses have been shown to express proteins that function as suppressors of RNA interference (for reviews see Kok and Jin, 2006; Zheng et al., 2005). These viral proteins bind double stranded RNA and are able to protect messenger RNA from degradation by sequestering siRNAs and preventing the formation of an active RISC. The capsid protein of VEEV contains regions that bind specifically to RNA (Strauss and Strauss, 1994). Mutations may have occurred in this protein that led to an increased ability to sequester siRNA and therefore an increased resistance to RNA interference. It has been reported that an additional mechanism for suppressing RNA interference is the production of RNAs that subvert host cell functions, including the inhibition of the RNA interference pathway (for a review see Sarnow et al., 2006). However, this type of RNA has so far only been found in viruses with DNA genomes making this an unlikely explanation for siRNA resistance in VEEV.

A number of methods for delivering siRNAs in vivo have been tested (Bitko et al., 2005; Ge et al., 2004; Geisbert et al., 2006; Giladi et al., 2003; Li et al., 2005; Sioud and Sørensen, 2003; Soutschek et al., 2004; Zimmermann et al., 2006). Liposome formulations are generally used (Bitko et al., 2005; Ge et al., 2004; Geisbert et al., 2006; Sioud and Sørensen, 2003; Zimmermann et al., 2006) but a simple intranasal administration of naked siRNAs has been shown to be effective against respiratory viruses (Bitko et al., 2005). The induction of immune responses by siRNAs is of concern for their future clinical use and the delivery method may have a role to play in this (Geisbert et al., 2006; Hornung et al., 2005; Judge et al., 2005; Sioud, 2005). Targeting siRNAs to specific cells would be therapeutically advantageous. Antibodies (Song et al., 2005; Zhang et al., 2004), peptide ligands (Schiffelers et al., 2004) and aptamers (McNamara et al., 2006) have been successfully used to target siRNAs to cancer cells in vivo.

To date, RNA interference has been shown to be effective in mouse models of hepatitis B virus (Giladi et al., 2003; Klein et al., 2003; McCaffrey et al., 2003), herpes simplex virus (Palliser et al., 2006), influenza virus (Ge et al., 2004; Tompkins et al., 2004), parainfluenza virus (Bitko et al., 2005) and Respiratory Syncytial virus (Bitko et al., 2005), against Ebola virus in a guinea pig model (Geisbert et al., 2006) and against SARS coronavirus in a Rhesus macaque model (Li et al., 2005). The high level of viral inhibition induced by the VEEV siRNA pool suggests that the siRNAs may be active in vivo and this is currently being examined. Although the level of inhibition induced by the siRNA pool was substantial, the transient nature of the inhibition suggests that a single administration of siRNAs could not be used to cure VEEV infection. Using multiple doses of siRNAs should be avoided as it risks inducing viral resistance. However, the temporary inhibition of viral replication may allow the infected host enough time to develop an immune response capable of overcoming VEEV. Alternatively, siRNAs could be used therapeutically in combination with other antiviral compounds to successfully treat the infection. With a disease such as VEEV, it may be necessary to deliver siRNAs to the brains of infected individuals. Direct injection of siRNAs into the brain has been effectively used to target a wide range of genes (Fountaine et al., 2005) but ideally siRNAs would be administered systemically and then be able to cross the blood–brain barrier. It has been shown that intravenous injections of pegylated immunoliposomes connected to targeting monoclonal antibodies are able to deliver siRNAs across the blood-brain barrier to successfully treat intracranial brain cancer (Zhang et al., 2004). Alternatively, it may be possible to link siRNAs to appropriate lipid molecules for delivery across the blood–brain barrier. This approach was shown to be effective at delivering cholesterol-conjugated siRNAs to the liver and jejunum (Soutschek et al., 2004).

The pool of four siRNAs described here offers a new approach for future antiviral therapies against VEEV. However, avoiding the generation of resistance and developing an effective delivery method will be crucial if siRNAs are to be used therapeutically.

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