Published in final edited form as:

Antivir Ther. 2010 ; 15(5): 727–736. doi:10.3851/IMP1593.

Control of African swine fever virus replication by small interfering RNA targeting the A151R and VP72 genes

Djénéba Keita¹, Livio Heath², and Emmanuel Albina^{1,*}

¹CIRAD, UMR CMAEE, Montpellier, France

²ARC-Onderstepoort Veterinary Institute, Transboundary Animal Diseases Programme, Onderstepoort, Pretoria, Republic of South Africa

Abstract

Background—African swine fever virus (ASFV) is the unique member of the *Asfarviridae* family and *Asfivirus* genus. It is an enveloped double-stranded DNA arbovirus that replicates in the cell cytoplasm, similarly to poxviruses. There is no vaccine and no treatment available to control this virus.

Methods—We describe the use of small interfering RNA (siRNA) targeting the A151R and VP72 (B646L) genes to control the ASFV replication in vitro.

Results—Results suggest that siRNA targeting the A151R and VP72 genes can reduce both the virus replication and its levels of messenger RNA transcripts. The reduction was up to 4 log₁₀ copies on the virus titre and up to 3 log₁₀ copies on virus RNA transcripts levels. The combination of multiple siRNA did not improve the antiviral effect significantly, compared to use of individual siRNAs.

Conclusions—The function of A151R gene product in the virus replication cycle is yet unclear, but is essential. We also demonstrate that it is possible to inhibit, using small interfering RNA, a virus that replicates exclusively in the cell cytoplasm in specific viral factories.

Introduction

African swine fever virus (ASFV) is a contagious and lethal viral disease of domestic pigs and European wild boars caused by a virus of the *Asfivirus* genus, the unique member of the *Asfarviridae* family [1, 2]. This virus is also an arbovirus, as soft ticks are biological vectors that can be infected and transmit the disease through biting. ASF pathogenesis varies from a generalized hemorrhagic fever to asymptomatic infections, depending on viral and host factors [3]. The mortality in severe cases in domestic pigs and European wild boars can be as high as 100%. There is currently no vaccine and no treatment available against this disease. The disease is endemic in the African continent and Sardinia. Recently, ASFV was introduced in Caucasus [4].

ASFV is a large double-stranded DNA virus encoding > 150 proteins. It replicates in the cell cytoplasm [5], similar to poxviruses. The virus is known to modulate the immune response in the infected host by expressing several proteins that act as virulence factors. It has been previously shown that ASFV can be attenuated by deleting genes involved in virulence [6-9]. In a strategy to produce an attenuated ASFV vaccine by deletion, our group identified

*Corresponding author emmanuel.albina@cirad.fr.

Disclosure statement

The authors declare no competing interests.

a region within the left variable part of the genome that includes virulence genes, such as A224L expressing an anti-apoptotic protein, and A238L, expressing a nuclear factor- κ B antagonist [10-13]. The A151R product function is unclear. To investigate whether this A151R gene is essential for virus replication, its expression was repressed using an RNA interference approach. Another gene (B646L) encoding the virus capsid protein VP72 was included in the study as a control of small interfering RNA (siRNA) repression, as it is likely that this gene is essential in the virus replication cycle.

RNA interference was first described in nematodes [14], but was later confirmed to be present in most eukaryotic cells [15-18]. It acts in the cell cytoplasm through siRNAs of 21–23 nucleotides that specifically bind to a messenger RNA (mRNA), triggering cleavage of the mRNA by a RNA-induced silencing complex (RISC) and resulting in the post-transcriptional down-regulation of the targeted gene [18-20]. In this study, we show that A151R and VP72 (B646L) mRNA are repressed by the introduction of complementary siRNAs, and that the down-regulation negatively affects viral replication.

Methods

The Spanish Vero-adapted strain of ASFV (Badajoz 1971, BA71V, [21]) was kindly provided by the Centro de Biología Molecular (Madrid, Spain). A virus stock was prepared in Vero cells maintained at 37°C and 5% CO₂ in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal bovine serum (FBS; Eurobio, Courtaboeuf, France) and 2 mM L-glutamin (Gibco, Life Technology, Paisley, UK). The virus stock was titrated as previously described [22] and expressed in medium tissue culture infective dose (TCID₅₀)/ml. The infections of Vero cells for the RNA interference studies were done at multiplicities of infection (MOI) of 0.1 or 0.01 TCID₅₀, respectively.

The nucleotide sequences of A151R and VP72 genes of BA71V strain (GenBank/EMBL accession number **ASU18466** [21]) were first verified by direct sequencing of the virus stock. Specific primers corresponding to the ends of each gene were used to amplify the genes by PCR (Table 1). The amplified products were purified and sent to GATC (Konstanz, Germany) for direct sequencing. Eleven double-stranded 21-nucleotide RNAs were designed by Cenix algorithm (Ambion/Applied Biosystems, Cergy-Pontoise, France) based on the verified sequences. Three RNAs, siRNA-A151R_1, _2 and _3, were designed to target the A151R. An additional set of eight RNAs, named siRNA-VP72_1 to _8 were designed against the VP72 gene. An irrelevant siRNA targeting the GAPDH gene (siRNA-GAPDH; Silencer™ GAPDH siRNA human control number 4605; Ambion/Applied Biosystems) was used as a negative control in all inhibition experiments. An additional irrelevant siRNA (siRNA-NPPR1) targeting the nucleoprotein gene of an animal morbillivirus was included in the initial tests: this siRNA represses the morbillivirus replication [23] and was selected because it has a base composition that is similar to siRNA-A151R_3 (A6, U5, G5 and C3). Because no differences were seen between siRNA-GAPDH and siRNA-NPPR1, only the former siRNA was kept as control in all test repetitions, for practical and economical reasons. To verify the specificity of the siRNAs, sequences were queried against the BLAST database.

For the most efficacious siRNA, nucleotide sequences were compared to ASFV sequences available in GenBank, in order to assess the conservation of the targeted regions. For the VP72 gene, a total of 58 sequences retrieved in GenBank. A total of 9 A151R gene sequences were retrieved from GenBank, and sequences were generated in this study for an additional 10 Kenyan and Malawi ASFV isolates (Kenya: KillealI/1959, KillealII/1959, MagadiW/H3/1959, MagadiW/H8/1959, KimakialI/1961, Kab1994/1, Ken/2001/2 and Malawi: Malawi/1978, Dedza/1986, Malawi/2002/1).

For transfection assays, 10^5 Vero cells in 500 μ l of EMEM supplemented with 10% FBS were seeded into a 24-well plate and incubated at 37°C. Different concentrations of siRNA were initially diluted in 100 μ l of culture medium without serum and then adjusted to a final concentration of 6.25, 12.5, 25, 50 and 100 nM in 600 μ l of transfection solution. An 8 μ l aliquot of HiPerfect Transfectant reagent (Qiagen, Courtaboeuf, France) were added to the diluted siRNA and mixed by pipetting. The mix was incubated 15 min at room temperature to allow the formation of transfection complexes, overlaid onto the cells and the cells gently mixed to ensure uniform distribution of the complexes. Cells were further incubated with EMEM supplemented with 10% FBS for 6h; the medium was then discarded and 1 ml of EMEM supplemented with 5% FBS was added. Unless otherwise stated, at 1 h or 24 h post-transfection, cells were infected with BA71V in 200 μ l of serum-free EMEM. When the antiviral effect of siRNA was measured on virus titres and cytopathic effect (CPE), two virus loads were tested for each: 0.1 and 0.01 MOI. By contrast, when the antiviral effect was assessed through the quantification of mRNA, the virus challenge could not be > 0.01 MOI, presumably because of RNA saturation that prevented the detection of any differences. At 1 h post-infection, cells were rinsed with 700 μ l of EMEM to remove virus inoculum and 1 ml of EMEM supplemented with 5% FBS was added. In some assays, cells were not rinsed to test the effect of residual infectivity on the siRNA efficacy. A quantity of 800 μ l of EMEM supplemented with 5% FBS was added to the virus inoculum. In all assays, cells were incubated for an extra period of 72 h. Controls consisted of non-transfected infected or transfected uninfected cells. Individual, as well as several combinations of siRNAs, were transfected. Combinations of siRNAs consisted of siRNA-A151R_1+2, siRNA-A151R_1+3, siRNA-A151R_2+3, siRNA-A151R_1+2+3, siRNA-A151R_2+VP72_8; siRNA-A151R_2+3+VP72_8. Dose effect was measured by transfecting cells with fourfold serial dilutions of siRNA starting at 100 nM.

CPEs were visualised by two different investigators using an inverted microscope. The two investigators had to characterize the importance of CPE by using a scale with four grades: 0 (no CPE), 33 (CPE 33%), 66 (CPE>33% and 66%), and 100 (CPE > 66%) as previously described [24]. The final CPE values were the averages of the two readers. This method was previously shown to give results with a comparable sensitivity to real-time PCR or flow cytometry [23, 24]. In addition, cells and supernatants harvested 72 h post-infection were kept at -70°C until used for virus titration or mRNA quantification. Infectious virus in cell supernatants was titrated using 10-fold serial dilutions inoculated onto Vero cells. The virus titre was expressed in TCID₅₀/ml. For assays in which the virus inoculum was not discarded and cells not rinsed after infection, a control was included to measure the residual infectivity of virus inoculum after incubation for 3 days. This control consisted of 200 μ l of virus inoculum and 800 μ l EMEM supplemented with 5% FBS laid on a plastic well with no cells and titrated after 72 h of incubation at 37°C.

Total cellular RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in 50 μ l of ultra-pure water and stored at -70°C for future use. To eliminate the presence of viral genomic DNA, 15 μ l of RNA were treated by DNase I digestion reagent (TURBO DNA-free™ Kit number AM1907; Ambion/Applied Biosystems). The mix was incubated for 30 min at 37°C and DNase inactivated by the addition of the inactivation reagent solution. Reaction was incubated for 2 min at room temperature and mixed 3x, centrifuged 5 min at 10,000 rpm, and supernatant collected in a new tube for real-time reverse transcriptase PCR.

Quantification of RNA transcripts were carried out by quantitative real-time PCR using primers designed for A151R and VP72 genes (Primer3 software; Table 1). Specific complementary DNA for each of the genes was synthesized by reverse transcription at 50°C for 30 min, followed by PCR amplification in the same tube using 1 μ l StrataScript Reverse

Transcriptase (Stratagene, Amsterdam, the Netherlands), 2 µl of extracted RNA, 12.5 µl of SYBR Green Master mix (2x; Stratagene), 2.5 µl of each primer (initial concentration 1 µM) and up to 4.5 µl with water. Thermocycling was performed using an Mx3005P machine (Stratagene). The PCR cycling programme consisted of a denaturation for 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 56°C and 30 s at 72°C, for VP72 and 18S genes and 30 s at 95°C, 1 min at 58°C and 30 s at 72°C for the A151R gene. PCR amplification was followed by one cycle of dissociation of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C.

To determine A151R and VP72 gene copy number, two standards were constructed by cloning the target genes into standard vector plasmids. Total DNA from 100 µl of BA71V supernatant was extracted with a GFX Genomic Blood DNA Column purification kit (GE Healthcare, Orsay, France) according to the manufacturer's protocol. PCR products of complete genes A151R (560 base pairs) and VP72 (2,277 base pairs) were amplified using 10 µl of BA71V DNA template, 0.5 µl of DNA polymerase in 25 µl of Premix C (FailSafe PCR System with PreMix Choice Kit, Epicentre, Tebu-Bio, Le Perray-en-Yveline, France) containing 1 µl at 25 pmol/µl of each couple of appropriate primers (Table 1) and 12.5 µl of water. Thermocycling was performed on an Applied Biosystems 2720 Thermal Cycler Instrument (Applied Biosystems, Cergy-Pontoise, France). The PCR cycling programme consisted of a denaturation step for 4 min at 94°C followed by 25 cycles of 30 s at 95°C, and 30 s at 55°C, with a final extension step at 72°C for 45 s for A151R gene and 2.5 min for VP72 gene. All PCR products included a final extension step of 7 min at 72°C. Purified products were cloned in suitable vector plasmids yielding pCR Blunt-A151R and pSC-B-VP72. Standard curves were prepared using plasmids linearized with BamH I restriction enzymes and used to determine copy numbers.

The gene encoding 18S ribosomal subunit was selected as a housekeeping gene to assess the quantity of total RNA extracted from each Vero cell preparation and to normalize the quantification of the other transcripts [25]. A standard consisting of five 10-fold serial dilutions of RNA extracted from 10⁶ Vero cells was included in all PCR assays. The copy numbers of A151R and VP72 genes were then adjusted against the copy number of 18S RNA.

The statistical significances of the results were determined using the Student's t-test. P-values were calculated for all transfected cultures compared with the positive control of virus replication.

Results

Verified sequences of A151R and VP72 genes were submitted to Ambion/Applied Biosystems for design of 11 siRNAs using proprietary Cenix software. Cell cultures were transfected with the different siRNAs and the effect on viral replication measured. When transfected 24 h before virus infection, all siRNA targeting the A151R gene inhibited the virus replication at all concentrations tested (between 1 nM and 100 nM) with maximal efficacy achieved at 100 nM. At 100 nM, the CPE caused by virus replication could be reduced by 80% at MOI 0.1 and 90% at MOI 0.01 (Fig. 1 and Table 2). Increasing the concentration beyond 100 nM did not increase inhibition significantly. Concentration of siRNAs beyond 400 nM also induced cell toxicity (DK *et al.*, data not shown).

A clear decrease of virus progeny was also observed with a reduction of virus titres >3 log₁₀ at MOI 0.1 and 0.01 compared with the positive control (Figure 1 and Table 2). Results were further confirmed by the real-time PCR on the corresponding gene transcripts for the tests at MOI 0.01. Levels of mRNA were reduced by 2 Logs in the presence of the three siRNA

targeting the A151R gene and siRNA-VP72_8 (Figure 1). Interestingly, the three siRNAs against A151R mRNA not only repressed the expression of A151R gene by 2 log₁₀ but also the expression of the late VP72 gene by 1.8 log₁₀. Serial fourfold dilutions of siRNA-A151R_2 showed a dose relationship on antiviral effects (Figure 2). The minimal effective dose of siRNA-A151R_2 was 0.39 nM resulting in a reduction of the virus titre by 1.2 log₁₀.

CPE and virus titres were significantly decreased by 70% and between 2 to 3 log₁₀ values ($P < 0.02$) by siRNA-VP72_7 and VP72_8, respectively. However, both siRNAs had more limited effects on the expression of VP72 mRNA (1–1.7 log₁₀) and A151R mRNA (1–1.3 log₁₀) compared to the levels of inhibition achieved by the siRNAs targeting A151R. The irrelevant siRNA-GAPDH and siRNA-NPPR1 (DK *et al.*, data not shown) and siRNA-VP72_1, siRNA-VP72_2, siRNA-VP72_5 had no effect on the CPE, virus titres, or the expression of the two genes. The siRNA-VP72_3, siRNA-VP72_4 and siRNA-VP72_6 were considered to have only a marginal effect (Figure 1). The effects of siRNA-A151R_2 or siRNA-VP72_8 were not different, irrespective of whether the virus inoculum was removed or not after the initial infection (DK *et al.*, data not shown). The virus was not removed in some trials in order to assess the residual contribution of the inoculum infectivity in the final virus titres. As mentioned in Methods, residual infectivity was determined by titration of the inoculum after 72 h at 37°C in the absence of cells. Interestingly, virus inoculums corresponding to MOI 0.1 and 0.01 when stored at 37°C for 3 days only decreased by 0.3 and 0.6 log₁₀, respectively. Because the virus titres in cells treated with siRNA were always higher (> 1 log₁₀) than the residual titre of the virus inoculum, we can conclude that none of our siRNAs could completely inhibit the virus replication.

When the most efficient siRNAs against the A151R and VP72 genes, were delivered 1 or 24 h after virus infection at MOI 0.1, the inhibition of virus replication was marginal (DK *et al.*, data not shown); however, when the inoculum was reduced to MOI 0.01, an inhibitory effect was observed irrespective of when infected cells were exposed to the siRNA (Table 2). As expected, the inhibition of CPE varied from 70% for siRNA against VP72 to 90% for siRNA-A151R_2 for cells infected 1 and 24 h after the siRNAs delivery. By contrast, the CPE was reduced by only 35–70% when the infection took place 24 and 1 h before the siRNA delivery. Similarly, the reduction of virus titres increased between 1.7 and 3.3 log₁₀ values when siRNAs were delivered 1 and 24 h before infection, whereas the reduction ranged between 0.1 and 2.5 log₁₀ values when siRNAs were delivered 24 and 1 h after infection (Table 2).

In an attempt to increase the levels of inhibition, cells were transfected with a combination of siRNAs. At 100 nM, the combination of multiple siRNA that target different regions of A151R gene or the two A151R and VP72 genes, increased the efficacy by only 0.2–0.9 log₁₀ values compared with the single siRNA-A151R_2 (Figure 3). The increase was only significant for the combined siRNA-A151R_1+_2+_3, siRNA-A151R_2+VP72_8, and siRNA-A151R_2+_3+VP72_8 ($P < 0.05$). Transfection of multiple siRNAs did not improve efficacy at lower concentrations compared with single siRNAs (Figure 2). In addition, the combination of siRNAs did not improve the antiviral effect when the delivery followed the virus infection (DK *et al.*, data not shown).

To assess whether the genetic variations could possibly affect the efficacy of the siRNAs, the sequence of the most effective siRNA were compared to A151R gene sequences of 19 ASFV isolates. The analysis of the four targeted regions showed 91, 65, 50, and 40% conservation, for siRNA-VP72_8, siRNA-A151R_1, siRNA-A151R_3 and siRNA-A151R_2, respectively (Table 3). The lowest conservation of the target sequence was

observed for siRNA-A151R_2, the siRNA targeting the A151R gene that resulted in the most significant inhibition of virus replication.

Discussion

In this study, the transfection of cells with siRNAs targeting the A151R and VP72 genes resulted in the reduction of A151R and VP72 mRNA levels, CPE and virus titres. This demonstrates that the A151R protein is essential for the virus replication. The function of the A151R gene product has not yet been fully characterized. The corresponding protein is expressed during both early and late stages of virus replication [26]. During the early stages of virus replication the protein is possibly involved in DNA precursor synthesis or a regulation of signal transduction. The observation that siRNAs targeting A151R down-regulate the expression of VP72 mRNAs suggests that the protein might also play a direct or indirect role in viral transcription. Dixon *et al.* (2008) [27] and Rodriguez *et al.* (2006) [26] have also suggested that this protein could possibly play a role in ASFV morphogenesis during the later stages of the viral life cycle. The A151R protein contains two cysteine residues in a conserved motif CXXC at amino acid positions 132–135, characteristic of proteins involved in the thioredoxin family pathway [28, 29]. It is suggested that the A151R gene encodes a non-structural protein that interacts with the non-structural protein B119L as part of the redox potential reduction pathway responsible for the formation of the disulfide bonds of E248R protein. The E248R protein is essential for the correct assembly of a mature virion in a reducing environment. Deletion of the B119L gene has been shown to significantly reduce virus morphogenesis, resulting in the decrease of virus titres and the increased formation of aberrant virions [26]. The results described herein support the hypothesis that the A151R protein might be involved in a critical step of virus morphogenesis.

To our knowledge, this work is the fifth demonstration of the efficacy of siRNA on a double-stranded DNA virus that replicates in the cell cytoplasm. The first three demonstrations were from the same research group working on the orthopoxvirus vaccinia [30, 31, 32]. By targeting the DNA-independent nucleoside triphosphatase gene (D5 protein) with 100 nM of specific siRNA, this research group was able to reduce the replication of vaccinia virus (VACV) by 96% (1.4 log₁₀). Whatever the virus load used for cell infection (MOI from 0.0001 up to 3), the maximum reduction of the virus titre in the earlier studies could not exceed 2 log₁₀ [30], compared with our 4 log₁₀ reduction (99.99% reduction) using an MOI of 0.1. In another study by this group, the VACV G7L gene was also targeted. It is an early gene and its product is required for virus morphogenesis, just as A151R protein is required for ASFV. At 24 h post-transfection of siRNAs against G7L, the VACV titre produced after infection with 0.1 MOI and incubation over 24 h was reduced by 99% [32], which is 1–2 log₁₀ values less than the reduction achieved in our work. By contrast, a fourth study targeting the A6R gene of Monkeypox virus showed an almost complete inhibition of virus replication when specific siRNAs were delivered at 40 nM, 18 h before infection at 0.1 MOI [33]. The antiviral therapeutic effect of siRNAs against 0.0001 MOI of VACV at 1, 8 and 24 h post-infection showed a reduction of VACV replication of 92.0%, 72.3% and 74.2% respectively [30]. In our present study, we could only successfully reduce CPE (by 70%) and titre (reduction of 2.5 log₁₀) when the siRNAs were delivered 1 h post-infection. At 24 h post-infection, the therapeutic effects of siRNAs were minimal; however, it must be stressed that our virus load for the infection was 100x higher and the incubation period 1 day longer. In a previous work carried out on morbilliviruses, we also showed that an efficient therapeutic effect from 24 h up to 144 h post-infection could be obtained when reducing the virus load from 0.1 to 0.001 MOI [23]. In conclusion, we provide evidence that the siRNAs identified in this study have excellent efficacy compared with previous works on orthopoxviruses; however, none of these siRNAs could completely inhibit the replication of

ASFV, in contrast to our results achieved on another virus, a negative strand RNA morbillivirus [23]. This might result from the finding that mRNA are less accessible to siRNAs in ASFV viral factories, or that the accumulation of mRNA overwhelms the siRNA production in the cell cytoplasm; however, it could also result from the lack of accessibility of the mRNA targeted region as mentioned by different authors [34–36]. A rapid analysis of the secondary structure of A151R and VP72 mRNAs by the bioinformatics tool (Rensselaer Polytechnic Institute, Troy, NY, USA) predicted a higher percentage of accessibility (83%) for the two siRNA-A151R_2 and siRNA-A151R_3 compared to the siRNA-VP72_8 and siRNA-A151R_1 (62%). The combined delivery of multiple siRNAs targeting A151R and VP72 genes did not significantly improve the overall control on the virus replication; however, the benefit from the use of combined siRNAs in previous studies depended on the virus used. In some cases it was improved [37] and in others it was not [38, 39]. Another reason for this discrepancy could be the saturation of RISC by the delivery of high concentrations of multiple siRNAs [39]. In order to clarify this hypothesis, we intend to generate Vero cells constitutively expressing active siRNAs to avoid the need to transfect high doses of siRNA into the cell cytoplasm. By generating cell cultures constitutively expressing the siRNAs, the problem of cell toxicity could be avoided, possibly resulting in the increased inhibition of virus replication.

Compared with other antiviral molecules tested so far on the ASFV, our siRNA provide similar inhibition. For example, the antitumoural drug lauryl gallate used at 10 μ M reduced the virus replication by a factor of 3–5 $\log_{10}$ [40]. Similar to our observations with siRNA, the administration of the drug 24 h after the infection did not significantly control the ASFV replication. This demonstrates that the therapeutic indication of both approaches is limited at the present, but that preventive application in case of emergency still valuable, especially when considering there is no vaccine against this highly fatal disease of swine. One of the advantages of the use of siRNA over the use of alternative antiviral drugs is their tight target specificity and minimal side effects. By contrast, it has been shown that a single mismatch can abolish the interference. Therefore, the efficacy of siRNAs is greatly influenced by the genetic variation within the target sequence. To assess the genetic diversity within the A151R gene, the sequences of the siRNAs were compared with that of several ASFV field isolates. Significant variations were found for some of the siRNA described here. To limit the risk of mismatch, siRNA can be used in combination to target different regions of interest [37, 41–43]. In our study, the combination of different siRNAs did not improve or reduce the inhibition of ASFV replication; therefore, such a combination could be safely used in our case in order to limit the risk of mismatch or virus escape mutant. For example, the combined use of siRNAs targeting the three active positions of A151R gene, in addition to the following sequences **GCCAGGAAGAACTTAAAGG**, **CTTCAGTCGGTCCTCATAT**, **CTAAAGCCGGTCCTTGAT**, **GCTTATAGAGTGCATCTAT** and **GCTTATAGAGTGCATTGAT**, where mutated nucleotides are shown in bold, would cover the genetic variation of 85 % of the viruses for which the A151R gene sequences are currently known.

Acknowledgments

We thank Yolanda Revilla and Marisa Salas (Centro de Biología Molecular, Spain) for providing the ASFV (Badajoz 1971, BA71V). We also acknowledge Geneviève Libeau and Catherine Cêtre-Sossah for their helpful discussions. This work was supported by Wellcome Trust (N°210183. 183, AHDW/03/04), the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement KBBE- 211691- ASFRISK, and also partially funded by the European Union through the Network of Excellence EPIZONE (number FOOD-CT-2006-016236).

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Figure 1.

Inhibitory effects of siRNAs

Vero cells were transfected with 100 nM of each small interfering RNA (siRNA) and, 24 h later, infected with BA71V, either at a Multiplicity of infection 0.1 median tissue culture infective dose (TCID₅₀)/cell (for cytopathic effect [CPE] and titre) or 0.01 TCID₅₀/cell (for targeted gene messenger RNAs [mRNAs]). The positive control consists of the virus BA71V alone inoculated onto Vero cells whereas siRNA-GAPDH was used as an irrelevant siRNA to test the specificity of interference. Numbers above the bars indicate the percentage of CPE induced by BA71V. Bars represent the virus titres in cell supernatants expressed in log₁₀ TCID₅₀/ml. Curves show the results of mRNA quantification by real time reverse transcriptase PCR. Error bars represent the standard deviations of 10 independent replicates, with the exception of siRNAs VP72_1, _2, _3, _4 and _5 (n=3). ^aSignificant difference compared to the positive control (P<0.05).

Figure 2.

Dose-dependent effects of single or multiple siRNAs on BA71V replication

Vero cells were transfected with fourfold serial dilutions of small interfering RNA (siRNAs) starting from 100 nM and infected 24 h later with BA71V at a multiplicity of infection of 0.1 median tissue culture infective dose (TCID₅₀)/cell. Supernatants were collected 3 days post-infection and titrated. The virus titres are expressed in log₁₀ TCID₅₀/ml. Positive control is the BA71V virus alone on Vero cells. siRNA-VP72_2 served as a non active siRNA (negative control). Results are representative of three repetitions.

Figure 3.

Inhibitory effects of single or multiple siRNAs

Vero cells were transfected for 24 h with 100 nM total each single or multiple small interfering RNA (siRNAs) and then infected 24 h later with BA71V at a multiplicity of infection of 0.1 median tissue culture infective dose (TCID₅₀)/cell (for cytopathic effect [CPE] and viral progeny) or 0.01 (TCID₅₀)/cell (for targeted gene messenger RNAs [mRNAs]). The positive control consists of the virus BA71V alone inoculated onto Vero cells whereas siRNA-GAPDH was used as an irrelevant siRNA to test the specificity of interference. Bars represent the virus titres in cell supernatants expressed in log₁₀ TCID₅₀/ml and are representative of two repetitions. Curves show the results of mRNA quantification by real time reverse transcriptase PCR. Error bars represent the standard deviations of 10 independent replicates. All single and multiple siRNA significantly reduced the virus titres and mRNAs compared to the positive controls. ^aSignificant differences between multiple siRNAs and single siRNA-A151R_2 (P<0.05).

Table 1

List and sequence of siRNA and primers used in this study

siRNA	Sequence	Gene	Nucleotide position
A15IR_1	5' GCAAAAGAAGUCUAAAGGtt 3' 5' CCUUUAAGAUUCUUCUUUGCtt 3'	A15IR	167 - 185
A15IR_2	5' CCUCAGUCGGUCUUGUAUtt 3' 5' AUACAAGGACCGACUGAGGtt 3'		353 – 371
A15IR_3	5' GCUUAUAGAGUGCAUCGAAtt 3' 5' UUCGAUGCACUCUAUAAAGCtt 3'		27 - 45
VP72_1	5' AAAACAUUCCGUAAACUGCtt 3' 5' GCAGUACGGAAAGUUUUtt 3'	VP72	329 - 311
VP72_2	5' AUGAAAAACUAAUGUCUCUGCtt 3' 5' GCAGACAUAAGUUUUUCAUtt 3'		587 - 569
VP72_3	5' GAACUUUACAUCAAUACCtt 3' 5' GGUUAUUGAUGUAAAAGUUCtt 3'		711 - 693
VP72_4	5' GAUGUAAUUGGAAAUUCCCtt 3' 5' GGGAAUUUCCAUUUACAUCtt 3'		1362 - 1344
VP72_5	5' UUUAAACGAGAACGUGAACCCtt 3' 5' GGUUCACGUUCUCGUUAAAtt 3'		933 - 915
VP72_6	5' CGCUUGUGCGCAAAUUUUGtt 3' 5' CAAAAUUUGCGCACAAAGCGtt 3'		1316 - 1298
VP72_7	5' GCAGGUAAACAAGAUAUUAtt 3' 5' UAAUAUCUUUGUUACCUGCtg 3'		1074 - 1056
VP72_8	5' CGUGAACCUUGCUAUUCCCtt 3' 5' CGUGAACCUUGCUAUUCCCtt 3'		922 - 904
GAPDH	5' AAGGUCAUCCAUGACAAACUtt 3' 5' AGUUGUCAUGGAUGACCUUtt 3'	GAPDH	
Real-time PCR Primers			
A15IR-166 sense	5'-AGCAAGAAGATCTTTAAAGGAGCA-3'		249 ^a
A15IR-414r antisense	5'-AAAGGATGTGCATTTAGTACACCA-3'		
VP72-80 sense	5'-GTAGTGATAGACCCACGTAATCC-3'		232 ^a
Vp72-311r antisense	5'-CTGCTCATGGTATCAATCTTATCG-3'		
18Sf	5'-TCAAGAACGAAAGTCGGAGG-3'		
18Sr	5'-GGACATCTAAGGGGCATCACA-3'		400 ^a
Conventional PCR Primers			

siRNA	Sequence	Gene	Nucleotide position
A151R-a sense	5'-CTGCGCAGTATTATTAAAAATCATGT-3'		560 ^a
A151R-b antisense	5'-ATCTAATTTATTGGGAATATATTGGGC-3'		
VP72-a sense	5'-AAITTTTGACGCCATTTTGCC-3'		2,277 ^a
VP72-b antisense	5'-CTTGGCATAAACCGCCATATTT-3'		

siRNA, small interfering RNA.

^aValue shown is the amplicon size measured in base pairs.

Table 2

Efficacy of five siRNAs when delivered at 100 nM at different time points before or after the virus infection

siRNA, 100 nM	Time of virus infection compared with siRNA delivery	BA71V CPE, (%)	Virus progeny, 10X TCID ₅₀ /ml
A151R_1	-24h	60	4.8
A151R_2		50	4.6
A151R_3		60	4.8
VP72_7		65	6.2
VP72_8		65	6
PC		100	6.3
A151R_1	-1h	50	4.5
A151R_2		30	4.8
A151R_3		50	4.9
VP72_7		60	Not tested
VP72_8		60	5.2
PC		100	6.3
A151R_1	+1h	30	4.2
A151R_2		10	3
A151R_3		25	4.2
VP72_7		30	4.6
VP72_8		30	4.4
PC		100	6.3
A151R_1	+24h	20	3.9
A151R_2		10	2.9
A151R_3		15	3.9
VP72_7		30	4.3
VP72_8		25	3.7
PC		100	6.2

The effect was measured by virus titration in supernatants and messenger RNA quantification in cell cytoplasm by real-time reverse transcriptase PCR. Multiplicity of infection was equal to 0.01. Positive control (PC) is the virus BA71V alone. CPE, cytopathic effect; siRNA, small interfering RNA; TCID₅₀, median tissue culture infective dose.

Table 3
Base variations of the four regions in A151R gene that are efficiently targeted by siRNA

siRNA regions	siRNA sequences in DNA										Number of strains analyzed	
siRNA-A151R_1	G	C	A	A	A	G	A	A	G	A	G	11
Translation sense	T											2 (Mkuzi 1979 and Warmbayhs)
												1 (Warthog 80)
												1 (Kenya 1950)
												3 (Malawi_Lil 1983, Malawi_78, Malawi Dedza 86)
												2 (Malawi 2002/1, Tengani 62)
siRNA-A151R_2	C	C	T	C	A	G	T	C	G	T	C	8
												2 (Malawi 2002/1, Tengani 62)
												6 (KAB 94/1, Kilelesh, J 59, Kilelesh, JI 59,
Translation sense	T											Magadi W/H3 59, Magadi W/H8 59, KEN 01/2)
												1 (Kenya 1950)
												1 (Malawi_Lil 1983)
												2 (Malawi Dedza 86, Malawi_78)
siRNA-A151R_3	G	C	T	A	T	A	G	A	G	T	C	10
												6 (KAB 94/1, Kilelesh, J 59, Kilelesh, JI 59,
Translation sense												Magadi W/H3 59, Magadi W/H8 59, KEN 01/2)
												1 (Kenya 1950)
												3 (Malawi_Lil 1983, Malawi_78, Malawi Dedza 86)
siRNA-VP72_8	C	G	T	G	A	A	C	C	T	T	C	53
Translation sense												5

Bold font denotes the base variations siRNA, small interfering RNA.