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Small interfering RNAs targeting viral structural envelope protein genes and the 5′-UTR inhibit replication of bovine viral diarrhea virus in MDBK cells

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Summary. – Bovine viral diarrhea viruses (BVDVs) are important pathogens of cattle that occur worldwide, and for which no antiviral therapy is available. In the present study, the inhibitory effect of small interfering (si) RNAs on bovine viral diarrhea virus 1 (BVDV-1) replication in cultured bovine cells was explored. Four synthetic siRNAs were designed to target structural envelope region genes (E^{rns}, E1, and E2) and one cocktail of siRNA was generated to target the 5′-UTR of the BVDV-1 genome. The inhibitory effects of siRNAs were assessed by determination of infectious viral titer, viral antigen and viral RNA. The siRNA cocktail and three of the synthetic siRNAs produced moderate anti-BVDV-1 effect *in vitro* as shown by 25%–40% reduction in BVDV-1 antigen production, 7.9–19.9-fold reduction in viral titer and 21–48-fold reduction in BVDV-1 RNA copy number. Our findings suggest that siRNA cocktail targeted at the 5′-UTR is a stronger inhibitor of BVDV-1 replication and the targets for siRNA inhibition can be extended to BVDV-1 structural envelope protein genes.

Keywords: bovine viral diarrhea virus; RNA interference; small interfering RNA

Introduction

BVDVs are economically important pathogens of cattle, which cause significant respiratory and reproductive disease worldwide. Despite several control measures, such as selective test and slaughter, eradication, vaccination or in various combinations, have been adopted, the disease is still endemic in many countries. Hence, the development of effective antiviral therapies may be useful in the future. BVDV-1, BVDV-2 along with the border disease virus (BDV) and classical swine fever virus (CSFV) belong to the genus *Pestivirus* in the family *Flaviviridae* (Fauquet *et al.,* 2005). Within *Flaviviridae*, BVDV-1 and BVDV-2 are more related to human hepatitis C virus (HCV) than flaviviruses. Due to the similarities in genome structure, organization, replication strategies and the ability to cause long-term infection, BVDV-1 has often been used as a surrogate model for HCV infection. The genome of both BVDV-1 and BVDV-2 consists of a single-stranded RNA of about 12.3 kb in length. A long ORF flanked by 5′-UTR and 3′-UTR is translated into a poly-protein of about 4000 amino acids and is cleaved into four structural (capsid (C) and three envelope (E^{rns}, E1, and E2) proteins) and 7-8 nonstructural proteins (N^{pro}, p7, NS2-3 or NS2 and NS3, NS4A, NS4B, NS5A, and NS5B) by viral and host cell proteases (Meyers and Thiel, 1996).

RNA interference (RNAi), a naturally occurring cellular mechanism of gene suppression is a promising approach to develop effective antiviral drugs. Since the discovery that small interfering RNAs (siRNA) upon direct transfection *in vitro* can selectively initiate gene suppression (Elbashir *et al.,* 2001), siRNAs have been shown to suppress replication of a variety of viruses that infect humans and animals such as hepatitis C virus (Randall *et al.,* 2003), influenza A virus (Zhou *et al.,* 2007), foot-and- mouth disease virus (Chen *et al.,* 2004), porcine reproductive and respiratory syndrome virus (He *et al.,* 2007) and classical swine fever virus (Xu *et al.,* 2008).

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Abbreviations: BVDVs = bovine viral diarrhea viruses; BVDV-1 = bovine viral diarrhea virus 1; BVDV-2 = bovine viral diarrhea virus 2; siRNA = small interfering RNA; IRES = internal ribosome entry site; CSFV = classical swine fever virus; HCV = hepatitis C virus; MAb = monoclonal antibody; p.i. = post infection

Inhibition of BVDV-1 replication in cell culture to a variable extent has been demonstrated recently by synthetic siRNAs targeting the 5′-UTR, capsid (C), NS4B and NS5A regions of the genome with maximum inhibition achieved by siRNAs targeting capsid and NS5A regions (Lambeth *et al.,* 2007). However, no effort has been made to study the inhibitory potential of siRNAs targeted at envelope protein genes, although the effect of siRNAs may take place during the viral progeny production and BVDVs envelope proteins play crucial roles in viral assembly and entry (Krey *et al.,* 2005). Additionally, since the 5′-UTR contains an internal ribosome entry site (IRES) that mediates translation of the ORF (Poole *et al.,* 1995), BVDV-1 replication inhibition by a cocktail of siRNAs targeting this region may be possible. Hence, the aim of this work was to determine the inhibitory effect of siRNAs targeting envelope protein genes and the 5′-UTR on the replication of BVDV-1 in MDBK cells.

Materials and Methods

Virus and cells. Pestivirus free MDBK cells (Cell Culture Collection of Veterinary Medicine, Friedrich-Loeffler Institute, Island of Riems, Germany) were grown in Eagle′s MEM (Sigma) supplemented with 10% FCS (Invitrogen) in a humidified atmosphere containing 5% CO_2 at 37°C. Indian cattle BVDV-1 noncytopathic strain Ind S-1449 (Mishra *et al.,* 2004) was propagated on MDBK cells using EMEM containing 5% FCS and the titer of the virus stock was determined following a standard method.

siRNAs. Four siRNA duplexes targeting E^{rns}, E1, and E2 regions of the Indian cattle BVDV-1 isolate Ind S-1449 genome (GenBank Acc. No. AY911670, Mishra *et al.,* 2006) were designed using the Hi Performance Design Algorithm (Novartis AG) and synthesized commercially (Qiagen). The oligonucleotide sequences used for siRNA synthesis and their target regions are shown in Table 1. The negative control siRNA was synthesized (Qiagen) using previously reported sequences (Lambeth *et al.,* 2007). To generate siRNA cocktails, the 5′-UTR of BVDV-1 strain Ind S-1449 was targeted. The templates for *in vitro* transcription were obtained by amplification of a 288 bp fragment (nt 108–395 of BVDV-1 strain NADL) using primers 324 and 326 (Vilcek *et al.,* 1994) appended to the T7 promoter sequence and Access RT-PCR kit (Promega). The siRNA cocktails were produced using the Silencer siRNA cocktail kit RNase III (Ambion). The negative control siRNA was labeled with Cy3 using silencer siRNA labeling kit (Ambion) for optimization of transfection.

Table 1. Characteristics of BVDV-1 specific siRNAs

siRNA (target)	Target	
	Sequence	Location (nt)[a]
si821 (E^{rns})	sense 5′-CAAUGGAACUUACGAGAUA-3′ antisense 5′-UAUCUCGUAAGUUCCAUUG-3′	823–841
si1518 (E1)	sense 5′-GGCUUGGUUACGUCGAUUA-3′ antisense -UAAUCGACGUAACCAAGCC-3′	1520–1538
si1921 (E1)	sense 5′-AGUAAUUAAGAUUGUCUUA-3′ sntisense 5′-UAAGACAAUCUUAAUUACU-3′	1923–1941
si2527 (E2)	sense 5′-GGCCGUUGUUCGAACGUAU-3′ antisense 5′-AUACGUUCGAACAACGGCC-3′	2529–2545
si-cocktail (5′-UTR)		108–395[b]

[a]Location corresponding to BVDV-1 isolate Ind S-1449 (GenBank Acc. No. AY911670). [b]Location corresponding to BVDV-1 strain NADL.

Testing of siRNAs. The transfection protocol and quantity of siRNAs (5 to 150 nmoles) that inhibit BVDV-1 replication without visible cytotoxicity were optimized using Cy3-labelled control siRNA, MDBK cells (90% confluency) and siPORT Amine agent (Ambion). Briefly, the transfection agent and siRNA complex was formed in serum-free Optimem 1 medium (Invitrogen). Transfection was carried out for 16 hrs in the presence of BVDV-1-specific synthetic and cocktail siRNAs at a concentration of 100 nmoles per well in 96-well plates. Following removal of the mixture and washing, the cells were infected with 100 $TCID_{50}$ BVDV-1 per well and incubated at 37°C for 48 hrs before testing for siRNA inhibitory effects. Healthy MDBK cells, negative control siRNA-transfected cells, BVDV-1-specific siRNA-transfected but not infected cells, and cells with only transfection agent added were kept as controls. All the experiments were conducted thrice in triplicates.

Virus titration. The infected cell supernatants collected at 48 hrs post infection (p.i.) following freezing and thawing were subjected to virus titration on MDBK cells to determine the reduction in virus titer under siRNA effect. The replication of BVDV-1 was demonstrated by immunochemistry and the virus titer was expressed as $\log_{10}$ $TCID_{50}$/ml.

Immunochemistry. The effect of siRNAs on viral antigen expression was assayed by immunochemistry using BVDV-1 E2-specific monoclonal antibody (MAb) 157. Briefly, the cells at 48 hrs p.i. were washed, heat fixed and incubated with 50 µl of MAb 157 for 1 hr at 37°C. After washing, the cells were reacted with 50 µl of peroxidase-conjugated anti-mouse IgG (Sigma) for 1 hr at 37°C followed by exposure to 3-amino-9-ethylcarbazole (AEC) substrate. The amount of viral antigen production (% of antigen positive cells) in cells transfected with BVDV-1 specific siRNAs was calculated by considering the value obtained by negative control siRNA as 100%.

Real-time RT-PCR. For assaying viral RNA, real-time RT-PCR was performed using Mx 3000 (Stratagene), SuperScript III quantitative real time RT-PCR system (Invitrogen), BVDV-1-specific primers and TaqMan probes as described earlier (Baxi *et al.,* 2006). The total RNA of cells transfected with various siRNAs and challenged with BVDV-1 for 48 hrs was extracted using the QIAamp viral RNA purification kit (Qiagen) and was used as template. Data analysis was performed using the Mx 3000 software to obtain log BVDV-1 RNA copy numbers.

Results

We used four synthetic siRNAs targeting E^{rns}, E1, and E2 regions and a cocktail siRNA preparation targeting 5′-UTR of BVDV-1 genome. An optimal level of transfection was observed

at 16 hrs post transfection onwards (data not shown). MDBK cells were transfected with BVDV-1-specific siRNAs and the inhibitory effects of siRNAs on BVDV-1 replication were studied by assaying the viral antigen, infectious virus and viral RNA.

The results of viral antigen expression (% of antigen-positive cells) by immunochemistry (Table 2) showed that the level of suppression of BVDV-1 antigen production by synthetic and cocktail siRNAs was variable. The highest suppression was achieved by si-cocktail(5′-UTR) (40%), followed by si2527(E2) (35%), si821(E^{rns}) (30%) and si1518(E1) (25%). However, only marginal suppression was evident with si1921(E1).

The influence of various siRNAs on the level of infectious virus titer ($TCID_{50}$/ml) was determined and the results (Table 2) showed that si-cocktail(5′-UTR), si2527(E2), si821(E^{rns}) and si1518(E1) reduced the virus titer by 7.9- to 19.9-fold, while no reduction in virus titer was achieved by si1921(E1). Similar to the results of suppression of BVDV antigen production, the highest reduction in virus titer was obtained with si-cocktail(5′-UTR), followed by si2527(E2).

To further test the inhibitory potential of these siRNAs, BVDV-1 viral RNA (log RNA copy number) levels were assessed and the results are shown in Table 2. The maximum inhibition of BVDV-1 RNA was again obtained with si-cocktail(5′-UTR) (48-fold), followed by si2527(E2) (31-fold), si821(E^{rns}) (27-fold) and si1518(E1) (21-fold), while no inhibition was evident with si1921(E1). Taken together, our results demonstrated that moderate anti-BVDV-1 effect on MDBK cells was achieved by si- cocktail(5'-UTR), si821(E^{rns}), si1518(E1) and si2527(E2).

Discussion

As conventional therapies for treating viral diseases of human and livestock have their limitations, and alternate treatments are urgently needed, RNAi technology can have potential applications in this regard. As a plus-strand RNA virus, BVDV-1 appears to be an attractive target for siRNA, since its genome functions as both replication template and mRNA. In this study, we determined antiviral effects of siRNAs against the non-cytopathic biotype of BVDV-1, as this biotype is preponderant in nature and responsible for development of persistently infected immunotolerant animals, which are considered to be the main source of BVDV transmission.

We used a siRNA cocktail instead of specific siRNAs targeting the 5′-UTR of BVDV-1 genome, since a previous study has shown the failure of IRES-directed siRNAs to induce marked anti-BVDV-1 response (Lambeth *et al.,* 2007). Our results demonstrated that siRNA cocktail is a better BVDV-1 inhibitor than the individual siRNAs targeting 5′-UTR. We hypothesize that a pool of siRNAs targeted at different regions within 5′- UTR including IRES might have been effective against BVDV replication. It is surprising that although the IRES sequence shares features among pestiviruses and distantly related hepatitis C virus in humans, siRNA targeted to HCV IRES exerted significant antiviral effect (Kanda *et al.,* 2007), while siRNAs targeted to BVDV IRES had little antiviral effect (Lambeth *et al.,* 2007). Further screening of siRNAs directed to IRES extending to N^{pro} coding region may be taken up in the future for obtaining a more efficient anti BVDV-1 response.

With the exception of the nucleocapsid protein (C), all the other structural proteins E^{rns}, E1, and E2 are glycoproteins and are part of the BVDV envelope. E2 is responsible for virus attachment, entry and generation of neutralizing antibodies (Donis and Dubovi, 1987; Krey *et al.,* 2005), while E^{rns} has the ability to bind to glycosaminoglycans, and E1 is assumed to be a membrane anchor for E2 (Rumenapf *et al.,* 1993). Our results demonstrated that moderate inhibition of BVDV-1 RNA replication in MDBK cells was achieved by si821(E^{rns}), si1518(E1) and si2527(E2). Although highly variable regions are poor targets of gene silencing, the results indicate that it is possible to target siRNAs at the conserved regions within the variable envelope region for obtaining a moderate anti-BVDV-1 effect. Our results also get support from an earlier study showing efficient inhibition of hepatitis C virus in human cells by siRNA targeting the E2 envelope region (Liu *et al.,* 2006). Although the causes of non-inhibitory effect of si1921(E1) have not been ascertained, the possible reasons include inaccessibility of the target RNA region to the RNA-induced silencing complex (RISC) or inability of siRNA to form RISC essential for gene silencing. Similarly, whether induction of interferon by single or cocktail siRNAs may play a role in the observed inhibition of viral replication cannot be fully excluded.

Efficient *in vitro* inhibition of BVDV-1 replication has been achieved by siRNAs targeting structural capsid region and nonstructural NS4B and NS5A regions (Lambeth *et al.,* 2007).

Table 2. Antiviral effects of siRNAs

Virus infection parameter[a]	siRNA					
	si821(E^{rns})	si1518(E1)	si1921(E1)	si2527(E2)	si-cocktail (5′-UTR)	Negative control
Viral antigen-positive cells (%)	70	75	95	65	60	100
Infectious virus titer (log $TCID_{50}$/ml)	4.0	4.1	5.0	3.8	3.7	5.0
Viral RNA (log copy number)	4.2	4.3	5.6	4.1	3.9	5.6

[a]The values are shown as means of three replicates of three independent experiments.

A more efficient inhibition of the closely related classical swine fever virus replication *in vitro* has recently been obtained by siRNAs targeting N^{pro} and NS5B genes (Xu *et al.,* 2008). Our results provide evidence that the siRNA cocktail targeting 5′-UTR is a stronger inhibitor of BVDV-1 replication and extends the targets for siRNA inhibition to BVDV-1 envelope protein genes. Study of the inhibitory potential of siRNAs targeting various other regions of BVDV-1 genome should, however, continue to obtain a more effective antiviral therapeutic.

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