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Developing an effective RNA interference strategy against a plus-strand RNA virus: silencing of coxsackievirus B3 and its cognate coxsackievirus-adenovirus receptor

Denise Werk[1], Steffen Schubert[1], Vanessa Lindig[2], Hans-Peter Grunert[2], Heinz Zeichhardt[2], Volker A. Erdmann[1] and Jens Kurreck[1,*]

[1] Institute for Chemistry (Biochemistry), Free University of Berlin, Thielallee 63, D-14195 Berlin, Germany
[2] Department of Virology, Institute for Infectious Diseases Medicine, Charité-University Medicine Berlin, Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany

* Corresponding author
e-mail: jkurreck@chemie.fu-berlin.de

Abstract

Coxsackievirus B3 (CVB-3) is a plus-strand RNA virus that is believed to be the most common causal agent of viral myocarditis. Since no specific treatment for CVB-3 infections is available to date, we and others have recently started to develop RNA interference (RNAi) approaches to prevent virus propagation. Here we describe our strategy for the development of efficient small interfering RNAs (siRNAs) against viral genomes. Initially, fusion constructs of a reporter (green fluorescent protein) and viral subgenomic fragments were employed to select active siRNAs against the virus. Moreover, in an attempt to achieve sustained virus silencing and reduce the risk of generating escape mutants, only highly efficient siRNAs directed against regions of the viral genome that are unlikely to tolerate mutations were considered for virus inhibition. Two siRNAs directed against the 3D RNA-dependent RNA polymerase were found to inhibit virus propagation by 80–90%. The protective effect of the efficient siRNAs lasted for several days. Furthermore, we have first evidence that inhibition of the cellular coxsackievirus-adenovirus receptor (CAR) by RNAi also reduces the virus titre. Our strategy is likely to be applicable to other (RNA) viruses as well.

Keywords: picornavirus; siRNA; small interfering RNA.

Introduction

Viruses with a single-stranded RNA genome in plus-strand orientation can be grouped into several families, including picornaviridae, flaviviridae, togaviridae, coronaviridae, caliciviridae and hepatitis E virus. The picornavirus family contains more than 200 species, among them numerous of major clinical relevance. Despite the differences in tropism and the broad variety of diseases caused by members of this family of viruses, they share common features with respect to their life cycle. Their genomic RNA serves as a template for the synthesis of one polyprotein that is subsequently processed to give rise to the mature viral proteins. In contrast to cellular mRNAs, the viral RNA does not carry a cap group at the 5′ end. Viral protein synthesis is therefore initiated by a cap-independent mechanism making use of the highly conserved internal ribosome entry site (IRES) in the 5′ untranslated region (UTR). The viruses encode RNA-dependent RNA polymerases to generate minus strands from the genomic RNA, which subsequently serve as a template for the synthesis of new, positive-stranded RNA for translation and virus assembly. All of these processes take place in the cytoplasm.

Coxsackieviruses belong to the genus *Enterovirus* within the picornavirus family (for a review, see Zeichhardt and Grunert, 2003). Coxsackievirus B3 (CVB-3) is of particular medical interest since it is believed to be the most common causal agent of viral myocarditis, which may persist chronically and lead to dilated cardiomyopathy (DCM). This disease may finally progress to terminal heart failure and frequently requires heart transplantation. Although treatment with interferon-β has been shown to eliminate cardiotropic viruses and improve heart function in patients with myocardial persistence of viral genomes (Kühl et al., 2003), no specific treatment for coxsackievirus infections is available to date. New approaches to treat infectious and inflammatory heart diseases are therefore desirable.

RNA interference (RNAi) is a phenomenon of sequence-specific gene silencing induced by double-stranded RNA molecules that was first described for the nematode *Caenorhabditis elegans* (Fire et al., 1998). To avoid the induction of an interferon response, RNA duplexes shorter than 30 nucleotides have to be employed for gene silencing in eukaryotic cells (Elbashir et al., 2001). These molecules, referred to as small interfering RNAs (siRNAs), are incorporated into a multimeric protein complex, named the RNA-induced silencing complex (RISC). After sequence-specific hybridisation to a complementary RNA, the Argonaute 2 protein of RISC cleaves the target and thus prevents its translation (for mechanistic details, see Meister and Tuschl, 2004).

It has been demonstrated that RNAi approaches can outperform traditional techniques for gene silencing (Grunweller et al., 2003). The method has therefore been widely used for functional studies. Furthermore, RNAi is believed to hold great potential for therapeutic purposes (Ryther et al., 2005; Shankar et al., 2005), and the first clinical trials employing siRNAs were initiated in late 2004. In addition to cancer and neurodegenerative diseases, viral infections are a focus of the development of RNAi applications. The strategy has been found to be

suitable for the inhibition of different types of viruses in cell culture and *in vivo* (for reviews, see Joost Haasnoot et al., 2003; Colbère-Garapin et al., 2005; Schubert and Kurreck, 2005). RNAi and related nucleic acids-based strategies have been proposed as promising new approaches to treat virus induced heart diseases (Poller et al., 2004).

We and others have recently started to explore the potential of RNAi approaches to inhibit CVB-3 (Schubert et al., 2005a; Merl et al., 2005; Yuan et al., 2005). Here, we describe strategies to overcome hurdles on the way to generate efficient siRNAs targeting plus-stranded RNA viruses. Subgenomic fragments of the viral RNA fused to a reporter gene can be used to screen for active siRNAs. Although the 5′ UTR is an attractive target region due to its high degree of conservation, we experienced difficulties in finding efficient siRNAs against this highly structured area, as has previously been reported in the literature (see the comprehensive summary in Joost Haasnoot et al., 2003). In contrast, we were able to identify siRNAs that silence the expression of the RNA-dependent RNA polymerase fused to green fluorescent protein (GFP) in the subnanomolar range. These siRNAs were capable of efficiently inhibiting replication of the infectious virus in HeLa cells, thus demonstrating the suitability of using a reporter gene fusion construct to initiate RNAi approaches. As a complementary strategy, we silenced the coxsackievirus-adenovirus receptor (CAR), which has been identified as a cellular protein involved in attachment and infection by group B coxsackieviruses and various adenoviruses (for reviews, see Philipson and Petterson, 2004; Coyne and Bergelson, 2005). Knockdown of the receptor was also found to reduce virus propagation.

Results

Picornaviruses consist of a single plus-strand RNA genome of approximately 7500 nucleotides in length. In theory, any region of the genomic RNA can be targeted by siRNAs. A major problem for long-term silencing of viruses is the emergence of escape mutants. After initial successful inhibition, virus titres have frequently been reported to increase at later time points (Gitlin et al., 2002; Boden et al., 2003; Das et al., 2004). The problem is particularly relevant for RNA viruses with an error-prone replication machinery, such as picornaviruses. It is therefore advisable to select conserved regions of the viral genome that are unlikely to tolerate mutations. The IRES is known to have a high degree of conservation between various strains and can thus be considered as an attractive target region for antiviral siRNAs.

We therefore started by evaluating a first set of siRNAs targeted against the 5′ UTR of CVB-3. To facilitate initial selection of active siRNAs we subcloned the target region downstream of GFP. siRNAs that induce cleavage of the subgenomic viral RNA will lead to reduced expression of the reporter, which can easily be analysed by fluorescence microscopy or simple Western blotting with GFP-specific antibodies. As can be seen in Figure 1A, none of the siRNAs under investigation resulted in satisfactory silencing of the fusion construct, even at a concentration of 10 nM.

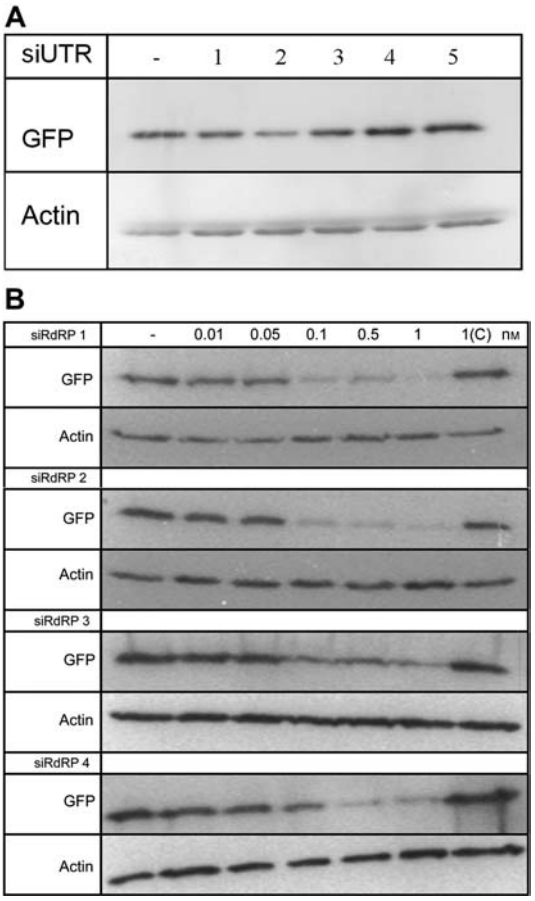

Figure 1 Selection of efficient siRNAs against subgenomic fragments of CVB-3 fused to GFP.
(A) Western blot of GFP translated from a fusion mRNA consisting of GFP and the 5′ UTR of CVB-3 with GFP-specific antibodies. Five siRNAs (siUTR 1–5) were cotransfected at a concentration of 10 nM. (B) Evaluation of four siRNAs targeting CVB-3 RdRP. The viral gene was cloned downstream of GFP and siRNAs were tested in a concentration-dependent manner. Western blotting was performed with a GFP-specific antibody. siUTR 2 was used as a control (C) that does not have any sequence homology to the target mRNA. Actin bands are shown as a loading control.

We therefore chose a distinct region of the coxsackievirus genome for analysis of a second set of siRNAs. Structural proteins are more likely to tolerate mutations than enzymes with catalytic activity. Therefore, four siRNAs targeting the 3D RNA-dependent RNA polymerase (RdRP) were selected on the basis of sequence criteria for siRNAs published by Reynolds et al. (2004). Again, a subgenomic fragment of the CVB-3 genome was cloned downstream of GFP. All four siRNAs were found to be active in a dose-dependent manner, even at subnanomolar concentrations (Figure 1B), siRdRP 3 being slightly inferior compared to the other RdRP siRNAs.

After having demonstrated the high efficiency of the siRNAs in an artificial system with the target gene fused to a reporter, we investigated the potential of the siRNAs in a cell viability assay with the infectious CVB-3

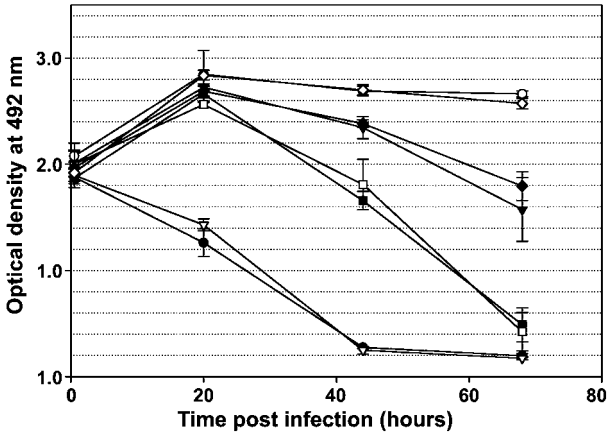

Figure 2 Viability assay of HeLa cells after infection with CVB-3.
Time-dependent cell viability is shown for untreated cells (open circles), mock transfected cells with lipofectamine only (open diamonds), virus infected cells (open triangles) and cells treated prior to virus infection with siRdRP 1 (filled triangles), 2 (filled diamonds), 3 (open squares), 4 (filled square) or unrelated control siRNA (filled circles).

(Figure 2). Untreated HeLa cells grow until they are confluent and reach a plateau phase. Loss of cell viability reflects virus reproduction leading to cytopathic alteration. After infection with CBV-3, cell lysis starts rapidly and 43 h after inoculation hardly any viable cells are left. All four siRNAs targeting viral RdRP exerted a significant protective effect on cell viability after infection with the virus. siRNAs 1 and 2 were found to be more potent as antiviral agents than the other two siRNAs. An unrelated control siRNA did not show any effect. We conclude from these results that our initial experiments with subgenomic fragments of the viral RNA fused to a reporter were a suitable system for selecting siRNAs that efficiently inhibit reproduction of the infectious virus.

The best siRNA (siRdRP 2) was further analysed in a time- and dose-dependent manner. As can be seen in Figure 3, this siRNA has antiviral activity, even at a low concentration of 0.1 nM, at 20 h post-inoculation. At later time-points, however, no protective effect was observed. In contrast, significant inhibition of virus propagation was observed even at 68 h post-inoculation with higher concentrations of the siRNAs. Small differences at early time points seem to have a severe impact on the later stages.

While cell viability tests give an indirect insight into virus reproduction, plaque inhibition assays provide direct and reliable quantitative measures of the inhibitory potential of antiviral agents. Reduction of virus titre with the four siRNAs ranged from 70% to almost 90% (Figure 4). Consistent with the results mentioned above, differences in the inhibitory efficiency between siRNAs 1 and 2 as measured by plaque reduction assay and the inferior siRNAs 3 and 4 are small compared to the strong effects observed in the cell viability assay 68 h post-inoculation (compare Figure 2 and 4).

As an alternative approach to direct silencing of the virus, we attempted to inhibit the expression of CAR, which is known to mediate CVB-3 entry into cells. We screened various siRNAs against the receptor that is endogenously expressed in HeLa cells, with the aim of isolating active siRNAs. The use of at least two independent siRNAs targeted to different sites in the same message is considered to be an important control to enhance confidence in RNAi data (Editorial, 2003).

siCAR2 and siCAR9 were found to almost completely silence target gene expression 48 h after transfection in a concentration range between 10 and 100 nM, whereas an unrelated control did not exert any effect (Figure 5A). Subsequently, we used these siRNAs in a plaque reduction assay with CVB-3 to test if silencing of CAR has an inhibitory effect on virus reproduction. These experiments showed that inhibition of CAR expression reduced the virus titre by approximately 60% (Figure 5B). Both CAR-specific siRNAs resulted in a consistent reduction of virus propagation, whereas the control siRNA gave higher variation between experiments, a phenomenon that has to be further investigated. As is discussed below in more detail, further reduction of virus propagation can

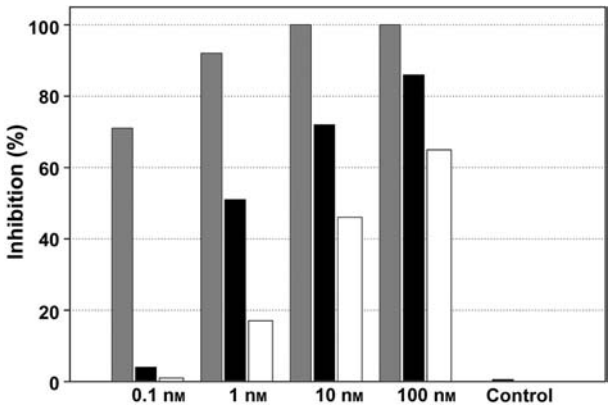

Figure 3 Concentration dependence of virus inhibition by siRdRP 2.
Percentage inhibition at 20 (grey bars), 44 (black bars) and 68 (white bars) h post infection relative to untreated cells is shown for cells pre-treated with increasing amounts of siRdRP 2 or 100 nM of an unrelated control siRNA.

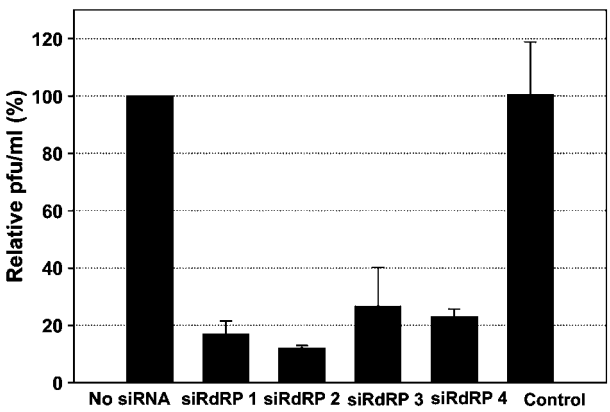

Figure 4 Reduction of virus titre by 100 nM siRdRP 1–4 in a plaque reduction assay.
The titre of CVB-3 is expressed as plaque-forming units (p.f.u.)/ml on HeLa cells relative to virus-only treated cells for cells pre-treated with either of the four siRNAs or an unrelated control siRNA.

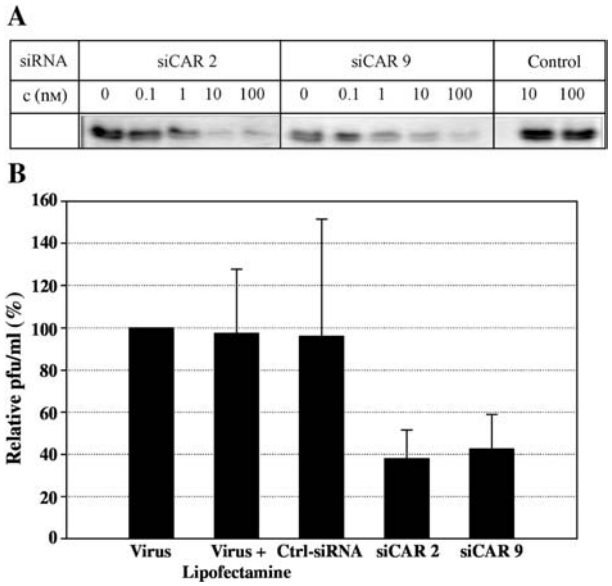

Figure 5 RNA interference approach against CAR.
(A) Western blot against endogenously expressed CAR with monoclonal RmcB antibodies. Lanes 1 and 6, untreated HeLa cells; lanes 2–5, increasing concentration of siCAR 2; lanes 7–10, increasing concentrations of siCAR 9; lanes 11 and 12, unrelated control siRNA. (B) Virus titre after knockdown of CAR. Titre of CVB-3 is expressed as plaque-forming units (p.f.u.)/ml on HeLa cells relative to virus-only treated cells. Virus titres after mock transfection with lipofectamine or pre-treatment with 50 nM control siRNA, siCAR 2 and siCAR 9 are shown. All data are means and standard deviations of six values, obtained from three independent experiments performed in duplicate.

be expected from simultaneous silencing of the viral co-receptor CD55.

Discussion

RNA interference is a promising novel technology not only for functional studies, but also for new therapeutic approaches. Cancer, neurological degeneration and genetic alterations are among the diseases that are currently the focus of many RNAi-based research projects (Ryther et al., 2005; Shankar et al., 2005). Furthermore, RNAi has been successfully used to inhibit virus propagation in cell culture and *in vivo* (for reviews see Joost Haasnoot et al., 2003; Colbère-Garapin et al., 2005; Schubert and Kurreck, 2005).

Picornaviridae are a large family of plus-strand RNA viruses with high medical relevance (for a review, see Zeichhardt and Grunert, 2003). CVB-3, a prominent representative of this virus family, is one of the most important myocardial pathogens. In two independent studies, RNAi approaches have recently been demonstrated to inhibit CVB-3 replication in cell culture (Schubert et al., 2005a; Yuan et al., 2005). Subsequently, siRNAs targeting the 2A protease were found to be suitable for inhibiting CVB-3 propagation *in vivo* and to prolong the survival time of highly susceptible mice (Merl et al., 2005). In the present study, we describe strategies to develop RNAi approaches that are likely to be generally applicable to the silencing of RNA viruses and analyse the mechanism of virus inhibition in more detail.

The generation of mutants due to the selection pressure imposed by the long-term use of a single siRNA is one of the major problems that have to be taken into account when developing RNAi-based strategies for virus inhibition. Extensive rearrangements in the viral genome have been observed upon silencing of HIV-1 (Das et al., 2004), but in some cases even a single point mutation was sufficient to escape silencing by siRNAs (Gitlin et al., 2002; Boden et al., 2003). Selection of conserved target sites will reduce the probability that mutations are tolerated without loss of infectivity. The IRES of RNA viruses is known to be highly conserved and has therefore been considered to be an attractive target region (Martinand-Mari et al., 2003). However, it cannot be excluded that suboptimal, but medically harmful virus strains with mutations in the IRES can accumulate even when targeting a conserved region.

Successful inhibition of virus replication with siRNAs targeting the 5′ UTR has been shown for hepatitis C virus, a plus-stranded RNA virus belonging to the flavivirus family (Yokota et al., 2003). In numerous other reports, including the present study, however, targeting of the non-coding regions has been found to be weakly active or inefficient (Wilson et al., 2003; Phipps et al., 2004; Yuan et al., 2005).

Variation in the silencing activity of several siRNAs targeting the same mRNA has already been reported by Holen et al. (2002) and was subsequently verified by others (Kawasaki et al., 2003; Vickers et al., 2003). Large-scale screening of siRNAs has been used to identify thermodynamic criteria inherent to active siRNAs (Khvorova et al., 2003) and – based on these findings – algorithms have been developed that help to predict the efficacy of an siRNA (Amarzguioui and Prydz, 2004; Reynolds et al., 2004; Ui-Tei et al., 2004). Without doubt, these calculations greatly contribute to the success of RNAi approaches. Still, some studies suggest that the structure of the target RNA has an important influence on the efficiency of siRNA-mediated silencing (Holen et al., 2002; Bohula et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003; Vickers et al., 2003; Luo and Chang, 2004; Overhoff et al., 2005). Based on a systematic analysis of silencing efficacy using intentionally designed target regions, we have recently proposed a two-step model to describe the crucial points of the RNAi pathway (Schubert et al., 2005b). First, asymmetric strand incorporation into the RISC determines whether an siRNA will be capable of inducing sequence-specific cleavage of its intended target strand at all, but secondly, inaccessibility of the target site can drastically diminish activity, even of efficiently loaded siRNA strands.

The highly ordered structure of the 5′ UTR of RNA viruses with stable stem-loop motifs might explain the above-mentioned difficulties in targeting this region. It might therefore be necessary to screen numerous siRNAs before identifying a highly active molecule. As an alternative, siRNAs targeting the coding region of the viral genome can be used. Mutations in structural proteins are more likely to maintain infectivity than substitutions in catalytically active enzymes that are essential for the viral life cycle. For the RNAi approaches against CVB-3 published to date, siRNAs have consequently been directed

against either the 2A protease (Merl et al., 2005; Yuan et al., 2005) or the 3D RdRP (Schubert et al., 2005b).

Since assays with infectious viruses are time-consuming, labour-intensive and have to be performed under elevated safety conditions, a simple method for the preselection of active siRNA species is desirable. In the present study, we demonstrate that fusion of subgenomic fragments of the viral genome downstream of a reporter gene such as GFP or luciferase is an appropriate method to screen for efficient siRNAs. The siRNAs isolated by this approach have been found to be suitable for virus silencing. Interestingly, the siRNA duplexes siRdRP 3 and 4 used in the present study are only slightly less active in inhibiting virus propagation at early time points than siRdRP 1 and 2, but they do not protect cells from viral lysis at late time points in a cell viability assay (Figure 2), while the latter siRNAs still maintain their antiviral effect.

Another strategy to circumvent the problem of the emergence of escape mutants upon prolonged virus inhibition by RNAi is to silence virus receptors on host cells. As an example, lentivirus-mediated delivery of siRNAs against the HIV-1 co-receptor CCR5 into human peripheral blood T-lymphocytes has been demonstrated to provide substantial protection from CCR5-tropic HIV-1 virus infection (Qin et al., 2003).

A receptor belonging to the superfamily of immunoglobulin-like surface molecules, named the coxsackievirus-adenovirus receptor (CAR), has been identified that mediates cellular entry of group B coxsackieviruses and certain adenovirus subtypes (Bergelson et al., 1997). CAR was found to be expressed in a highly variable manner during development and after myocardial infarction (Fechner et al., 2003). In particular, it is known to display a high level of expression in patients with dilated cardiomyopathy (for a review, see Poller et al., 2002). We therefore attempted to knockdown the expression of CAR by RNAi as a possible approach in treating infectious heart diseases.

Generally, use of two siRNAs targeting different sites on the mRNA is considered to be a valuable confirmation showing that the antiviral effect is indeed due to RNAi (Editorial, 2003). This independent proof is particularly important for viruses such as the coxsackievirus that are susceptible to interferon (Kühl et al., 2003), as siRNAs have been found to induce interferon response in some cases (summarised in Jackson and Linsley, 2004). We thus used two independent siRNAs to silence CAR, one of which has previously been used to analyse the interactions of CAR with other proteins in the tight junction (Coyne et al., 2004). Both siRNAs inhibited CAR expression in a concentration-dependent manner almost completely and reduced the virus titre in a plaque assay by approximately 60% (Figure 5). The failure to achieve further reduction of the virus titre by CAR-specific siRNAs might be explained by the finding that the decay accelerating factor (DAF/CD55) functions as a co-receptor for CVB-3 (Selinka et al., 2004) and it will be interesting to investigate whether the combined use of siRNAs against CAR and DAF will enhance the protective effect. Unexpectedly, the control siRNA without homology to human or coxsackieviral genes resulted in high variation between experiments. At higher concentrations it even inhibited virus propagation by an unspecific mechanism that remains to be analysed in more detail (data not shown).

Taken together, we and others have shown that RNAi is a suitable strategy for inhibition of the heart pathogenic coxsackievirus B3 in cell culture (Schubert et al., 2005a; Yuan et al., 2005) and *in vivo* (Merl et al., 2005). Since testing that was too narrow has been the cause of numerous artefacts in the traditional antisense field (Stein, 2001), we used various experimental proofs (fusion constructs, cell viability assays, virus titres, Western blots) in our present study that are concordant with each other and thus enhance the reliability of the findings. Different basic strategies have been presented that are likely to reduce the risk of the emergence of escape mutants after long-term virus inhibition by RNAi: selection of conserved and essential target regions that will not tolerate mutations, and silencing of virus receptors on the host cell. These approaches exemplified for coxsackievirus in the present study are likely to be of general applicability to all (RNA) viruses.

Materials and methods

Cell culture

Cos-7 (African green monkey kidney fibroblasts) and HeLa cells (human cervix carcinoma; Wisconsin strain, kindly provided by R. Rueckert, University of Wisconsin, USA) were grown in monolayers in Dulbecco's modified Eagle's medium (PAA laboratories, Coelbe, Germany) containing 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were cultivated at 37°C in a humidified atmosphere with 5% CO_2. FCS content was reduced to 5% for cell viability and plaque reduction assays.

GFP fusion constructs

Fusion constructs of GFP and viral subgenomic fragments were obtained by TOPO cloning into pc3.1/NT-GFP according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). We found improved GFP expression when introducing a TGA stop codon between the GFP cDNA and the viral cDNAs located downstream, which then gave a new artificial untranslated region. The CVB-3 5′ UTR ranged from nucleotide 1 to 742 (according to GenBank accession No. M33854), and RdRP ranged from nucleotide 5911 to 7297.

siRNAs and transfection

The siRNAs with two nucleotide overhangs used in the present study (Table 1) were purchased from MWG Biotech (Ebersberg, Germany) or as a SMARTpool package from Dharmacon (Lafayette, CO, USA). A negative control siRNA was obtained from Qiagen (Hilden, Germany) which has no matches either in the viral or the human genome. For transfection, cells were plated in 24-well plates at a density of 0.7×10^5 cells/well in a volume of 500 μl without antibiotics. The next day, cells were transfected with the appropriate amount of siRNA and 2.5 μl of Lipofectamine 2000 (Invitrogen) per well, following the manufacturer's instructions. Co-transfection experiments with GFP-5′ UTR and GFP-RdRP fusion construct were performed with Cos-7 cells, whereas RNAi experiments with endogenously expressed CAR were performed in HeLa cells.

Table 1 List of siRNAs used in the present study.

siRNA	Target sequence
siUTR 1	ACA GCC UGU GGG UUG AUC C
siUTR 2	GUA ACA CAC ACC GAU CAA C
siUTR 3	GGA GAA AGC GUU CGU UAU C
siUTR 4	UAC AGA CAU GGU GCG AAG A
siUTR 5	CCG ACU ACU UUG GGU GUC C
siRdRP 1	GUA CAA AAC UUU CCA CCU A
siRdRP 2	CUA AGG ACC UAA CAA AGU U
siRdRP 3	GCA CGA AUA UGA GGA GUU C
siRdRP 4	GUA CAG GGA UAA ACA UUA C
siCAR 2	GCU ACA UCG GCA GUA AUC A
siCAR 9	GGU GGA UCA AGU GAU UAU U
Control siRNA	UUC UCC GAA CGU GUC ACG U

The sequence of siCAR 9 was taken from Coyne et al. (2004).

Western blotting

At 24 h after transfection, cells were lysed in 24-well plates with lysis buffer [125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 1.4 M β-mercaptoethanol, 25% (v/v) glycerol, 0.05% (w/v) bromophenol blue]. For blotting of the CAR protein, the cells were lysed under non-reducing conditions with the same lysis buffer, except for omission of β-mercaptoethanol. After boiling the lysate at 95°C for 5 min, proteins were separated on a 12.5% (w/v) polyacrylamide gel. Transfer of proteins to PVDF membranes (GE Healthcare/Amersham, Freiburg, Germany) was performed with a semi-dry blotter (Peqlab, Erlangen, Germany). Subsequently, membranes were incubated with either rabbit GFP antiserum (1:5000; Invitrogen) or anti-CAR monoclonal antibody (clone RmcB; 0.2 μg/ml; Upstate, Charlottesville, VA, USA) overnight in dry milk at 4°C. Secondary antibodies conjugated with alkaline phosphatase (Chemicon, Temecula, CA, USA) were used at a dilution of 1:5000. Detection by chemiluminescence was achieved using CDP-Star (Roche, Mannheim, Germany). To confirm equal loading of samples, membranes were stripped and re-probed with a monoclonal mouse antibody (1:5000) specific for actin (Chemicon).

CVB3 infection and cell viability assay

HeLa cells (1.5×10^5) were plated in 96-well plates (Costar, Cambridge, MA, USA) at a density of 2.5×10^4 cells/well in minimal essential medium (MEM)-Eagle (Invitrogen/GIBCO) containing 5% FCS without antibiotics. Cell monolayers were transfected with the respective siRNA in quadruplicate. After 4 h, the transfected cells were infected with a multiplicity of infection (m.o.i.) of 0.1 plaque-forming unit (p.f.u.)/cell of the CVB-3 (Nancy, ATCC No.VR-30). At 1, 20, 44, and 68 h post-inoculation, cell viability was determined with the Cell Proliferation Kit II (Roche), following the manufacturer's instructions. The absorbance measured at 492 nM directly correlates to the cell viability.

Plaque reduction assay

HeLa cells (1.5×10^5) were seeded in 24-well plates, incubated overnight to give monolayers and transfected with siRNAs as described above in duplicate. At 4 h after transfection, cells were infected with CVB-3 in 10-fold dilutions, resulting in a m.o.i. of 10–50 p.f.u./well, for 30 min according to Dulbecco (1952). Subsequently, cells were overlaid with agar containing Eagle's MEM. After 3 days of incubation at 37°C, cells were stained with 0.025% (w/v) neutral red. Virus titres (p.f.u./ml) were determined by plaque counting. To investigate the antiviral effect of siRNAs against CAR, cells were transfected with siRNA 3 days prior to virus infection to allow complete degradation of the receptor.

Values given are means and standard deviation of at least two independent experiments, each performed in duplicate.

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References

Amarzguioui, M. and Prydz, H. (2004). An algorithm for selection of functional siRNA sequences. Biochem. Biophys. Res. Commun. *316*, 1050–1058.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horowitz, M.S., Crowell, R.L., and Finberg, R.W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science *275*, 1320–1323.

Boden, D., Pusch, O., Lee, F., Tucker, L., and Ramratnam, B. (2003). Human immunodeficiency virus type 1 escapes from RNA interference. J. Virol. *77*, 11531–11535.

Bohula, E.A., Salisbury, A.J., Sohail, M., Playford, M.P., Riedemann, J., Southern, E.M., and Macaulay, V.M. (2003). The efficacy of small interfering RNAs targeted to the type 1 insulin-like growth factor receptor (IGF1R) is influenced by secondary structure in the IGFR1 transcript. J. Biol. Chem. *278*, 15991–15997.

Colbère-Garapin, J., Blondel, B., Saulnier, A., Pelletier, I., and Labadie, K. (2005). Silencing viruses by RNA interference. Microbes Infect. *7*, 767–775.

Coyne, C.B. and Bergelson, J.M. (2005). CAR: a virus receptor within the tight junction. Adv. Drug Deliv. Rev. *57*, 869–882.

Coyne, C.B., Voelker, T., Pichla, S.L., and Bergelson, J.M. (2004). The coxsackievirus and adenovirus receptor interacts with the multi-PDZ domain protein-1 (MUPP-1) within the tight junction. J. Biol. Chem. *279*, 48079–48084.

Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R., and Berkhout, B. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol. *78*, 2601–2605.

Dulbecco, R. (1952). Production of plaques in monolayer tissue cultures by single particles animal virus. Proc. Natl. Acad. Sci. USA *38*, 747–752.

Editorial (2003). Whither RNAi? Nat. Cell Biol. *5*, 489–490.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Fechner, H., Noutsias, M., Tschoepe, C., Hinze, K., Wang, X., Escher, F., Pauschinger, M., Dekkers, D., Vetter, R., Paul, M., et al. (2003). Induction of coxsackievirus-adenovirus-receptor expression during myocardial tissue formation and remodeling: identification of a cell-to-cell contact-dependent regulatory mechanism. Circulation *107*, 876–882.

Fire, A., Xu, S.Q., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.

Gitlin, L., Karelsky, S., and Andino, R. (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. Nature *418*, 430–434.

Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V.A., and Kurreck, J. (2003). Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and siRNA. Nucleic Acids Res. *31*, 3185–3193.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., and Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. *30*, 1757–1766.

Jackson, A.L. and Linsley, P.S. (2004). Noise amidst the silence: off-target effects of siRNAs? Trends Genet. *20*, 521–524.

Joost Haasnoot, P.C., Cupac, D., and Berkhout, B. (2003). Inhibition of virus replication by RNA interference. J. Biomed. Sci. *10*, 607–616.

Kawasaki, H., Suyama, E., Iyo, M., and Taira, K. (2003). siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. Nucleic Acids Res. *31*, 981–987.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNA and miRNA exhibit strand bias. Cell *115*, 209–216.

Kretschmer-Kazemi Far, R. and Sczakiel, G. (2003). The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. Nucleic Acids Res. *31*, 4417–4424.

Kühl, U., Pauschinger, M., Schwimmbeck, P.L., Seeberg, B., Lober, C., Noutsias, M., Poller, W., and Schultheiss, H.-P. (2003). Interferon-β treatment eliminates cardiotropic viruses and improves left ventricular function in patients with myocardial persistence of viral genomes and left ventricular dysfunction. Circulation *107*, 2793–2798.

Luo, K.Q. and Chang, D.C. (2004). The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. Biochem. Biophys. Res. Commun. *318*, 303–310.

Martinand-Mari, C., Lebleu, B., and Robbins, I. (2003). Oligonucleotide-based strategies to inhibit human hepatitis C virus. Oligonucleotides *13*, 539–548.

Meister, G. and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343–349.

Merl, S., Michaelis, C., Jaschke, B., Vorpahl, M., Seidl, S., and Wessely, R. (2005). Targeting 2A protease by RNA interference attenuates coxsackieviral cytopathogenicity and promotes survival in highly susceptible mice. Circulation *111*, 1583–1592.

Overhoff, M., Alken, M., Far, R.K., Lemaitre, M., Lebleu, B., Sczakiel, G., and Robbins, I. (2005). Local RNA target structure influences siRNA efficacy: a systematic global analysis. J. Mol. Biol. *348*, 871–881.

Philipson, L. and Petterson, R.F. (2004). The coxsackie-adenovirus receptor – a new receptor in the immunoglobulin family involved in cell adhesion. Curr. Top. Microbiol. Immunol. *273*, 87–111.

Phipps, K.M., Martinez, A., Lu, J., Heinz, B.A., and Zhao, G. (2004). Small interfering RNA molecules as potential anti-human rhinovirus agents: *in vitro* potency, specificity and mechanism. Antiviral Res. *61*, 49–55.

Poller, W., Fechner, H., Noutsias, M., Tschoepe, C., and Schultheiss, H.-P. (2002). Highly variable expression of virus receptors in the human cardiovascular system. Z. Kardiol. *91*, 978–991.

Poller, W., Fechner, H., Kurreck, J., Pauschinger, M., Kühl, U., Erdmann, V.A., Lamers, J.M.J., and Schultheiss, H.-P. (2004). Nucleic acid-based modulation of cardiac gene expression for the treatment of cardiac diseases. Z. Kardiol. *93*, 171–193.

Qin, X.-F., An, D.S., Chen, I.S.Y., and Baltimore, D. (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. USA *100*, 183–188.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., and Khvorova, A. (2004). Rational siRNA design for RNA interference. Nat. Biotechnol. *22*, 326–330.

Ryther, R.C.C., Flynt, A.S., Phillips, J.A., and Patton, J.G. (2005). siRNA therapeutics: big potential from small RNAs. Gene Ther. *12*, 5–11.

Schubert, S. and Kurreck, J. (2005). Oligonucleotide-based antiviral strategies. In: RNA Towards Medicine, V.A. Erdmann, J. Brosius and J. Barciszewski, eds. (Berlin/Heidelberg, Germany: Springer-Verlag), in press.

Schubert, S., Grunert, H.-P., Zeichhardt, H., Werk, D., Erdmann, V.A., and Kurreck, J. (2005a). Maintaining inhibition: siRNA double expression vectors against coxsackieviral RNAs. J. Mol. Biol. *346*, 457–465.

Schubert, S., Grünweller, A., Erdmann, V.A., and Kurreck, J. (2005b). Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. J. Mol. Biol. *348*, 883–893.

Selinka, H.-C., Wolde, A., Sauter, M., Kandolf, R., and Klingel, K. (2004). Virus-receptor interactions of coxsackie B viruses and their putative influence on cardiotropism. Med. Miocrobiol. Immunol. *193*, 127–131.

Shankar, P., Manjunath, N., and Lieberman, J. (2005). The prospect of silencing disease using RNA interference. J. Am. Med. Assoc. *293*, 1367–1373.

Stein, C.A. (2001). The experimental use of antisense oligonucleotides: a guide for the perplexed. J. Clin. Invest. *108*, 641–644.

Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., and Saigo, K. (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. *32*, 936–948.

Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., and Baker, B.F. (2003). Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. J. Biol. Chem. *278*, 7108–7118.

Wilson, J.A., Jayasena, S., Khvorova, A., Sabatinos, S., Rodrigue-Gervais, I.G., Arya, S., Sarangi, F., Harris-Brandts, M., Beaulieu, S., and Richardson, C.D. (2003). RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc. Natl. Acad. Sci. USA *100*, 2783–2788.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M., and Mizusawa, H. (2003). Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. *4*, 602–608.

Yuan, J., Cheung, P.K.M., Zhang, H.M., Chau, D., and Yang, D. (2005). Inhibition of Coxsackievirus B3 replication by small interfering RNAs requires perfect sequence match in the central region of viral positive strand. J. Virol. *79*, 2151–2159.

Zeichhardt, H., and Grunert, H.-P. (2003). Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses and Enteroviruses 68–71. In: Infectious Diseases, 2nd Ed., J. Cohen, W.G. Powderly and S.M. Opal, eds. (London, UK: Elsevier Health Sciences).

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