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Antiviral activity of highly potent siRNAs against echovirus 30 and its receptor

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RNA interference (RNAi) has been shown to be suitable to inhibit viruses in experimental setups and is considered a promising antiviral strategy that is currently being tested in various clinical trials. The present study provides an approach to design siRNAs with high potency against a virus-specific target gene. In recent years, several outbreaks of aseptic meningitis caused by an echovirus 30 (EV-30) infection have been described. Based on an initial set of 30 *in silico* designed siRNAs, six siRNAs targeting the 3D RNA-dependent RNA-Polymerase ($3D^{Pol}$) of EV-30 were selected. All but one of them showed high efficiency in both, reporter and virus assays. A second aim of the study was to re-investigate the relevance of the decay-accelerating factor (DAF, also known as CD55) as cellular entry receptor of EV-30 by means of RNAi, a question which had been under debate in previous studies. Knockdown of DAF inhibited drastically infection by EV-30 indicating that DAF plays an important role either as an attachment factor or as a receptor.

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1. Introduction

RNA interference (RNAi) is a post-transcriptional silencing process triggered by double stranded (ds)RNA (Fire et al., 1998). Since long dsRNA induces an interferon response in mammals (Stark et al., 1998), small interfering RNA (siRNA) molecules shorter than 30 nucleotides are usually employed to inhibit target genes in mammalian cells (Elbashir et al., 2001). siRNA duplex molecules synthesized chemically are typically 21 nucleotides (nt) long with 2-nt overhangs at their 3′ ends. A protein complex referred to as RNA-induced silencing complex (RISC) incorporates the antisense or guide strand of the siRNA, whereas the complementary sense or passenger strand is degraded. Activated RISC then binds to the target mRNA in a sequence-specific manner and induces its cleavage by the Argonaute 2 (AGO2) protein. The target mRNA is further degraded, while RISC is recycled for several rounds of cleavage (for current reviews see Kim and Rossi, 2007; Rana, 2007).

In principle, both, sense and antisense strand, are capable of serving as the guide strand. However, two independent studies showed an asymmetric preference for RISC incorporation of the two siRNA strands (Khvorova et al., 2003; Schwarz et al., 2003), a finding that has been confirmed by several other groups (Amarzguioui and Prydz, 2004; Ui-Tei et al., 2004). The strand with lower relative thermodynamic stability at its 5′ end is incorporated preferentially into RISC to serve as the guide strand. Based on these studies, a scoring system for the design of efficient siRNAs was developed (Reynolds et al., 2004). Currently, most of the available siRNA design tools use these (or similar) criteria. In addition, structural properties of the target RNA were found to influence the siRNA efficiency (Luo and Chang, 2004; Overhoff et al., 2005; Schubert et al., 2005b). According to these studies, accessible target sites are a prerequisite for efficient RNAi-mediated inhibition of gene expression. Furthermore, the secondary structure of the guide strand was reported to influence its efficiency (Patzel et al., 2005). Stable stem-loop structures decreased the potency, whereas siRNAs with antisense strands that do not form any structure were very efficient for silencing their target gene.

RNAi has not only become an important research tool within a few years, but is also considered to offer new therapeutic options. Among other applications, RNAi has been found to inhibit efficiently viruses (Fabani et al., 2006; Haasnoot et al., 2007) and clinical trials to treat infections with the respiratory syncytial virus, the human immunodeficiency virus and the hepatitis B virus have been initiated (Haussecker, 2008).

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Enteroviruses are members of the picornavirus family with a single-stranded RNA genome (~7200–8500 bases) in positive orientation (Racaniello, 2007). The 5′ UTR of the genome contains an Internal Ribosome Entry Site (IRES) for cap-independent initiation of translation. The RNA-dependent RNA polymerase (RdRP or $3D^{Pol}$), which is one of the most conserved polypeptides throughout the picornavirus family (Whitton et al., 2005), is required for replication of the RNA virus genome. Echoviruses (enteric human orphan virus) are a large group within the enterovirus genus which are responsible for a broad variety of diseases including aseptic meningitis (Lee and Davies, 2007), neonatal carditis, encephalitis, hepatitis, and diseases of the upper respiratory tract (Barnard, 2006). Recently, EV-30 was reported to be the most frequent serotype isolated from samples of patients from aseptic meningitis outbreaks in France and Spain (Brunel et al., 2008; Cabrerizo et al., 2008), but it has also been identified as the prevalent serotype for this disease in East Asia in certain years (Tseng et al., 2007).

Since current prospects for treatment are limited, RNAi has been explored as a new approach to inhibit enteroviruses. Several groups have employed siRNAs to inhibit poliovirus (Gitlin et al., 2005; Saulnier et al., 2006), coxsackievirus B3 (Ahn et al., 2005; Merl et al., 2005; Schubert et al., 2005a; Yuan et al., 2005), and enterovirus 71 (Lu et al., 2004; Tan et al., 2007). An siRNA against the highly conserved cis replication element was found to inhibit various coxsackieviruses as well as echoviruses 6 and 7 (Lee et al., 2007). An alternative to direct targeting of the viral RNA is to inhibit expression of host factors, which are essential for the virus. Several studies have shown that silencing of the coxsackievirus–adenovirus receptor prevents spread of coxsackievirus B3 (Coyne and Bergelson, 2006; Fechner et al., 2007; Werk et al., 2005).

To the best of our knowledge, RNAi-approaches against EV-30 have not been reported so far. While pre-designed or even validated siRNAs with high silencing efficiency are available for most cellular targets, the design of siRNAs against viruses still remains a challenging task. The present study describes an approach including thermodynamic features of the siRNA, structure of the guide strand and accessibility of the target site to obtain active siRNAs with high antiviral activity. Furthermore, the question is addressed, which receptor is primarily used by EV-30, a topic that has not been unequivocally determined. For entry into a host cell, many echoviruses use the decay-accelerating factor (DAF/CD55) (Bergelson et al., 1994), but the $\alpha_2\beta_1$-Integrin (VLA-2) was found to be important for some echovirus serotypes as well (for an overview, see Racaniello, 2007). Host cell receptors for other echoviruses, however, remain to be identified. Previous research with monoclonal antibodies against DAF and soluble variants of DAF did not yield a clear picture of the main entry receptor of EV-30. This virus is considered to bind to DAF, but to recognize an alternative cellular receptor with higher affinity (Powell et al., 1998). RNAi is a new and rapid method to address this question and the present study shows that silencing of DAF drastically inhibits EV-30 infection of Rhabdomyosarcoma (RD) cells.

2. Materials and methods

2.1. Cell culture

RD (Rhabdomyosarcoma) cells and HEK293 (human embryonic kidney) cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, non-essential amino acids, and the antibiotics penicillin and streptomycin. FCS content was reduced to 5% for the cell viability and virus propagation assay on RD cells.

2.2. Cloning of echovirus 30 $3D^{Pol}$ and DAF

The sequence of the EV-30 $3D^{Pol}$ was defined by alignment with the homologous sequence of EV7. The cDNA of $3D^{Pol}$ was obtained by reverse transcription and PCR of viral RNA using the PCR primers *TGA* GGT GAA ATC GAA TTC ATT and AAA AGA GTC TAG CCA CTT CC, corresponding to bases 5952–5969 and 7337–7318 of the EV-30 sequence, respectively (GenBank accession no. AF311938).

The coding sequence of DAF was obtained by reverse transcription and PCR of HeLa RNA using the PCR primers *TGA* ATG ACC GTC GCG CGG CCG and CTA AGT CAG CAA GCC CAT GG, corresponding to bases 247–264 and 1392–1373 of the human DAF (CD55) mRNA sequence, respectively (GenBank accession no. NM_000574).

The bases in italics represent an added translational stop codon upstream of $3D^{Pol}$ or DAF that resulted in improved GFP expression.

2.3. siRNAs and oligonucleotides

siRNA duplexes against EV-30 with symmetrical two nucleotide overhangs were purchased from MWG Biotech (Ebersberg, Germany). The siRNAs targeting DAF were a Dharmacon SMARTpool package (Thermo Fisher Scientific, Lafayette, CO, USA, Catalog # MQ-004573-01). Design of the siRNAs follows the company's hidden algorithm. A negative control siRNA with no matches either in the viral or human genome was obtained from Qiagen (Hilden, Germany). DNA oligonucleotides for PCR and cloning procedures were purchased from Tib Molbiol (Berlin, Germany).

2.4. Transfection protocols

Transient transfection of RD cells with the target-plasmid and siRNA was performed in 24 well plates using the Lipofectamine™ 2000 transfection reagent (Invitrogen). Each well was seeded with 1.5×10^5 RD cells in a volume of 500 μl DMEM (supplemented with 10% FCS, but without antibiotics) and transfected 1 day after seeding by the following procedure: 0.8 μg of the plasmid DNA and the indicated amount of siRNA (the indicated molar concentrations are related to the final volume of 600 μl per well) were diluted in Opti-MEM (Invitrogen) to a volume of 50 μl. Subsequently, 2 μl Lipofectamine transfection reagent were premixed with 48 μl Opti-MEM, and incubated for 5 min at room temperature. Finally, the Lipofectamine and the DNA/siRNA mixtures were combined, incubated for at least 20 min at room temperature, and added to the cells.

Transfection of RD cells and HEK293 cells with siRNAs for the virus propagation assay was performed as described for transient transfection, but without transfection of target plasmid.

2.5. Western blot

24 h after transfection, cells were harvested with lysis buffer [62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 50 mM DTT, 10% (v/v) glycerol, and 0.01% (w/v) bromophenol blue]. After boiling the lysate at 95 °C for 5 min, proteins were separated on a 15% (w/v) SDS-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 rabbit GFP antiserum (1:5000, Invitrogen, Karlsruhe, Germany) diluted in TTBS overnight at 4 °C. Secondary antibodies conjugated with horseradish peroxidase (Pierce, IL, USA) were used at a dilution of 1:5000. Chemiluminescent signals were detected with the ECL Western Blotting substrate from Pierce (IL, USA). To confirm equal loading of the samples membranes were stripped and re-probed with a monoclonal mouse antibody specific for actin (Chemicon, Temecula, CA, USA) at a dilu-

Table 1
List of siRNAs used in the present study. The siRNAs consisted of unmodified RNA nucleotides (except for the deoxythymidine overhangs as indicated).

siRNA	Sequence 5′–3′	Position
siRdRP1	S: AUU AGC AAC UCU CGA UAU C [TT] AS: GAU AUC GAG AGU UGC UAA U [TT]	6203–6221
siRdRP2	S: ACU UGA GGA UGC AGU AUA C [TT] AS: GUA UAC UGC AUC CUC AAG U [TT]	6239–6257
siRdRP3	S: AAA GAC CUG GCG AAA UUG A [TT] AS: UCA AUU UCG CCA GGU CUU U [TT]	6357–6375
siRdRP4	S: AAA GGC UAU GGU UUG AUA A [TT] AS: UUA UCA AAC CAU AGC CUU U [TT]	6996–7014
siRdRP5	S: UAA GGA GAC UAA CUA CAU A [TT] AS: UAU GUA GUU AGU CUC CUU A [TT]	6731–6749
siRdRP6	S: GCU UAU GGU GAU GAC GUU A [TT] AS: UAA CGU CAU CAC CAU AAG C [TT]	6927–6945
siDAF2	S: GAA GAG UUC UGC AAU CGU A [NN][a] AS: UAC GAU UGC AGA ACU CUU C [NN][a]	517–535
siDAF3	S: UAA CAC AGG GUA CAA AUU A [NN][a] AS: UAA UUU GUA CCC UGU GUU A [NN][a]	816–834
siDAF5	S: GGC AGU CAA UGG UCA GAU A [NN][a] AS: UAU CUG ACC AUU GAC UGC C [NN][a]	496–514
siDAF6	S: AAU ACG AAA UGG UCA GAU U [NN][a] AS: AAU CUG ACC AUU UCG UAU U [NN][a]	750–768
Control siRNA	S: UUC UCC GAA CGU GUC ACG U [TT] AS: ACG UGA CAC GUU CGG AGA A [TT]	

S: sense strand of siRNA; AS: antisense strand of siRNA; Position: position within the target sequence.

[a] The SMARTpool package from Dharmacon consisted of siRNAs against DAF with UU (RNA) overhangs. These siRNAs were used for initial screening experiments. Reordered siRNAs with TT (DNA) overhangs were used for all subsequent experiments. No differences were observed for the different types of overhangs.

tion of 1:5000. Quantitation of Western Blots was carried out with Quantity One software (Biorad, Munich, Germany). Values of three independent experiments are given as the mean ± the standard deviation (SD).

2.6. FACS analysis

RD cells were seeded in 12 well plates at a density of 1.75×10^5 cells per well in a volume of 1 ml medium. The next day, cells were transfected with 100 nM of respective siRNAs targeting DAF (Table 1). Cells were harvested 3 days later with 0.2% EDTA in PBS and immunolabelled with 0.1 μg of the specific antibody CBL-511 (Chemicon, CA, USA), diluted in PBS with 3% BSA, for at least 12 h at 4 °C. Cells were then washed three times with ice-cold PBS and incubated with 1 μg of detection antibody FITC goat anti mouse (Upstate, VA, USA) for at least 30 min at room temperature. Cells were washed again and resuspended in PBS with 3% BSA prior to analysis by FACS scan (Beckmann Coulter Epics XL MCL, Krefeld, Germany). Data were evaluated with WINMDI 2.9.

2.7. Cell viability assay

RD cells were seeded in 96 well plates at a density of 3×10^4 cells per well in a volume of 100 μl medium without antibiotics. The next day, cells were transfected with 100 nM of the respective siRNA in quadruplicate. 4 h after transfection, cells were infected with EV-30 (Bastianni, ATCC no. VR-322) at a multiplicity of infection (m.o.i.) of 0.1 plaque forming units (p.f.u.)/cell. At the indicated time points post infection (h.p.i.), cell viability was determined with the Cell Proliferation Kit II (Roche, Mannheim, Germany), following the manufacturer's instructions. The absorbance measured at 492 nm directly correlates to the cell viability. RNAi experiments, which involved the transfection of siRNAs targeting DAF, were carried out under modified conditions: RD cells were seeded at a density of 1×10^4 cells per well in a volume of 100 μl medium. After transfection, cells were incubated for at least 48 h before infection.

2.8. In silico siRNA design and RNA structure analysis

The following, freely available siRNA design tools were used: MWG Biotech (http://www.mwg-biotech.com), Dharmacon (www.dharmacon.com) and Invitrogen (www.invitrogen.com). Secondary structures of the antisense strand of siRNAs as well as of the target mRNAs were calculated by using mfold 3.2 (Zuker, 2003) which is available at: http://www.bioinfo.rpi.edu/applications/mfold (as of September 2007).

2.9. Virus propagation assay

For transfection, RD cells were seeded into 24 well plates at a density of 8.75×10^4 cells per well in a volume of 500 μl medium containing 5% FCS. HEK293 cells were seeded into 24 well plates at a density of 1.25×10^5 cells per well in a volume of 500 μl medium containing 10% FCS. The next day, cells were transfected with 100 nM of siDAF2, control siRNA (ctr siRNA) or without siRNA (Lipo) as described in 2.4. After 3 days of incubation, cells were infected with EV-30 at a multiplicity of infection of 0.1 p.f.u./cell. Viral replication was stopped 1 day later by subjecting the cells to 3 freeze–thaw cycles.

For determination of EV-30 yield in infected cells, samples were titrated on confluent cells. To this end, RD cells were seeded into 24 well plates at a density of 1.25×10^5 cells per well in a volume of 500 μl medium containing 5% FCS. HEK293 cells were seeded into 12 well plates at a density of 5×10^5 cells per well in a volume of 1 ml medium containing 10% FCS. The next day, cells were infected with serial tenfold dilutions of the virus-cell samples in medium without FCS. After incubation for 30 min at 37 °C, supernatants were removed and cells were overlaid with 500 μl (1 ml for HEK293 cells) of Eagle's medium containing 0.73% agarose. After 2 days, cells were fixed with 10% trichloroacetic acid for 10 min at room temperature. Subsequently, the overlay was removed and cells were stained with 0.5% crystal violet for 5 min at room temperature. Finally, plates were washed with phosphate-buffered saline (PBS) and dried prior to photography or plaque counting.

3. Results

3.1. Identification of potent siRNAs targeting the echovirus $3D^{Pol}$ gene

The design of efficient siRNAs against viruses still remains a challenging task. Different features are considered to be relevant for the activity of siRNAs. While some studies suggest that properties of siRNA itself determine its RNAi efficiency, others have found a correlation between the accessibility of the target RNA structure and extent of silencing (this controversy is summarized in Kurreck, 2006). The present study provides a straightforward approach that combines various criteria to identify efficient siRNAs against EV-30 (Fig. 1).

As a first step, three freely available Internet design tools (from Invitrogen, Dharmacon and MWG Biotech) were employed to obtain a set of 30 siRNAs (10 siRNA per tool) targeting the $3D^{Pol}$ of EV-30. All siRNAs were assessed according to a scoring system, which takes into account the relative thermal stability of both ends as well as base preferences of active siRNAs in certain positions (Reynolds et al., 2004). All siRNAs designed with the tools provided by Dharmacon and MWG had a score of at least six points, which is considered

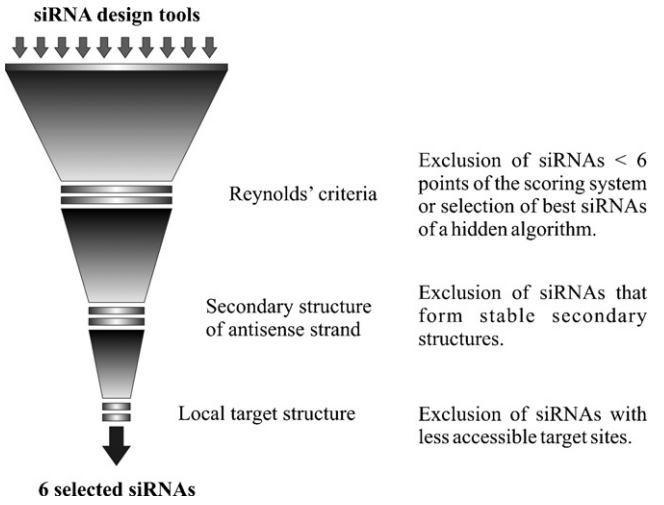

Fig. 1. Systematic selection of siRNAs against the 3D^{Pol} of echovirus 30. As a first step, siRNAs were scored according to a system based on the thermodynamic features of the siRNA (Reynolds et al., 2004). siRNAs with guide strands that will form stable structures were excluded. Finally, the accessibility of the target site in the viral RNA was tested.

the threshold for active siRNAs. The Invitrogen design tool uses a different, hidden algorithm as can be concluded from the results obtained with it.

Since structured guide strands were reported to have a reduced silencing activity (Patzel et al., 2005), the antisense strand of each of the proposed siRNAs was analysed by mfold 3.2 (Zuker, 2003) and $\Delta G_{antisense}$ was determined. All siRNAs whose antisense strands were predicted to form secondary structures (negative $\Delta G_{antisense}$) were excluded.

A further round of selection took properties of the target RNA into consideration. The complete RNA sequence of the EV-30 3D^{Pol} was folded by mfold and the local free energy (ΔG_{local}) of each target site motif was determined by using the "Free energy determination tool" of mfold 3.2. Low absolute ΔG_{local}-values indicate accessible target sites (Schubert et al., 2005b; Westerhout and Berkhout, 2007) and were therefore preferred. Based on the three-step selection scheme, six siRNAs named siRdRP1–6 against the 3D^{Pol} of echovirus 30 were identified which have a high theoretical chance of good antiviral activity (the sequences are assembled in Table 1; properties of the siRNAs are summarized in Table 2).

For an initial evaluation, the six selected siRNAs were tested in a GFP reporter assay. To this end the cDNA of 3D^{Pol} of echovirus 30 was cloned downstream of the GFP reporter gene (Fig. 2a). For improved GFP expression, a stop codon was introduced upstream of the 3D^{Pol} cDNA. Since RNAi results in complete degradation of the target RNA following the initial cleavage step, this construct still assures that successful targeting of the gene-of-interest will reduce expression of the reporter.

Table 2
Characteristics of selected siRNAs targeting the 3D^{Pol} of echovirus 30.

siRNA	Tool	Score (Reynolds)	$\Delta G_{antisense}$ (kcal/mol)	ΔG_{local} (kcal/mol)	Inhibition	
					Reporter	Virus
siRdRP1	M	6	+1.6	−5.21	48%	23%
siRdRP2	M	6	+0.9	−6.61	90%	66%
siRdRP3	D	7	+1.6	−1.4	89%	56%
siRdRP4	D	7	+2.3	−4.9	94%	83%
siRdRP5	D	8	+2.2	−23.3	72%	82%
siRdRP6	I	4	+2.2	−3.3	90%	86%

M: MWG Biotech; D: Dharmacon; I: Invitrogen. Inhibition of echovirus 30 was determined at 43 h.p.i.

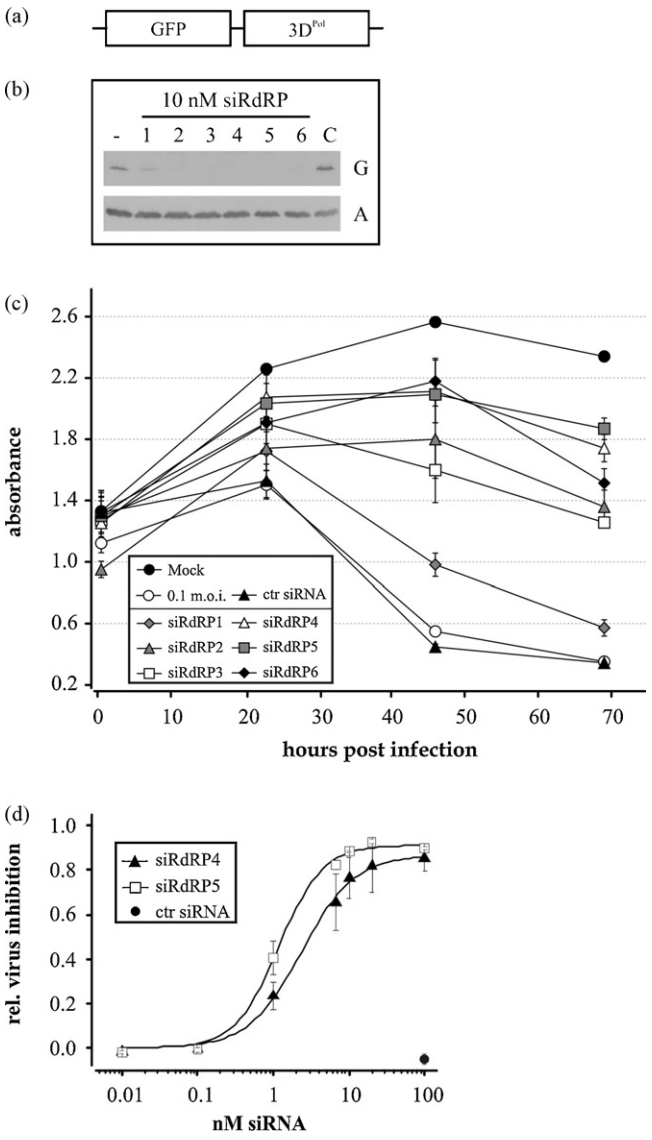

Fig. 2. Antiviral activity of siRNAs against EV-30 3D^{Pol}. (a) Test in a reporter assay. The viral sequence encoding the 3D^{Pol} was cloned downstream of GFP. Both open reading frames were separated by a stop codon to improve expression of the reporter. (b) Western Blot of GFP reporter. RD cells were transfected with the GFP-3D^{Pol} plasmid and siRNAs targeting the 3D^{Pol} of echovirus 30. Decreased GFP expression (G) indicates degradation of 3D^{Pol}. Actin bands (A) are shown as a loading control. C: cells transfected with control siRNA; -: untransfected cells. (c) Viability of EV-30 infected RD cells treated with virus-specific siRNAs. RD cells were transfected with 100 nM siRNA targeting the 3D^{Pol} of EV-30 and subsequently inoculated with 0.1 m.o.i. of the virus. Absorbance measured at 492 nm directly correlates to the cell viability. Mock: untransfected and uninfected cells; ctr siRNA: control siRNA. (d) Dose–response curve of siRNAs Nos. 4 and 5. Cells were transfected with increasing concentrations of siRNAs prior to inoculation with 0.1 m.o.i. of EV-30. The value for the control siRNA at the highest concentration used is shown for comparison. Cell viability was determined and normalized to the viability of uninfected cells. Averages and standard deviations of two independent experiments each performed in quadruplicate are shown.

Reporter constructs were co-transfected with each of the six siRNAs (at a concentration of 10 nM). As shown in the Western blot (Fig. 2b) all siRNAs targeting the 3D^{Pol} significantly reduced GFP expression, while the control siRNA had no effect. Five of the siRNAs (siRdRP2–6) reduced GFP expression virtually to completion, whereas siRdRP1 only lead to partial inhibition of target gene expression. Densitometric analysis of blots from three independent experiments revealed approximately 90% reduction in GFP level for siRdRPs no. 2, 3, 4, and 6; 72% reduction for no. 5 and ~50% inhibition for no. 1.

The crucial test is the evaluation of the antiviral activity of these siRNAs in assays with infectious virus. Therefore, the efficiency of the siRNAs to inhibit propagation of EV-30 was investigated with RD cells which were transfected with 100 nM of either siRNA and inoculated with 0.1 m.o.i. of EV-30 4 h later. Cell viability was determined at 0.5, 23, 46 and 69 h post infection (h.p.i.). Infected, untreated cells were rapidly lysed by the virus, resulting in a drastic decrease of cell viability after 2 days (Fig. 2c). Cells treated with control siRNA behaved the same as untreated cells and were lysed by the virus. In contrast, virus-specific siRNAs had a significant protective effect and maintained cell viability for up to almost 90% 46 h.p.i. Interestingly, a good correlation was observed between the reporter assay and the cell viability assay with the infectious virus. Five of the siRNAs, siRdRP2–6, were potent inhibitors of GFP-3D^{Pol} expression and had a pronounced antiviral activity; in contrast, siRdRP1 was not only less active in the reporter assay, but also had a significantly lower protective effect on cell lysis by EV-30 as well.

To have a more quantitative measure, dose–response experiments were performed for the most effective siRdRPs 4 and 5. As can be seen in Fig. 2d, both siRNAs inhibit virus replication in a dose-dependent manner. IC_{50}-values, defined as the concentration of half-maximal inhibition of cell lysis by the virus, of 2.35 ± 0.25 nM and 1.18 ± 0.07 nM were obtained from sigmoidal fits for siRdRP4 and 5, respectively. The control siRNA does not exert any antiviral activity even at the highest concentration used. The characteristics of the selected siRNAs and their inhibitory effects in GFP reporter assays as well as in virus assays are summarized in Table 2.

3.2. Downregulation of the receptor DAF

Viruses can escape long-term RNAi-mediated silencing by enriching escape mutants. A single nucleotide mutation in the centre of the target site can be sufficient for a virus to become resistant against the respective siRNA (Boden et al., 2003; Gitlin et al., 2005; von Eije et al., 2008). In order to prevent the emergence of escape mutants, host factors essential for the viral life cycle can be targeted and silenced instead. It is, for example, highly unlikely that a virus will circumvent inhibition of its receptor in the host cell by adapting to a new entry route. For various enteroviruses, DAF was shown to be the major entry receptor (Bergelson et al., 1994), but it is not quite clear whether this is true for EV-30 as well (Powell et al., 1998).

To address this question, a set of four pre-designed siRNAs (SMARTpool package from Dharmacon) directed against DAF (named siDAF 2, 3, 5 and 6) was used. In analogy to the experiments described above, the efficiency of these siRNAs to inhibit DAF was first tested in a GFP-reporter assay. Western blot analysis confirmed a high potency of all siRNAs as indicated by a strong decrease of GFP expression as compared to cells transfected with control siRNAs (Fig. 3a).

To investigate the downregulation of endogenously expressed DAF, FACS analysis was performed on RD cells which were transfected with 100 nM of each siRNA of the SMARTpool. With this method, a substantial variation in the activity of the siRNAs was observed. siDAF2 and 5 reduced the DAF expression to approximately 30% and 40%, respectively (Fig. 3b), whereas siDAF3 and 6 showed moderate efficiency of inhibiting the endogenous expression of DAF.

3.3. Virus inhibition by knockdown of DAF

The next aim was to investigate whether silencing of DAF prevents spread of EV-30 in RD cells. Cells were transfected with either of the siRNAs targeting DAF and inoculated with EV-30 after 3 days. Inhibition of DAF expression resulted in a significant protection of the cells against EV-30 as shown by cell proliferation assays (Fig. 4a).

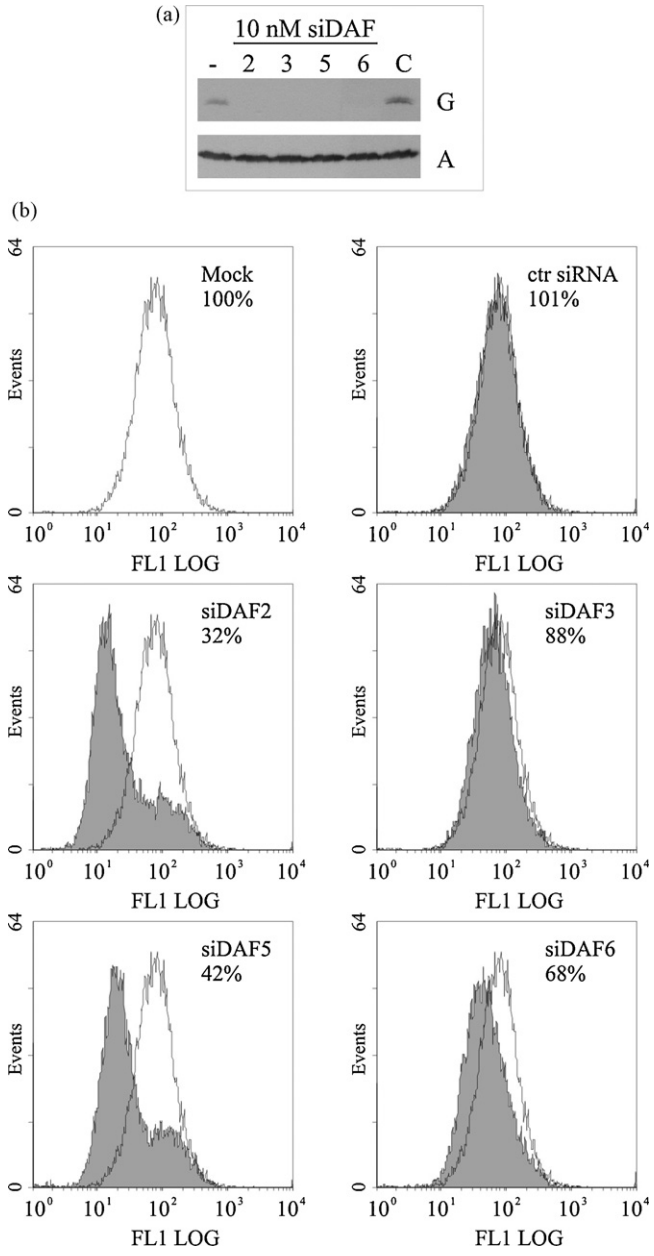

Fig. 3. Knockdown of DAF. (a) For initial experiments, DAF was fused to a GFP reporter as described for the viral 3D^{Pol}. RD cells were co-transfected with the GFP-DAF plasmid and siRNAs targeting DAF. Decreased GFP expression (G) indicates degradation of DAF. Actin bands (A) are shown as a loading control. C: cells transfected with control siRNA; -: untransfected cells. (b) FACS analysis of downregulation of endogenously expressed DAF in RD cells. Transparent curve: DAF expression in untransfected cells; filled curve: siRNA transfected cells.

The protective effect qualitatively correlated with the knockdown activity of the siRNAs. siDAF2, which was the most efficient siRNA, resulted in 70% silencing of the expression of the target gene and maintained high cell viability up to 3 days. The second best siRNA, siDAF5, initially had a comparable effect, but cell viability started to decrease drastically on day two. The two siRNAs which reduced DAF expression by less than 50% (siDAF3 and 6) had only a very weak effect on cell viability.

In order to investigate the effect of DAF knockdown on EV-30 replication more directly, virus propagation assays were carried out. RD cells were pre-treated with a control siRNA or siDAF2 and infected with EV-30 3 days thereafter. Although EV-30 does not form countable plaques, Fig. 4b clearly shows an approximately

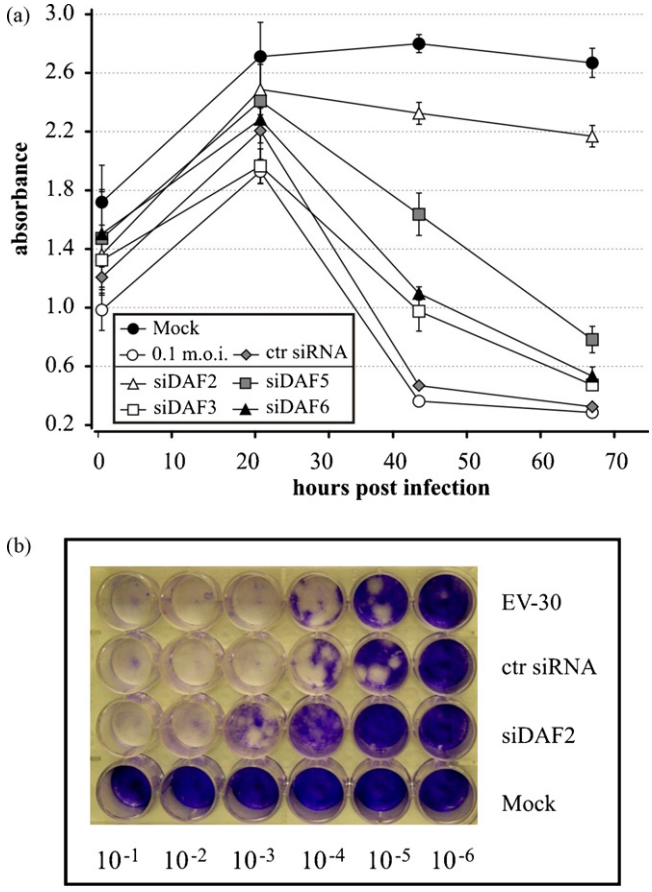

Fig. 4. Antiviral activity of siRNAs against DAF. (a) Viability of EV-30 infected RD cells treated with siRNAs against DAF. RD cells were transfected with 100 nM siRNA targeting DAF and subsequently inoculated with 0.1 m.o.i. of EV-30. Absorbance measured at 492 nm directly correlates to the cell viability. (b) Virus propagation assay of EV-30 on RD cells. Monolayers were infected with 10-fold dilutions of cell lysates, which were treated with 100 nM of the control siRNA and the DAF siRNA, respectively (lanes 2 and 3), prior to infection with 0.1 m.o.i. of EV-30. Mock: untransfected and uninfected cells; ctr siRNA: control siRNA.

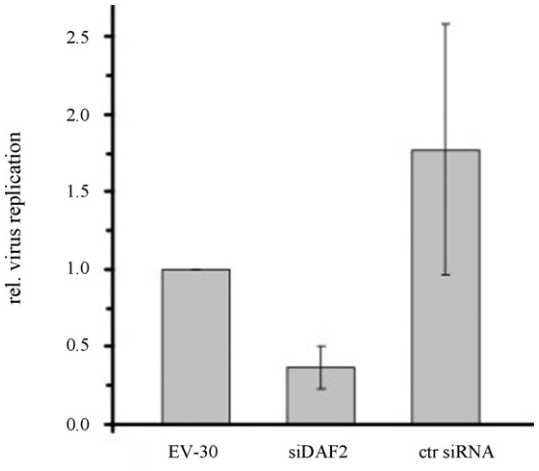

Fig. 5. Effect of DAF knockdown on EV-30 replication in HEK293 cells. Cells were transfected with 100 nM siDAF2 and subsequently infected with 0.1 m.o.i. of EV-30. Cell lysates were titred on confluent HEK293 cells. Averages and standard deviations of three experiments are shown.

1-log shift of the virus load. These experiments quantitatively confirm that knockdown of DAF inhibits virus propagation. To verify this result with an independent cell line, human embryonic kidney cells were chosen and a strong reduction of virus replication after knockdown of DAF by siDAF2 was observed (Fig. 5).

Taken together, silencing of DAF was a much easier task than direct inhibition of the virus, since pre-designed or even validated siRNAs exist for virtually any cellular target. Based on the results obtained in the present study, it can be concluded that DAF is essential for EV-30, since efficient silencing of DAF had a pronounced antiviral effect. The protection of RD cells against lysis by EV-30 even correlated with the extent of DAF silencing. Inhibition of DAF expression can therefore be considered as a new option for an antiviral approach against EV-30.

4. Discussion

In recent years, RNAi has proven to be an efficient tool to inhibit viruses (Haasnoot et al., 2007). Since the options for a specific treatment of enterovirus infections are limited, RNAi has also been applied to this class of viruses (see above). The present study extends these efforts to echovirus 30 (EV-30), which is associated with aseptic meningitis.

While siRNAs against cellular targets are available commercially, the design of efficient siRNAs against viruses still remains a challenging task. Due to their high degree of conservation, the untranslated regions (UTR) of picornaviruses are attractive targets for RNAi approaches. However, various groups reported the failure of efforts to target the 5′ UTR of coxsackievirus B3, an enterovirus that is closely related to EV-30 (Kim et al., 2007; Merl and Wessely, 2007; Werk et al., 2005; Yuan et al., 2005). These difficulties are most likely due to the tight structure of the IRES and intensive efforts are necessary to solve this problem (Dutkiewicz et al., 2008). For the coding region, sequences encoding non-structural proteins are less likely to tolerate escape mutations than structural protein-encoding sequences. The sequence encoding the 3D RNA-dependent RNA polymerase ($3D^{Pol}$) was therefore chosen as the target region.

Various different criteria for the design of siRNAs have been proposed (summarized in Kurreck, 2006). A number of studies indicate that thermodynamic features of the siRNA determine their efficiency (e.g. Khvorova et al., 2003; Schwarz et al., 2003). According to these models, the relative thermal stability of the two ends of a given siRNA will determine which of the two strands will be incorporated into RISC. Other groups observed an important impact of the target RNA structure on silencing efficiency (e.g. Overhoff et al., 2005; Schubert et al., 2005b). In addition, unstructured guide strands were reported to be more efficient than structured ones (Patzel et al., 2005).

In order to increase the success rate for efficient siRNAs and to avoid an expensive screening campaign, all of these parameters were taken into consideration. Based on tools for the design of siRNAs available through the Internet, a set of 30 siRNAs was assessed according to a scoring system for efficient siRNAs (Reynolds et al., 2004). Only those siRNAs, which passed this first filter, were evaluated further by calculating the free energy of their antisense strands $\Delta G_{antisense}$. A positive value indicates that the strand is unlikely to form detrimental secondary structures. Finally, the secondary structure of the target RNA was predicted and the local free energy ΔG_{local} of the target site was calculated. High ΔG_{local} values, preferentially above −15 kcal/mol, were shown to result in maximal RNAi efficiency (Schubert et al., 2005b; Westerhout and Berkhout, 2007).

In reporter assays, all of the pre-designed siRNAs showed high silencing activity at low concentrations, with one of the six siRNAs being slightly less active. This lower activity had significant impact on virus inhibition, indicating that it is essential to have highly potent siRNAs for antiviral applications. The best siRNA designed according to the approach described above had a very

low IC_{50}-value of ~1 nM for protecting cells against lysis by the virus.

Since viruses can become resistant against prolonged siRNA treatment by the accumulation of mutations, various strategies to circumvent this problem have been developed. One approach is to inhibit the major virus receptor, since it is highly unlikely that the virus will adapt to other routes of cellular entry. A prerequisite for this strategy, of course, is that the receptor is dispensable for normal cellular function. For example, knockdown of CCR5 resulted in protection of macrophages against HIV-1 infection (Anderson and Akkina, 2007). For the coxsackievirus B3, knockdown of the coxsackievirus–adenovirus receptor was shown to inhibit virus spread (Coyne and Bergelson, 2006; Fechner et al., 2007; Werk et al., 2005).

Most echoviruses use DAF as an entry receptor (Bergelson et al., 1994), but some serotypes were found to follow other entry routes. For example, EV1 and EV8 bind to a subunit of the integrin VLA2 (Bergelson et al., 1992, 1993). The situation for EV-30 is not fully clear. Experiments with a DAF-specific monoclonal antibody and soluble DAF indicate that EV-30 can bind DAF, but it seems to recognize an alternative cell receptor with higher affinity (Powell et al., 1998). This question can now be addressed again with RNAi as a new technology. Knockdown of DAF resulted in drastic inhibition of EV-30, a finding indicating that DAF is the primary receptor for this serotype as well. This result was confirmed in two different cell lines (RD and HEK293). The hypothesis is supported further by the observation that the extent of virus inhibition correlated with the degree of DAF silencing. The existence of a co-receptor or alternative receptors, however, cannot be excluded. Interestingly, HeLa cells are difficult to infect with EV-30 even though more DAF is expressed on their cell surface than on RD cells (data not shown). This indicates that interactions between membrane-bound receptors on the cell surface may play an important role.

The present study provides a straightforward, rapid and low cost method to use RNAi for the protection against viruses. By taking into consideration different design criteria for siRNAs, a high success rate with five out of six siRNAs efficiently preventing cell lysis by the virus was achieved. While this strategy was exemplified for EV-30 in the present study, it can be considered generally applicable for any type of virus. Furthermore, the finding that siRNA-mediated knockdown of DAF protects cells against EV-30 confirmed this receptor as a promising target for new approaches to develop antivirals against EV-30 and related viruses.

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