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RNA interference against Enterovirus 71 infection

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Abstract

Enterovirus 71 (EV71) is a highly infectious major causative agent of hand, foot, and mouth disease (HFMD) which could lead to severe neurological complications. There is currently no effective therapy against EV71. In this study, RNA interference (RNAi) is employed as a therapeutic approach for specific viral inhibition. Various regions of the EV71 genome were targeted for inhibition by chemically synthesized siRNAs. Transfection of rhabdomyosarcoma (RD) cells with siRNA targeting the 3′UTR, 2C, 3C, or 3D region significantly alleviated cytopathic effects of EV71. The inhibitory effect was dosage-dependent with a corresponding decrease in viral RNA, viral proteins, and plaque formations by EV71. Viral inhibition of siRNA transfected RD cells was still evident after 48 h. In addition, no significant adverse off-target silencing effects were observed. These results demonstrated the potential and feasibility for the use of siRNA as an antiviral therapy for EV71 infections.

Keywords: Enterovirus 71; RNA interference; 3′UTR; siRNA; Viral inhibition

Introduction

Enterovirus 71 (EV71) belongs to the family Picornaviridae under the genus *Enterovirus* and is a *Human enterovirus A* species (Miller, 1997). It is a positive single-stranded RNA virus with a non-enveloped capsid and a genome size of 7411 nucleotides. It has caused significant mortality worldwide in recent years (Ho et al., 1999). EV71 is one of the main etiological agents of hand, foot, and mouth disease (HFMD). HFMD is a form of mild exanthem which mainly affects young children (<6 years of age), resulting in the appearance of vesicular rashes on hands, feet, and buccal mucosa. EV71-infected children could develop severe neurological complications that lead to rapid clinical deterioration and death (Chang et al., 1999; Lum et al., 1998; McMinn et al., 2001). With the emergence of EV71 in Asia as a major causative agent of HFMD fatalities in recent years and the ineffectiveness of anti-enteroviral therapy, there is a need to find a specific antiviral therapy. RNA interference (RNAi) holds promise as the inhibition of replication of poliovirus (Gitlin et al., 2002) and rhinovirus (Phipps et al., 2004) was observed in infected tissue culture cells.

RNAi is a native and specific post-transcriptional gene silencing mechanism. The process is initiated by double-stranded RNA (dsRNA) molecules (Fire et al., 1998). The dsRNA is processed by the RNase III-like enzyme Dicer into small interfering RNAs (siRNA) of 21–23 base pairs. The siRNA molecule then induces the formation of the

Abbreviations: EV71, Enterovirus 71; HFMD, hand, foot and mouth disease; RD, rhabdomyosarcoma; RISC, RNA-induced silencing complexes; RNAi, ribonucleic acid interference; siRNA, small interfering ribonucleic acids.

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RNA-induced silencing complexes (RISCs) which recognize and degrade homologous target mRNAs. Long dsRNA will induce the interferon response in mammalian cells which lead to the inhibition of protein synthesis by the activation of the PKR pathway and the activation of RNase L (Sledz et al., 2004). To circumvent the use of dsRNA in RNAi, Elbashir et al. (2001a, 2001b) had successfully used siRNA for silencing genes in mammalian system (Elbashir et al., 2001a, 2001b).

In this study, we tested chemically synthesized siRNAs for the specific inhibition of replication of EV71. Viral RNA, viral protein, plaque forming units, and cytopathic effects of infected rhabdomyosarcoma cells were examined as the indicators of targeted gene silencing by siRNA. We demonstrated that chemically synthesized siRNAs targeting at the 3′UTR, 2C, $3C^{pro}$, or $3D^{pol}$ of the EV71 genome are capable of inhibiting viral replication in a specific and dose-dependent manner.

Results

Selection of EV71 specific siRNA and transfection efficiency

The design of the siRNAs was based on general guidelines (Elbashir et al., 2001a, 2001b). Four synthetic 19-nucleotide siRNA duplexes targeting the non-translated regions and non-structural genes within the EV71 genomic domains: 3′UTR, 2C, $3C^{pro}$, and $3D^{pol}$ were designed (Table 1). Non-specific scrambled sequences with the same sequence composition as si-3′UTR served as a specificity control.

Fluorescein-labeled si-3′UTR duplexes were used to assay for the transfection efficiency of siRNA into the RD cell lines under study. Different concentrations of labeled siRNA were used to check for the transfection efficiency. At 24 h post-transfection with 1 nM, 50 nM, and 100 nM labeled si-3′UTR, the percentage of fluorescein-labeled RD cells was 72 ± 1%, 83 ± 1%, and >95%, respectively. The fluorescein-labeled siRNA was localized in the cytoplasm of the cell (data not shown).

Cell growth and viability assay

Cell growth and viability were used as parameters to determine whether the transfection agent or siRNAs introduced into the cells could result in any non-specific cytotoxic effects. Using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) reagent, cell viability assays were performed at 1 and 2 days post-transfection. All the siRNAs used in the experiment did not have any deleterious effects on cell growth and viability (data not shown).

siRNAs delayed cytopathic effects and increased cell viability in EV71-infected RD cells

RD cells were transfected with chemically synthesized siRNAs to evaluate their inhibitory effects on viral replication. In microscopic observations of RD cells treated with si-3′UTR, the siRNA targeting at the 3′UTR showed that cytopathic effects (CPE) in RD cells were delayed by up to 48 h post infection (h.p.i). RD cells that were treated with increasing concentrations of si-3′UTR were found to have decreased CPE (Fig. 1). The dosage-dependent viral inhibitory effect when using siRNAs such as si-2C, si-3C, and si-3D was also evident in RD cell lines, delaying CPE for up to 48 h.p.i. The 3′UTR scrambled siRNA failed to protect against CPE in EV71-infected RD cells, even when it was used at 100 nM. The treatment of RD cells with the sense or antisense strand of si-3′UTR at 100 nM also resulted in total CPE at 48 h.p.i. The protective effect of the siRNAs over a period of time after EV71 infection was also evaluated. At 24 h.p.i, the viability of cells transfected with the various siRNAs was comparable to the uninfected control cells. At 48 h.p.i, approximately 72% of si-2C, 82% of si-3D, and 70% of si-3′UTR transfected cells were viable at 48 h.p.i. In contrast, the untreated and mock-transfected cells had significantly lowered viability at 48 h.p.i. However, despite the initial protective effect, prolonged incubation up to 72 h.p.i led to total cell death, even after the transfection with the various siRNAs.

Table 1
Chemically synthesized siRNA molecules corresponding to the indicated segments of EV71 genome with the sense strand and corresponding antisense strand

siRNA	Nucleotide sequence	Nucleotide location	Gene
si-3′UTR	5′-UCU GGU CGU GUU AAU GAC U TT-3′ 5′-AGU CAU UAA CAC GAC CAG A TT-3′	7361–7379	3′UTR
si-2C	5′-A ACC AGA UCA CGA ACU UGG TT-3′ 5′-CCA AGU UCG UGA UCU GGU U TT-3′	4251–4269	2C
si-3C	5′-GAG CUA CUU UGC GAG UAU GCA TT-3′ 5′-UGC AUA CUC GCA AAG UAG CUC TT-3′	5918–5940	$3C^{pro}$
si-3D	5′-GCU ACU UUG GGA UGC A TT-3′ 5′-UGC AUA CUC GCA AAG UAG C TT-3′	7304–7322	$3D^{pol}$
Scrambled scr	5′-AGU UAC CGA UAA CCC AAG A TT-3′ 5′-UCU UGG GUU A UCG GAU ACU TT-3′	–	–

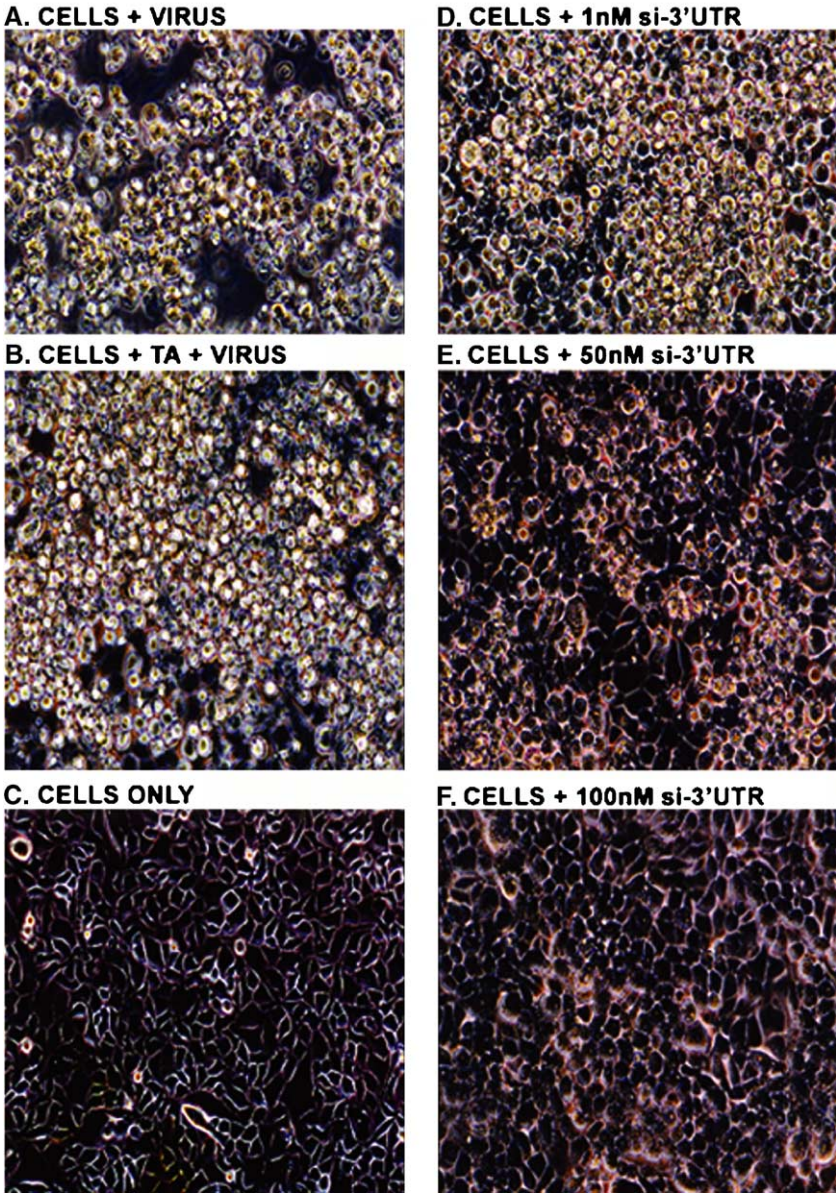

Fig. 1. RD cells infected with EV71 showing CPE. RD cells were transfected with chemically synthesized siRNA targeting at the 3′UTR and then infected with 60 pfu of EV71. CPE was first observed at 20 h.p.i., and the pictures were taken at 48 h.p.i. In panel A, cells were not pretreated with siRNA and were subsequently infected with EV71. Panel B showed RD cells treated with Lipofectamine 2000 and were infected with EV71. Panel C showed uninfected healthy RD cells. Panels D to F are EV71-infected cells after transfection with 1 nM si-3′UTR D, 50 nM si-3′UTR E, and 100 nM si-3′UTR F.

siRNA-treated cells reduced plaque formations

Plaque assay was carried out to evaluate the effectiveness of siRNA in reducing viable virus count. RD cells were infected with 60 pfu of EV71. Plaque assays showed that RD cells transfected with si-3′UTR, si-2C, si-3C, or si-3D had reduced plaque formations (Fig. 2). Mock-transfected cells did not exhibit any reduction in plaques. Cells transfected with the siRNA targeting at the $3D^{pol}$ gene were found to exert the most potent antiviral effect in concentrations being tested followed by siRNA targeting the $3C^{pro}$, 2C, and 3′UTR (Table 2). When 50 nM or 100 nM of single-stranded antisense siRNA was targeted at the 3′UTR, slight protection against viral infections was observed. The sense strand of si-3′UTR did not reduce plaque formations significantly at 50 nM and 100 nM. The non-specific scrambled duplex siRNA of 3′UTR did not reduce plaque formation (Fig. 3).

siRNA treatment of RD cells reduced EV71 specific RNA

Using real-time reverse transcription PCR based on hybridization probes targeting at the VP1 region of EV71, we quantified the reduction in viral titer after subjecting the RD cells to treatment with various siRNAs. Treatment of RD cells with siRNAs targeting the 2C, 3C, $3D^{pol}$, and 3′UTR genes of EV71 resulted in a dose-dependent decrease in

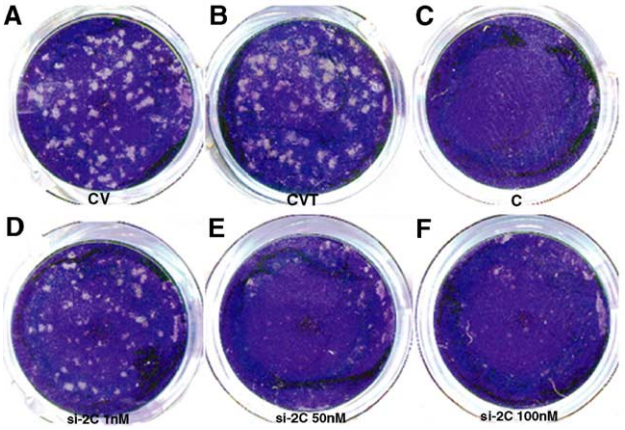

Fig. 2. RD cells treated with the siRNA targeting at the 2C gene (si-2C) demonstrated reduced plaque formations in a dosage-dependent manner. Transfection with siRNA was carried out 24 h prior to infecting the cells with 60 pfu of EV71. In panel A, cells were left untreated and then infected (CV). In panel B, mock-transfected cells were infected with EV71 (CVT). In panels D, E, and F, cells were treated with 1 nM, 50 nM, and 100 nM of si-2C, respectively and then challenged with EV71. Panel C shows non-infected cell monolayer. RD cell monolayer was stained with 1% crystal violet. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

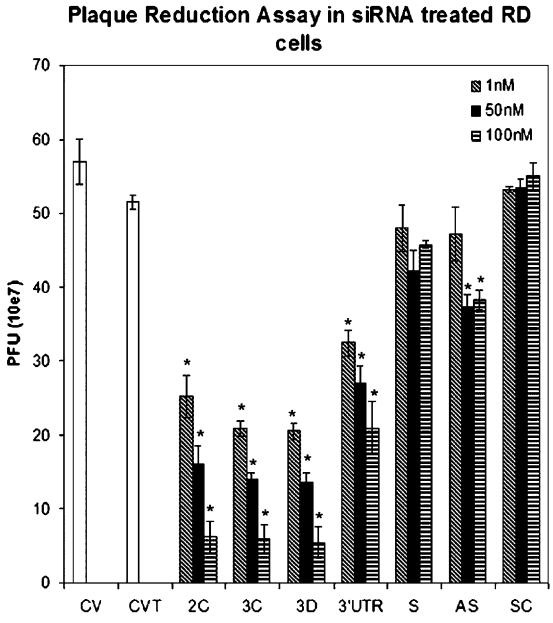

Fig. 3. Dose-dependent inhibitory effects of various siRNAs on plaque formation in RD cells. Cells were left untreated and infected with 60 pfu of EV71 (CV), or transfected with Lipofectamine 2000 and subsequently infected (CVT), or transfected with 1–100 nM of si-2C, or si-3C, or si-3D, or si-3′UTR, or the sense strand of 3′UTR (S), or the antisense strand of 3′UTR (AS), or the scrambled siRNA sequences (SC) at 24 h prior to EV71 infection. Plaque reductions were assayed at 48 h.p.i. The data shown represent the mean ± SD (n = 4). *P < 0.01. ANOVA analysis, for comparison between CVT and other treatments.

absolute viral copy numbers (Table 2). The transfection of RD cells with 100 nM of si-2C, si-3C, si-3D, and si-3′UTR resulted in viral titer reductions of 54.0 ± 4.0%, 76.1 ± 4.0%, 77.5 ± 2.1%, and 44.1 ± 5.9%, respectively (Table 2). The transfection of RD cells with the antisense sequence of 3′UTR at 50 nM resulted in a slight reduction of approximately 18.5 ± 3.5%. However, the inhibition effect was not enhanced when the antisense 3′UTR sequence was used at 100 nM (Table 2). In contrast, the sense and scrambled sequences of 3′UTR did not decrease viral RNA.

siRNA-treated RD cells reduced EV71 specific proteins

In order to investigate whether the reduction in specific viral RNA correlated with a down-regulation in specific viral protein, Western blotting using monoclonal antibody against EV71 VP1 structural protein was carried out. We chose si-3D and si-2C to assay for the subsequent reduction in viral proteins. It is evident from the results that the siRNA targeting at the 2C and $3D^{pol}$ genes of the EV71 genome significantly reduced the amount of detectable VP1 in a dose-dependent manner (Fig. 4). In contrast, the transfection of RD cells with the siRNA carrying the non-specific scrambled sequences of 3′UTR failed to yield any reduction in the synthesis of VP1. Similar trend was observed when si-3C was used (data not shown). These results were consistent with the data obtained at the RNA level by real-time PCR-based assays.

The specificity of the siRNA-mediated viral inhibition effect

To test for the specificity of RNAi, the effects of si-2C, si-3D, and si-3′UTR on the expression of endogenous PKR were assessed. Western blot analysis with antibodies against

Table 2
Comparative percentage reduction of viral load between data from real-time PCR and plaque assay

siRNA	1 nM		50 nM		100 nM	
	Viral RNA (mean ± SD %)	Plaque reduction (%)	Viral RNA (mean ± SD %)	Plaque reduction (%)	Viral RNA (mean ± SD %)	Plaque reduction (%)
si-2C	–	50.9 ± 5.4	50.8 ± 1.9	68.5 ± 4.8	54.0 ± 4.0	87.8 ± 4.1
si-3C	18.0 ± 3.0	58.7 ± 2.0	56.0 ± 2.0	71.1 ± 2.0	76.1 ± 4.0	88.7 ± 3.8
si-3D	18.3 ± 2.1	60.1 ± 2.1	59.9 ± 1.5	73.8 ± 2.9	77.5 ± 2.1	89.3 ± 4.0
si-3′UTR	–	36.9 ± 3.4	12.0 ± 2.1	47.6 ± 4.6	44.1 ± 5.9	59.2 ± 6.9
ss-3′UTR	–	6.7 ± 5.8	–	17.9 ± 6.0	18.5 ± 9.7	10.0 ± 1.0
as-3′UTR	–	8.2 ± 6.9	18.5 ± 3.5	27.2 ± 2.9	18.5 ± 3.5	25.7 ± 2.5
sc-3′UTR	–	–	–	–	–	–

–: Denotes no reduction.

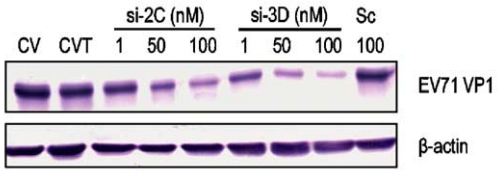

Fig. 4. Western blot analysis of EV71 VP1. Approximately 1.0×10^6 RD cells were transfected with si-2C or si-3D at various concentrations and subsequently infected with the virus. The amount of EV71 structural protein, VP1, was reduced upon treatment of RD cells with si-2C and si-3D. VP1 decrease was determined through Western blotting using anti-EV71 monoclonal antibody (Chemicon) (upper panel). Internal loading control β-actin was detected using anti-β-actin (Sigma) (lower panel).

PKR showed no increase in endogenous PKR expression at 30 min, 60 min, and 90 min after siRNA entry. The cells transfected with the various siRNAs showed similar levels of PKR as the control cells (C) at various time-points post-transfection (Fig. 5).

Discussion

The development and use of antivirals, like enviroxime (Heinz and Vance, 1995), pleconaril (Pevear et al., 1999), and 3C protease inhibitors (Patick et al., 1997) for treating enterovirus infections showed variable effectiveness against the neurotropic EV71 (Robart et al., 1998). As a result, there is a great interest in exploring new antiviral therapeutic strategies against EV71 infection. RNAi has been successfully applied as a potent antiviral against the human immunodeficiency virus (Capodici et al., 2002), hepatitis B virus (Li et al., 2004), poliovirus (Gitlin et al., 2002), and rhinovirus (Phipps et al., 2004).

The selective inhibition of EV71 has been demonstrated using plasmid-based shRNA (Lu et al., 2004). The use of recombinant vector producing siRNAs targeting EV71 was shown to inhibit viral replication in both HeLa and Vero cells (Lu et al., 2004). However, plasmid-derived shRNAs are widely used as inexpensive proof-of-concept studies and are unlikely to be a method employed for viral gene therapy. The main obstacle in achieving in vivo gene silencing by RNAi against EV71 is its delivery across the blood–brain barrier (BBB) into the CNS, which is highly immunogenic (Trulzsch and Wood, 2004). Although the expression of plasmid-derived shRNAs can be tightly regulated and also tissue-specific expression cassettes can be incorporated, the intrinsic size of plasmid-derived shRNAs and thus its problems in delivery are drawbacks for its use as a possible antiviral therapy. Furthermore, the difficulty to directly control the transcriptional efficiency and dosage of the plasmid-derived shRNAs intracellularly poses a problem for pharmacokinetics studies. Viral-vector derived shRNAs have been successfully employed for gene knockdowns. The need for specific delivery to the target cells can also be addressed by the use of viral-based vectors, however, the use of viral vectors is plagued with problems like immunogenicity and random integration into host-genome, especially for adeno-viral-based vector and lentivirus-based vector, respectively.

As viral infection is transient in nature, it is unnecessary to have a long-lasting viral knockdown effect, which might result in non-specific adverse effects. Chemically synthesized siRNA used to mediate RNAi against viruses could be the most relevant strategy for genetic antiviral therapy as it is straightforward and non-immunogenic (Trulzsch and Wood, 2004). In addition, chemically synthesized siRNA is amenable to various chemical modifications or conjugations to increase its stability and specificity for cellular uptake. Chemically stabilized and cholesterol-conjugated siRNAs have been shown to silence endogenous genes by means of a systemic delivery of the modified siRNA into mouse models (Soutschek et al., 2004). This is especially relevant to EV71 infection in which the systemic delivery of the modified synthetic siRNAs could be engineered to be highly tissue-specific.

In this study, by targeting the non-structural genes of EV71, namely 2C, $3C^{pro}$, $3D^{pol}$, and 3′UTR, four separate lines of evidence have established that RNAi can be used to suppress EV71 replication. These are: (1) chemically synthesized siRNA significantly reduced viral RNA in infected cells; (2) EV71 specific protein (VP1) was visibly reduced in cells treated with the siRNAs; (3) viable viral count and infectivity measured by plaque assay were significantly reduced upon siRNA treatment; (4) the cells

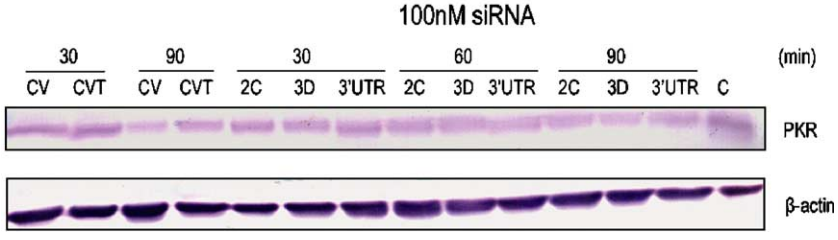

Fig. 5. Western blot analysis of dsRNA-activated protein kinase (PKR) (top panel) showing no significant changes in the expression level of endogenous PKR at various time-points after transfection. Cells were transfected with 100 nM of si-2C, si-3D, or si-3′UTR of the EV71 genome. Untreated EV71-infected RD cells (CV) and EV71-infected mock-transfected RD cells (CVT) were used as positive controls for interferon activation. Untreated cells (C) were used as negative control for interferon activation. At 30, 60, and 90 min post-transfection and infection, the total cell lysates were collected and analyzed for PKR and β-actin expression using specific antibodies. The β-actin Western blot demonstrates equal loading of total protein (lower panel).

transfected with the siRNAs were protected against the formation of cytopathic effects (CPE) for up to 48 h.

The use of siRNA as an antiviral agent could lead to a selective pressure on the siRNA target, which might result in the appearance of escape variants due to the changes in the target sequence as has been observed with poliovirus (Gitlin et al., 2002). To minimize this possibility, EV71 target sequences that were chosen for the design of the siRNA were those that were highly conserved and are therefore less likely to alter and generate escape mutants. All four chemically synthesized siRNAs, namely, si-3′UTR, si-2C, si-3C, and si-3D, were capable of eliciting a dosage-dependent inhibition of EV71 replication. The dosage-dependent viral inhibitory effect of the siRNAs correlated with their transfection efficiencies into the cells.

The plaque assay data correlated well with that from the real-time PCR assay, the most effective inhibition of viral replication was observed with si-3D followed by si-3C, si-2C, and si-3′UTR. The $3D^{pol}$ gene encodes the viral RNA-dependent RNA polymerase, which oligomerizes into a form most suited to the elongation and RNA-binding activity of the polymerase. As the $3D^{pol}$ gene product acts in concert with other viral and cellular factors to be the indispensable component facilitating viral replication, it is therefore expected that its down-regulation would produce the most effective inhibitory effect on viral replication. The 3C region encodes for a protease, which performs majority of the secondary processing steps in the polyprotein. Studies from polioviruses have indicated two cleavages being carried out with 3CD, which is the precursor of 3C. Whether EV71 exhibits the same specific 3CD proteolytic activity is still unknown, however, our inhibition data suggest that siRNA targeting either at the $3C^{pro}$ or the $3D^{pol}$ region in the viral genome elicited comparable and most effective viral inhibition. In EV71, specific functions of the 2C protein and the 3′UTR have yet to be identified. The 2C protein has been implicated to be involved in the initiation of negative and positive-strand synthesis and is known to associate with the 3′UTR of negative-strand viral RNA replication intermediates (Banerjee et al., 1997). Our results also correlate with the finding by Lu et al. (2004) who used plasmid-derived shRNA for viral inhibition studies. Both studies showed that targeting at the viral RNA-dependent RNA polymerase, $3D^{pol}$, is the most effective in inhibiting viral replication. We have further established that targeting at the other EV71 non-structural regions like $3C^{pro}$, 2C, and 3′UTR is also effective in inhibiting viral replication.

It is important to assess whether single-stranded sense siRNA or antisense siRNA could elicit similar inhibitory effect. Using single-stranded 5′-phosphorylated sense or antisense siRNA, only slight inhibitory effect (<20%) was observed at high concentrations (100 nM) of the antisense strand and is not as effective when compared with the use of duplex siRNA at the same concentration. This is due to the inefficient reconstitution of RISC when single-stranded siRNA was used (Martinez et al., 2002).

The protection of RD cells against CPE by various siRNAs over a time-course of EV71 infection was assessed. The CPE was delayed by up to 48 h for the cells transfected with the four siRNAs. However, despite the initial protection, prolonged incubation for up to 72 h.p.i resulted in cell death. The loss of protection conferred by the siRNA is likely to be due to the decrease in the siRNA concentration as the cell population divides.

In principle, it is possible that the inhibition of viral replication is caused by an interferon-mediated response, which can be activated by short dsRNA (Sledz et al., 2004). It is therefore important to find out whether the siRNAs used in the study are capable of eliciting off-target effects, like the activation of the interferon (IFN) response. A Western blot conducted with a monoclonal antibody against protein kinase R (PKR) showed that the IFN pathway is not engaged after siRNA transfection. Cell viability was used as a global indicator to check for the adverse effects of IFN activation, and it was not compromised during the 48 h time period post-transfection with all the siRNAs used in the experiment. The lack of the IFN response at the global level lends optimism to the hope that siRNA of 21 nt targeted to EV71 could be a promising antiviral for EV71 infections.

In conclusion, this study demonstrates that RNAi mediated by chemically synthesized siRNA can be used as a possible therapeutic strategy to inhibit EV71 replication in human cells. RNAi using chemically synthesized oligonucleotides si-2C, si-3C, si-3D, and si-3′UTR might offer an important alternative for treatments against EV71. Given the rapid strides being made in the field, especially regarding in vivo delivery methods, the use of siRNA as an antiviral therapeutic approach has great potential.

Materials and methods

Cell culture

Rhabdomyosarcoma (RD) cells were routinely grown in minimum essential medium (Gibco, Boston, MA, USA) supplemented with 5% fetal calf serum, 1% sodium pyruvate, and 1.5% sodium bicarbonate.

Design and synthesis of double stranded short interfering RNA (siRNA)

21-nt double-stranded siRNA sequences were designed with 3′-dTT extensions against 3′UTR, and 2C, $3C^{pro}$, and $3D^{pol}$ of EV71. A scrambled sequence with the same base composition as si-3′UTR and single-stranded sequence of the sense and antisense of 3′UTR-31 were used as controls. All the siRNAs and control sequences were listed in Table 1. Fluorescein-labeled si-3′UTR was also synthesized. The label was on the 3′ end of the sense strand. The siRNA molecules were synthesized by Proligo, Singapore.

Table 3
Probes and primers used for detection of EV71

Name	Nucleotide sequence	Nucleotide location
VP1-Fwd	5′-GAG AGT TCT ATA GGG GAC AGT-3′	2466–2486
VP1-Rev	5′-AGC TGT GCT ATG TGA ATT AGG AA-3′	2669–2647
EVpprot_FL	5′-GTG TGT CTT GGC CGG TAG GTG CCG GT-3′	2538–2513
EVpprot_LC	5′-GAG CTC GGG TGA GGG CTC TGC TCA C-3′	2511–2487

Transfection and infection

Rhabdomyosarcoma cells (5×10^4) were seeded into each well of a 24-well plate and allowed to recover for 24 h, and the growth medium in each well was replaced with 500 μl of pre-warmed reduced serum medium, OPTI-MEM I (Gibco, Boston, MA,USA), for another 24 h. Before transfection, the appropriate amount of siRNAs (1, 50, or 100 nM) was diluted in 50 μl of OPTI-MEM I. In another tube, 1 μl of Lipofectamine 2000 was also diluted in 50 μl of OPTI-MEM I. After incubation at room temperature for 5 min, these two solutions were mixed gently and incubated for 20 min at room temperature to allow the siRNA:lipofectamine 2000 complexes to form. Then, 100 μl of the complexes was added to each well and mixed gently by rocking the plate back and forth. Mock transfections were carried out as described above but with the omission of siRNA. After 24 h, each of the wells was infected with EV71 at an MOI of 10. At 48 h post-infection, total RNA was extracted from each well using TRIzol LS reagent (Invitrogen Life Technologies, Carlsbad, CA).

To estimate transfection efficiency, cells were grown on Labtek Permanox Chamberslide (Nunc, Germany). At 24 h post-transfection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. The cells were then washed again with PBS, and a drop of Vectashield mounting medium (Vector Laboratories, USA) containing propidium iodide was then added. Cells were observed using Olympus BX60 fluorescent microscope. The percentage of fluorescein-labeled cells in five randomly selected fields from three independent experiments was determined.

Cell viability assay

To assay for cell viability due to the toxicity of the transfection agent, RD cells were seeded in a 24-well plate. Transfection and treatment of cells were performed following the methods already described above. At 1 and 2 days post-transfection, cells were trypsinized, and a 100 μl aliquot of the resuspended cells was transferred to 96-well plate in triplicates. 20 μl of MTS/PMS reagent (Promega, USA) was then added into each well. After incubation at 37 °C for 1 h, the absorbance at 490 nm was measured. All assays were performed in triplicate and as two independent experiments.

Real-time reverse transcription (RT) PCR detection of EV71 using hybridization probes

Primers and hybridization probes for the real-time RT-PCR were designed, and a BLAST search was conducted against Genbank sequences. The probes and primers were synthesized by Proligo, Singapore (Table 3). Following reverse transcription, the cDNA was subjected to 40 PCR cycles of amplification at 60 °C for 15 s and at 72 °C for 17 s in the Roche LightCycler (Roche Molecular Biochemicals, Germany). Absolute quantitation of viral RNA was calculated using the standard curve previously created.

SDS-PAGE and Western blot analysis

RD cells were grown in 6-well plate according to the experimental set up described earlier. At 48 h post-transfection, the cells were harvested for total proteins. The cells were lysed using 125 μl of CelLytic M Cell Lysis Reagent (Sigma, USA). An aliquot of 30 μl of each lysate was electrophoresed in a denaturing 10% polyacrylamide gel. Western blotting was performed following standard procedures. The detection procedure based on chromogenic method made use of monoclonal EV71 antibody (Chemicon International, USA), anti-PKR (Sigma, USA), and β-actin antibody (Sigma, USA).

Plaque assay of EV71-infected cells

To determine the viral infectivity of EV71 after siRNA treatment, plaque assays were carried out after the transfection protocol. One milliliter of 1% w/v carboxymethylcellulose (CMC) in 2× growth medium was layered onto the infected cell monolayer. The plates were incubated at 37 °C in for 48 h. Plaques of EV71 viruses were observed by fixing with 20% formalin in PBS and staining with 1% crystal violet for 30 min at room temperature.

Acknowledgments

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