Inhibition of Enterovirus 71 in Virus-infected Mice by RNA Interference

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Enterovirus 71 (EV71) is the main causative agent of hand, foot, and mouth disease (HFMD) in young children. It is often associated with neurological complications and has caused high mortality levels in recent outbreaks in the Asia Pacific region. Currently, there is no effective antiviral therapy against EV71 infections. In this study, we have evaluated and compared the efficacies of three different forms of small interfering RNAs (siRNAs) in inhibiting EV71 replication in a murine model. We have shown that both synthetic 19-mer siRNAs and plasmid-borne short hairpin RNAs (shRNAs) targeted at the conserved 3D domain region were able to inhibit EV71 infections in suckling mice when delivered with or without lipid carrier via the systemic route. The treated mice did not exhibit hind limb paralysis and weight loss, as was observed in untreated mice. EV71 replication was significantly reduced as revealed by real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot. In addition, no evidence of interferon (IFN) induction was detected in the intestinal tissues harvested from the mice as a result of siRNA administration. However, the chemically synthesized 29-mer shRNA did not protect the suckling mice from EV71 infections despite being more potent in the in vitro system. Our results indicate that RNA interference (RNAi) may be a promising therapeutic approach for fighting EV71 infections.

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INTRODUCTION

Enterovirus 71 (EV71) is a positive single-stranded RNA virus with a non-enveloped capsid and a genome size of ~7.5 kilobase. It is one of the main etiological agents of hand, foot, and mouth disease (HFMD), a mild childhood disease that mainly affects young children (<6 years of age). HFMD is characterized by 3–4 days of fever, development of rashes, and vesicles on the limbs as well as multiple ulcers in the mouth. Although other enteroviruses such as CA16, CA5, CA9, and Echo 7 are also able to cause HFMD, serious neurological manifestations such as brainstem and/or cerebellar encephalitis, acute flaccid paralysis, and aseptic meningitis were observed in many children with acute EV71 infections during the large scale HFMD outbreaks in the Asia Pacific region. These neurological manifestations were frequently accompanied by complications such as pulmonary edema and myocarditis and sometimes lead to death. At present, there is no effective antiviral therapy or vaccine available to treat the disease. It is important to note that most deaths from HFMD caused by EV71 occur within 24 hours, due to children developing complications associated with HFMD. With rising concern about the virulence of EV71, there is a need to develop a specific antiviral therapy against EV71.

RNA interference (RNAi) is a defense strategy that was first described in Caenorhabditis elegans. Since then, RNAi has been widely applied as cellular defense against viruses, and has shown promise as a potential therapeutic strategy to fight against infectious diseases. It is a multi-step process of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNA molecules. The double-stranded RNAs are processed by the RNase III-like enzyme, Dicer, into small interfering RNAs (siRNAs) of 21–23 nucleotides. The siRNA will induce the formation of the RNA-induced silencing complexes that subsequently recognize and degrade homologous target messenger RNAs.

By targeting viral transcripts and/or host genes which produce co-factors critical for viral replication, the RNAi approach has been shown to be effective against a wide range of viruses such as the human immunodeficiency virus, hepatitis C virus, and the influenza virus A in the in vitro systems. This approach has also been used successfully against viruses of the Picornaviridae family, including the poliovirus, as well as the Coxsackievirus B3. Several studies have shown that siRNAs were effective in inhibiting replication of viruses such as hepatitis B and influenza virus A, and the respiratory syncytial virus in murine model. Thus, the RNAi strategy possesses great potential for being developed as antiviral therapy to fight against EV71.

Previous investigations have shown that both chemically synthesized 19-mer siRNAs and DNA vector-based short hairpin RNA (shRNA) producing systems targeted at specific regions of EV71 were able to significantly inhibit replication of EV71.
in the \textit{in vitro} systems without inducing the interferon (IFN) response.\textsuperscript{19,20} In this study, we have evaluated and compared the efficacies of chemically synthesized 19-mer siRNAs, psiStrike plasmids expressing shRNAs, and the chemically synthesized 29-mer shRNAs in inhibiting EV71 infections in a murine model. The siRNAs were designed to target the RNA-dependent RNA polymerase, 3D\textsuperscript{pol} region of the EV71 genome, and we have shown that both chemically synthesized siRNAs and shRNAs produced from psiStrike plasmids were equally effective in inhibiting EV71 infections in mice. However, despite showing enhanced potency in inhibiting EV71 infections in the \textit{in vitro} system, the chemically synthesized 29-mer shRNAs failed to protect the mice from EV71 infections.

\section*{RESULTS}
\subsection*{Designing siRNAs against EV71 infection}
We have shown previously that the chemically synthesized 19-mer siRNAs (19mer-3D) and the 29-mer shRNAs (29mer-3D) designed to target the 3D\textsuperscript{pol} region of the EV71 genome were highly effective in inhibiting EV71 replication in rhabdomyosarcoma cell line.\textsuperscript{20,21} In this study, the oligonucleotide bearing the same sequence as the chemically synthesized 19-mer siRNAs (19mer-3D) was cloned into the psiStrike shRNA expression vector and a recombinant plasmid expressing 19mer-shRNAs (psi-3D) was constructed. Our results showed that the 19-mer shRNAs expressed from the recombinant psiStrike plasmids (psi-3D) were highly effective in inhibiting EV71 replication in the \textit{in vitro} system (data not shown).

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.pdf}
\caption{Prophylactic effect of different forms of small interfering RNAs (siRNAs) in protecting mice ($n = 5$) from Enterovirus 71 (EV71)-infection as shown by changes in weight and hind limb paralysis. (a) EV71 infected suckling mice treated with 10 nmol of chemically synthesized 19-mer siRNAs (19mer-3D). (b) EV71 infected suckling mice treated with 50 µg of psiStrike plasmids expressing short hairpin RNAs (shRNAs) (psi-3D). (c) EV71 infected mice treated with chemically synthesized 29-mer shRNAs (29mer-3D). The untreated mice and mice treated with scrambled siRNAs (19mer-scr, psi-scr or 29mer-scr) were used as controls. All the data represent the mean weights ± SD of four independent experiments. The analysis of variance (ANOVA) for comparison of weight changes between untreated infected mice and mice treated with 10 nmol of 19mer-3D was $P < 0.01$. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 50 µg of psi-3D was $P < 0.01$. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 10 nmol of 29mer-3D was $P < 0.05$. (d) A representative picture of a suckling mouse treated with 10 nmol 19mer-3D or 50 µg psi-3D compared to a suckling mouse treated with scrambled siRNAs (on the left-hand side). (e) A representative picture of an untreated mouse showing hind limb paralysis (indicated by the arrow) and significant weight loss caused by EV71 infection on day 6 post infection. (f) A representative picture of an age-matched healthy uninfected sucking mouse. All the observations represent the results from four independent experiments.}
\end{figure}

\subsection*{siRNA-mediated inhibition of EV71 in vivo}
To test the prophylactic activity of the various siRNAs, the suckling mice were first injected with 10 nmol of chemically synthesized 19-mer siRNAs (19mer-3D), 10 nmol of 29-mer shRNAs (29mer-3D) or 50 µg of psiStrike plasmid expressing 19mer-shRNAs (psi-3D) coupled with Oligofectamine via the intraperitoneal (IP) route. After 24 hours, the suckling mice were infected with 10$^4$ of a 50% tissue culture infective dose (TCID$_{50}$) of EV71. The suckling mice that served as controls were either not treated with any siRNAs or treated with siRNAs with scrambled sequences (19mer-scr, psi-scr or 29mer-scr) prior to infection. Results showed that the sucking mice treated with 10 nmol of 19mer-3D or 50 µg of psi-3D showed significant protection against EV71 infection since the treated sucking mice showed no signs of significant weight loss (Figure 1a and b) or acute flaccid paralysis of the hind limbs (Figure 1d) after 14 days of EV71 infection. In contrast, the sucking mice that received no siRNA treatment or treated with the scrambled siRNAs showed significant weight loss and hind limb paralysis on day 6 post infection, and all the infected mice died between day 12 and 14 post infection (Figure 1e). Surprisingly, the sucking mice that were treated with 29mer-3D did not recover from the EV71 infection (Figure 1c and d). Thus, the administration of both synthetic 19-mer siRNAs and the psiStrike plasmids expressing shRNAs were able to inhibit EV71 replication in a murine model.

To examine the therapeutic potential of 19mer-3D, psi-3D, or 29mer-3D, the mice were first infected with 10$^4$ of a TCID$_{50}$ of EV71. After 24 hours, the infected sucking mice were
administered with 10nmol of 19mer-3D, 10nmol of 29mer-3D or 50µg of psi-3D coupled with Oligofectamine via the oral or the IP route. It was observed that the infected mice that received no siRNA treatment or treated with the scrambled siRNAs showed significant weight loss on day 6 post infection (Figure 2) and all died between day 12 and 14 post infection. When the infected suckling mice were administered with 10nmol of 19mer-3D or 50µg of psi-3D via the IP route, significant protection against EV71 infection was observed as these treated suckling mice showed no signs of weight loss or hind limb paralysis after 14 days of EV71 infection (Figure 2a and b). In contrast, the infected suckling mice did not recover from the EV71 infection when they were treated with 29mer-3D (Figure 2c). All the treated suckling mice were further monitored for another 2 weeks for any delayed EV71 infection. Since none of them showed any signs of hind limb paralysis or weight loss, this is indicative of full recovery from the EV71 infection. Notably, similar protective effects from EV71 infections were observed in the suckling mice when 19mer-3D and psi-3D were administered without using Oligofectamine as the lipid carrier (Figure 3). This indicated that the siRNAs were capable of providing significant protection against EV71 infections even when they were administered without being coupled with a lipid carrier. However, the suckling mice failed to be protected from EV71 infections when they were treated with either 19mer-3D or psi-3D via the oral route (data not shown).

**Efficiency of siRNA delivery to mice tissues**

We next determined the uptake efficiencies of the three different forms of siRNAs by the intestinal cells by injecting the suckling mice with 10nmol of 19mer-3D, 10nmol of 29mer-3D or 50µg of psi-3D without being coupled with a lipid carrier. However, the suckling mice failed to be protected from EV71 infections when they were treated with either 19mer-3D or psi-3D via the oral route (data not shown).

**Figure 2 Changes in weight in Enterovirus 71 (EV71)-infected mice (n = 5) treated with different forms of small interfering RNAs (siRNAs) coupled with Oligofectamine.** (a) EV71 infected suckling mice treated with 10nmol of 19mer-3D. (b) EV71 infected suckling mice treated with 50µg of psi-3D. (c) EV71 infected mice treated with 29mer-3D. Untreated mice and mice treated with scrambled siRNAs (19mer-scr, psi-scr or sh-scr) were used as negative controls. All the data represent the mean weights ± SD of four independent experiments. The analysis of variance (ANOVA) for comparison of weight changes between untreated infected mice and mice treated with 10nmol of 19mer-3D was P < 0.01. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 50µg of psi-3D was P < 0.01. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 10nmol of 29mer-3D was P < 0.05.

**Figure 3 Changes in weight in Enterovirus 71 (EV71)-infected mice (n = 5) treated with different forms of small interfering RNAs (siRNAs) not coupled with Oligofectamine.** (a) EV71 infected suckling mice treated with 10nmol of 19mer-3D. (b) EV71 infected suckling mice treated with 50µg of psi-3D. (c) EV71 infected mice treated with 29mer-3D. Untreated mice and mice treated with scrambled siRNAs (19mer-scr, psi-scr or sh-scr) were used as negative controls. All the data represent the mean weights ± SD of four independent experiments. The analysis of variance (ANOVA) for comparison of weight changes between untreated infected mice and mice treated with 10nmol of 19mer-3D was P < 0.01. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 50µg of psi-3D was P < 0.01. The ANOVA for comparison of weight changes between untreated infected mice and mice treated with 10nmol of 29mer-3D was P < 0.05.
Inhibition of EV71 by RNAi in Murine Model

Figure 4 Delivery efficiencies of the various forms of small interfering RNAs (siRNAs) by the intestinal cells. The uptake efficiencies (%) of (a) 19mer-3D, (b) psi-3D, and (c) 29mer-3D by the intestinal cells were compared to untreated intestinal cells. The delivery efficiencies of the scrambled siRNAs, namely (d) 19mer-scr, (e) psi-scr and (f) 29mer-scr were also investigated. The results represent the mean percentages from two independent experiments.

obtained even when the siRNAs were administered without Oligofectamine (data not shown).

Dosage dependency of siRNAs in inhibition of EV71 replication

To determine the dose–response kinetics and the optimal concentrations of 19mer-3D, 29mer-3D, and psi-3D in inhibiting EV71 replication, we injected the suckling mice with different concentrations of 19mer-3D, 29mer-3D, or psi-3D. On day 14 post infection, the intestinal cells were harvested from the suckling mice and the inhibitory effects of each of the three different forms of siRNAs on EV71 replication were analyzed by using the real-time hybridization probe, reverse transcription polymerase chain reaction (RT-PCR) and Western blot methods. It was observed that treatment with 1 nmol of 19mer-3D led to ~40% decrease in viral transcripts while treatment with 10 nmol of 19mer-3D led to 100% reduction in the intestines (Figure 5a). Similar dosage dependency effects were observed when the concentration of psi-3D was increased from 10 to 50 µg (Figure 5a). However, there was no significant decrease in the EV71 viral RNA transcripts in the suckling mice when they were treated with 29mer-3D (Figure 5a). To corroborate the results obtained from the real-time RT-PCR, the Western blot method was carried out to analyze the EV71 viral proteins in the intestinal cells harvested from the suckling mice that had earlier been treated with 19mer-3D, psi-3D, or 29mer-3D. Our results showed that when the concentration of 19mer-3D was increased from 1 to 10 nmol, the VP1 viral protein levels decreased correspondingly (Figure 5b).

Similar dosage dependency effects were observed when the mice were treated with 10, 25, or 50 µg of psi-3D (Figure 5b). There was, however, no significant decrease in the VP1 protein levels observed in the intestinal cells harvested from the suckling mice treated with 29mer-3D (Figure 5b). No significant decrease in the VP1 protein levels or EV71 RNA transcripts was observed either, when the suckling mice were treated with 10 nmol of the chemically synthesized scrambled siRNAs (19mer-scr and 29mer-scr) or 50 µg of psiStrike plasmids expressing scrambled shRNAs (psi-scr).

These results put together helped us conclude that both the chemically synthesized 19-mer siRNAs (19mer-3D) and the psiStrike plasmid expressing shRNAs (psi-3D) were effective in inhibiting EV71 replication in suckling mice in a dosage dependent manner, and the most effective inhibitory concentrations of 19mer-3D and psi-3D were 10 nmol and 50 µg, respectively. On the other hand, the chemically synthesized 29-mer shRNAs (29mer-3D) failed to protect the suckling mice from EV71 infections despite showing enhanced potency in inhibiting EV71 replication in the in vitro system.

We next conducted a kinetic study to determine the inhibitory effects of 19mer-3D and psi-3D (that lead to protecting the suckling mice from EV71 infection) by treating the infected suckling mice with 19mer-3D or psi-3D any time from day 1 to 4 after they...
Figure 6 Histological changes in Enterovirus 71 (EV71)-infected suckling mice (n = 5) under ×40 magnification. Immunohistochemical staining was carried out with specific anti-EV71 antibody. (a) Presence of EV71 was detected (indicated by arrow) at the epithelia of the intestines on day 7 post infection. (b) Extensive damage to the epithelia and the intestinal cells caused by EV71 on day 14 post infection. (c) Presence of EV71 and extensive damage in the intestines harvested from mice treated with chemically synthesized 29-mer short hairpin RNAs (29mer-3D). (d) No presence of EV71 was detected when the suckling mice were treated with either 19mer-3D or psi-3D. (e) Normal morphology of the intestines in healthy suckling mice that served as negative controls. The results represent the observations from two independent experiments.

Immunohistochemistry staining for presence of EV71 in the organs of the treated suckling mice

The effects of 19mer-3D, 29mer-3D, and psi-3D on EV71 replication were determined histologically by immunohistochemical staining of the intestines harvested from the mice on day 7 and 14 post EV71 infection. On day 7 post infection, presence of EV71 was observed in the intestines harvested from untreated suckling mice (Figure 6a), and there was extensive damage done to the intestinal epithelia and intestinal cells after 14 days of infection with EV71 (Figure 6b). Similar extensive intestinal damage was observed in the intestines harvested from the suckling mice treated with 29mer-3D (Figure 6c). In contrast, no presence of EV71 was observed in the intestines when the suckling mice were treated with either 10 nmol 19mer-3D or 50 µg of psi-3D (Figure 6d). These results highlighted the significance of both 19mer-3D and psi-3D for their ability to inhibit EV71 replication in vivo.

siRNAs do not activate IFNs

To provide further evidence that EV71 inhibition was due to the specific antiviral effects exerted by both 19mer-3D and psi-3D and not due to activation of IFNs, we measured the levels of IFN-α and IFN-β in the homogenates of the intestines harvested from the treated suckling mice. The results showed no activation of IFN-α or IFN-β and we therefore concluded that the EV71 inhibition in the suckling mice was mediated specifically through RNAi (Figure 7).

DISCUSSION

Currently, most cases of HFMD are managed with symptomatic treatment. There are a number of other antiviral agents that may be effectively used against enteroviruses; however, these are still under study. One of the "WIN" compounds—pleconaril—is found to have significant therapeutic effects in aseptic meningitis, acute flaccid paralysis, and encephalitis resulting from enteroviral infections. However, pleconaril was found to have only limited effectiveness against EV71, especially EV71-associated neurological complications. Thus, it is of great interest to develop therapeutic strategies against EV71 infections.

The success of RNAi has been widely reported in in vitro systems, and has gradually led to growing interest in in vivo
applications and could eventually lead to its validation as a potential drug therapy for use against infectious diseases. In this study, the 3D region of EV71 was selected as the target site since it has been previously shown that siRNA targeted at the 3D region exerted the most potent inhibitory effect on EV71 replication.19-21 The 3D region of EV71 encodes the viral RNA-dependent RNA polymerase and is being highly conserved, is less likely to mutate and generate mutants. This is important when designing siRNAs to target viruses that mutate frequently such as poliovirus.24

When used either as a prophylactic or as a treatment drug, we demonstrated that both synthetic 19-mer siRNAs and psiStrike plasmids expressing shRNAs were effective in inhibiting EV71 replication in a murine model. The growth of the treated sucking mice was essentially comparable to that of the uninfected mice, showing that both the synthetic 19-mer siRNAs and psiStrike plasmids expressing shRNAs were actually able to protect the sucking mice from developing EV71 infections instead of just delaying the onset of infection. The inhibitory effects on EV71 replication exerted by both forms of siRNAs were dosage dependent as shown by Western blot and real-time RT-PCR analysis. The shorter lasting time of the effects of the chemically synthesized 19-mer siRNAs in the sucking mice (3 days) as compared to plasmid-borne shRNAs (4 days) may be due to the transient delivery nature of the chemically synthesized 19-mer siRNAs, unlike the recombinant DNA-vector system which can express shRNAs endogenously.

In this study, the chemically synthesized 29-mer shRNAs (29mer-3D) failed to protect the sucking mice from EV71 infection despite demonstrating enhanced potency in inhibiting EV71 replication in the rhabdomyosarcoma cell line.25 Since the flow cytometric analysis showed similar uptake efficiencies of chemically synthesized 19-mer siRNAs (19mer-3D), 29-mer shRNAs (29mer-3D) and the psiStrike plasmids expressing shRNAs (psi-3D) by the intestinal cells, the lack of protective effect exerted by the 29-mer shRNAs cannot be attributed to poor siRNA uptake by the mice tissues; we suggest, therefore, that the sucking mice might lack certain mechanisms or cellular proteins which facilitate the delivery of the 29-mer shRNAs to the Dicer so as to be processed into the functional 19-mer siRNAs.

Delivering siRNAs to animal tissues is a complicated process and has been a challenging one. This is due to the fact that the accessibility of the siRNAs to different tissue types makes it almost impossible to have a universal in vivo delivery system.25 In this study, we have compared two systemic methods of delivering siRNAs into the infected sucking mice. The systemic administration approach was used in this study since EV71 infections can affect multiple organs, including the intestines and the brainstem.26 Our results showed that both forms of siRNAs were able to protect the infected sucking mice from EV71 infection when administered via the IP route. In contrast, delivery of the siRNAs via the oral route failed to protect the sucking mice from EV71 infections. This could be due to the degradation of the siRNAs in the gastrointestinal tract; this might have resulted in their inability to exert their antiviral effects. We have also shown in this study that the sucking mice were protected from EV71 infections when the siRNAs were administered even without coupling with any transfection agent. This is a noteworthy result. Even though coupling the siRNAs with cationic lipids has been shown to enhance the siRNA delivery via systemic routes, it has been reported that this can also trigger the cellular IFN pathway.27 To date, an effective mechanism to deliver siRNAs to the central nervous system remains to be elucidated. Thus, early detection and treatment of the EV71 infections is essential so that the siRNAs can be delivered effectively to inhibit the viral replication before irreversible damage to the central nervous system occurs.

It has been established that long double-stranded RNAs (~30 nucleotides) and/or high concentrations of siRNAs can elicit a non-specific IFN response, resulting in the expression of a large number of IFN-stimulated genes that cause the pleiotropic effects of IFN; these include interference with viral replication and modulation of host immune response.27 Thus, it is important to use a judicious concentration of siRNAs which can optimally inhibit EV71 replication, while at the same time not eliciting any immune responses. Our data showed no activation of IFN responses.

<table>
<thead>
<tr>
<th>siRNA</th>
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<th>Nucleotide location</th>
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<tr>
<td>psi-3D</td>
<td>5′-ACCG-GAAATTTGGCTCGAATGTTCATTGCTGGTA(\ast) AACAATTTCGAGCGGCTTCTCCTGTCA(\ast)</td>
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<td>psi-scr</td>
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</tr>
<tr>
<td>19mer-scr</td>
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<td>—</td>
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<tr>
<td>29mer-3D</td>
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<td>7303–7331</td>
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<tr>
<td>29-mer-scr</td>
<td>5′-AGAAGACCUCUUAAGACCCGUGG-3′ 3′-UUUUUUGGUAAUUGG-3′</td>
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Abbreviations: siRNA, small interfering RNA; shRNA, short hairpin RNA.

\(\ast\)Overhang sequences to allow cloning into the psiStrike expression vector. \(\ast\)Stem loop sequence. \(\ast\)U6 termination sequence. \(\ast\)Stem loop sequence.
indicating that both chemically synthesized siRNAs and psiStrike plasmid expressing shRNAs were specific in inhibiting EV71 replication and do not cause undesirable side effects. In conclusion, since this study has shown that RNAi can be used to inhibit EV71 replication in a murine model, it may prove to be a potential therapeutic strategy for use against EV71 infections in children.

MATERIALS AND METHODS

Cell culture and virus strain. The EV71 strain, designated as 5865/SIN/00009 (GenBank accession no. AF316321), isolated from a fatal case during the outbreak in Singapore in October 2000, was used in this study. The virus was cultured in rhabdomyosarcoma cell lines and purified by sucrose gradient centrifugation.

psiStrike plasmid expressing shRNAs and synthetic siRNAs. The chemically synthesized 19-mer siRNAs and the 29-mer shRNAs were designed to target the 3D\textsuperscript{NS} region of the EV71 genome and were designated as 19mer-3D and 29mer-3D, respectively, as described previously.\textsuperscript{20,21} The same oligonucleotide sequence of the 19-mer-3D was also cloned into psiStrike U6 shRNA expression vector according to the manufacturer’s instructions (Promega, Madison, WI). A recombinant plasmid was constructed and designated as psi-3D. The vector contained the human U6 promoter that allowed endogenous transcription of the 19-mer shRNAs. These were subsequently processed into 19-mer siRNAs by the Dicer inside the cells. The delivery efficiency of the psiStrike plasmid vector could also be determined by the presence of a green fluorescent protein, which was driven by the cytomegalovirus promoter. A scrambled sequence with the same base composition as the 19-mer-3D and 29-mer-3D (designated as 19mer-scr and 29mer-scr, respectively) was used as a negative control. The same oligonucleotide sequence of 19mer-scr was also cloned into the psiStrike plasmid vector and the recombinant plasmid expressing scrambled sequences (psi-scr) was used as a negative control. The sequences of 19mer-3D, 29mer-3D, psi-3D, and the scrambled siRNAs are shown in Table 1. Integrated DNA Technologies synthesized all the siRNA oligonucleotides.

siRNA treatment in vivo. For all the in vivo experiments, 1-day-old Balb/c suckling mice were used. The suckling mice (n = 5) were first infected with 10\textsuperscript{4} of a TCID\textsubscript{50} of EV71 via the IP route. The first group of infected suckling mice was used as positive control. 24 hours post infection, six other groups of infected suckling mice were treated with 1, 5, or 10 nmol of 19mer-3D, 1, 5, or 10 nmol of 29mer-3D, 10, 25, or 50 µg of psi-3D, 10 nmol of 19mer-scr, 10 nmol of 29mer-scr or 50 µg of psi-scr via the oral or IP route. All the siRNAs were complexed with Oligofectamine (Invitrogen, Carlsbad, CA) and made up to 100 µl with Opti-MEM (Gibco Life Technologies, Invitrogen, Carlsbad, CA) prior to administration. One group of suckling mice (n = 5) was not infected by EV71 and was used as a negative control. When the siRNA was used without Oligofectamine, the Opti-MEM was substituted with 1× phosphate-buffered saline. During the course of the experiment, the weights of all the suckling mice were monitored every alternate day. On day 14 post infection, the intestines were harvested from the suckling mice, and the mouse intestinal cells were extracted according to the method described by Zhang et al. (2003).\textsuperscript{28} The extracted intestinal cells were stored under –20 °C until further testing. All the mice experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the National University of Singapore, and were repeated four times independently to ensure good reproducibility.

RT-PCR assay. Total RNA was extracted from the supernatants using the RNaseasy extraction kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. The total RNA extracted were then analyzed for presence of EV71 using the real-time hybridization probe RT-PCR for detection of EV71 RNA transcripts, as described previously.\textsuperscript{29} Briefly, the real-time hybridization probe RT-PCR, was carried out using the LightCycler real-time PCR system and the LightCycler RNA Amplification Hybridization Probe kit (Roche Molecular Biochemicals, Mannheim, Germany). Each 10 µl reaction contained 300 ng of RNA, 5 mmol/l MgCl\textsubscript{2}, 0.5 µmol/l of the forward primer, 0.3 µmol/l of the reverse primer, 0.2 µmol/l of each hybridization probe, 2.0 µl hybridization probe reaction mix, 0.2 µl enzyme mix and water. The complementary DNA was first synthesized from the RNA template by reverse transcription for 20 minutes at 95 °C and subsequently amplified for 40 cycles at 95 °C for 35 seconds, 55 °C for 15 seconds and 72 °C for 9 seconds.

Immuno blot. The extracted intestinal cells were then lysed using 200 µl of Celllytic M Cell Lysis Reagent (Sigma, California). An aliquot of 20 µl of each lysate was electrophoresed in a denaturing 10% polyacrylamide gel. Western blotting was then performed following standard procedures. The detection procedure was based on the chromogenic method described previously using EV71 VP1 monoclonal antibody (Chemicon International, Madison, WI) and the β-actin antibody (Sigma, California).\textsuperscript{20,21}

Immunohistochemical analysis. On day 7 and 14 post infection, the intestines of the infected and treated suckling mice were harvested and stored immediately at –80 °C. Cryosections of 4 µm from the frozen tissues were made and fixed on poly-L-lysine glass slides. Immunostaining of the fixed tissues was carried out by incubation with 0.2% Triton for 10 minutes. EV71 was detected using the mouse monoclonal antibody against EV71 (Chemicon International, Madison, WI). After washing with 1× phosphate-buffered saline, the tissues were incubated with biotinylated anti-mouse secondary antibodies (Zymed Laboratories, San Francisco, CA). A red colored peroxidase stain was developed using aminoethyl carbazole substrate and counterstained with hematoxylin (Zymed Laboratories, San Francisco, CA).

IFN-α and IFN-β assay. The suckling mice were first injected with 10 nmol 19mer-3D or 50 µg psi-3D via the IP route. After 24 hours, homogenates of the intestines were prepared. The levels of IFN-α and IFN-β in the homogenates were then measured by the enzyme-linked immunosorbent assay (PBL Biomedical Laboratories, Piscataway, NJ) according to the manufacturer’s instructions.

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Inhibition of EV71 by RNAi in Murine Model


