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Enhanced potency and efficacy of 29-mer shRNAs in inhibition of Enterovirus 71

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Abstract

Enterovirus 71 (EV71) is the main causative agent of hand, foot, and mouth disease (HFMD) in young children. It has been associated with severe neurological complications and has caused significant mortalities in large-scale outbreaks in Asia. In this study, we demonstrated an enhanced silencing of EV71 through the use of chemically synthesized 29-mer shRNAs. The 29-mer shRNAs were designed to target three highly conserved regions of EV71 genome. Transfection of rhabdomyosarcoma (RD) cells with the 29-mer shRNAs significantly inhibited EV71 replication in a dose-dependent manner as demonstrated by reduction of viral RNA, VP1 protein and plaque forming units. The inhibitory effects were more potent and were achieved at 10-fold lower concentrations when compared to 19-mer siRNAs reported previously [Sim, A.C.N., Luhur, A., Tan, T.M.C., Chow, V.T.K., Poh, C.L., 2005. RNA interference against Enterovirus 71 infection. Virology 341, 72–79]. The viral inhibitory effects lasted 72 h post-infection and there was no adverse off-target silencing effect. Gene silencing by 29-mer shRNAs targeted at the $3D^{pol}$ region (sh-3D) was the most effective, achieving 91% viral inhibition. Further evaluation found that no enhanced inhibitory effects were observed when sh-3D was cotransfected with each of the other two candidates. This study showed an improvement in triggering RNAi using the more potent 29-mer shRNAs, indicating its therapeutic potential against EV71.

Keywords: Enterovirus 71; Hand, foot and mouth disease (HFMD); RNA interference; 29-mer shRNAs; Enhanced silencing effects; Potential antiviral therapy

1. Introduction

Enterovirus 71 (EV71) belongs to the human Enterovirus A species of the Picornaviridae family. It is a positive single-stranded RNA virus with a non-enveloped capsid and a genome size of 7411 bases. It was first discovered and isolated from the stool of an infant who was suffering from encephalitis in California in 1969 (Schmidt et al., 1974). EV71 infections manifest most frequently as hand, foot, and mouth disease (HFMD), a mild childhood exanthem which mainly affects young children (<6 years of age), and is characterized by 3–4 days of fever, development of vesicular exanthema on the buccal mucosa, tongue and gums. The clinical symptoms caused by EV71 are indistinguishable from Coxsackievirus A16 (CA16), another common causative agent of HFMD. However, EV71 is more often associated with severe neurological diseases such as acute flaccid paralysis, cerebellar encephalitis, aseptic meningitis and even death (Lum et al., 1998; McMinn et al., 2001). Significant mortalities in recent HFMD outbreaks in the Asia Pacific region have raised public concerns about the virulence of EV71 (Ho et al., 1999). The current use of antivirals such as pleconaril has been shown to be effective for most enteroviruses but not for EV71 (Robart et al., 1998; McMinn, 2002). Thus, there is a need to develop a specific antiviral therapy against EV71.

Over the last few years, RNA interference (RNAi) has emerged as a promising approach to develop potential therapeutic agents based on specific gene silencing. The siRNA is processed by the RNase III-like enzyme known as the Dicer into smaller interfering RNAs (siRNAs) of 21–23 nucleotides. The antisense strand of the siRNA will serve as the template and induce the formation of the RNA-induced silencing complexes (RISCs) which subsequently recognize and degrade complementary target mRNAs (Fire et al., 1998). Majority of the studies which use RNAi strategies involve the use of 21 nucleotides which contain the 19-mer core sequences and 2d-TT at the 3′ ends. It was reported recently that 29-mer short hairpin RNA

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(shRNAs) have enhanced potency and are more efficient than 19-mer siRNAs in silencing genes without inducing any interferon response (Siolas et al., 2005). Therefore, the use of 29-mer shRNAs may prove to be even more efficient as potential therapeutic agents against EV71 in comparison to the two different forms of 19-mer siRNAs reported previously (Lu et al., 2004; Sim et al., 2005). Lu et al. (2004) reported a plasmid-derived shRNA system producing siRNAs which targeted the VP1 and $3D^{pol}$ regions of the EV71 genome that were able to inhibit EV71 replication in both HeLa and Vero cell lines. In a separate study, chemically synthesized 19-mer siRNAs targeted at the 2C, 3C, $3D^{pol}$ and 3′UTR regions of the EV71 genome have also shown significant inhibition of EV71 replication in the RD cell line. Both studies showed that siRNAs targeting the RNA-dependent RNA polymerase, $3D^{pol}$ were the most effective in inhibiting EV71 replication (Sim et al., 2005).

In this study, we demonstrated that the chemically synthesized 29-mer shRNAs targeted at the 2C, $3C^{pro}$, and $3D^{pol}$ regions of the EV71 genome were more potent than the chemically synthesized 19-mer siRNAs and the plasmid-derived shRNAs in inhibiting EV71 replication in RD cells.

2. Methods and materials

2.1. Cell culture and virus strain

The EV71 strain isolated from a fatal case during the outbreak in Singapore in October 2000, designated as 5865/SIN/00009 (Genbank accession no. AF316321) was used in this study. Rhabdomyosarcoma (RD) cells were routinely grown in minimum essential medium (Gibco, Boston, MA, USA) supplemented with 5% fetal calf serum, HEPES, 1% sodium pyruvate, and 1.5% sodium bicarbonate.

2.2. Design and synthesis of double stranded 29-mer shRNAs

The 29-nt double-stranded shRNAs were designed to target specific sequences within the 2C, $3C^{pro}$, and $3D^{pol}$ of the EV71 and designated as sh-2C, sh-3C and sh-3D, respectively. The design of the shRNAs was based on the general guidelines as described (Elbashir et al., 2001a,b). The sequences were subjected to BLAST search of the National Center for Biotechnology Information database to ensure that they target the desired viral genes specifically. The shRNA oligonucleotides were synthesized with 3′-dUU extensions. A scrambled sequence (scr) with the same base composition as the sh-3D was also used as a negative control. The sequences of the chemically synthesized siRNAs were listed in Table 1. For comparative purpose, the chemically synthesized 19-mer siRNAs targeted at $3D^{pol}$ region (designated as 19mer-3D) of EV71 reported previously (Sim et al., 2005) was included in this study. All the siRNA oligonucleotides were synthesized by Dharmacon, USA.

2.3. Transfection and infection

Transfection was carried out under optimal conditions. Briefly, RD cells (5×10^4) were seeded into each well of a 24-well plate and grown at 37 °C overnight. The growth medium in each well was then replaced with 500 μl of pre-warmed reduced-serum medium, OPTI-MEM I (Gibco, Boston, MA, USA) for another 24 h. Upon reaching 80% confluency, the cells were transfected with complexes containing shRNAs and Lipofectamine 2000CD (Invitrogen Life Technologies, Carlsbad, CA). After 48 h, each well was infected with EV71 at an MOI of 10 for 1 h. The cells were then overlaid with the growth medium and incubated at 37 °C in 5% CO_2. At different time points post-infection, cell lysates and cell supernatants were collected and stored at −80 °C for further studies. To evaluate the therapeutic potentials of the 29-mer shRNAs, the RD cells were infected with EV71 (MOI of 10) and at each hourly interval (up to 5 h) after infection, the RD cells were then transfected with 29-mer shRNAs.

2.4. SDS-PAGE and Western blot

RD cells were grown in wells of a six-well plate and transfection was performed according to the experimental set up

Table 1
Chemically synthesized 29-mer shRNAs sequences and their corresponding nucleotide positions in EV71 genome

siRNA	Nucleotide sequence	Nucleotide location	Gene
sh-2C	5′-GAACCAGAUCACGAACUUGGAGCAGTCCG **UUGG**[a]; 3′-UUCUUGGUCUAGUGCUUGAACCUCGUCAGGC Molecular weight: 20,519.3 g/mol	4250–4278	2C
sh-3C	5′-AGCUACUUUGCGAGUAUGCAAGGUGAGAU **UUGG**; 3′-UUUCGAUGAAACGCUCAUACGUUCCACUCUA Molecular weight: 20,474.3 g/mol	5917–5949	$3C^{pro}$
sh-3D	5′-AGAAAUUGGCUCGAAUUGUUUUAAUAUUA **UUGG**; 3′-UUUCUUUAACCGAGCUUAACAAAAUUAUAAU Molecular weight: 20,384.2 g/mol	7303–7331	$3D^{pol}$
scr	5′-AAAGAACCUUAAGACCUCGGUUAAUAUUA **UUGG**; 3′-UUUUUCUUGGAAUUCUGGAGCCAAUUAUAAU Molecular weight: 20,414.2 g/mol	–	–

[a] Stem loop sequence.

as described. At 24 h post-infection, the cells were harvested for determination of total proteins. The cells were first lysed using 200 μl of CelLytic M Cell Lysis Reagent (Sigma, USA). 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 as described previously using EV71 VP1 monoclonal antibody (Chemicon International, USA), the anti-PKR antibody (Sigma, USA), the anti-phospho PKR antibody (Sigma, USA) and the β-actin antibody (Sigma, USA) (Sim et al., 2005).

2.5. Viral plaque assay

To determine the viral infectivity of EV71 after siRNA treatment, plaque assays were carried out after the transfection protocol. After 24 h of transfection, 1% (w/v) carboxymethylcellulose (CMC) in 2× growth medium was layered onto the infected cell monolayer. The plates were then incubated at 37 °C for 48 h. Plaques of EV71 were observed by fixing with 20% formalin in PBS and staining with 1% crystal violet for 30 min at room temperature.

2.6. Real-time reverse transcription (RT) PCR

At 48 h post-infection, total RNA was extracted from each well using TRIzol LS reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. The efficiency of the various shRNAs in inhibiting EV71 replication were then analysed using real-time RT-PCR Hybridization probe-based assay for the detection of EV71 RNA as described previously (Tan et al., 2006). Briefly, the real-time RT-PCR Hybridization probe assay was carried out using the LightCycler real-time machine and the LightCycler RNA Amplification Hybridization Probe kit (Roche Molecular Biochemicals, Germany). Each reaction was performed in a reaction capillary tube by mixing the reagents, followed by spinning down the mixture briefly with the help of a special LightCycler centrifuge adapter. Each 10 μl reaction contained 1.0 μl of RNA, 5 mM $MgCl_2$, 0.5 μM of the forward primer, 0.3 μM of the reverse primer, 0.2 μM of each hybridization probe, 2.0 μl hybridization probe reaction mix, 0.2 μl enzyme mix and water.

The cDNA was first synthesized from the RNA template by reverse transcription for 20 min at 95 °C and subsequently amplified for 40 cycles at 95 °C for 35 s, 55 °C for 15 s and 72 °C for 9 s. Melting curve analysis was carried out after the PCR reaction. A rapid thermal ramp to 95 °C was achieved for complete denaturation of the PCR amplicons, followed by hybridization at 60 °C for 10 s. A slow steady transition from 60 to 95 °C was then performed. Subsequently a graph was plotted using dF/dT against temperature which would show a peak corresponding to the T_m of the PCR amplicon.

2.7. Cell viability assay

To assay for cell viability due to the toxicity of the 29-mer shRNAs, RD cells were seeded in a 24-well plate and the transfection of cells were performed as described above. At 1 and 2 days post-transfection, cells were trypsinized, and 100 μl of the resuspended cells were transferred to the wells of a 96-well plate in triplicates. A total of 20 μl of the MTS/PMS reagent (Promega, USA) were 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 triplicates and carried out as two independent experiments.

3. Results

3.1. Protection of RD cells from EV71-induced CPE by 29-mer shRNAs

Three 29-mer shRNAs were designed to target specific sequences in the non-structural genes such as the 2C, $3C^{pro}$ and $3D^{pol}$ of EV71. A BLAST search showed that the 29-mer shRNAs designed were specific for the three targets and there was no homology with human genes.

The efficacy of the 29-mer shRNAs in inhibiting EV71 replication were first evaluated by transfecting the RD cells with sh-3D at a final concentration of 1 and 10 nM. At 48 h post-transfection, the RD cells were infected with EV71 at a MOI of 10. Significant inhibition of EV71-induced CPE was observed with as low as 1 nM of the sh-3D. This is in sharp contrast to the RD cells treated with the scrambled shRNAs which showed 100% CPE. No CPE was observed in RD cells transfected with 10 nM of sh-3D. The inhibition of EV71-induced CPE was observed to last by up to 72 h post-infection (hpi). The result showed that the 29-mer shRNAs targeted the $3D^{pol}$ region of EV71 genome was able to inhibit EV71-induced CPE in RD cells (Fig. 1).

To ascertain that there is no cytotoxicity induced by the chemically synthesized shRNAs, the MTS assay which measured the viability of cells was carried out. The assay was performed at 48 h post-transfection and it was observed that the three 29-mer shRNAs did not cause any cytotoxic effects on the growth and viability of the RD cells (Fig. 2).

3.2. Inhibition of EV71 replication by 29-mer shRNAs

After demonstrating the inhibition of EV71-induced CPE in RD cells by the 29-mer shRNAs, we next determined the potency of the 29-mer shRNAs by studying the inhibitory effects of different concentrations of shRNAs on EV71 replication. The RD cells were transfected with graded concentrations of each of the three 29-mer shRNAs (1, 5 and 10 nM), followed by infections with EV71 at a MOI of 10. The inhibitory effects of each of the three 29-mer shRNAs on EV71 replication were analyzed by Western blots, plaque assays and real-time RT-PCR Hybridization probe assay, respectively. Our results showed that when the concentration of each of the 29-mer shRNAs was increased from 1 to 10 nM, VP1 viral protein levels were observed to decrease correspondingly. Similar observations were obtained when the RD cells were treated with 1, 5 and 10 nM of 19-mer siRNAs. However, the decrease in the VP1 protein levels were not as significant as what was observed in the RD cells transfected with

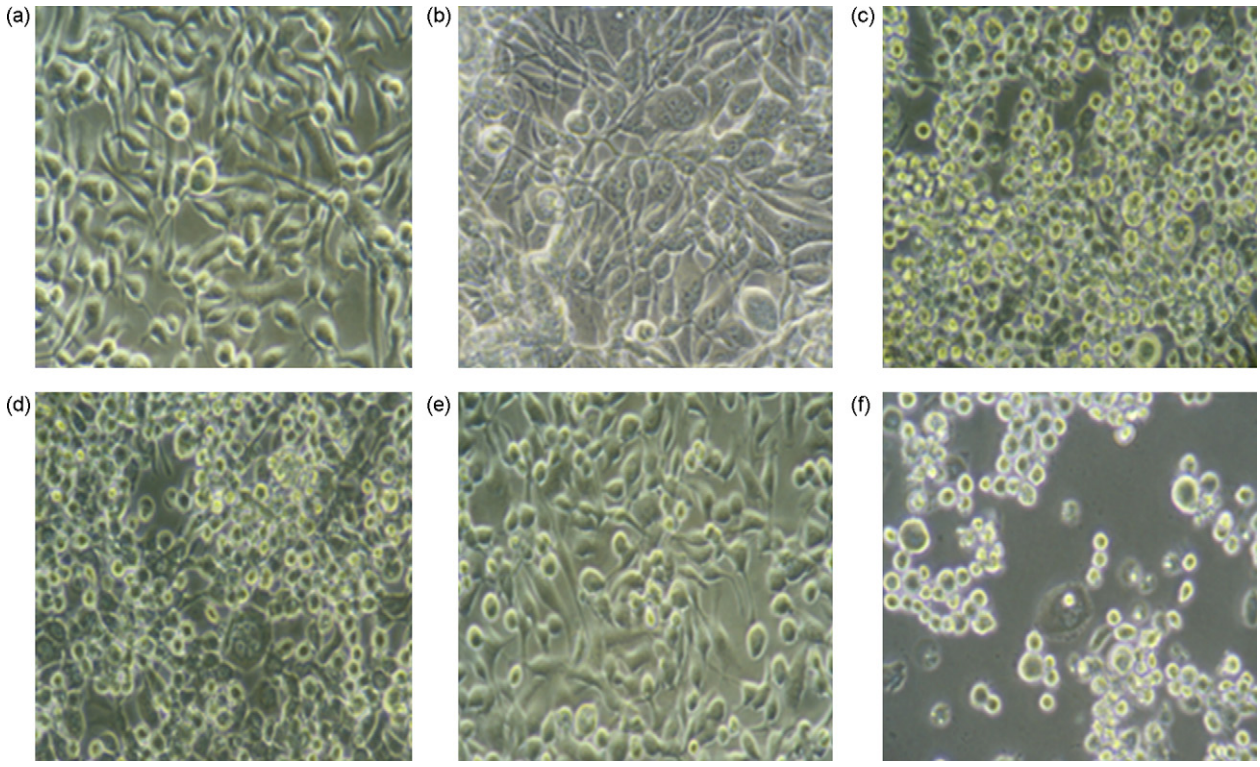

Fig. 1. Inhibition of EV71-induced CPE by 29-mer shRNAs. The RD cells were first transfected with 29-mer shRNAs and then infected with EV71 at a MOI of 10. The morphological changes of the RD cells were observed at 72 hpi under the light microscope at 40× magnification. (a) Uninfected RD cells; (b) uninfected RD cells transfected with 10 nM sh-3D; (c) infected RD cells in the absence of sh-3D; (d) EV71-infected RD cells treated with 1 nM of sh-3D; (e) EV71-infected RD cells treated with 10 nM of sh-3D; (f) RD cells treated with 10 nM scrambled 29-mer shRNA. The tests were carried out in two independent experiments.

the 29-mer shRNAs. As expected, there was no decrease in the VP1 protein levels in the RD cells treated with the scrambled 29-mer shRNAs (Fig. 3a). To corroborate with the significant decrease in VP1 viral proteins expression, the infectious titre of EV71 in the RD cells transfected with 29-mer shRNAs was determined by plaque assay. Transfection of RD cells with sh-2C resulted in inhibition of EV71 by 52%, 73% and 87% when the concentrations of sh-2C were increased from 1 to 10 nM. Similar dosage dependency effects were observed when the RD cells were treated with 1, 5 and 10 nM of sh-3C (60%, 77% and 89%) and sh-3D (64%, 81% and 91%). In contrast, when the RD cells

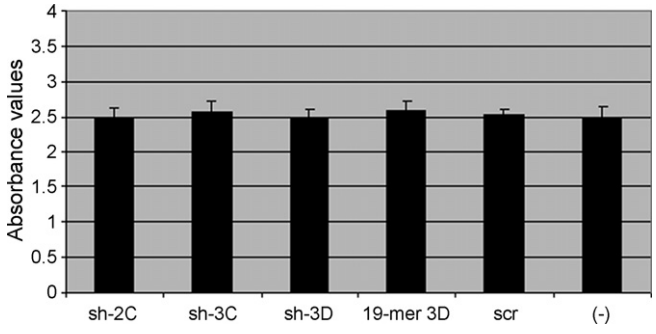

Fig. 2. Viability of RD cells was determined by MTS assay after transfection with 10 nM of 29-mer shRNAs targeted at the 2C (sh-2C), $3C^{pro}$ (sh-3C) and $3D^{pol}$ (sh-3D) genes of the EV71 genome for 48 h. Absorbance values were obtained by measuring at 490 nM. The data shown represent the mean ± S.D. from two independent experiments. Ten nanomolars of scrambled shRNAs (scr) and untransfected (−) RD cells were used as controls.

were treated with the 1 nM, 5 nM and 10 nM of 19-mer siRNA targeted at the $3D^{pol}$ region, the efficiency of EV71 inhibition was found to be only 60%, 66% and 71%, respectively (Fig. 3b).

The amount of EV71 viral transcripts in the RD cells treated with different concentrations of sh-2C, sh-3C and sh-3D was determined by real-time RT-PCR Hybridization probe assay. Compared to untreated RD cells which were infected with EV71, a 2.5 log unit reduction in viral RNA transcripts was observed when the RD cells were treated with 10 nM of sh-3D. The RD cells treated with 10 nM of sh-2C and sh-3C showed a 1.5 log unit decrease in viral RNA transcripts. In the case of the 19-mer siRNAs targeting at the $3D^{pol}$ region, a 0.8 log unit decrease in the EV71 viral transcripts was observed (data not shown). Based on the results, we demonstrated that the 29-mer shRNAs targeted at the specific sequences of the 2C, $3C^{pro}$ and $3D^{pol}$ of EV71 were effective in inhibiting EV71 replication in RD cells in a dosage dependent manner. The 29-mer shRNAs targeting at the $3D^{pol}$ region (sh-3D) of EV71 was found to exhibit the most potent anti-EV71 effects in RD cells. It is also established in this study that a much lower concentration of the 29-mer shRNAs was able to inhibit EV71 replication more effectively as compared to the chemically synthesized 19-mer siRNAs.

The efficacy of 29-mer shRNAs in inhibiting an on-going EV71 infection was tested by infecting the RD cells at a MOI of 10 over a period of 5 h. At every hour interval, the RD cells were transfected with 10 nM of the sh-2C, sh-3C or sh-3D and were observed for CPE at 48 hpi. Significant CPE was observed in the untreated EV71-infected cells. In contrast, survival of the

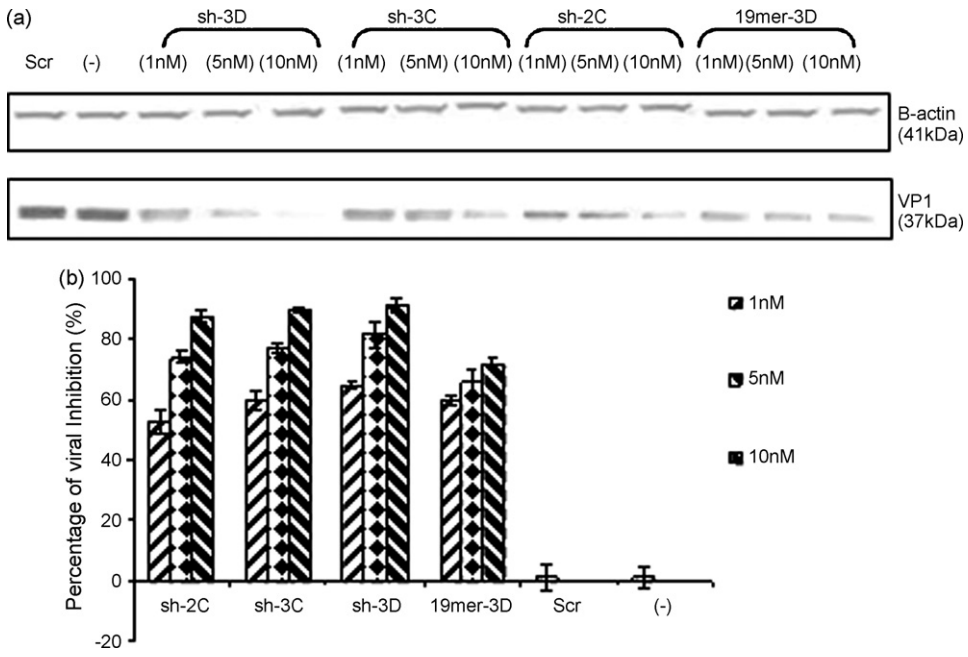

Fig. 3. Dose-dependent inhibition of EV71 replication with 29-mer shRNAs targeted at the 2C (sh-2C), $3C^{pro}$ (sh-3C) and $3D^{pol}$ (sh-3D) genes of the EV71 genome. The RD cells were transfected with 1, 5 and 10 nM of sh-2C, sh-3C or sh-3D, followed by infections with EV71 at a MOI of 10. The RD cells treated with 1, 5 and 10 nM of 19-mer siRNAs (19mer-3D), 10 nM of scrambled shRNAs (scr) and untransfected (−) RD cells were used as controls. The cell lysates and cell supernatants were harvested to determine viral titres and VP1 protein levels. (a) Western blot analysis was performed using monoclonal antibodies against the VP1 structural proteins of EV71. β-Actin was used as an internal control, using anti-β-actin monoclonal antibodies. The tests were carried out in two independent experiments. (b) Viral titres were determined by plaque assay. The data shown represent the mean ± S.D. from two independent experiments. As high as 91% inhibition of the infectious EV71 titre was observed. The ANOVA analysis for comparison between different concentrations of each 29-mer shRNA was found to be $P < 0.05$. The ANOVA analysis for comparison between the untransfected RD cells and the treated RD cells was $P < 0.01$. The ANOVA analysis for comparison between the sh-3D and the 19mer-3D was $P < 0.01$.

RD cells treated with 10 nM of each of the 29-mer shRNAs was still observed 3 h post-infection. Thus, administration of 29-mer shRNAs to RD cells after EV71 infection can still effectively inhibit viral replication.

3.3. No enhanced inhibitory effects on EV71 replication by combinations of two 29-mer shRNAs

It has been reported that an enhanced antiviral effects of combinations of siRNAs was observed in HIV infection (Ji et al., 2003). To test whether cotransfection with two specific siRNAs targeted at different regions of the EV71 genome will improve the antiviral effect in this study, two 29-mer shRNAs were cotransfected into the RD cells, followed by infection with EV71 as described in part 2.3. A combination of sh-3D and each of the remaining two 29-mer shRNAs (sh-2C and sh-3C) were evaluated at a combined final concentration of 10 nM. The efficacy in inhibiting EV71 replication was measured by real-time RT-PCR Hybridization probe assay. Results by the real-time RT-PCR showed that similar inhibitory efficacy on EV71 replication was observed as compared to the 29-mer shRNAs when used individually (data not shown). This suggests that there is no enhanced antiviral effect when a combination of 29-mer shRNAs was used.

3.4. No activation of interferon pathway when RD cells were treated with 29-mer shRNAs

There is a possibility that inhibition of viral replication is due to activation of PKR and induction of the interferon response.

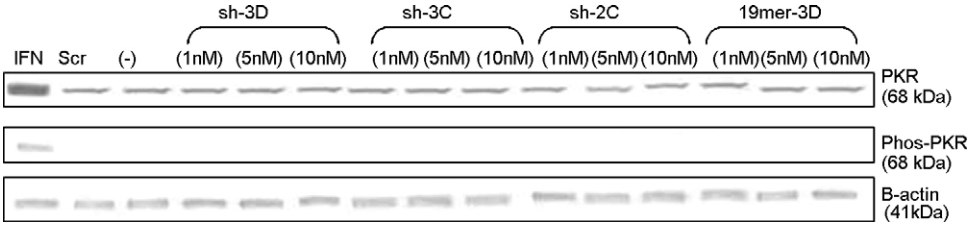

Fig. 4. PKR and phosphor-PKR protein levels in the RD cells were shown by Western blotting. The RD cells were transfected with various concentrations of sh-2C, sh-3C and sh-3D for 48 h, followed by EV71 infections at a MOI of 10. The RD cells treated with alpha interferon, 19-mer siRNAs (19mer-3D), scrambled shRNAs (scr) and untransfected (−) RD cells were used as controls. Western blot analysis was performed using monoclonal antibodies against the PKR or phospho-PKR. β-Actin was used as an internal control, using anti-β-actin monoclonal antibodies. The tests were carried out in two independent experiments.

To rule out such possibility, we carried out Western blot using specific monoclonal antibodies against endogenous PKR. As a positive control for the induction of PKR, the RD cells were treated with human alpha interferon (Sigma, USA) as described previously (Kanda et al., 2004). Our results showed that there was no increase in PKR expressions in the RD cells after they were transfected with each of the three 29-mer shRNAs for 48 h. However, IFN specifically stimulated the PKR expression (Fig. 4). It is well-established that activation of the interferon response is characterized by the transient autophosphorylation of PKR in the early stages of the initiation process (Khabar et al., 2003). We thus analysed the expression of phopho-PKR in the transfected RD cells by Western blot using specific monoclonal antibodies against phospho-PKR (Sigma, USA). Other than the positive control, we did not observe any phospho-PKR expression in the RD cells treated with each of the 29-mer shRNAs (Fig. 4). These observations indicated that each of the three 29-mer shRNAs did not elicit interferon response and we conclude that viral inhibition was mediated through RNAi.

4. Discussion

RNAi technique has been extensively exploited as potential therapeutic approaches to treat infectious diseases such as polioviruses (Gitlin et al., 2002), HIV (Capodici et al., 2002), Hepatitis C virus (Yokota et al., 2003) and Hepatitis B virus (Li et al., 2004). Currently, antivirals have been developed against some members of the enteroviruses, but none of them was found to be effective against EV71. The 'WIN' group of compounds was found to be the most promising among these antiviral agents. One of the "WIN" compounds, pleconaril, was found to be effective in treating aseptic meningitis and encephalitis caused by some enteroviruses but showed limited therapeutic benefit against EV71 (McMinn, 2002). Thus, it is of great interest to develop therapeutic strategies against EV71 infections.

In this study, specific sequences within the 2C, $3C^{pro}$ and $3D^{pol}$ regions of EV71 were chosen as the target sites as they are highly conserved and are less likely to mutate to generate mutants. This is an important feature for the design of siRNAs and is more significant when targeting viruses that mutate frequently such as poliovirus (Gitlin et al., 2005). We observed ~90% inhibitory effects on EV71 replication when 10 nM of 29-mer shRNAs targeted at each of the three specific sites were used. The inhibitory effects exhibited by the each of the three 29-mer shRNAs on EV71 replication were more potent than the siRNAs expressed from the plasmid-derived shRNA vector system (Lu et al., 2004) and the chemically synthesized 19-mer siRNAs (Sim et al., 2005). In both studies, at least 10 fold higher concentrations of siRNAs were needed to exert significant inhibition of EV71 replication. In assessing the protection of the transfected RD cells against CPE caused by EV71, we observed that all three 29-mer shRNAs at 10 nM were able to protect the RD cells from EV71-induced CPE by up to 72 h. This is in contrast to only 48 h of protection of RD cells conferred by the siRNAs reported by Lu et al., 2004 and Sim et al., 2005. In this study, we demonstrated that the 29-mer shRNAs were more potent than the 19-mer siRNAs in inducing RNAi. The enhanced potency of the 29-mer shRNAs could be due to higher affinity of the 29-mer shRNAs for the Dicer enzyme (Rusk, 2005; Siolas et al., 2005). An alternative explanation is that the hairpin RNAs may interact with specific cellular proteins which facilitate the delivery of these 29-mer shRNA substrates to the Dicer (Siolas et al., 2005). The loss of the protective effects conferred by the 29-mer shRNAs after 72 h post-infection is likely due to the decrease in the 29-mer shRNA concentration as the RD cells divide.

All the three chemically synthesized 29-mer shRNAs examined in this study showed a dosage dependent inhibition of EV71 replication. Among the three 29-mer shRNAs, sh-3D was shown to be the most potent in inhibiting EV71 replication, followed by sh-3C and sh-2C. This supported the findings by Lu et al., 2004 and Sim et al., 2005 which also showed that targeting at the viral RNA-dependent RNA polymerase, $3D^{pol}$, is the most effective in inhibiting EV71 replication. The specific function of 2C has yet to be identified. The 3C region encodes a protease which performs majority of the secondary processing of the polyprotein. The 3D region encodes the viral RNA-dependent RNA polymerase which oligomerizes into a complex and subsequently binds to the viral RNA. Since the $3D^{pol}$ gene and the other cellular factors form an important component in facilitating viral replication, its down-regulation could produce the most potent inhibitory effect on EV71 replication.

It has been shown that cotransfection of cells with two or more siRNAs targeted at different sites resulted in enhanced gene silencing when compared to single siRNA is used (Ji et al., 2003). The underlying mechanism of enhanced gene silencing with multiple siRNAs has yet to be elucidated. It was postulated that the binding of one siRNA may change the secondary structure of the target RNA, thus result in more accessible sites for another siRNA (Ji et al., 2003). However, in this study, we did not observe any enhancement effects when we cotransfected the RD cells with different combinations of the three 29-mer shRNAs. The possible reason for the discrepancy may be because the 29-mer shRNAs developed in this study did not affect the secondary structure of the EV71 mRNA, and thus do not increase accessibility to other 29-mer shRNAs. Nevertheless, although the mechanism of the enhanced gene silencing with multiple siRNAs is unknown, it may be a good approach for treatment of viral infections, especially for viruses which mutate and escape from RNA interference.

Longer dsRNAs (~30 nts) can elicit non-specific interferon response, leading to subsequent inactivation of the cellular transcriptional machinery and resulting in death of the mammalian cells (Persengiev et al., 2004; Sledz et al., 2004). The interferon response is also responsible for protection against viral infections (Khabar et al., 2003). The main mechanism in the interferon pathway is the induction of dsRNA-dependent protein kinase R (PKR). PKR is a serine–threonine kinase which is normally inactive. Upon activation by interferon or dsRNA, PKR are phosphorylated and the levels are upregulated. The activated PKR will then phosphorylate the cellular proteins, notably eIF2α, which are downstream of the interferon pathway, and subsequently leading to translational arrest (Khabar et al., 2003; Persengiev et al., 2004; Sledz et al., 2004). Thus, during the

design of RNAi strategy, the siRNAs must be long enough for effective gene silencing, but short enough to prevent the induction of the interferon response. Our study used a much lower concentration of siRNAs and showed a higher efficacy in silencing EV71. This minimized the chances of "off target effects" due to the interferon response. Since no increase in the endogenous PKR and phospho-PKR levels was detected, our data indicated no engagement of the interferon pathway in the RD cells when they were transfected with each of the three 29-mer shRNAs.

In conclusion, our study showed an improvement in triggering RNAi in mammalian cells using the more potent 29-mer shRNAs. We have shown that increased RNAi potency was observed for all the three-targeted sites (2C, $3C^{pro}$ and $3D^{pol}$) when compared to the inhibitory effects shown by the 19-mer siRNAs. The improved efficacy of the 29-mer shRNA at $3D^{pol}$ indicated its high potential to be developed as an antiviral therapeutic agent against EV71.

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