Development of potential antiviral strategy against coxsackievirus B4

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\textbf{A B S T R A C T}

Coxsackievirus B4 (CVB4) can cause a broad range of diseases such as aseptic meningitis, meningoencephalitis, myocarditis, hepatitis, pancreatitis, gastroenteritis, necrotizing enteroculitis, pneumonia and sudden death in the neonates. CVB4 has also been implicated as a possible etiological agent for type 1 insulin dependent diabetes mellitus (IDDM). In this study, the possibility of RNA interference (RNAi) as a potential therapeutic approach to treat CVB4 infection was explored. The results showed that the Rhabdomyosarcoma (RD) cells treated with 19-mer siRNAs displayed high specificity against CVB4 replication without displaying any sign of target effects. The siRNA targeting the 3C\textsuperscript{pro} region of CVB4 genome was also established to be the most effective in inhibition of CVB4 replication in RD cell line in a dosage dependent manner, indicating its potential to be developed as an antiviral strategy against CVB4.

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1. Introduction

Coxsackievirus B4 (CVB4) is a member of the \textit{Enterovirus} family and is one of the six serotypes of coxsackievirus group B (CVB) (Reuckert, 1990). Like all CVBs, CVB4 has been known to elicit a wide variety of diseases in the world. The most common result of general CVB infection is asymptomatic infection, undifferentiated febrile illness, or mild upper respiratory symptoms (Pallansch, 1990). Like all CVBs, CVB4 has been known to elicit a wide variety of diseases in the world. The most common result of general CVB infection is asymptomatic infection, undifferentiated febrile illness, or mild upper respiratory symptoms (Pallansch, 1990). However, the most significant chronic disease associated with CVB4 infection is the juvenile onset of insulin dependent diabetes mellitus (IDDM) (Pallansch, 1997). Severe disease symptoms are readily recognised by the presence of fever, chest pain, pleural inflammation, headache and sore throat. Infections by CVB4 have also been known to cause aseptic meningitis, encephalitis, pleurodynia, myocarditis, and pericarditis (Crowell and Landau, 1997). Although most of the diseases caused by CVB4 are also commonly seen in infections with other enteroviruses, nevertheless, clinical symptoms such as myopericarditis and pleurodynia (Bornholm disease) are still distinct and are associated only with CVB4 infections (Modlin and Rotbart, 1997). However, the most significant chronic disease associated with CVB4 infection is the juvenile onset of insulin dependent diabetes mellitus (IDDM) (Pallansch, 1997). The ability of CVB4 to induce glucose intolerance, resulting in viral-induced insulin dependent diabetes mellitus (IDDM) has long been suspected and demonstrated both in vitro (Yoon et al., 1979; D’Alessio, 1992; Fohlman and Firman, 1993) and in vivo (Yoon et al., 1986; Horwitz et al., 1998; Richardson et al., 2009).

There are currently no available vaccines for non-polio enteroviruses (Pallansch, 1997) and no antiviral therapy for enterovirus infections. By far, the only available treatment is directed towards alleviating the symptomatic effects as a result of CVB4 infections. There has been successful identification of candidate drugs exhibiting antiviral activity against enteroviruses in tissue culture and animals (Rozhon et al., 2005). Several of this class of drugs are currently in development and in early clinical trials (McKinlay and Steinberg, 1986; O’Connell et al., 1995). However, they have not been evaluated in the treatment of diseases in animal model systems of both myocarditis and diabetes (Ilback et al., 1993; See and Tilles, 1993). Hence, there is a need for an antiviral therapy which is specific for treating CVB4 infections. RNA interference (RNAi) is an evolutionary conserved mechanism that has been observed across all eukaryotic cells (Dykxhoorn et al., 2006). RNAi plays an important role in the endogenous silencing of repetitive or transposable genetic elements for the maintenance of cellular homeostasis (Sijjen and Plasterk, 2003). RNAi has also been shown to confer protection to human cells from infections caused by viruses by inhibiting the transcription of viral transcripts (Gitlin et al., 2002; Lecellier et al., 2005). In this study, we evaluated RNAi as potential antiviral strategy against CVB4 in an in vitro system.

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2. Materials and methods

2.1. Virus strains

The ATCC CVB4 J.V.B strain (Accession number X05690) which was used in this study, was provided by Department of Microbiology, National University of Singapore for a research project undertaken by APY Wong during his undergraduate course.

2.2. Design of chemically synthesized 19-mer small-interfering RNAs (siRNAs)

The siRNAs were designed to target the 2A, 3C and 3D regions which encode for non-structural genes of the CVB4 genome (2Apro, 3Cpro and 3Dpol) based on the general guidelines described by Elbashir et al. (2001). Using the cluster alignment function of the DNASTAR program, highly conserved regions within the three regions were identified and entered into the siRNA Target Finder software (Ambion, Inc.). Successfully identified siRNA targets were then subjected to analysis by the BLAST algorithm. Non-specific scrambled sequence of the siRNA targeting different regions of 3Dpol region was designed and used as a control. All siRNAs were synthesised by Sigma Proligo, USA (Table 1).

2.3. Transfection and infection

Rhabdomyoscarcoma (RD) cells were maintained in growth medium (MEM supplemented with 5% FCS; Gibco, USA), 2% HEPES and 1.5% NaHCO3. When the growth of the RD cells reached confluence, the cell monolayer was rinsed with phosphate buffered saline (PBS) to remove dead cell and debris before treatment with 1X trypsin-EDTA to detach the cells. Upon detachment of the RD cells from the bottom of the flask, fresh growth medium was added to stop the reaction. The cells were then passaged at a ratio of 1:4 in T75 tissue culture flasks and maintained in a 37 °C incubator with 5% CO2. For RNAi studies, RD cells were seeded at a 5 x 10^5 cells/well concentration into a 24-wells microtiter plate. Transfection of RD cells with siRNAs was based on a serum starvation protocol using LipofectamineTM 2000® (Invitrogen, USA). The transfection protocol was optimised according to manufacturer’s instructions. The growth medium was replaced with 500 µl of OPTI-MEM® (Gibco, USA) 24 h after the cells were seeded into the microtiter plate. After 24 h of starvation with OPTI-MEM®, the RD cells were transfected with a transfection mixture containing 1.2 µl of LipofectamineTM 2000CD diluted in OPTI-MEM® containing different concentrations of siRNAs (0, 25, 50 and 100 nM). The transfection mixtures were added into individual wells and incubated for 48 h. Transfection was carried out without the addition of siRNAs as the negative control. At 48 h post-transfection, the transfected RD cells were infected with CVB4 at 30 plaque forming units (pfu) for 1 h. Different time points post-infection, cell supernatant was first collected. The RD cells were then lysed using CellLytic buffer (Invitrogen, USA) and centrifuged for the cell lysate. Both the cell lysates and cell supernatants were stored at −80 °C for further studies.

2.4. Observation of transfection efficiency

RD cells were seeded onto Labtech Permanox® Chamberslide (Nunc, Germany) and transfected with varying concentrations (0, 10, 50 and 100 nM) of the fluorescein labelled siRNA (siRNA_3DF). As a control, one of wells was treated with 100 nM of siRNA_3DF without LipofectamineTM 2000CD. At 48 h post-transfection, the cells were washed twice with PBS and fixed with methanol for 10 min. The cells were washed again with PBS before addition of Vectashield Mounting Medium with propidium iodide (Vectorlabs, USA). The cells were then observed using the Olympus BX60 fluorescent microscope.

2.5. Cell viability assay

Cell viability assay was carried out to determine cell viability using the CellTiter 96® AQueous Non-radioactive Cell Proliferative Assay (Promega, USA). The MTS/PMS reagent of the assay is composed of a tetrazolium compound that is reduced by dehydrogenases within viable RD cells into a formazan product. Briefly, at 48 h post-transfection, the RD cells were trypsinized and transferred into 96-well microtiter plate in triple aliquots of 100 µl. Subsequently, 20 µl of MTS/PMS reagent were added into each 100 µl aliquot of cells. After incubation at 37 °C for 3 h in the dark, the absorbance of formazan was measured at OD 490 nm. Hence, the amount of formazan formed is directly proportional to the number of viable cells.

2.6. Plaque assay

To determine the viral infectivity of CVB4 after siRNA treatment, plaque assays were carried out after the transfection protocol. In brief, 48 h after infection with 30 pfu (plaque forming units) of CVB4, the plaque media was removed and the RD cells fixed with 7.5% of parafomaldehyde. Once the cells were fixed, the plaques were visualised by staining the RD cells with 1% crystal violet for half an hour at room temperature.

2.7. Real-time RT-PCR

At 48 h post-infection, the RD cells were harvested and total viral RNA extraction was carried out by addition of 150 µl of CellLytic M Cell Lysis Reagent (Sigma, USA) into the individual wells containing the RD cells. Viral RNA was then extracted using the QIAamp® Viral RNA Mini Kit according to the manufacturer’s instructions (Qiagen, USA).

The efficiency of the various siRNAs in inhibiting CVB4 replication were then analysed using the real-time TaqMan RT-PCR assay. A pair of primers (namely CVB_3DF and CVB4_3DR) and a TaqMan probe was designed from the highly conserved regions within the 3D region (Table 2). The real-time RT-PCR TaqMan assay was carried out using the LightCycler® (Roche® Molecular Biochemicals, Germany). The LightCycler® machine achieves efficient heat transfer to the amplification mixture through the use of air currents and glass capillary reaction vessels. The sensitive fluorescent detectors allow real-time monitoring of amplification process through changes in emission of fluorescence.

The LightCycler® RNA Amplification Hybridization Probes kit (Roche® Molecular Biochemicals, Germany) were used in this study. The test kit allows a one-step RT-PCR in glass capillaries using the LightCycler instrument. The enzyme mix contains a mixture of

Table 1: Nucleotide sequences of the 19-mer siRNAs.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Region</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA_2A</td>
<td>2A</td>
<td>5′-GAG AGT GAG TAT TAC CCC A TT-3′ (Sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-TGG GTA AAT ACT CAC TCT C TT-3′ (Anti-sense)</td>
</tr>
<tr>
<td>siRNA_3C</td>
<td>3C</td>
<td>5′-GCT GTG CTC GCC ATT AAC A TT-3′ (Sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-TGG TAA TGG CGA CGA CAG C TT-3′ (Anti-sense)</td>
</tr>
<tr>
<td>siRNA_3D</td>
<td>3D</td>
<td>5′-ATT GAA GGA ATG TAT GGA C TT-3′ (Sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GTC CAT ACA TTC CTT CA A TT-3′ (Anti-sense)</td>
</tr>
<tr>
<td>siRNA_5C</td>
<td>Scrambled</td>
<td>5′-GGA ATT TAT GAA ATG CGG A TT-3′ (Sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-TCC CTT CAT AAA TTC C TT-3′ (Anti-sense)</td>
</tr>
<tr>
<td>siRNA_3DF</td>
<td>3D</td>
<td>5′-(Fluo)ATT GAA GGA ATG TAT GGA C TT-3′ (Sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-(Anti-sense)</td>
</tr>
</tbody>
</table>
reverse transcriptase and “Faststart” Taq Polymerase that allows reverse transcription of RNA template and subsequent cDNA amplification. Each 10 μl reaction contained 1.0 μl of RNA, 5 mM MgCl2, 0.5 μM each of CVB3DF and CVB3DR, 0.2 μM of TaqMan probe, 2.0 μl reaction buffer mix, 0.2 μl enzyme mix and made up to 10 μl with water. cDNA was first synthesized from the RNA by reverse transcription for 10 min at 55 °C and subsequently amplified for 45 cycles at 95 °C for 30 s, 56 °C for 15 s and 72 °C for 15 s.

2.8. Western blot analysis

At 24 h post-infection, the RD cells which were infected with 30 pfu of CVB4 were lysed with 150 μl of CellLytic™ M Cell Lysis Reagent (Sigma, USA). Aliquots of 30 μl of each cell lysate were electrophoresed with 12% polyacrylamide gel and Western blot was performed according to standard protocol. Detection of proteins was based on the WesternBreeze® Chromogenic Western Blot Immunodetection Kit (Invitrogen, USA) in conjunction with the use of the anti-PKR antibody (Sigma, USA), the anti-phospho-PKR antibody (Sigma, USA) and the β-actin antibody (Sigma, USA).

3. Results

3.1. High transfection efficiency of the siRNAs using Lipofectamine™ 2000CD

Fluorescein labelled siRNAs targeting at the 3D region of the CVB4 genome (siRNA3DF) was used for the determination of transfection efficiency of the siRNAs into RD cells using Lipofectamine™ 2000CD. Different concentrations of siRNA3DF (10, 50 and 100 nM) were used for transfection into RD cells. At 24 h post-transfection, the fluorescein labelled siRNA3DF could be seen localized in the cytoplasm of the RD cells (Fig. 1). It was also observed that with increasing concentrations of siRNA3DF being transfected into RD cells, there was corresponding increase in the amount of labeled siRNA3DF in the cytoplasm RD cells (Fig. 1). On the other hand, no fluorescence was observed when the siRNAs were transfected in the absence of Lipofectamine™ 2000CD. This observation showed that the fluorescence was not due to random adherence of the siRNAs to the surface of the RD cells, verifying the successful delivery of siRNAs into the RD cells by the transfection agent.

3.2. No induction of cell toxicity on the RD cells by the 19-mer siRNAs

Ideally, the amount of Lipofectamine™ 2000CD used in this study should mediate high transfection efficiency and yet exhibiting none or minimal cytotoxic effects. Hence, cell viability assay was carried out 48 h post-transfection to check for cytotoxicity of Lipofectamine™ 2000CD. Results showed that all the siRNAs used in this study, regardless of the concentrations, did not exhibit any non-specific cytotoxic effects which could affect the growth and viability of the RD cells (Fig. 2).

3.3. RD cells transfected with siRNA reduced plaque formation

Plaque reduction assay was carried out to enable a quantitative evaluation of the efficiency of siRNAs targeting the 2A, 3C and 3D regions of CVB4. An effective siRNA would cause inhibition of the replication of CVB4 and hence a reduction in viable virus titre. This is reflected by observing a reduction in the number of plaques formed on the RD cell monolayer from the original 30 pfu. The plaque assays were able to show that RD cells transfected with the three different siRNAs (siRNA2A, siRNA3C and siRNA3D) displayed a reduction in number of plaques formed (Fig. 3). None of the mock transfected RD cells (0 nM of siRNA) exhibited any reduction in number of plaques. RD cells which were transfected with siRNA targeting the 3C region of the CVB4 genome exerted the most potent effect in inhibition of CVB4 replication with dosage dependency effect. This was followed by siRNA targeting the 3D region. siRNA targeting the 2A region was found to be the least effective with limited reduction in the number of plaques despite the increase in siRNA2A concentrations (Fig. 4). The reduction in number of plaques was shown to be significant as the t-distribution test had a p-value ranging from 4.0 × 10^{-5} to 9.14 × 10^{-3}. The non-specific scrambled siRNA in contrast was not able to exhibit any inhibitory effects on CVB4 replication. This was supported by the student’s t-distribution test where the p-value (0.749) was shown to be non-significant for comparison between the negative control and RD cells transfected with scrambled siRNAs. To evaluate the therapeutic effects of the designed 19-mer siRNAs, the RD cells were first infected with CVB4, followed by treatment with 100 nM of siRNA2A, siRNA3C or siRNA3D at each hourly interval post-infection for up to 6 h. Results showed that there were significant reductions in plaque formation when the infected RD cells were treated with the various 19-mer siRNAs up to 4 h post-infection (data not shown).

3.4. Reduction in CVB4 RNA in RD cells treated with siRNAs

The amount of CVB4 RNA transcripts after treatment with the various concentrations of the siRNAs were analysed by real-time TaqMan RT-PCR assay. Results showed that treatment of RD cells with siRNAs targeting the 2A, 3C and 3D regions of CVB4 resulted in a dosage dependent decrease of CVB4 RNA transcripts (Fig. 5). The siRNA targeting the 3C region of CVB4 exhibited the highest inhibition of replication by causing the greatest reduction in copies of CVB4 RNA transcripts detected by the real-time TaqMan RT-PCR assay. A decrease of 49.59, 66.90 and 75.44% of CVB4 RNA transcripts was observed following the transfection of RD cells with 25, 50 and 100 nM of siRNA3C, respectively (Fig. 5). The siRNAs targeting the 3D region of CVB4 was also fairly efficient in the reduction of CVB4 RNA transcripts. Consistent with the results obtained from the reduction in the plaque formation, the siRNA targeting 2A region in contrast was not as effective as the other two regions targeted as there was only a 32.98% reduction in CVB4 RNA transcripts when the RD cells were treated with 100 nM of siRNA2A (Fig. 5). The inhibitory effects of the siRNAs targeting the three different regions of the CVB4 genome was also shown to be target specific as reductions in CVB4 RNA transcripts was observed to be negligible in RD cells transfected with 100 nM of non-specific scrambled siRNA (siRNA3C).
3.5. No enhanced inhibitory effects on CVB4 replication by combinations of two 19-mer siRNAs

So far, our results have shown that both siRNA_3C and siRNA_3D were more effective in inhibiting the replication of CVB4 as compared to siRNA_2A. 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 CVB4 genome will improve the antiviral effect in this study, two 19-mer siRNAs (siRNA_3C and siRNA_3D) were cotransfected into the RD cells at a combined final concentrations of 100 nM, followed by infection with CVB4 as described in Section 2.3. The efficacy in inhibiting CVB4 replication was measured by real-time RT-PCR assay. Results by the real-time RT-PCR showed that similar inhibitory efficacy on CVB4 replication was observed as compared to the siRNA_3D when used individually (data not shown). This suggests that there is no enhanced antiviral effect when a combination of 19-mer siRNAs was used.
3.6. Specificity of siRNA-mediated inhibition of CVB4 replication

Inhibition of viral replication could be due to the induction of non-specific interferon pathways within RD cells. The protein kinase (PKR) that could be activated by dsRNA is one of the several enzymes that could be induced in Type 1 interferon pathway. Hence, to eliminate the participation of non-specific interferon response in inhibition of viral replications, levels of PKR were detected using the anti-PKR monoclonal antibodies. 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 siRNAs for 48 h (Fig. 6). It is also 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 phospho-PKR in the transfected RD cells by western blot using specific monoclonal antibodies against phospho-PKR (Sigma, USA). Similarly, other than the positive control, we did not observe any phospho-PKR expression in the RD cells treated with each of the 19-mer siRNAs (Fig. 6). These results indicated that the 19-mer siRNAs did not elicit any interferon response and the inhibition of CVB4 replication is mediated through RNAi.
Fig. 4. Inhibition of CVB4 replication of various siRNAs showing dosage dependency on the plaque formation in RD cells. Negative control RD cells were not treated with siRNA but infected with 30 pfu of CVB4 (Neg). RD cells were transfected with various concentrations of siRNAs (0, 25, 50 and 100 nM) targeting 2A, 3C and 3D regions (represented by 2A, 3C and 3D) then infected with 30 pfu of CVB4. 100 nM of scrambled sequence siRNA were transfected into RD cells which were subsequently infected with 30 pfu of CVB4 as a non-specific control. The data shown represent the mean value of three independent experiments with corresponding standard deviations.

Fig. 5. Comparative reductions of CVB4 RNA transcripts by siRNAs targeting different genomic regions by the real-time TaqMan RT-PCR assay. The data shown represent the levels of CVB4 RNA transcripts in percentages. Viral RNA were extracted from RD cells transfected with different concentration of siRNA (0, 25, 50 and 100 nM) targeting the different regions of CVB4 genome (2A, 3C and 3D). The data represents the results obtained from three independent experiments.

Fig. 6. Western blot analysis of protein kinase (PKR) and phospho-PKR. The RD cells were transfected with various concentrations of the siRNAs targeting the different genes (2A, 3C and 3D) of CVB4 for 48 h, followed by EV71 infections at a MOI of 10. The RD cells treated with alpha interferon, scrambled siRNAs (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 loading control, using anti-β-actin monoclonal antibodies. The tests were carried out in two independent experiments.

4. Discussion

It has been well known that RNAi acts as a natural antivi-
ral mechanism in eukaryotic cells especially against RNA viruses (Gitlin and Andino, 2003). Before the discovery by Fire (1999), mammalian cells were not thought to possess any active RNA silencing machinery (Fire, 1999). As a form of defense against viruses, mammalian cells were known to induce a non-specific interferon mediated antiviral response in the presence of long dou-
ble stranded RNA (>30 nt) (Gitlin et al., 2002). The RNAi mechanism operates at the post-transcriptional level by specific cleavage of target mRNA. To date, studies using RNAi as a potential antiviral
therapy has shown many promising results. Viruses which have shown to be successfully inhibited by RNAi includes the human immunodeficiency virus (Lee et al., 2002), hepatitis B virus (Li et al., 2004), hepatitis C virus (Kapadia et al., 2003), poliovirus (Gitlin et al., 2002), coxsackievirus B3 (Yuan et al., 2005) and enterovirus 71 (Sim et al., 2005; Tan et al., 2007). In the RNAi study of coxsackievirus B3 (CVB3) (Yuan et al., 2005), siRNA were designed to target the 5′ UTR, the AUG start codon, VP1, 2A and 3D region of the CVB3 genome. It was shown that the siRNA targeting the 2A region of the CVB3 genome was the most effective followed by the VP1, 3D, 5′UTR and the start codon. In another recent RNAi study of enterovirus 71 (EV71) (Sim et al., 2005), four regions of the EV71, 3′UTR, 2C, 3C and 3D were targeted for RNAi. Similarly, the 3D regions of EV71 were shown to be the most effective in inhibition of EV71 replication, and this was further supported by Tan et al. (2007), who also showed that 29-mer short hairpin RNAs (shRNAs) targeted at the 3D region was the most effective.

In this study, three siRNAs were designed to target the 2A, 3C and 3D regions of the CVB4 genome. The 2A and 3C regions encode for the viral protease 2Apro and 3Cpro while the 3Dpol region encodes for the viral RNA-dependent RNA polymerase 3Dpol (Leong et al., 2002). All three regions encode for non-structural proteins of the CVB4 virus which plays an important role in the process of CVB4 replication. Therefore, cleavage of these RNA transcripts would effectively result in more potent inhibition of the virus replication process. As the 2A, 3C and 3D regions are non-structural proteins, they have lesser tendency to mutate. This is of utmost importance in the design of siRNA especially for CVB4. Since it is a RNA virus, it can mutate easily and has the potential to generate escape mutants (Gitlin et al., 2005).

The efficiency of RNAi for the three target regions was demonstrated by the results of the plaque assay and the real-time TaqMan RT-PCR assay. Both results showed good correlations in the trend of inhibition of viral replication. The most effective inhibition was observed for the siRNA targeting the 3C region (siRNA3C). This was followed by the siRNA which targeted the 3D region (siRNA3D) and lastly the siRNA targeting the 2A region (siRNA2A). The difference in efficiency may be due to the differences in function of the non-structural enzymes which are encoded by these regions. The 3C region encodes for a protease (3Cpro) which is responsible for major role of the cleavage of the translated viral polyprotein (Leong et al., 2002). Besides functioning as a protease, 3Cpro as well as its precursor 3CDpol also plays an important role at the level of viral replication (Parsley et al., 1999). 3Cpro has been shown to be critical for interacting with the cloverleaf structures found at the 5′ UTR of the viral genome to deliver the viral RNA polymerase 3Dpol to the replication complex (Leong et al., 1993). Since the function of 3Cpro is required prior to 3Dpol, a down-regulation of 3Cpro would have a detrimental effect as available 3Dpol would not be able to carry out replication of the CVB4 genome.

As mentioned earlier, the 3D region encodes for the RNA-dependent polymerase (3Dpol). Although 3Dpol must interact with other viral proteins (e.g. 3Cpro) to dock at the appropriate site for initiation of genome replication, 3Dpol by itself is the central component for the replication machinery. Therefore, a reduction in the levels of 3Dpol would also result in the inhibition of CVB4 replication. In contrast, the 2A region which encodes for a protease (2Apro) is only responsible for the cleavage of the viral polyprotein in cis at its own amino terminus (between P1 and P2 region). Instead, 2Apro has been recognized to have a dramatic effect on the host cellular cap-dependent translation process by cleaving the eIF4G (eukaryotic initiation factor 4G) (Leong et al., 2002). Hence, the reduction in levels of 2Apro could only exhibit limited effect on the inhibition of CVB4 replication. Since 3Cpro and 3Dpol are of utmost importance to the replication of CVB4, the down-regulation of these two proteins could therefore produce more potent inhibitory effects on the replication of CVB4.

The difference in efficiency of siRNAs targeting the three different regions of CVB4 genome could also be due to different positional accessibility to the respective target mRNA. Steric hindrance caused by secondary structures or protein binding to the site of cleavage by RNAi could account for the different extent of inhibitory effects observed. The possibility of secondary structures playing a role in RNA interference is still debatable. However, previous studies have suggested that binding of cellular proteins to certain target sites may affect the efficiency of the siRNAs in the cells by preventing access of the RISC complex to the target site (Yuan et al., 2005).

RNA viruses like CVB4 are particularly prone to genetic mutations, due to the lack of proof reading capability of RNA polymerase used in the replication process of the virus (Elena and Sanjuán, 2005). Hence, it is possible that a siRNA can become ineffective after prolonged exposure to the same siRNA. To tackle the high mutation rate associated with RNA virus and escape mutants, it has been reported that combinations of siRNA targeting different genes could increase the chance of inhibiting viral replication (Ji et al., 2003; Kanda et al., 2004). However, in this study, we did not observe any enhancement effects in inhibiting CVB4 when both siRNA3C and siRNA3D were cotransfected into RD cells. Similar observations were also obtained in another study by Tan et al., 2007, who also showed no enhanced inhibitory effects on EV71 by combinations of two 29-mer shRNAs. It was suggested that this may be because the 19-mer siRNAs did not affect the secondary structure of the viral mRNA, and thus do not increase accessibility to other 19-mer siRNAs (Tan et al., 2007). Nevertheless, combination of siRNAs is still a good approach against viruses which mutate frequently.

In order for the siRNA to be used as a potential antiviral therapy, an optimal dosage of siRNA must successfully be delivered into the cytoplasm of the cells, the site of viral replication and the RNAi machinery. In this study, the delivery of siRNA into the RD cells was achieved by complexing the siRNAs with Lipofectamine™ 2000CD. However, lipid based formulations have occasionally been associated with high levels of cell toxicity (Tousignant et al., 2000), and induction of profound IFN response in vitro and in vivo (Judge et al., 2005). Since the RD cells remained viable after 48 h for the siRNAs to exhibit its antiviral effect, the direct delivery of siRNA into the site of viral infection could be investigated further in animal models.

It has been shown by Sledz et al. (2003) that it is possible for the viral replication to be inhibited by interferon mediated antiviral response that is activated by short dsRNAs (Sledz et al., 2003). Hence, it is important to find out if the inhibition of CVB4 replication in this study is indeed due to RNAi by the specific targeting of siRNA and not due to off-target effects, like the activation of interferon response. Levels of PKR detected by Western blot showed that the interferon pathway was not activated by the introduction of siRNA into the RD cells. The activation of interferons can also lead to cell death; therefore cell viability was also used as an indicator for monitoring adverse effects due to interferon response. Since cell viability was not compromised at 48 h post-transfection, it implied that all the siRNAs used in this study exhibited no significant levels of cytotoxicity.

The ability for the siRNAs to persist within the cytoplasm of the cell and not be degraded by endogenous nucleases is also an important factor in the determining the efficiency of siRNA on the inhibition of viral replication (Behlke, 2006). In two separate studies of RNAi of EV71 (Sim et al., 2005; Tan et al., 2007), it was shown that the direct introduction of siRNAs and shRNAs inhibited the replication of EV71 for a maximum of 48–72 h. In this study, RD cells which were treated with siRNAs directly were allowed to propagate for 48 h before being challenged with CVB4 virus. It was observed that after 48 h, the siRNAs could still inhibit the replication of CVB4.
in RD cells. However, in the clinical setting, a longer persistence of siRNA within the patient’s cells would be more promising as therapeutic approach. This is because only a low dosage of siRNA would be required to inhibit the replication of CVB4 and the patient would not be required to undergo consistent administration of the siRNA throughout the course of treatment.

It has been suggested that the sequence of dsRNA can be expressed inside the cell using DNA-directed RNAi (ddRNAi) expression cassettes that direct the synthesis of the siRNAs (Behlke, 2006). This method of introducing siRNA into the cell could theoretically produce a sustained RNAi effect which might even be permanent. These prolonged effects are largely dependent on the expression levels of the vectors used. Currently it has been shown that the ddRNAi expression cassettes can be introduced into the cell using either plasmid or viral vectors, such as retrovirus (Brummelkamp et al., 2002) and adenovirus (Shen et al., 2003). However, the use of plasmid or viral vectors presents problems which are parallel to those encountered in gene therapy (Behlke, 2006; Cekola et al., 2006). One such problem would be the poor control over the insertion site on the host genome which can lead to triggering of oncogenes (Cekola et al., 2006). Research is currently still ongoing to counter the problems in regards to the use of these vectors.

In conclusion, siRNA targeting the 3C region of the CVB4 genome exhibited the highest efficiency in inhibiting the replication of CVB4 in RD cells. Since RD cells treated with the siRNA displayed no signs of cell toxicity or off-target silencing effect, future studies should investigate RNA interference of CVB4 in mice to further evaluate the in vivo efficiency of viral inhibition. In vivo studies will serve as a better indication of the effective dosage required as well as side effects if siRNA is to be adopted as a potential antiviral therapy against CVB4.

References