Virology 399 (2010) 39–45

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Targeting enteroviral 2A protease by a 16-mer synthetic peptide: Inhibition of 2A^{pro}-induced apoptosis in a stable Tet-on HeLa cell line

Nader Maghsoudi [a,*,1], Narges Kh. Tafreshi [a,b,1], Fariba Khodagholi [a], Zahra Zakeri [c], Mitra Esfandiarei [d], Hamid Hadi-Alijanvand [e], Marjan Sabbaghian [e], Amir Hossein Maghsoudi [a], Mahnaz Sajadi [f], Mastaneh Zohri [f], Maryam Moosavi [g], Mehdi Zeinoddini [h]

[a] Neuroscience Research Center, Shahid Beheshti University of Medical Science, Tehran, Iran
[b] H. Lee Moffitt Cancer Center, Tampa, FL, USA
[c] Department of Biology, Queens College and Graduate Center of the City University of New York, Flushing, NY, USA
[d] Child & Family Research Institute, Department of Anesthesiology, Pharmacology & Therapeutics, University of British Columbia, Vancouver, Canada
[e] Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[f] Department of Biology, Tofigh Daru Co., Tehran, Iran
[g] Department of Physiology and Neuroscience Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[h] School of basic science, Tarbiat Modares University, Tehran, Iran

ARTICLE INFO

Article history:
Received 22 September 2009
Returned to author for revision
16 October 2009
Accepted 15 December 2009
Available online 22 January 2010

Keywords:
CVB3
2A protease
Apoptosis
Caspase-3

ABSTRACT

Enteroviridae such as coxsackievirus are important infectious agents causing viral heart diseases. Viral protease 2A (2A^{pro}) initiates the virus life cycle, and is an excellent target for developing antiviral drugs. Here, to evaluate the validity of the 2A^{pro} as a proper therapeutic target, and based on the existing information and molecular dynamics, a 16-mer peptide was designed to specifically target the active site of protease 2A^{pro} in order to block the activity of CVB3 2A^{pro}. We showed that the peptide could compete with endogenous substrate in a concentration-dependent manner. Further, we established a HeLa cell line that expressed 2A^{pro}. Expression of 2A^{pro} resulted in significant morphological alteration and eventual cell death. Western blot and viability assay showed that the 16-mer peptide (200 μg/ml) could significantly block 2A^{pro} activity and its cytotoxic effect. Future modification of the 16-mer peptide can improve its affinity for 2A^{pro} and therefore develop effective antiviral drug.

Introduction

Coxsackievirus B3 (CVB3) is considered as the most important infectious agent that can cause severe heart complications. CVB3 is associated with both acute and chronic forms of myocarditis (inflammation of the myocardium), a life threatening disease (Reyes and Lerner, 1985; Woodruff, 1980). Chronic forms of this disease may lead to dilated cardiomyopathy (DCM), for which the only available and effective treatment is the highly cost heart transplantation procedure. Entroviruses, in general, and CVB3, in particular, are responsible for causing up to 30–45% of acute forms of myocarditis and 25% of DCM (Bowles et al., 1986; Frisk et al., 1984). In an infected cardiomyocyte, cleavage of dystrophin by viral 2A^{pro} is considered an important mechanism for CVB3-induced cardiac injury (Badorrf et al., 1999). CVB3 infection is also associated with programmed cell death and apoptosis in myocardial tissue (Goldstaub et al., 2000; Olivetti et al., 1997).

CVB3 is a picornavirus, a family of small positive-stranded RNA viruses associated with a large variety of human and animal diseases. Following virus entry, viral RNAs are translated, and newly synthesized viral proteins are released within the cytoplasm of infected host as large a polyprotein, which will be then processed and further cleaved by virus-specific protease 2A and 3C to generate mature structural and nonstructural viral proteins (Krausslich and Wimmer, 1988; Skern and Liebig, 1994). Viral replication depends on this proteolytic cleavage. Although the newly synthesized viral polyprotein is processed mainly by 3C^{pro}, the primary cleavage event separating the structural protein precursor from the nonstructural one is performed by 2A^{pro} (Hanecak et al., 1982; Toyoda et al., 1986). Viral protease 2A is a multifunctional chymotrypsin-like cysteine proteinase that catalyzes the cleavage of the viral polyprotien at a tyrosine-glycine pair between the C-terminus of VP1 and its own N-terminus (Rueckert, 1996; Toyoda et al., 1986). Both 2A^{pro} and 3C^{pro} are also responsible for the cleavage of the other cellular proteins such as the eukaryotic Initiation Factor-4G (eIF4G) and the cytoplasmic protein dystrophin. Cleave of eIF4G (formerly known as p220) leads

* Corresponding author. Fax: +982122431624.
E-mail address: nmaghsoudi@sbmu.ac.ir (N. Maghsoudi).
[1] Both authors have equally contributed to this manuscript.

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.12.017

to the shut-off of the host cell protein synthesis machinery (Hellen et al., 1991; Seipelt et al., 2000; Ventoso et al., 1998). Another protein of the cellular translation apparatus, poly-ADP-ribose-polymerase (PARP), is also cleaved by $2A^{pro}$ and $3C^{pro}$ (Calandria et al., 2004).

Due to the unique protein structure and essential role played by $2A^{pro}$ and $3C^{pro}$ in viral replication and disease progression, they have been considered as potential therapeutic targets to block virus replication and virus-induced cell injury (Krausslich and Wimmer, 1988; Sablina and Antonov, 1991). Despite considerable efforts in recent years, no promising antiviral compound for CVB3-induced heart diseases has been developed. In the present study the cleavage motif of CVB3 $2A^{pro}$ substrate was used to generate a potent competitor peptide that potentially competes with and blocks $2A^{pro}$ activity and subsequent viral replication.

In order to further study the effect of the newly designed peptide on $2A^{pro}$ activity, we used a tetracycline-dependent (Tet-on) expression HeLa cell line, in which the expression of viral $2A^{pro}$ was conditionally controlled. Such tightly controlled system provided us with the proper tool to monitor the direct interaction between conditionally overexpressed viral 2A protease and the synthesized competitive peptide in a timely manner.

Results

In vitro activity of the purified $2A^{pro}$

To ensure the integrity of the purified $2A^{pro}$ and the efficiency of the cloning and transformation, $2A^{pro}$ gene was approved by sequencing in pET22b. After purification, to assay the activity of the $2A^{pro}$, the cleavage of eIF4G, as the specific substrate of $2A^{pro}$ and a component of eIF4F, was analyzed. As shown in Fig. 1, addition of the purified enzyme resulted in complete cleavage of eIF4G, indicating that the purified $2A^{pro}$ was active.

Isolation of HeLa cell clones expressing coxsackievirus B3 $2A^{pro}$ under the control of Tet-on expression system

The Tet-On inducible gene expression system provides ready access to a tightly regulated, high-level gene expression system as described by (Gossen et al., 1994) with modifications made by (Urlinger et al., 2000). In the Tet-On system, expression is activated by the addition of doxycycline (Dox), a tetracycline derivative, to the culture medium. This permits gene expression to be tightly regulated a specific stimulus (e.g., doxycycline). Unlike other inducible mammalian expression system, gene regulation in the Tet-on/off systems is highly specific, so results are not complicated by pleiotropic effects or nonspecific inductions.

A stable double-transformed HeLa Tet-On/$2A^{pro}$ cell was established as described in the Material and methods section. After selection and cell cloning, hygromycin resistant cell clones were screened for expression of $2A^{pro}$ by RT–PCR. Two clones, 4 and 5, were clearly positive in this method (Fig. 2). Dox was added to 80% confluent double-stable cells as well as noninducible HeLa cells (Fig. 3a) and 1 day after addition of Dox to selected clones, cells round up (Fig. 3b, Tet-on HeLa cells), and after 48 hours of incubation less than 1% of cells remained viable and attached to the culture plate (Fig. 3c, Tet-on HeLa cells). In the presence of doxycycline, these clones displayed a high expression of viral $2A^{pro}$, PARP protein cleavage, and an increased level of apoptosis.

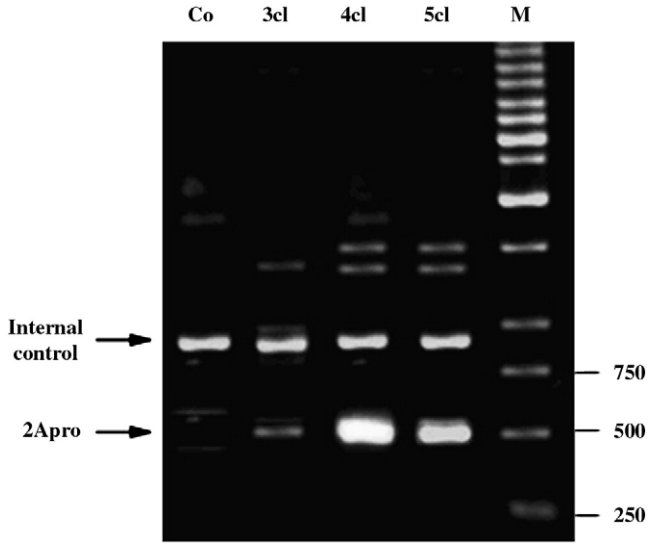

Fig. 2. Cells were induced by 300 ng/ml Dox (Sigma) for expression of $2A^{pro}$ and 48 hours later, induced Hygromycin resistant clones were collected and total RNA extraction was done and extracted RNA reverse-transcribed and amplified using gene specific primers for $2A^{pro}$ and β-actin. An 840 bp RT–PCR product corresponding to the amplified β-actin (internal control) fragment was observed in all clones. In contrast, the 500 bp RT–PCR product corresponding to the amplified $2A^{pro}$ fragment was only observed in positive clones (positive clones have been shown in this figure). Clones #4 and #5 were selected for further analyses. M: 1 kb DNA ladder, Fermentas.

Homology modeling and structure prediction

Multiple sequence alignment of CVB3 and HRV2 $2A^{pro}$ revealed that conserved residues are spread along the whole sequence but N-terminal regions are the least conserved residues (Maghsoudi et al., 2008). Judging from such a high sequence homology, a high-quality 3D structure of CVB3 $2A^{pro}$ can be expected in homology modeling. It is indeed well known that a homology-modeled structure of a target protein can be accurate enough to be used in a docking study once the sequence identity between target and template approaches 40% (Baker and Sali, 2001).

Topology of predicted homology model of CVB3 $2A^{pro}$

To determine the 3D structure of the protein, an ensemble of 25 model structures was generated. Table 1 presents a comparison between the accessible surface area (ASA) obtained using HM- and PHD-derived results. The overall ASA HM structure and PHD derived ASA are similar. Therefore, the HM-predicted structure is confirmed by PHD data. The overall tertiary structure of CVB3 $2A^{pro}$ strongly resembles that of its template HRV2 $2A^{pro}$. Fig. 4a shows the schematic representation of a homology model of CVB3 $2A^{pro}$ showing arrangement of structural elements. The tertiary structure comprises beta barrel globally. This domain type is common among proteases and it resembles trypsin-like protease.

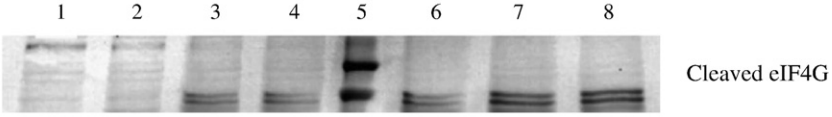

Fig. 1. Activity of recombinant $2A^{pro}$. 10 μg of the recombinant $2A^{pro}$ incubated with S10(eIF4G) at 0 (lane 2), 2 (lane 3), 6 (lane 4), 8 (lane 6), 12 (lane 7), and 24 (lane 8) hours. Reactions stopped by addition of sample buffer and were subjected to 7.5% SDS–PAGE and Western blot using anti-17CV antibody. Negative control for S10, and molecular weight marker have been shown in lanes 1 and 5, respectively.

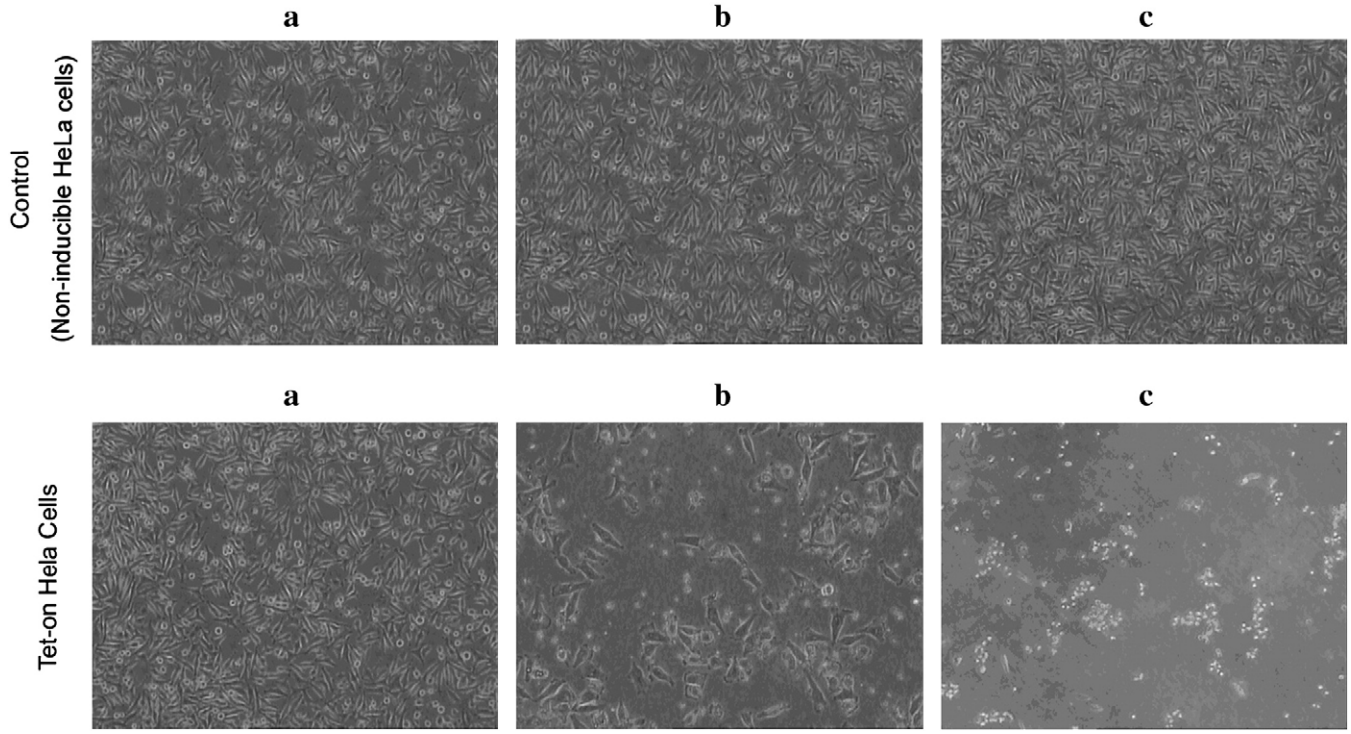

Fig. 3. Morphological alteration of stable HeLa cell expressing coxsackievirus B3 $2A^{pro}$ under the control of tet (clone number 5). Phase-contrast microscopy of HeLa cells at different time points after induction of $2A^{pro}$: (a) absence of viral $2A^{pro}$ (0 hour), (b) 28 hours after induction of viral $2A^{pro}$ expression, and (c) 48 hours after induction of viral $2A^{pro}$ expression. Noninducible HeLa cells were treated with Dox with the same concentration. Dox does not have any nonspecific effects on the non-inducible cells.

Inhibition of CVB3 $2A^{pro}$ by peptide

In order to understand the specificity of CVB3 $2A^{pro}$, an oligopeptide structurally determined by molecular dynamics was docked into the substrate binding site. The cleavage site for both HRV2 and CVB4 $2A^{pro}$ corresponding to GRTTLSTR↓GPPRGGPG in human eIF4G (Lampheaer et al., 1993). We studied to determine whether CVB3 $2A^{pro}$ has the same cleavage site since HRV2 and CVB3 $2A^{pro}$ have a strong similarity in their active sites. In addition, Wang et al. (1998) reported that HRV2 $2A^{pro}$ cleaves a peptide corresponding to its own cleavage site *in vitro*.

To design inhibitors with great potency against enzymes, substrate sequence was used. The nomenclature Pn-P1-P1′-Pn′, of substrate or inhibitor was reported by Lampheaer et al. (1993) with the scissile peptide bond lying between P1 (Arg) and P1′ (Gly). As shown in Fig. 4b, among different binding possibilities, this peptide has high affinity for two amino acids located in the enzyme active site (binding energy of −402.6 kJ/mol). Such affinity cannot be found at the other sites. Replacing Arg in P1 with Asp resulted in a significant decrease in affinity for enzyme (data not shown).

Electrostatic complementarities of interacting surfaces are important factors for protein-protein interaction. In our previous investigation, we showed that CVB3 $2A^{pro}$ is a negatively charged protein (Maghsoudi et al., 2008), which makes it more possible for a substrate with positive potential to enter the active site. In Fig. 4c, the global electrostatic potentials of the residues have been mapped on the protein surface, which has been found to be a negatively charged protrusion, symbolized as a red bump. These calculations indicate that the best ligand–receptor complementary electric field appears in one ligand binding site, which was confirmed by HEX output as the most stable one as well.

In a separate investigation, the inhibitory effect of this peptide on $2A^{pro}$–substrate (eIF4G) was determined experimentally by an *in vitro* cleavage assay. As shown in Fig. 4d, this peptide could significantly decrease eIF4G cleavage by viral $2A^{pro}$.

In addition, Western blot analysis has shown that the proteases 2A can cleavage the poly-ADP-ribose-polymerase (PARP). The cleavage is partially blocked by 50 μg/ml of designed peptide, suggesting an inhibitory effect of the synthesized peptide on viral $2A^{pro}$ activity (Fig. 5). Results from the cell viability assay (MTT) have also shown that at the concentration of 100 μg/ml, our synthesized peptide can completely blocks $2A^{pro}$ activity and subsequent cell death whereas the scrambled peptide (Arg in P1 was replaced by Asp in its sequence) could not inhibit cell death significantly (Fig. 6). The docking of $2A^{pro}$-substrate along with our experimental data would thus support a direct cleavage of eIF4G that continues to be the subject of much debate (Bovee et al., 1998).

Discussion

Coxsackievirus B3 (CVB3) is the most common cause of inflammatory heart diseases and the subsequent end-stage cardiomyopathy

Table 1
Comparison of the accessible surface area (ASA) obtained using HM and PHD.

```
experi: eeeeeeeibbibbeiibbieeeieeebeeeieieieibbbbbeeieeeieiebbebeieeiibibeeeeeeieiebeeeeeb
predi :  eeeeeee b b  b b b  b eeee ee b    e  bbb    eeee  bb bebeebbbbb eeee bbb beeee   b
experi:  eeeeeieeeebeibbibeeeeeeieeiiibeieiibbbbiiiieeiibbbbbbiebbeieeiieee
predi :   eee e bb bbbbb b  ee  bbb b be bbbbbbbbeee  bbbb b eb e eeee ee
```

e: exposed, i: intermediate, b: buried.

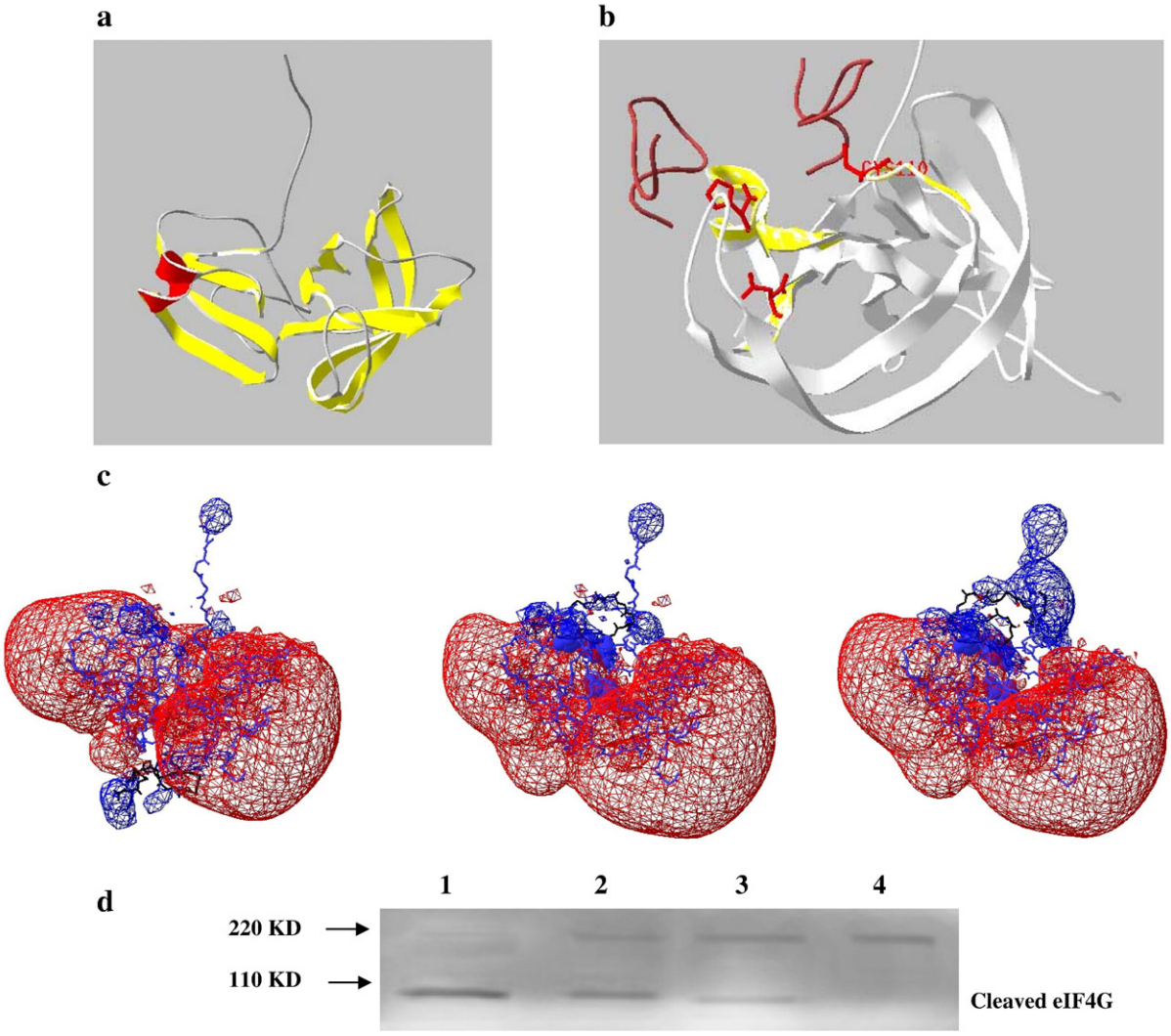

Fig. 4. (a) The 3D structure of CVB3 2A ^{pro}. Ribbon diagram of the overall structure was generated using the Swiss pdb viewer program. Helix and sheet are shown in red and yellow, respectively. (b) A model of substrate binding in CVB3 2A^{pro}. Enzyme, active site and substrate (16-mer peptide) are donated by white, yellow, and dark red colors, respectively. The three important amino acids in active site that interact with substrate are shown by light red color. (c) Electrostatic complementarities between CVB3 2A^{pro} and an oligopeptide substrate. Enzyme and substrate characterized by negative and positive potentials highlighted by red and blue colures, respectively. (d) The inhibitory effect of peptide on 2A^{pro}–substrate interaction. About 10 μg/ml of the purified CVB3 2A^{pro} incubated with S10 (eIF4G) in the presence of 0 (lane 1), 1 (lane 2), 3 (lane 3), and 5 mM (lane 4) peptide. After 8 hours, the reaction was stopped by addition of sample buffer and was subjected to 7.5% SDS–PAGE and Western blot analysis using anti-17CV antibody.

(Gillum, 1986; Reyes and Lerner, 1985; Sugrue et al., 1992; Woodruff, 1980). CVB3 genome encodes two proteases, 2A and 3C, which are responsible for processing the viral polyprotein, and therefore, for production of all mature viral particles (Krausslich and Wimmer, 1988; Skern and Liebig, 1994). In addition, they are involved in the cleavage of host cells proteins essential for normal cellular homeostasis and functions such as transcription, translation and cytoskeletal integrity (Badorrf et al., 1999; Hellen et al., 1991; Seipelt et al., 2000; Ventoso et al., 1998). Due to their unique protein structures and essential roles in viral replication, 2A and 3C proteases have been considered as suitable targets for blocking virus replication and its destructive effect within the host cell (Krausslich and Wimmer, 1988; Sablina and Antonov, 1991). Considering their crucial roles during virus life cycle, inhibition of these proteases may be an excellent strategy to restrain viral replication and resulting detrimental effects on the heart and other infected organs.

Several compounds targeting 2A^{pro} of polioviruses and rhinoviruses have been reported including the inhibition of 2A^{pro} by *o*-phenanthroline, leupeptin, zVAD.fmk [benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone] (Deszcz et al., 2006; Konig and Rosenwirth, 1988). However, their effects are not specific to viral proteins and may include some host cell proteins as well. One example is the nonspecific binding of Z-VAD-fmk or related caspase inhibitors to nonviral proteases that contain a cysteine in their active sites like cathepsin B or H (Schotte et al., 1999).

During the last decade, the need for an antiviral drug with no side effect on host cells normal physiological functions has been increasing. The main challenge is to design an effective and highly specific inhibitor capable of blocking viral replication, preferably during the early phase of virus life cycle. A rational strategy to develop a specific protease inhibitor should be based on the structure and function of the protease. Unfortunately, no report of the three-dimensional structure of CVB3 2A^{pro} has been published. We therefore predicted the 3D structure of CVB3 2A^{pro} based on the comparative homology modeling. In the absence of a crystal structure, our study can provide a rational alternative for structure-based drug design. So, in the present study, we have focused on designing a highly specific competitor peptide in an attempt to block the activity of CVB3 2A^{pro} in a HeLa cell culture model.

To analyze the effect of the synthesized peptide on 2A^{pro}, we used a tetracycline (Tet)-dependent expression system. This system can constitute an easy and rapid screening method for identification of

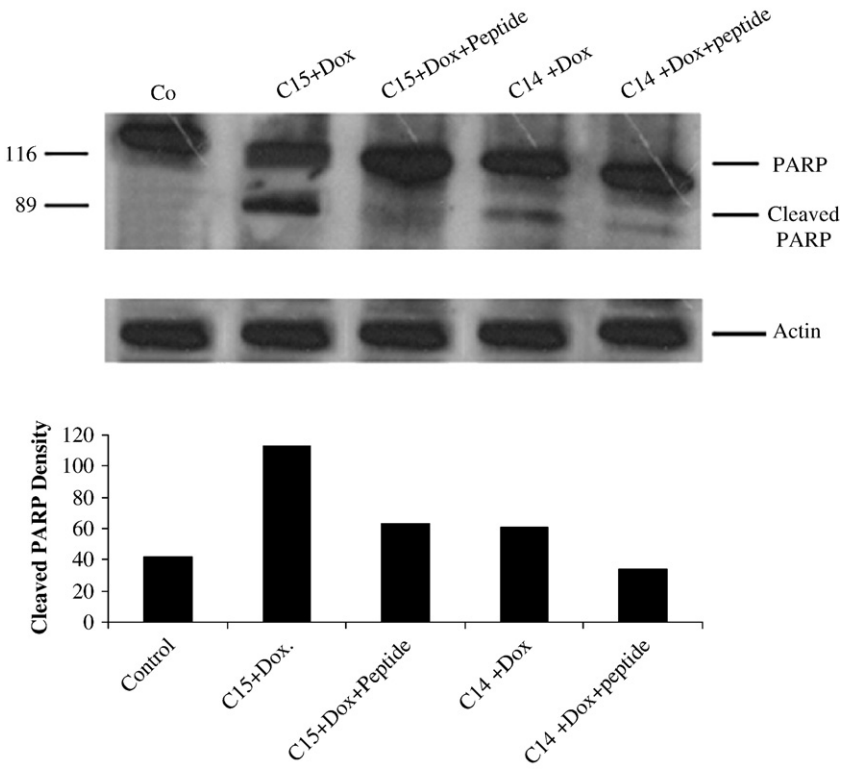

Fig. 5. PARP cleavage in HeLa cell line inducibly expressing the $2A^{pro}$ and the effect of peptide on PARP cleavage. Two clones (number 4 and 5) were selected based on RT–PCR results. Positive clones of HeLa cells grown in flasks were treated by Dox (300 ng/ml) to express $2A^{pro}$, and were then treated with 50 μg/ml of peptide. PARP cleavage was measured by Western blot analysis using anti-PARP monoclonal antibody.

antiviral agents targeted towards $2A^{pro}$. In this system, overexpression of viral $2A^{pro}$ induces apoptosis that is associated with cleavage of caspase-3 and PARP proteins confirming that our Tet/$2A^{pro}$ system mimics the actual CVB3 infection in a HeLa cell culture. Treatment of the Tet/$2A^{pro}$ system with our synthetic inhibitor peptide rescued HeLa cells and blocked cell apoptosis caused by $2A^{pro}$. Our results have shown that $2A^{pro}$ activity is inhibited more than 60% by 50 μg/ml peptide; therefore, the peptide is likely to be structurally very similar to $2A^{pro}$ substrate and can be effective and specific tool for inhibiting of $2A^{pro}$ action, preventing the apoptotic effect on host cells, and suggesting an effective and feasible therapeutic approach for viral myocarditis.

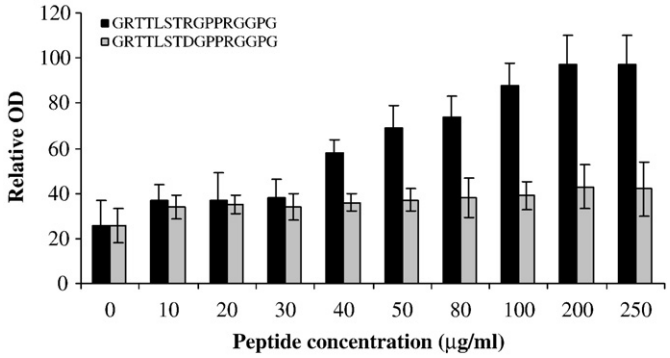

Fig. 6. Effect of the 16-mer peptides as well as scrambled peptide on cell viability. HeLa cell line expressing coxsackievirus B3 $2A^{pro}$ were treated with the synthetic peptides at the indicated concentration and then HeLa cell viability was determined by MTT assay. Cell viability is calculated as the percentage of living cells in treated cultures to those in control cultures. Data are represented as means ± SEM of three independent experiments.

There are other approaches to control CVB3 replication, amongst which are reports on application of siRNA to block its replication (Yuan et al., 2005; Merl et al., 2005). However, the main challenge for siRNA is still the problem with the targeted delivery and nonspecific bindings (Zhang et al., 2009). Another limitation is that siRNA targeting CVB3 mRNA strand is probably more effective during the very early stages of virus replication and is more practical when virus infection is detected early. The efficiency of such approach during the chronic phase of infection, in which most of the cytotoxic effects caused by virus in due to the cleavage of host cell proteins by viral proteases, is a matter of question. Such limitation in siRNA delivery warrants further investigation to establish inhibitory peptides that are more specific in binding to the protein of interest, and easier to deliver.

In conclusion, this study is a proof of principle that demonstrates a well-established, tightly controlled and consistent *in vitro* model for studying and monitoring the inhibition of viral protease 2A functions and destructive effects. Furthermore, the observation that our 16-mer peptide appears to be highly modifiable will provide us with possibilities in the future to improve its structure to achieve a higher-affinity or a potent noncompetitive and specific inhibitor for protease 2A. The ultimate goal is to test the inhibitory peptide in an *in vivo* animal model of viral myocarditis.

Materials and methods

Viral RNA preparation

Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was used for growth and maintenance of HeLa cells cultures. Subconfluent cell cultures were infected with 10^5 plaque-forming units (PFU) CVB3 in a medium containing 1% fetal bovine serum. Cell lysates were then collected at 24 hrs post-infection

by centrifugation at 300×*g* for 10 min and total RNA was extracted by RNX solution as described by the manufacturer (Cinagen, Iran). Briefly, after homogenizing the cells, 1 ml of RNX solution was added to the tube and incubated at room temperature for 5 min. Chloroform was added to the solution and centrifuged for 15 min at 12,000 RPM. The upper phase was then transferred to another tube and an equal volume of isopropanol was added. The mixture was centrifuged for 15 min at 12,000 RPM and the resulting pellet was then washed in 70% ethanol and dissolved in DEPC-treated water.

Construction of expression vector

cDNA that was generated from PCR product of $2A^{pro}$ was cloned into pET22b vector, and the direction of cloning was confirmed. For expression, pET-$2A^{pro}$ recombinant vector was transferred to *Escherichia coli* BLR(DE3)pLysS strain and IPTG was used to express $2A^{pro}$ as described previously (Maghsoudi et al., 2008). Finally, purification was performed by Q-Sepharose column (Pharmacia, Sweden).

The ability of purified $2A^{pro}$ to cleave eIF4G, a natural substrate of $2A^{pro}$, was assayed on postmitochondrial HeLa cell extracts (S10) that were obtained as described by Brown and Ehrenfeld (1979). The S10 extract was incubated with 5 μg of purified $2A^{pro}$ protein for 2 hours at 37 °C. The reaction was stopped by adding 1/4 volume of 4× sample buffer (640 mM Tris–HCl pH 6.8, 8% SDS, 20% 2-mercaptoethanol, 40% glycerol, 4 mM PMSF, and 0.015% bromophenol blue). The samples were analyzed by Western blotting using a polyclonal antibody against eIF4G. Production of rabbit antiserum against eIF4G was carried out as previously described (Maghsoudi et al., 2008).

Generation of stably transformed HeLa cells bearing the CVB3 $2A^{pro}$ gene

The Tet-On system is based on two mutant "reverse" Tet repressors (rTetRs) that bind to tetO in the presence of doxycycline (Sigma, USA). These rTetRs have been fused to VP16 AD. The resulting trans-activators (rtTA) activate transcription from a TRE as a consequence of doxycycline treatment (Gossen et al., 1994; Hillen and Berens, 1994; Urlinger et al., 2000). To establish a stably transformed HeLa cell line, $2A^{pro}$ gene was subcloned in pBI-G Tet vector (Clontech, USA). The pBI-G Tet Vector contains the bidirectional promoter Pbi-1, which is responsive to the tTA and rtTA regulatory proteins in the Tet-Off and Tet-On systems, respectively.

Construction of pBI-G-$2A^{pro}$

The pET22b plasmid containing $2A^{pro}$ gene was used as the template and PCR was performed. One pair of primers was used for this part of preparation: F-2ak containing the *Pst*I restriction site (underlined) and Kozak sequence (5′-ATC*CTGCAG*ACCACCATGGGCG-CATTTGGACAAC-3′), and R-2a containing *Sal*I restriction site (underlined) (5′-ACG*GTCGAC*TCACTGTTCCATTGCATCATC-3′).

PCR was carried out by PFU polymerase under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles (94°C for 1 min, 68 °C for 1 min, and 72 °C for 1 min), and a final extension for 5 min at 72 °C. The PCR products digested by *Pst*I/*Sal*I were inserted into the *Pst*I/*Sal*I restriction sites of digested/dephosphorylated pBI-G vector (Clontech, USA) and ligated mixtures were transformed into competent cells of *E. coli* DH5α using the electroporation method.

Preparation of a stable Tet-On HeLa cell line

Increasing concentrations (0, 50, 100, 200, 400, and 800 μg/ml) of Geneticin (G418) (MP Biomedicals, USA) were tested on HeLa cell cultures to find a suitable concentration for developing the stable cell line. HeLa cells were transfected with 5 μg of pWHE134 containing neoR using the electroporation technique. Successfully transfected colonies were selected based on their response to G418, which resulted in massive cell death in ~5 days. Two to four weeks following treatment, G418-resistant HeLa cell colonies appeared. Large, healthy colonies were selected and transferred to individual plates. HeLa Tet-On Cells were grown and maintained in DMEM supplemented with 10% FBS and 150 μg/ml of G418 (GIBCO BRL, USA).

Development of a double-stable Tet-On HeLa cell line

A 60-mm-diameter plate seeded with 10^6 HeLa Tet-On cells was cotransfected with 5 ng of pBI-G-$2A^{pro}$ encoding the CVB3 $2A^{pro}$ under the control of the tet-responsive promoter, and 250 ng of linear hygromycin marker (Clontech, USA). The transfection was carried out by electroporation. Double-transfected HeLa cells were selected in the presence of 200 μg/ml hygromycin B (Invitrogen, USA) and 150 μg/ml of G418. Large and healthy-looking colonies were isolated and expanded to individual plates. Cells were induced by 300 ng/ml doxycycline for expression of $2A^{pro}$ and 48 hours later hygromycin-resistant clones were collected and total RNA was extracted using RNX plus solution according to the manufacturer's protocol (Cinnagen, Iran). Complementary DNA synthesis reactions were performed using 1 μg of RNA and MMLV reverse transcriptase (Gibco BRL, Germany) with oligo(dT)18 priming in a 20-ml reaction as described by manufacturer. PCR was carried out using F-2ak and R-2a primers as described before and β-actin (internal control) primers as follows: forward primer; 5′-CACCACACCTTCTACAATGAGCTGCG-3′ and reverse primer; 5′-TACTCCTGCTTGCTGATCCACATCTGC-3′. The PCR amplification was performed for 35 cycles. The cycling conditions for β-actin were as follows: 94 °C for 30 s, 58.5 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 5 min. A suitable double-stable Tet-On HeLa cell line was frozen to ensure a renewable source for future use.

Treatment of cells with 16-mer peptide and measurement of cell viability

The established double stable Tet-on HeLa cell line was maintained as monolayer in DMEM medium supplemented with 5% fetal bovine serum, 200 μg/ml hygromycin B, and 150 μg/ml of G418. At the time of experiment, transfected HeLa cells were induced by 300 ng/ml doxycycline, then treated with increasing concentrations of peptide (10–250 μg/ml), and cell viability was measured after 48 hours by the conventional MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay according to the manufacturer's protocol (RDSystem, USA). Results were reported as the percentages of cell viability, where the viability of the control group was arbitrary set as 100%.

Western blot analysis

To determine the activity of conditionally expressed viral $2A^{pro}$ protein in stably transfected Tet-on HeLa cells, cell lysates was subjected to the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot analysis. Following various treatments, cells were collected and cell lysates were prepared using the lysis buffer containing 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris pH 8.0. After centrifugation to remove cell debris, the lysates were prepared using sample buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.04% bromophenol blue, 60 mM Tris–HCl, pH 6.8), and then loaded onto a 15% PAGE in a buffer containing 0.1% sodium dodecyl sulfate. The proteins were transferred to a nitrocellulose transfer membrane (Millipore, USA), and then were probed with specific antibodies (Cell Signaling, USA). β-Actin protein was detected as a control housekeeping protein to ensure equal protein loading in all experiments. Protein bands were detected by chemiluminescence using enhanced electrochemiluminescence (ECL) reagent (Amersham Bioscience, USA) and autoradiography. Data analysis was done by Labwork data analysis (Cambridge, UK).

Computational analyses

Sequence alignment was performed using the Psi-Blast sequence alignment (Altschul et al., 1997). Protein tertiary structure predictions were performed with the protein homology modeling Swiss modeler server using the crystal structure of HRV2 $2A^{pro}$ (1z8r) (Petersen et al., 1999) as a template. This program is completely automated and is capable of generating energy minimized protein models by satisfying spatial restrains on bond distance and dihedral angles extracted from the template PDB file. The input for the program modeler consisted of the aligned sequence of CVB3 and HRV2 $2A^{pro}$, a steering file that gives all the necessary commands to the modeler and pdb file of the template. Many runs of modeler were carried out to obtain the most plausible model. The program was implemented using standard parameters and a database of protein with known 3D structure. To ensure the reliability of the alignment and modeling of variable surface loops, structural investigations on the graphic screen using 3D visualization program Swiss pdb viewer was performed. Docking study was performed with HEX 4.5 program (Ritchie and Kemp, 2000). The substrate structure was estimated with molecular dynamic (MD) simulation (Kohlbacher and Lenhof, 2000; Moll et al. 2006). A 10-ps short MD under AMBER force filed was sufficient for a short peptide and BALL output used for docking studies as ligand.

Acknowledgment

We would like to thank the research council of Shahid Beheshti University (M. C.) for the financial support of this work.

References

Altschul, S.F., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.
Badorff, C., et al., 1999. Enteroviral protease 2A cleaves dystrophin: evidence of cytoskeletal disruption in an acquired cardiomyopathy. Nat. Med. 5, 320–326.
Baker, D., Sali, A., 2001. Protein structure prediction and structural genomics. Science 294, 93–96.
Bovee, M.L., Marissen, W.E., Zamora, M., Lioyd, R.E., 1998. The predominant eIF4G-specific cleavage activity in poliovirus-infected HeLa cells is distinct from 2A protease. Virology 245, 229–240.
Bowles, N.E., Richardson, P.J., Olsen, E.G., Archard, L.C., 1986. Detection of coxsackie-B-virus-specific RNA sequences in myocarditis and dilated cardiomyopathy. Lancet 1 (8490), 1120–1123.
Brown, B.A., Ehrenfeld, E., 1979. Translation of poliovirus RNA *in vitro*: changes in cleavage pattern and initiation sites by ribosomal salt wash. Virology 97, 396–405.
Calandria, C., Irurzun, A., Barco, A., Carrasco, L., 2004. Individual expression of poliovirus $2A^{pro}$ and $3A^{pro}$ induces activation of caspase-3 and PARP cleavage in HeLa cells. Virus Res. 104, 39–49.
Deszcz, L., Cencic, R., Sousa, C., Kuechler, E., Skern, T., 2006. An antiviral peptide inhibitor that is active against picornavirus 2A proteinases but not cellular caspases. J. Virol. 80, 9619–9627.
Frisk, G., Torfason, E.G., Diderholm, H., 1984. Reverse radioimmunoassay of IgM and IgG antibodies to coxackie B viruses in patients with acute myopericarditis. J. Med. Virol. 14, 191–200.
Gillum, R.F., 1986. Idiopathic cardiomyopathy in the United States, 1970–1982. Am. Heart J. 111, 752–755.
Goldstaub, D., et al., 2000. Poliovirus 2A protease induces apoptotic cell death. Mol. Cell. Biol. 20, 1271–1277.
Gossen, M., Bonin, A.L., Freundlieb, S., Bujard, H., 1994. Inducible gene expression systems for higher eukaryotic cells. Curr. Opin. Biotechnol. 5, 516–520.
Hanecak, R., Semler, B.L., Anderson, C.W., Wimmer, E., 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. U. S. A. 79, 3973–3977.
Hellen, C.U., Facke, M., Krausslich, H.G., Lee, C.K., Wimmer, E., 1991. Characterization of poliovirus 2A proteinase by mutational analysis: residues required for autocatalytic activity are essential for induction of cleavage of eukaryotic initiation factor 4F polypeptide P220. J. Virol. 65 (8), 4226–4231.
Hillen, W., Berens, C., 1994. Mechanisms underlying expression of Tn10-encoded tetracycline resistance. Annual. Rev. Microbiol. 48, 345–369.
Kohlbacher, O., Lenhof, H.P., 2000. Rapid software prototyping in computational molecular biology. Bioinformatics 16, 815–824.
Konig, H., Rosenwirth, B., 1988. Purification and partial characterization of poliovirus protease 2A by means of a functional assay. J. Virol. 62, 1243–1250.
Krausslich, H.G., Wimmer, E., 1988. Viral proteinases. Annu. Rev. Biochem. 57, 701–754.
Lampheaer, B.J., et al., 1993. Mapping the cleavage site in protein synthesis initiation factor eIF-4 gamma of the 2A proteases from human coxsackievirus and rhinovir. Biol. J. Chem. 268, 19200–19203.
Maghsoudi, N., Khodagholi, F., Sadjadi, M., Zeinodini, M., Sabaghian, M., 2008. Purification and partial characterization of coxsackievirus B3 2A protease expressed in *E. coli*. Int. J. Biol. Marcomol. 43, 238–244.
Merl, S., Michaelis, C., Jaschke, B., Vorpahl, M., Seidl, S., Wessely, R., 2005. Targeting 2A protease by RNA interference attenuates coxsackieviral cytopathogenicity and promotes survival in highly susceptible mice. Circulation 111, 1583–1592.
Moll, A., Hildebrandt, H.P., Lenhof, Kohlbacher, O., 2006. BALLView: a tool for research and education in molecular modeling. Bioinformatics 22, 365–366.
Olivetti, G., Anversa, P., et al., 1997. Apoptosis in the failing human heart. N. Engl. J. Med. 336, 1131–1141.
Petersen, J.F.W., et al., 1999. The structure of the 2A proteinase from a common cold virus: a proteinase responsible for the shut-off of host-cell protein synthesis. EMBO J. 18, 5463–5475.
Reyes, M.P., Lerner, A.M., 1985. Coxsackievirus myocarditis – with special reference to acute and chronic effects. Prog. Cardiovasc. Dis. 27, 373–394.
Ritchie, D.W., Kemp, G.J.L., 2000. Docking large proteins using spherical polar Fourier correlations. Proteins 39, 178–194.
Rueckert, R.R., 1996. Picornaviridae: the viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology. Lippincott-Raven Publishers, Philadelphia, pp. 609–654.
Sablina, E.P., Antonov, V.K., 1991. Recombinant poliovirus 3C protease: the enzyme application to protein specific fragmentation. FEBS Lett. 283 (2), 291–294.
Schotte, P., Declercq, W., Van Huffel, S., Vandenabeele, P., Beyaert, R., 1999. Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett. 8, 117–121.
Seipelt, J., Liebig, H.D., Sommergruber, W., Gerner, C., Kuechler, E., 2000. 2A proteinase of human rhiovirus cleaves cytokeratin 8 in infected HeLa cells. J. Biolog. Chem. 275 (26), 20084–20089.
Skern, T., Liebig, H.D., 1994. Picornains 2A and 3C. Methods Enzymol. 244, 583–595.
Sugrue, D.D., Rodeheffer, R.J., Codd, M.B., Ballard, D.J., Fuster, V., Gersh, B.J., 1992. The clinical course of idiopathic dilated cardiomyopathy: a population-based study. Ann. Intern. Med. 117, 117–123.
Toyoda, H., et al., 1986. A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45, 761–770.
Urlinger, S., et al., 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc. Natl. Acad. Sci. U.S.A. 97 (14), 7963–7968.
Ventoso, I., MacMillan, S.E., Hershey, J.W., Carrasco, L., 1998. Poliovirus 2A proteinase cleaves directly the eIF-4G subunit of eIF-4F complex. FEBS Lett. 435 (1), 79–83.
Wang, Q.M., Johnson, R.B., Sommergruber, W., Shepherd, T.A., 1998. Development of invitro peptide substrates for human rhinovirus-14 2A protease. Arch. Biochem. Biophys. 356, 12–18.
Woodruff, J.F., 1980. Viral myocarditis. A review. Am. J. Pathol. 101, 425–484.
Yuan, J., Cheung, P.K., Zhang, H.M., Chau, D., Yang, D., 2005. Inhibition of coxsackievirus B3 replication by small interfering RNAs requires perfect sequence match in the central region of the viral positive strand. J. Virol. 79 (4), 2151–2159.
Zhang, H.M., Su, Y., Guo, S., Yuan, J., Lim, T., Guo, P., Yang, D., 2009. Targeted delivery of anti-coxsackievirus siRNAs using ligand-conjugated packaging RNAs. Antiviral Res. 83 (3), 307–316.