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Inhibition of coxsackievirus infection in cardiomyocytes by small dsRNA targeting its cognate coxsackievirus adenovirus receptor

Mirnalini Sharma[1], Baijayantimala Mishra[1], Uma Nahar Saikia[2], Ajay Bahl[3] & R.K. Ratho[1]

Departments of [1]Virology, [2]Histopathology & [3]Cardiology, Postgraduate Institute of Medical Education & Research, Chandigarh, India

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***Background & objectives*: Coxsackievirus B (CVB), a member of human *Enterovirus* group, is the most common cause of viral myocarditis. Coxsackievirus adenovirus receptor (CAR) is identified as a key determinant for the entry of CVB in the target cells. Thus, blockade of receptor by RNA interference (RNAi) may inhibit the entry and pathogenesis of CVB in cardiac cells. The present study was aimed to determine the effect of CAR small dsRNA (siRNA) on coxsackieviral load and CAR expression in coxsackievirus-infected cardiomyocytes.**

***Methods*: Transfection efficiency in rat cardiomyocytes (H9c2) was determined by the fluorescent microscopy and flow cytometry. CAR siRNA dose was optimized based on cell viability and relative CAR messenger RNA (mRNA) expression. Cardiomyocytes were transfected with CAR siRNA followed by infection with 100 multiplicity of infection of CVB, which were harvested after 24, 48 and 72 h post-infection (p.i.). RNA was extracted for relative CAR mRNA expression. Cells were freeze-thawed thrice for estimating coxsackieviral load.**

***Results*: The efficiency of transfection was optimized to be >80 per cent and CAR siRNA dose of 60 pmol was standardized. The knockdown of CAR by siRNA decreased its expression twice the expression in normal cardiomyocytes after 24 h p.i. of CVB. The treatment with CAR siRNA resulted in significant two log reduction of CVB load in cardiomyocytes infected with CVB at 24 h p.i. and retained till 72 h p.i.**

***Interpretation & conclusions*: The inhibition of CAR by siRNA was found to be effective against CVB in cardiomyocytes. However, this treatment strategy has to be evaluated *in vivo* to develop a new treatment strategy for patients suffering with viral myocarditis.**

Key words Coxsackievirus - coxsackievirus adenovirus receptor - gene silencing - relative expression - RNA interference - small dsRNA - viral load

Coxsackievirus B (CVB) is a non-enveloped, small positive-sense single-stranded RNA virus which belongs to family Picornaviridae and genus *Enterovirus* (EV). CVB is of high clinical relevance since it can cause febrile illness, meningoencephalitis and pancreatitis and is a common cause of viral myocarditis. The virus can persist to cause chronic myocarditis which can progress to dilated cardiomyopathy (DCM) and heart failure. The persistence of CVB characterized by the presence of genomic RNA and VP1 antigen

in myocardial tissues has been observed in DCM patients[1,2]. Although many antivirals such as pleconaril and interferon β have been used to eliminate EVs, but till date no specific clinical therapy is available for CVB.

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism where small double stranded (ds)RNA (siRNA) molecules bind to a protein complex RNA-induced silencing complex (RISC). The complex hybridizes to the complementary RNA sequence, and argonaute protein of RISC cleaves the target sequence[3,4]. RNAi is used for the treatment of virus-induced heart diseases. CVB3 has been found to be inhibited by RNAi-targeting RNA-dependent RNA polymerase in the previous studies[5,6]. However, CVB interacts with coxsackievirus adenovirus receptor (CAR) through which they initiate their pathogenesis; therefore, it is an attractive target for the inhibition by siRNA. Thus, it can be another approach to prevent viral entry into the host cell and progression of viral myocarditis to DCM. The present study was undertaken to investigate the potential antiviral efficacy of CAR siRNA on CVB load in coxsackievirus-infected rat cardiac myocytes cell line.

Material & Methods

Cell lines & virus: H9c2 (2-1) rat cardiac myocytes cell line was obtained from National Centre for Cell Science, Pune (India). The cell line was used to study the effect of CAR inhibition *in vitro*. Cells were sub-cultured and grown in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10 per cent fetal bovine serum (FBS, Sigma-Aldrich, USA) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (HiMedia Laboratories, India) at 37°C in 5 per cent CO_2. CVB5 reference strain was obtained from King Institute of Preventive Medicine and Research, Chennai. The virus strain was used to study the effect of gene silencing by CAR siRNA on CVB viral load *in vitro*.

Determination of viral load of reference strain: The viral load of CVB stock was estimated by EV real-time reverse transcription polymerase chain reaction (RT-PCR) using TaqMan assays containing the primers and probe targeting 5'UTR of EV genome as described previously[7]. Viral RNA was extracted from the virus stock using Viral RNA Extraction Kit (Qiagen, GmBH, Germany) following the procedure as described by the manufacturer and subjected to EV TaqMan real-time RT-PCR targeting 5'UTR region of EV (ABI, Foster City, USA)[7].

Relative coxsackievirus adenovirus receptor (CAR) messenger RNA (mRNA) expression in CVB-infected cells: In a 24-well plate, 10^5 H9c2 cells per well were seeded in 2 ml DMEM supplemented with 10 per cent FBS. When the cells became 70-80 per cent confluent, these were washed twice with PBS. Cells were infected with 0.1 ml of the reference CVB strain with 10 multiplicity of infection (m.o.i.) using the standard protocol as described earlier[8]. The cells were incubated at 35°C and harvested after 6, 12, 15, 18 and 24 h from triplicate wells. Mock-infected cell culture was used as cell control.

RNA was extracted from the cells harvested at different time intervals using RNeasy Mini kit (Qiagen, GmBH, Germany) and 1 µg of it was reverse-transcribed to complementary DNA (cDNA) using Moloney Murine Leukemia Virus reverse transcriptase (MBI Fermentas, USA) following the manufacturer's recommendations. The cDNA was subjected to SYBR Green Real-Time RT-PCR using β-actin as an internal control for normalization of messenger RNA (mRNA) expression and CAR-specific primers as previously described[9]. The cycle threshold (C_T) values of CAR and β-actin were compared and relative quotient was calculated.

Small dsRNA (siRNA): CAR siRNA (Cat No. 4392420) (Ambion, USA) was directed against the extracellular amino terminal domain D1 of CAR, the binding site of both CVB and adenovirus (AdV) was used for inhibition of CAR *in-vitro*.

Block-iT Fluorescent Oligo, a fluorescein isothiocyanate (FITC)-labelled control siRNA (Cat No.13750062) (Invitrogen, USA) having no homology with human, rat, mouse and viral genome, was used to optimize the transfection protocol.

Optimization of transfection: In a 24-well plate, 10^4 H9c2 cells were seeded in one ml antibiotic-free normal growth medium supplemented with 10 per cent FBS and incubated at 37°C in a CO_2 incubator for 18-24 h. The cells were transfected with 20, 40, 60, 80 and 100 pmol of FITC-labelled control siRNA (Invitrogen, USA) using lipofectamine-2000 (Invitrogen, USA) as transfection reagent to optimize transfection following the protocol recommended by the manufacturer. The transfected cells were observed under fluorescent microscope (Olympus, Japan) for apple-green fluorescence and studied by FACS for intensity of green fluorescence after 12 h post-transfection. The untransfected cells were used as cell control.

Optimization of CAR siRNA dose: H9c2 cells were transfected with 20, 40, 60, 80 and 100 pmol of CAR siRNA (Ambion, USA) using lipofectamine-2000 following the manufacturer's transfection protocol. The cells were analyzed for the number of viable cells to determine the toxicity of siRNA dose as well as the relative CAR mRNA expression after transfection. The CAR siRNA dose which decreased the CAR mRNA expression to the greatest extent along with the minimum loss of cell viability was selected as optimal dose for further gene inhibition studies.

Determination of cell viability by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: In a 96-well plate, 10^3 H9c2 cells per well were seeded in 100 µl antibiotic-free DMEM supplemented with 10 per cent FBS and incubated at 37°C in a CO_2 incubator for 18-24 h. The cells were transfected with diluted CAR siRNA (20, 40, 60, 80 and 100 pmol) in triplicates using the above-mentioned transfection protocol. Ten µl of 5 mg/ml MTT (Sigma-Aldrich, USA) was added in 100 µl medium in each well and incubated in dark at 37°C for four hours; 100 µl of diethyl sulphoxide was added to dissolve the purple-coloured precipitates. Absorbance was read at 570 nm in ELISA Reader (ThermoScientific, USA). The uninfected cells were included as controls and only medium as blank.

Relative CAR messenger RNA (mRNA) expression in CAR siRNA-transfected cells: H9c2 (10^4) cells were seeded in the 24-well plate and transfected with different doses of CAR siRNA (Ambion, USA) using the transfection protocol. After 18-24 h of transfection, cells were harvested by trypsinization and washed twice with PBS. Untransfected cells were used as cell control for CAR siRNA transfection. The relative CAR mRNA expression was studied by SYBR Green real-time RT-PCR as described earlier. The C_T values of CAR in transfected cells were compared with untransfected cells (cell control) to determine the relative CAR expression by $\Delta\Delta C_T$ method[10].

Gene silencing by CAR siRNA in coxsackievirus-infected cells: In a 12-well plate, 10^5 H9c2 cells per well was seeded in one ml antibiotic-free normal growth medium supplemented with 10 per cent FBS. Cells were transfected with 60 pmol of CAR siRNA and incubated in CO_2 incubator for 18 h using the transfection protocol. The transfected cells were infected with 100 m.o.i. of CVB using the standard protocol described earlier. Cells were observed for cytopathic effect and harvested after 24, 48 and 72 h post-infection (p.i.) from quadruplicate wells. Cells harvested from two wells were subjected to SYBR Green Real-Time RT-PCR to study the decrease in CAR expression. Cells harvested from other duplicate wells were freeze-thawed thrice, and virus isolated was subjected to EV real-time RT-PCR as described before. The untransfected and uninfected cells were used as cell control and CVB infected untransfected cells were used as positive control for virus.

Statistical analysis: The statistical analysis was carried out using Statistical Package for Social Sciences (SPSS Inc., version 16.0 for Windows, Chicago, IL, USA). To study the effect of gene silencing by CAR siRNA at different time intervals paired *t* test was applied.

Results

CAR expression in CVB-infected cell line: CAR expression was observed in uninfected as well as CVB-infected H9c2 cells harvested after 6, 12, 18 and 24 h p.i. It was observed that relative to uninfected H9c2 the CAR mRNA expression was observed to increase at six hours post-CVB infection which increased gradually at 12 h p.i. and then further decreased at 24 h p.i. The CAR expression was observed to increase to two log (100 times) relative to uninfected H9c2 at 12 h p.i. (Fig. 1).

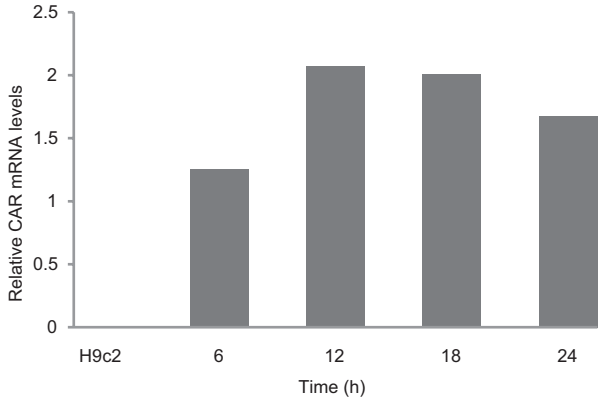

Fig. 1. Relative coxsackievirus adenovirus receptor (CAR) messenger RNA expression in coxsackievirus B-infected H9c2 cells harvested after different time intervals. The C_T values of CAR and β-actin/18S were compared and relative quotient (RQ) was calculated (grey bars). The CAR expression was observed to increase relative to uninfected H9c2.

Optimization of transfection: The efficiency of transfection in H9c2 was determined qualitatively by fluorescent microscopy and quantitatively by flow cytometry. Untransfected cells served as reference sample in the siRNA transfection experiments. Apple-green fluorescence was observed in the H9c2 transfected with siRNA. The number of cells showing fluorescence was observed to increase with the increase in siRNA dose. Thus, the transfection efficiency was found to be qualitatively highest at 100 pmol siRNA in H9c2. However, at 20 pmol concentration of FITC-labelled control siRNA, the transfection was recorded in 0.7 per cent H9c2 cells whereas the siRNA was incorporated into 81.3 per cent of the cell population at 60 pmol concentration (Table and Fig. 2). Along with the quantification of transfected cells, the mean fluorescent intensity (MFI) was also measured in transfected cells by FACS (Table). Quantitatively, the transfection efficiency was observed to be highest at 60 pmol dose of control siRNA in H9c2 cells.

Optimization of CAR siRNA dose: Dose-dependent inhibition experiments were done to determine the effect of siRNA on cell viability and CAR expression in transfected cells. Decrease in cell viability was observed with increase in the siRNA dose. The cell viability was reduced to 50 per cent at the dose of 60 pmol which was further reduced to 20 per cent at 100 pmol of CAR siRNA in H9c2 (Fig. 3A). Thus, for H9c2 based on the cell viability assay, the optimum CAR siRNA dose was selected as 60 pmol. After the determination of the effect of siRNA dose on cell viability, the effect of the siRNA dose on CAR expression was studied. No change in CAR mRNA expression was observed at 20 pmol; however, the relative CAR mRNA expression was reduced by 0.42 log at 40 pmol and 0.54 log at 60 pmol in H9c2 cells (Fig. 3B). Thus, 60 pmol was observed to be the optimized CAR siRNA dose which decreased the CAR mRNA expression by 72 per cent in H9c2.

Table. Small dsRNA (siRNA) transfection efficiency and mean fluorescent intensity in H9c2 transfected with different doses of control small dsRNA

siRNA concentration (picomole)	Transfection efficiency in H9c2 (%)	MFI
20	0.71	51.71
40	72.12	119.98
60	81.37	169.42
80	79.35	138.9
100	74.10	113.58

MFI, mean fluorescent intensity

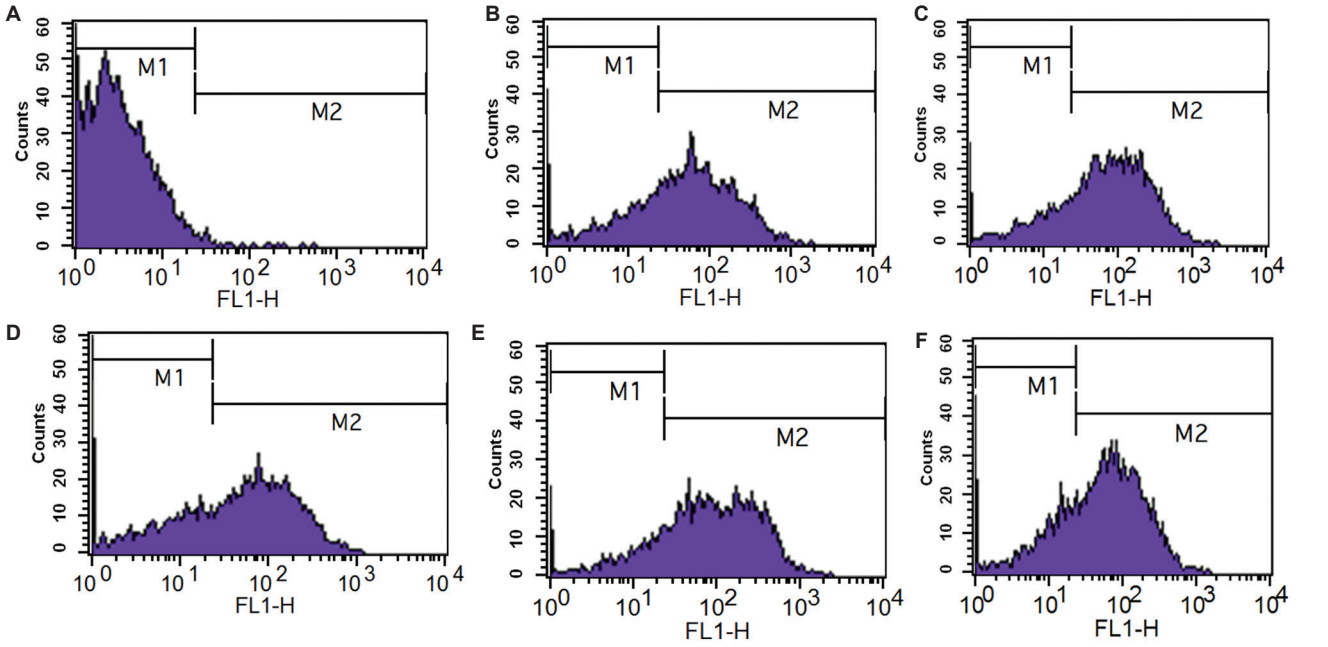

Fig. 2. Flow cytometry histograms showing H9c2 cell population transfected with fluorescein isothiocyanate (FITC)-labelled control small dsRNA. (**A**) Untransfected cell control; (**B**) cells transfected with 20 pmol small dsRNA; (**C**) cells transfected with 40 pmol small dsRNA; (**D**) cells transfected with 60 pmol small dsRNA; (**E**) cells transfected with 80 pmol small dsRNA; (**F**) cells transfected with 100 pmol small dsRNA.

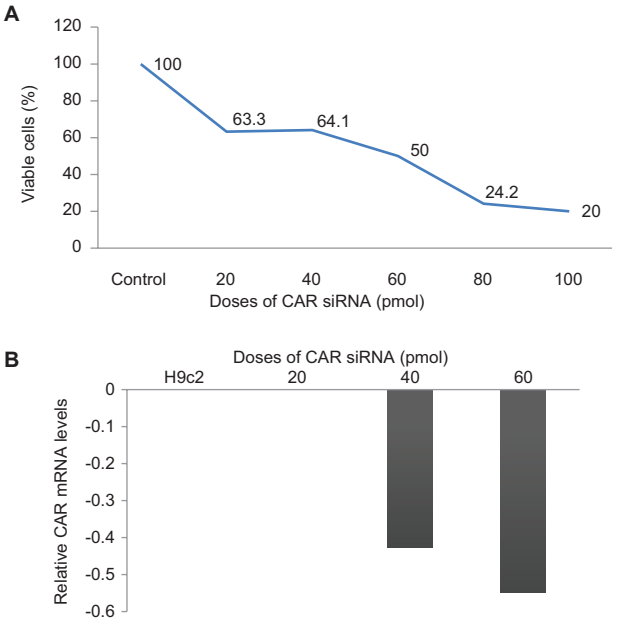

Fig. 3. (**A**) Cell viability in H9c2 cell line after transfection with 20, 40, 60, 80 and 100 pmol of coxsackievirus adenovirus receptor (CAR) small dsRNA; (**B**) CAR messenger RNA inhibition in H9c2 cells transfected with 20, 40 and 60 pmol CAR small dsRNA relative to untransfected cells. The C_T values of CAR and β-actin/18S were compared and relative quotient (RQ) was calculated (grey bars).

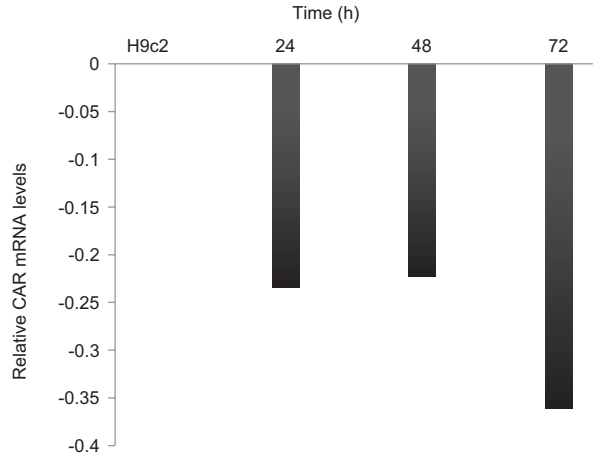

Fig. 4. Coxsackievirus adenovirus receptor messenger RNA (CAR mRNA) expression in H9c2 cells transfected with 60 pmol coxsackievirus adenovirus receptor small dsRNA and after 18 h of transfection infected with coxsackievirus B and harvested after 24, 48 and 72 h post-infection. The C_T values of CAR and β-actin/18S were compared and relative quotient (RQ) was calculated (grey bars). It was observed that relative to untransfected and uninfected cells the CAR mRNA expression was decreased.

Gene silencing by CAR siRNA in coxsackievirus-infected cardiac myocytes: It was observed that relative to untransfected and uninfected cells the CAR mRNA expression was decreased by 0.23 log in H9c2 24 h p.i.

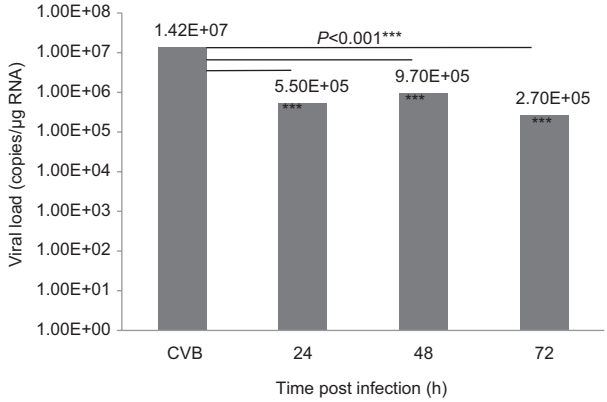

Fig. 5. Viral load in H9c2 cells transfected with 60 pmol coxsackievirus adenovirus receptor small dsRNA and after 18 h of transfection infected with coxsackievirus B and harvested after 24, 48 and 72 h post-infection (p.i.).

The inhibition of CAR mRNA expression was observed to reduce 48 h p.i. by 0.22 log which increased at 72 h p.i. (Fig. 4). The cytopathic effect of CVB in H9c2 was characterized by rounding, shrinking, degeneration of cells and detachment from surface 72 h p.i. The cytopathic effect observed after 72 h of infection suggested that the increased downregulation of CAR expression as observed could be due to cell death or degeneration and not the effect of CAR siRNA.

Fig. 5 shows the viral load of the stock virus used to infect H9c2 and CAR siRNA-transfected and CVB-infected H9c2 cells harvested after 24, 48 and 72 h. As compared to the stock virus which was used to infect the cell line, the CVB load was reduced significantly to two log in CAR siRNA treated H9c2 cells 24 h p.i. (P<0.001) which showed no significant change thereafter at 48 and 72 h p.i. (Fig. 5).

Discussion

CVB and AdV have been implicated as the most common causative agents of viral myocarditis and DCM. A major development occurred with the discovery that short dsRNA could mediate specific gene silencing in mammalian cells without evoking the interferon (IFN) response[11]. RNAi has been found suitable for inhibition of CVB by targeting different viral proteins including VP1, protease 2A, 2B, 3C and RNA polymerase[5,6,12-14].

Both CVB and AdV use common receptor (CAR), so blockade of this receptor appears to be a promising therapeutic approach in the management of viral myocarditis in DCM patients. In the present

study, CAR inhibition by gene silencing was studied in cardiac cells, the actual target cells of CVB3. Efficient gene silencing by RNAi depends on the activity of siRNA and the efficient delivery of siRNA into cells. Hence, high transfection efficiency at low amounts of siRNA is desirable for efficient gene silencing, especially *in vitro*. By forming complex nanoparticles with a size of about 200 nm, intact siRNAs could be readily delivered into cells by an endocytosis process while avoiding nuclease-induced degradation[15].

The present study optimized the siRNA concentration at which ≥80 per cent of cells can be transfected by the lipid mediated transfection. Fluorescent-labelled (FITC) control siRNA was used in our study to track siRNA delivery and to determine the transfection efficiency. Lipofectamine-2000 was used as the transfection reagent and transfection efficiency was measured both qualitatively by fluorescent microscopy and quantitatively by flow cytometry. The transfection efficiency was >80 per cent qualitatively and 81 per cent (max) with mean fluorescent intensity of 169 in H9c2 transfected with 60 pmol of control siRNA.

To determine the dose of CAR siRNA which causes significant reduction in CAR expression as compared to untransfected cells, dose-dependent experiments were carried out in H9c2. Based on the cell viability and level of CAR expression, the optimal CAR siRNA dose was determined. The inhibition of CAR mRNA expression was observed to be dose dependent and 71.2 per cent inhibition was observed in H9c2 at 60 pmol concentration of CAR siRNA. In an earlier study, nanomolar concentrations of CAR siRNA were used to study the inhibition of CVB in cell culture, and the authors reported inhibition of CAR expression by 10 and 100 nm CAR siRNA reduced the virus titre of CVB3 by approximately 60 per cent[5]. It has been studied that cells have a limited capacity to assemble the RNAi machinery and high siRNA concentrations might saturate the cellular machinery[16,17]. Thus, we used sub-nanomolar concentrations of CAR siRNA and demonstrated that optimal CAR inhibition (71.2%) was observed at 60 pmol CAR siRNA and this dose was used for further experiments.

A real-time PCR assay was used to quantitate CVB load to assess the efficiency of CAR gene silencing. Real-time RT-PCR was used for quantitation of viral load with primers and probe specific for 5'UTR region of EV.

The efficacy and stability of siRNA were evaluated over time to have an idea how long the siRNA will remain stable and effective if translated into therapy. siRNAs are highly susceptible and remain vulnerable to degradation by intracellular RNAses. Large size and negative charge of naked siRNAs thwart their diffusion across the plasma membrane and prevent intracellular accumulation[18]. In the present study, the viral load was estimated after transfection of CVB infected H9c2 with CAR siRNA 24, 48 and 72 h p.i. The CAR siRNA was found to be effective till 72 h in H9c2 where a two-fold decrease in CAR expression was observed 24 and 48 h p.i., respectively, and a significant reduction of CVB load (two log times) was determined till 72 h p.i. These results were consistent with the previous report where CAR expression was completely silenced at 48 h and viral titer was reduced to 60 per cent approximately[5]. In another study by the same group, the curative potential of 3Dpol-siRNAs and sCAR-Fc for CVB-3-infected cardiac cells was investigated in myocardial fibroblast cell culture. The antiviral effect of a single treatment lasted for up to one week and could be extended by repeated applications. Each of the single treatments initially reduced the virus titre by approximately one-log, whereas the combination of both approaches resulted in four-log inhibition[19]. However, vector-mediated silencing of CAR could result in 97 per cent inhibition of CVB3 in cardiac cells[10]. The failure to achieve further reduction of the virus titre by CAR-specific siRNAs might be explained by the finding that the decay accelerating factor (DAF/CD55) functions as a co-receptor for CVB-3[20] and it will be interesting to investigate whether the combined use of siRNAs against CAR and DAF will enhance the protective effect.

It is important to consider the possible side effects which may occur due to the ablation of normal cellular functions of the receptor after its downregulation or knockout. It is previously studied that cardiac tissue-specific deletion of CAR in mouse model resulted in severe cardiac abnormalities during embryonic development; however, CAR knockdown during late embryonic stages caused no abnormalities and animals survived[21,22]. Thus, cardiac cells should tolerate the treatment with CAR siRNA over considerable period. In our study, H9c2 cells could tolerate 60 pmol of CAR siRNA dose without causing change in cell growth and morphology and inhibit viral replication. These studies suggest that CAR siRNA can be administered during late stages of development and to the adults for treatment of viral myocarditis.

The limitation of the study is that effect of siRNA on CAR expression was not studied at protein level.

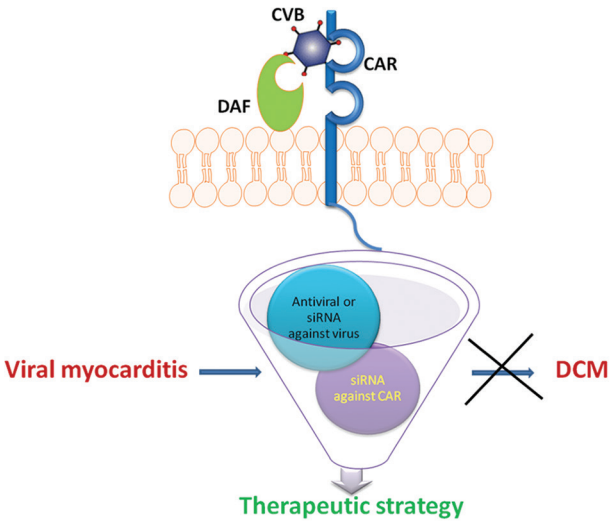

Fig. 6. RNA interference against coxsackievirus adenovirus receptor (CAR) and coxsackievirus B (CVB) can be a new therapeutic approach for the treatment of viral myocarditis and prevent its progression to dilated cardiomyopathy (DCM).

In conclusion, high-efficiency gene silencing of CAR by siRNA in cardiac cell line was observed against CVB. However, this strategy needs to be evaluated *in vivo* for any side effects over prolonged period so as to develop a new therapeutic approach targeting both the virus and receptor against viral myocarditis and prevent its progression to DCM (Fig. 6).

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***Conflicts of Interest*:** None.

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Reprint requests: Dr Baijayantimala Mishra, Department of Microbiology, All India Institute of Medical Sciences, Sijua, Patrapada, Bhubaneswar 751 019, Odisha, India
e-mail: bm_mishra@hotmail.com