CONSERVED REGIONS FROM COXSACKIEVIRUS GENOME AS TARGETS FOR GENE SILENCING (RNAi)

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ABSTRACT

Coxsackievirus infection is typically the respiratory or gastrointestinal at the beginning that may result in a secondary infection. Such secondary spread of virus to the central nervous system can result in aseptic meningitis, encephalitis, paralysis, pleurodynia or myocarditis. Disseminated infection can lead to exanthems, nonspecific myalgias, or severe multiple-organ disease in neonates.

No anti-Coxsackievirus drugs are currently licensed and treatment is directed toward ameliorating symptoms. This places the need of new tools to control the virus infection. Posttranscriptional gene silencing (PTGS, RNAi) is one of the promising new techniques for specific blocking the virus replication. Enteroviruses are RNA and possess high mutation rate during replication of their genomes. For this reason searching and analysis of conserved regions from the virus genome is essential for effective RNAi based drug development. In the present study we offer some conserved sequences from Coxsackievirus B genome which may result as potential targets against the virus infection.

Key words: *Coxsackieviruses, RNAi, PTGS*

Introduction

The Coxsackieviruses were discovered in 1948 by Gilbert Dalldorf in Albany, New York. Using newborn mice as a vehicle, he attempted to isolate such protective viruses from the feces of polio patients. In carrying out these experiments, he discovered viruses that often mimicked mild or non-paralytic polio. The virus he discovered was eventually given the name Coxsackie, for the town of Coxsackie, New York, a small town on the Hudson River where Dalldorf had obtained the first fecal specimens. The Coxsackie viruses subsequently were found to cause a variety of infections, including epidemic pleurodynia, aseptic meningitis, fever, herpangina, non-paralytic poliomyelitis, respiratory infections (Carpenter, C. et al., 1952).

Subtype B coxsackieviruses (CVB) represent the most commonly identified infectious agents associated with acute and chronic myocarditis. Damage to the heart is induced both directly by virally mediated cell destruction and indirectly due to the immune and autoimmune processes reacting to virus infection (Fechner, H.et al., 2011).

Coxsackievirus B3, is generally considered the primary etiological agent of myocarditis (Blauwet et al., 2010; Kuhl et al., 2005a; Mahrholdt et al., 2006). CVB3 infection of the heart is often persistent and enters the chronic phase, leading to dilated cardiomyopathy, viral myocarditis characterized by ventricular chamber dilation, increased wall thickness, weaker beating and abnormal heart function. Patients with dilated cardiomyopathy eventually develop into congestive heart failure. (Andreoletti et al., 2009; Cooper, L. et al., 2009; Kuhl et al., 2005b; Yajima& Knowlton, 2009).

To date, there is no clinically proven specific treatment available for viral myocarditis and cardiomyopathy. Patients with dilated cardiomyopathy eventually need heart transplantation as the final treatment (Schultz et al., 2009). However, some compounds like Disoxaril (WIN 51711) and Pleconaril, interact with Picornavirus antireceptor and block viral entry of the host cells (Groarke& Pevear, 1999; Kaiser et al., 2000; Reisdorph et al., 2003).

Several studies demonstrated naturally occurring mutants resistant to pleconaril and, moreover, pleconaril treatment seems to result in rapid emergence of resistant CVB3 mutants

(Groarke, J. et al., 1999; Schmidtke, M et al., 2005). Such WIN mutants predominantly contain a single amino acid substitution, Ile-1092 Leu/Met in the hydrophobic pocket of the canyon which prevents efficient binding of the compound, while it does not impair binding of the virus to cellular receptors (Schmidtke, M et al., 2005). In order to overcome virus resistance, new pleconaril derivatives have recently been synthesized and successfully tested against pleconaril-resistant mutants (Schmidtke, M. et al., 2009). A series of compounds, such guanidine hypochloride, HBB, MRL-1237 and TBZE-029 interact with the viral protein 2C, resulting in inhibition of the viral RNA synthesis and leading to protection of cells from virus-induced cell lyses (de Palma, A. et al., 2008; de Palma, A. et al., 2007). Their antiviral activity against coxsackieviruses was only investigated in *in vitro* experiments. These experiments, however, revealed that a single mutation in the viral 2C protein is sufficient to confer resistance against the antiviral treatment (de Palma, A. et al., 2008).

There is no vaccine to prevent Coxsackievirus infection. Due to a rapid development of drug resistance of initially drug-sensitive viruses anti-enteroviral chemotherapeutics for clinical use are not registered so far. This places the need of new tools to control the virus infection.

One of these tools is posttranscriptional gene silencing (PTGS, RNAi). Double strand RNA (dsRNA) has been shown to be an effective trigger of gene silencing in vertebrate, invertebrates and plant systems (Waterhouse *et al.,* 2001).

First, RNA nucleases (DICER) bind to a large dsRNA and cleave it into discrete ~21 to 25nt dsRNA fragments (siRNAs). In the second step, these siRNAs join a multinuclease complex, RISC, which finds complementary targeted fragment and degrades the homologous single stranded mRNAs. The dsRNA alone cannot degrade mRNA, but requires the assistance of two enzymes namely, Dicer and RISC. Dicer, which was first discovered by Bernstein *et al.* (2001), is a complex enzyme belonging to the RNase III family. RISC is the component of the RNAi machinery that uses siRNAs to track down and degrade the target mRNAs. The degradation process is initiated once the successful location and cleavage of the complementary mRNA occurs by the siRNA/RISC complex. In case of translational repression pathway, small RNAs direct RISC to bind to target mRNA (partial complementarity) and repress its translation process, rather than cleavage. Animal miRNAs typically, but not always, mediate translational repression rather than cleavage. Host cell targets for RNAi have been used to inhibit HIV-1 (Nekhai and Jerebtsova, 2006). Successful inhibition of tumour cell growth by RNAi aimed at oncogenes *in vitro* and *in vivo* supports the enthusiasm for potential therapeutic application of RNAi (Gartel and Kandel, 2006; Pai *et al.,* 2006). The dsRNAs that trigger RNAi may be usable as drugs. Therapies based on RNAi suggest that, the lack of interaction with DNA may alleviate some patients concerns about alteration of their DNA (as practiced in conventional gene therapy). This RNAi method of treatment would likely be safer than taking any prescription drug.

Material and methods

Referent compounds: 1/Disoxaril (WIN 51711) 5-[7-[4-(4,5-dihydro-1,3-oxazol-2-yl)phenoxy]heptyl]-3-methyl-1,2-oxazole; from Sanofi Winthrop Inc (Malvern, Pennsylvania, USA); 2/dsRNA of S segment of bacteriophage phi6 (2948bp).

Viruses: Coxsackievirus B1 (CVB1) (Connecticut 5) from collection of Institute of microbiology - BAS, Coxsackievirus B3 strain Nancy (CVB3), neurothropic, from Institut für Virologie und Antivirale Therapie, Klinikum der FSU, Jena, Germany.

Cell culture: In vitro experiments were done in monolayers of HEp-2 cells in 96-well plates.

Production of dsRNA specific for conserved region of the Coxsackievirus genome: dsRNA is synthesized by combined in vitro transcription and replication from a DNA template (according to Replicator RNAi Kit, Finnzymes, Finland). The DNA template is produced by PCR using Phusion High-Fidelity DNA polymerase. The PCR primers are designed so that the resulting PCR product contains the target sequence (conserved central part of VP1 (450bp) of Coxsackieviruses) flanked

by T7 promoter sequence at the 5' end and phi6 qRdRP promoter sequence at the 3'end. The PCR product is purified and transcribed into single-stranded RNA by T7 RNA polymerase. This single-stranded RNA is replicated into double-stranded RNA by phi6 RdRP in the same incubation step.

Sequences of the used primers: dsRNA 1 (5'-TAA TAC GAC TCA CTA TAG GGG GCC CAG TGG AAG AAT CGG T -3'), dsRNA 2 (5'-GGA AAA AAA ACA CTG GTA GCG GTA CTG G -3').

Results and discussion

The new technology designed by us can produce dsRNAs, specific to the conserved part of VP1 gene region of Coxsackieviruses, for induction of gene silencing. Thereby, the replication and spread of the virus into the host cells was blocked.

From the analyzed sequences from our virus collection of Coxsackievirus B1, compared with the sequences from NCBI data base we decided to target a comparatively conserved region from the central part of VP1 of Coxsakieviruses B1 and B3. The designed primers contain central part of 450 bp fragment from VP1 gene of Coxsackievirus B1, flanked by T7 and phi 6 qRdRP promoter sequences at both ends.

The choice of gene fragment plays a crucial role in target specific gene silencing. The gene fragments ranging from 50bp to 1000bp were used to successfully silence genes (Helliwell *et al.,* 2002). Two factors can influence the choice of length of the fragment, shorter the fragment the less effective silencing will be achieved, but very long fragments increases the chance of recombination. The effectiveness of silencing also appears to be gene dependent and could reflect accessibility of target mRNA or the relative abundance of the target mRNA. Fragment length of between 300 and 600bp is a suitable size to maximize the efficiency of silencing. The other consideration is the part of the gene to be targeted (Helliwell *et al.,* 2002).

Using the Replicator RNAi Kit, Finnzymes, Finland we optimized a PCR which resulted 450 bp specific dsRNA for VP1 (fig.1).

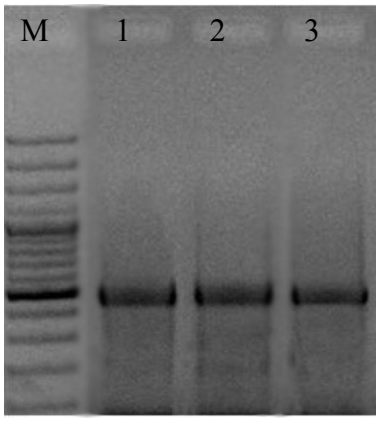

Fig.1 dsRNAs specific for VP1 of Coxsackieviruses.
M-100 bp ladder, 1- purified dsRNA, 2-unpurified dsRNA, 3-partially purified dsRNA

The resulting dsRNA is purified in two steps with 2M and 4M LiCl and presented in Fig. 1(1,3). For testing the effect of dsRNA monolayers of HEp-2 cells were inoculated with the resulting dsRNAs. Cytopathic effect (CPE) inhibition test used confluent cell monolayers of HEp-2 cells in 96 well-plates infected with 100 $CCID_{50}$ in 0.1 ml. CPE was observed and counted under light microscope and the inhibition effect in percentage was evaluated by comparison with the controls not treated with any substance (fig.2).

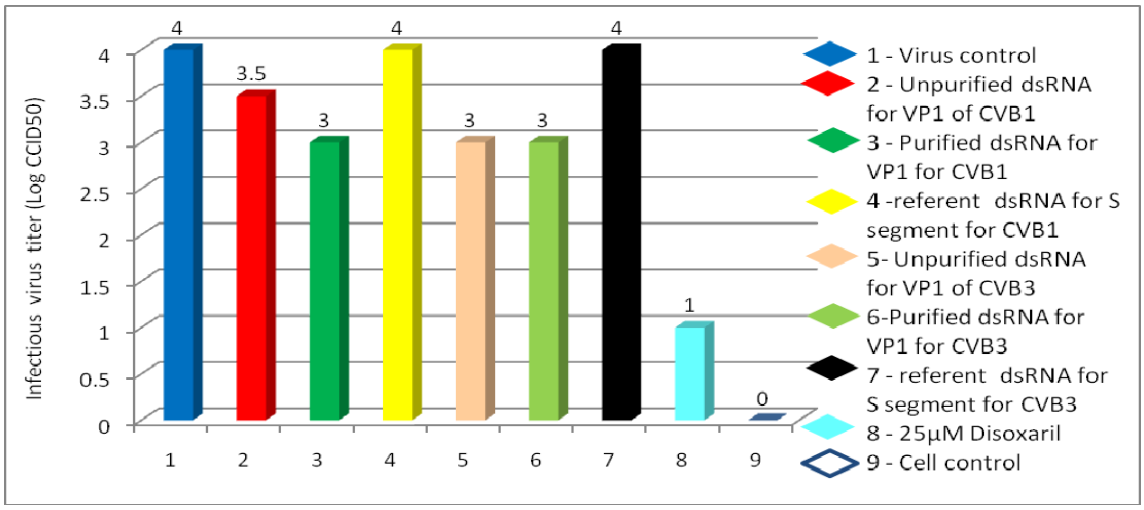

Fig.2 Effect of different dsRNAs against CVB infection in HEp-2 cells compared with Disoxaril

By mutational analysis of the mechanism of siRNA action, it functioned by targeting the positive strand of the virus and required a perfect sequence match in the central region of the target, but mismatches were more tolerated near the 3' end than the 5'end of the antisense strand (Yuan et al., 2005). siRNA targeting CVB3 2A by Merl and co-workers evaluated antiviral activity of siRNA-2A (nts 3637-3657) *in vitro* and in highly susceptible type I interferon receptor-knockout mice. They found that siRNA-2A led to significant reduction of viral tissue titers, attenuated tissue damage and prolonged survival of mice (Merl et al., 2005). *In vitro* studies demonstrated that WIN 54954 can reduce picornavirus titers by 1 to 2 orders of magnitude (Heim, A., et al., 1998; Woods, M., et al., 1989).

Unpurified dsRNA specific for conserved part of VP1 of CVB1 induced very slight effect on the CVB1 (fig.2) and reduced virus titer from 4 to 3.5 Log $CCID_{50}$. The same dsRNA reduced the CVB3 titer with 1 log. Purified dsRNA with LiCl had slightly larger effect on CVB1, reduction of the virus titer with 1 log, and the same effect on CVB3. Refferent dsRNA produced from S genome segment of bacteriophage phi6 had no effect on the virus replication in the cell monolayer. This suggests that unspecific or too large dsRNAs can not influence the Coxsackievirus B1 and B3 virus replication in vitro. As a referent compound we used Disoxaril which reduced the CVB1 virus titer with 3 log $CCID_{50}$.

The weak inhibitory effect of dsRNAs may be due to the low transfection rate of cell cultures in the absence of Lipofectamine or Olygofectamine.

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