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Small Interfering RNAs against Human Enteroviral Infections

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Abstract

Much is known about the enteroviral life cycle, however, because of the emergence of resistant mutants, no effective specific drugs are available to treat enteroviral infections. RNA interference (RNAi) is a post-transcriptional, sequence-specific, gene-silencing defense mechanism of eukaryotic cells against viruses and transposons. It is an evolutionarily conserved mechanism for regulating gene expression which has the potential to be a therapeutic alternative to antiviral small molecule drugs. Small interfering RNAs (siRNAs) induce RNAi and are critical for the inhibition of RNA virus replication in the host cell. siRNAs are also important regulators of virus-host cell interactions. In this investigation we describe a relatively novel approach for using RNAi against CBVs by creating a siRNA pool covering different conserved CBV gene sequences. Many viruses have evolved mechanisms to inhibit RNAi cell defenses. A single nucleotide substitution in the RNAi target site within the viral genome can result in complete loss of interference, depending on the location and the nature of the resulting mismatch. Using polls of siRNAs inhibit the viruses which may escape single-site siRNA silencing by point mutations, overlapping the mutated target region. The polymerase complex of bacteriophage phi 6 is used to synthesize specific dsRNAs complementary to target CBV genes. The dsRNAs are cleaved using Dicer to siRNA pool and introduced to cells by transfection. We produced specific dsRNAs and siRNA pools targeted to 5′UTR, 2A and 3D genes of CVB3 that inhibit completely virus replication *in vitro* in HEp-2 cells. Cytotoxic effects were not observed in the used concentrations.

Key words: RNAi, siRNAs, enteroviruses

Резюме

Много се знае за ентеровирусния жизнен цикъл, обаче, поради появата на резистентни мутанти, няма ефективни специфични лекарства на разположение за лечение на ентеровирусните инфекции. РНК интерференция (RNAi) е пост-транскрипционен, комплементарно-специфичен защитен механизъм на генно мълчание на еукариотните клетки, срещу вируси и транспозони. Това е еволюционно консервативен механизъм за регулиране на генната експресия, който има потенциала да бъде терапевтична алтернатива на нискомолекулните антивирусни средства. Малките интерфериращи РНК (миРНКи, siRNAs) индуцират RNAi и са критични за инхибиране на РНК вирусна репликация в клетката-гостоприемник. миРНКи са също важни регулатори на взаимодействията между вируса и клетката гостоприемник. В това изследване представяме сравнително нов подход за използване на RNAi срещу CBV чрез създаване на миРНК пул, обхващащ различни консервативни CBV генни последователности. Много вируси имат еволюирали механизми за инхибиране на RNAi клетъчната защита. Единично нуклеотидно заместване в прицелното RNAi място във вирусния геном може да доведе до пълна загуба на генно мълчание, в зависимост от разположението и естеството на полученото несъответствие. Използването на пулове от миРНКи инхибира вирусите, които могат да избегнат генното мълчание чрез единична място специфична точкова мутация, като припокриват мутиралия прицелен регион. Полимеразният комплекс на бактериофага Phi6 се използва за синтезиране на специфични двРНКи, комплементарни на прицелни CBV гени. двРНКи се нарязват

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с помощта на Dicer до пул от миРНКи и се въвеждат в клетките чрез трансфекция. Получихме специфични прицелни двРНКи и миРНКи пулове спрямо 5′UTR, 2A и 3D гени на CVB3, които инхибират напълно вирусната репликация *in vitro* в HEp-2 клетки. При използваните концентрации не бяха наблюдавани цитопатични ефекти.

Introduction

Coxsackieviruses belong to the *Enterovirus* genus within the *Picornaviridae* family. (Van Regenmortel *et al*., 2000). Clinical manifestations of the Coxsackievirus group B (CVB) infections include a wide range of diseases and tend to infect the heart, pleura, pancreas, and liver, causing pleurodynia, myocarditis, pericarditis, nonspecific febrile illnesses, rashes, upper respiratory tract disease, insulin-dependent diabetes, aseptic meningitis and hepatitis (Lim *et al*., 2013). Approximately 10 million symptomatic enteroviral infections are estimated to occur annually in the United States. From 2002-2004, an estimated 16.4-24.3% of these illnesses were attributed to coxsackievirus serotypes. For 2 of the 3 years, coxsackievirus B1 was the predominant serotype. Enteroviruses are responsible for approximately 30,000-50,000 hospitalizations per year (Kadambari et *al*., 2014). The disease is often self-limiting and subclinical, but some acute infections are severe and lethal. Enteroviruses, and CBVs in particular, may have a role in the pathogenesis of type 1 diabetes (Hyoty and Taylor, 2002).

The coxsackievirus genome is a linear molecule of ssRNA, owing to its positive polarity. The ssRNA comprises an open reading frame (ORF), flanked on both 3′ (~100 bases) and 5′ (~800 bases) termini untranslated regions (UTRs). The ORF contains genes encoding 11 proteins (Huber *et al*., 1998). The protein capsid is composed of 4 structural proteins - VP1, VP2, VP3 and VP4. There are seven other non-structural proteins like two viral proteases (2A, and 3C), an RNA-dependent-RNA-polymerase (3D), two proteins involved in RNA synthesis (2B, and 2C), a primer of initiation of RNA synthesis (3AB) and a small polypeptide (VPg) of 20–24 amino acids derived from gene 3B. The 5′ UTR of viral RNA is not linked to eukaryotic 7-methylguanosine triphosphate cap structure associated with eukaryotic mRNA, but the 5′ UTR covalently binds to VPg (Flanegan *et al*., 1977).

There are no virus-specific therapies for enterovirus infections. Galabov and colleagues examined a novel approach consisting of a consecutive and alternating, not simultaneous, application of the compounds in a combination on the model of an enterovirus infection in newborn mice (Vassileva-Pencheva and Galabov, 2010). They used 3 compounds differing in their mode of antiviral action selected for consecutive and alternating application: disoxaril, guanidine hydrochloride and oxoglaucine. The triple combination for consecutive application of disoxaril, guanidine. HCl and oxoglaucine (DGO), applied in the order of mentioning, shows a marked *in vivo* efficacy, expressed in a reduction of the mortality rate and lengthening of the mean survival time (Vassileva-Pencheva and Galabov, 2010). Thus, it has been demonstrated for the first time that preservation of the antiviral activity is possible if a triple combination of anti-enteroviral inhibitors is applied in a consecutive and alternating manner (not simultaneously) (Vassileva-Pencheva and Galabov, 2010).

Enterovirus infections, such as poliovirus and CBV3 infections have been successfully inhibited using RNAi in cell culture and in animal models (Gitlin *et al*., 2002, Merl *et al*., 2005; Tan *et al*., 2007; Fechner *et al*., 2008; Kim *et al*., 2008). These studies have employed sequence specific siRNAs, synthesized chemically.

Since identifying target sequence candidates for RNAi can be difficult and expensive, it would be beneficial to find an alternative way of producing siRNAs. One mechanism would be to digest long dsRNA into siRNA pools representing multiple sequences of the viral gene or genome.

Materials and Methods

Virus

Coxsackievirus B3, neurotropic strain Nancy stock for *in vitro* experiments, was obtained from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria. It was grown in monolayer HEp-2 cells with an infectious titer of $10^{4.5}$ $CCID_{50}$.

Cells

Monolayer cell cultures of human epithelial carcinoma cells (HEp-2) were grown in DMEM medium containing 5 % fetal bovine serum in 96-well plates in CO_2 incubator at 37 °C/ 5% CO_2.

Reference compounds

dsRNAs and siRNAs of the S segment of bacteriophage phi6 (2948bp) (obtained from Dennis Bumford, University of Helsinki, Finland) were

applied.

Extraction of total RNA

We used RNEasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Touch-down

RT-PCR (Don *et al.,* 1991**)** for amplification of a specific conserved fragment from 5′UTR, 2A and 3D of CVB3 genome. Used primers: 5′UTR For - ACC TTT GTG CGC CCT GTT; 5′UTR Rev - CAC GGA CAC CCA AAG TA; 2A For - GGA CAA CAA TCA GGG GCA; 2A Rev - TCC ATT GCA TCA TCT; 3D For- TCT CAT AGC ATT TGA TTA C; 3D Rev – ACG TGA CAC GTT CGG AGA AT; 3Da Pol For - TGG GGA TCC ATG TTG GCG GGA; 3Da Pol Rev - ACC CCC ACT GCA CCG TTA TCT.

Synthesis of copy

DNA: included denaturation of RNA (0.05-0.5 μg) at 95°C for 5 min with 7 μl of appropriate primer in a final volume of 10 μl; incubation of a master mix (5 μl 5×MMLV-buffer, 2 μl dNTPs (2mM), 0.5 μl M-MuLV reverse transcriptase (200 U/μl), 7.5 μl DNase and RNase free H_2O at 42°C for 60 min.

PCR

Preparation of a master mix at a final volume of 25 μl (6 μl cDNA, 2.75 μl10 × PCR buffer, 2.2 μl $MgCl_2$ (25 mM), 2.2 μl dNTPs (2 mM), 1 μl of each primers (10 μM), 1 μl proofreading Pfu DNA-polymerase (5 U/μl), 8.85 μl H_2O) in Auto-Q Server (LKB, UK)

Gel electrophoresis

According to Sambrook and Rassel (2001): DNA fragments were separated in 2% agarose gel in TAE buffer with ethidium bromide (0.2 μg/ml) at 80-150V for 1 h. PCR products were visualized in trans-illuminator GenoPlex (VWR) with λ = 315 nm.

In vitro dsRNA production system

The produced PCR fragments were used as templates. Polymerase promoter sequences needed for the dsRNA synthesis were added to both sides of the target sequence using PCR. The PCR primers were designed so that they contained RNA polymerase promoter sequences at their 5′-ends. Thus, in the PCR product, RNA polymerase promoter sequences flanked the target sequence. In addition to promoter sequences, each primer should contain 17-22 nucleotides of target gene-specific sequence at the 3′-end (Fig.1).

dsRNA was synthesized by the combination of *in vitro* transcription and replication of DNA template (according to the instructions of Replicator RNAi Kit, Finnzymes, Finland).

Generation of siRNA pools by Dicer- digestion

We used the enzyme Power Cut Dicer, which is a recombinant endo-ribonuclease from *Giardia intestinalis*. It effectively cut dsRNA, yielding fragments with a length of 25-27 nucleotides, which resulted in accumulation of a pool of siRNAs.

Transfection of cells

We transfected a cell monolayer with Oligofectamine™ Reagent (Invitrogen, USA), according to the manufacturer's instructions.

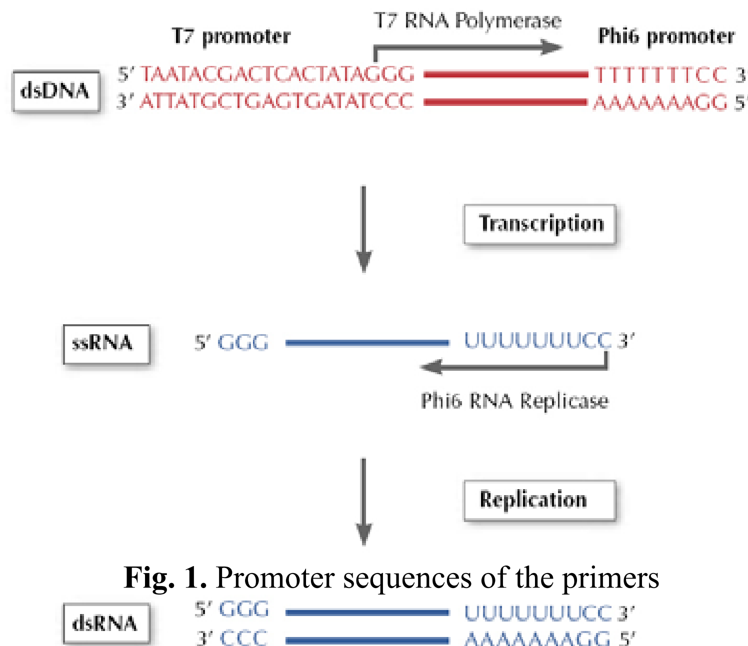

Fig. 1. Promoter sequences of the primers

Determination of cytotoxicity of dsRNAs and siRNA pools on monolayer culture HEp-2 by staining with neutral red (NR)

Cells were cultured in 96 well plates (Cellstar) having an output density $2.5x10^5$ cells/ml. Cells were incubated at 37°C/5% CO_2 until confluent monolayer formation. Each of the samples was repeated four times. Cells were incubated for 72 hours, and then washed with 0.15 ml/well NR. Cells were incubated for 3 hours at 37°C/5% CO^2 for the dye to penetrate viable cells. The dye was removed and cells were washed again with 0.15 ml/well of PBS, then were added in 0.1 ml/well of extraction solution (1% glacial acetic acid, 50% ethanol and 49% distilled water). With this solution, the plates were placed on a shaker for 10 minutes, and afterwards the absorbance of the samples from each well was read on ELISA Reader (Organon, Germany) at a wavelength of 540 nm and a reference value of 620 nm. The survival of the cells of the analyzed samples was calculated as % relative to control cells (which was assumed to be 100%) by the formula:

$$\text{cell viability\%} = \frac{\text{number of living cells per sample}}{\text{number of live cells in control x 100}}$$

The obtained values were used to build “dose-survival” curves, which identify 50% cytotoxic concentration (CC_{50}) of the individual substances.

Determination of the antiviral effect of the dsRNAs and siRNA pools obtained by inhibition of the cytopathic effect and staining with NR

Cells were cultured in 96-well plates with an output frequency of $2.5x10^5$ cells/ml in the formation of a confluent monolayer. The growth medium was aspirated and the cells were inoculated with a range of virus dilution corresponding to a multiplicity of infection (MOI) 0.004. After expiration of adsorption, the unabsorbed virus was removed and 0.1 ml/well of the appropriate dilution of the substance was added. As controls, we used non-infected cells cultured under the same conditions as the viral control, untreated control substance and toxicity for each dilution of the substance-free virus. Each of the samples was repeated four times. The plates were incubated for 72 hours at 37°C/5% CO_2. The inhibition of the cytopathic effect was determined by NR uptake by viable cells the dye uptake of NR viable cells. Decant the middle of the plate and the cells were washed once with 0.15 ml /well of PBS (without Ca^{2+} and Mg^{2+}). The phosphate buffer solution was decanted and treated drop wise with a 0.1 ml/well of dye (1% glacial acetic acid, 50% ethanol and 49% distilled water). The plate was incubated for 10 min with this solution, and afterwards the light absorbance by the sample was read at 540 nm (Borenfreund and Purner, 1985). The obtained values were used to calculate the percentage of protection of the cells, using the formula:

$$[OD_{(sample)} - OD_{(virus\ control)} / OD_{(toxicity\ control)} - OD_{(virus\ control)}] \times 100,$$

where: OD – optical density (absorption)

The resulting value was used to determine the concentration of the substance that inhibited the preparation of an infectious viral progeny by 50% (IC_{50}).

Statistics

The results were processed by determining the standard deviation using the program Microsoft Office Excel 2007 and Clustal Omega.

Results and Discussion

We optimized a new method that can produce dsRNAs, specific to the conserved parts of different gene regions of CVB3, for induction of posttranscriptional gene silencing and blocking the translation of the target genes. Thereby, the replication and spread of the virus into the host cells were blocked. From the analyzed sequences from our virus collection of CVB3, compared with the sequences from NCBI data base, we decided to target comparatively conserved regions from 5′UTR, 2A, and 3D. The designed primers flanked 396 bp and 1544 bp (3Da) fragments from 3D, 440 bp fragment from 2A and 520 bp fragment from 5′UTR genes of CVB3 (Fig. 2).

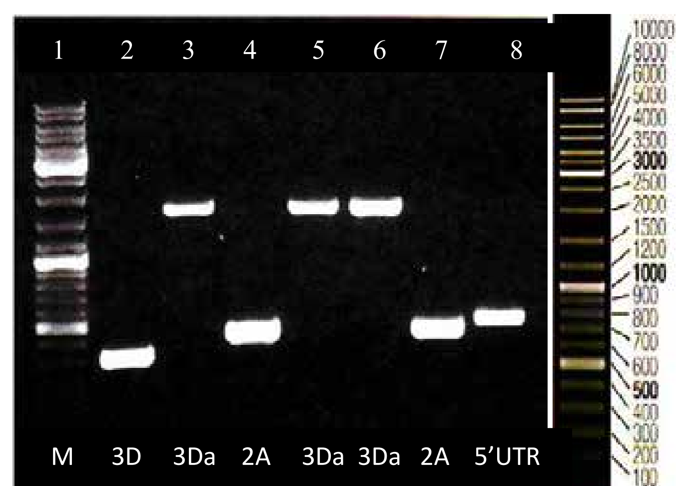

Fig. 2. dsRNAs of the conserved fragments from CVB3 genome

Legend: 1 - 100 bp ladder, 2 - 3D (396 bp), 3 - 3Da (1544 bp), 4 - 2A (440 bp), 5 - 3Da (1544 bp), 6 - 3Da (1544 bp), 7 - 2A (440 bp), 8 - 5′UTR (520 bp)

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 the shorter the fragment, the less effective silencing will be achieved, but very long fragments increase 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).

We chose 3D, 2A and 5′UTR to silence for the following reasons: the enteroviral RNA-dependent RNA polymerase 3D is one of the major components of the viral RNA replication complex and exhibits elongation activity (Van Dyke and Flanegan, 1980). It can uridylylate VPg and use VPg-pUpU as a primer during viral RNA replication (Paul *et al.*, 2003). The viral genome contains also a conserved 5′UTR, which is important to translation and RNA replication (Rohll *et al*., 1994). Gamarnik and Andino (1998) have suggested that the binding of 3CD to the cloverleaf at the 5′ end of the viral genome promotes the replication of RNA, rather than its translation. Furthermore, they found that PCBP2 binds to stem-loop IV in enterovirus translation (Gamarnik and Andino, 2000). 2A protease cleaves dystrophin protein, which is the factor leading to CVB3 cardiomyopathy (Badorff *et al*., 1999). It cleaves also TBP *in-vitro* (Yalamanchili *et al*., 1997) and cleaves eIF4GI and eIF4GII, which lead to the shut off of host translation (Sommergruber *et al*., 1994). The cleavages of poly(A) binding protein (PABP) by 2A protease contributes to the inhibition of cellular translation (Kuyumcu-Martinez *et al*., 2002).

The produced dsRNA fragments we digested with Power Cut Dicer and received siRNA pools (Fig. 3).

To determine the cytotoxicity of the produced fragments we use 12 different concentrations of dsRNAs (Fig. 4) and siRNAs (Fig.5): 72, 24, 8, 2.7, 0.9, 0.3, 0.2, 0.15, 0.1, 0.05, 0.03, 0.01 μM to silence 4 target genes of CVB3.

All the concentrations of the four used fragments (5′UTR, 2A, 3D and larger 3D called 3Da), even the highest (72 μM) showed no significant toxicity for HEp-2 cell monolayer (Fig. 4, 5). Even CC_{50} cannot be calculated. From 0.01 to 0.35 μM

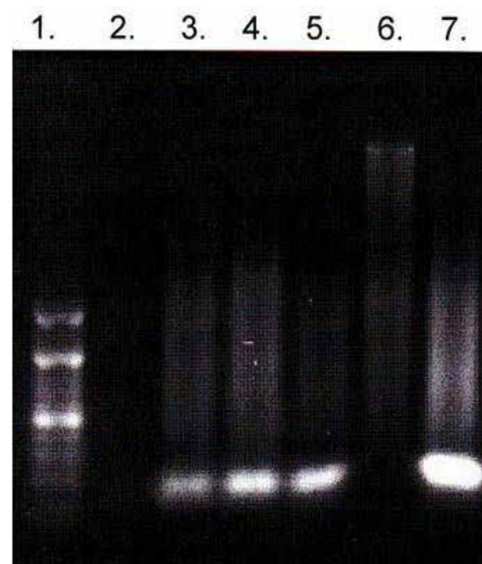

Fig. 3. siRNA pools received from dsRNAs
Legend: 1 - 100 bp ladder,2 - empty, 3- 3D, 4 - 3Da, 5 - 2A, 6 – PCR mix, 7 - 5'UTR

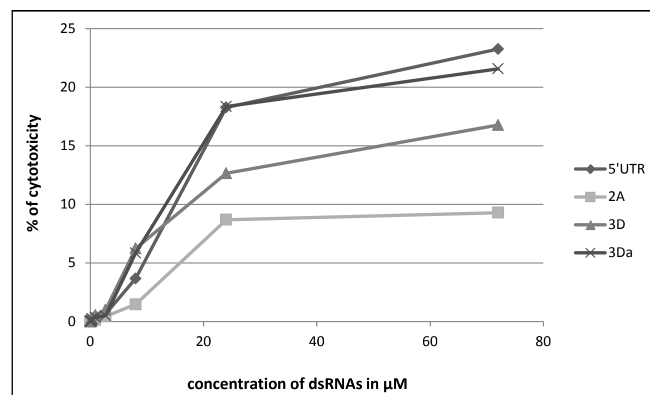

Fig.4. Cytotoxicity assay of sdRNAs for HEp-2 cells

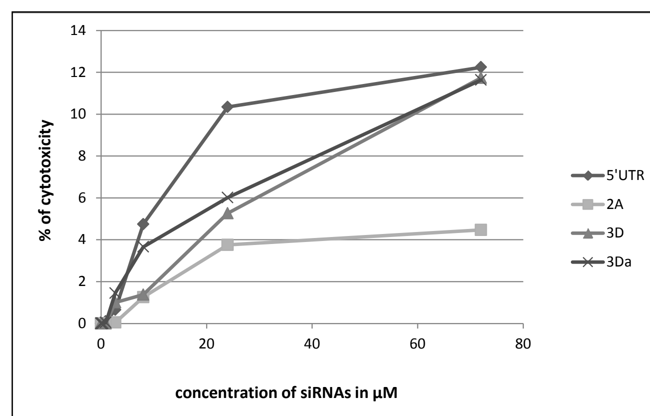

Fig. 5. Cytotoxicity assay of siRNA pools for HEp-2 cells

absolutely no cytotoxicity was observed for the used dsRNAs (Fig. 4) and siRNAs (Fig. 5). Weak cytotoxicity was observed in the range of 10 to 25 % when concentrations of dsRNAs of the target gene fragments above 25 μM were used (Fig. 4). Even the highest used concentration (72 μM) induced very litle cytotoxicity of 12 % (Fig. 5).

In our experiments aiming to determine the antiviral effect of both dsRNAs and siRNA pools we used only seven concentrations that gave absolutely no cytotoxic effect on cell monolayer: 0.3, 0.2, 0.15, 0.1, 0.05, 0.03, 0.01 μM (Fig. 6, 7).

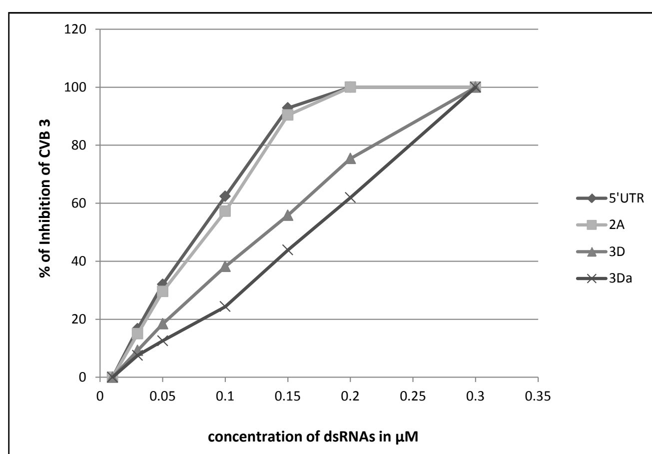

Fig. 6. Antiviral effect of dsRNAs against CVB3

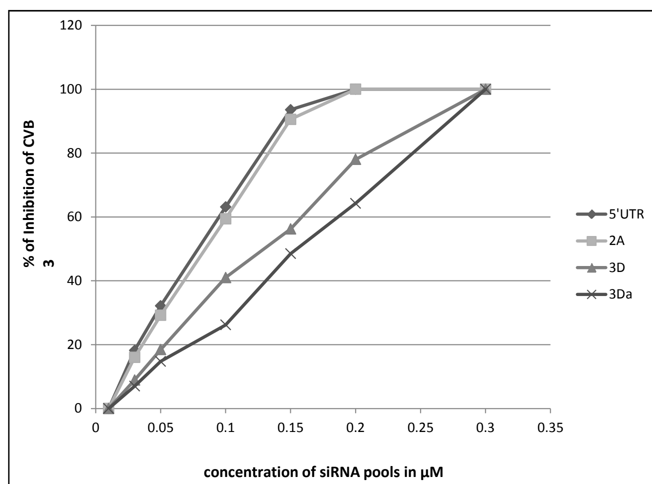

Fig. 7. Antiviral effect of siRNA pools against CVB3

Using 0.3 μM of all tested dsRNAs (Fig. 6) and siRNA pools (Fig. 7) completely inhibit CVB3 replication *in vitro* in HEp-2 cells. It was established a 100% inhibition of the virus content at MOI 0.04. 5′UTR and 2A siRNA pools at concentration of 0.2 μM also inhibited by 100 % the virus titer of CVB3 (Fig. 7). These are the most efficient fragments.

Various RNAi applications have been used to investigate gene functions, to target disease-related genes and as antiviral agents in cell culture and *in vivo*. The RNAi approach also has potential for antiviral therapy of virus infections. Treatment with siRdRP2 reduced the number of CBV3-infected cells. No off-target effects were detected that could be attributed to the w6–siRNA pools, probably due to the low concentration of each individual siRNA within the pool (Nygardas *et al.*, 2009).

Conclusion

We optimized a new technique for production of specific dsRNAs and siRNA pools targeted at 5'UTR, 2A and 3D genes of CVB3 genome. With these small RNA pools we efficiently inhibit CVB3 replication *in vitro* in HEp-2 cells. 0.3 μM of all tested sdRNAs and siRNA pools completely supress CVB3 replication *in vitro* in HEp-2 cells: a 100% inhibition of the virus titer at MOI 0.04. 5′UTR and 2A siRNA pools at concentration of 0.2 μM also inhibited by 100 % the virus titer of CVB3. Cytotoxic effects were not observed in the used concentrations. It seems that the use of pooled siRNAs is a favorable means to target viral infections and may offer a viable alternative for single-site siRNAs.

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