Inhibition of hepatitis C virus using siRNA targeted to the virus and Hsp90

Ana Claudia Silva Braga¹ · Bruno Moreira Carneiro^{1,2} · Mariana Nogueira Batista¹ ·
Mônica Mayumi Akinaga¹ · Paula Rahal¹

Received: 28 July 2016 / Revised: 24 October 2016 / Accepted: 8 November 2016 / Published online: 17 November 2016
© Cell Stress Society International 2016

Abstract Hepatitis C (HCV) is a viral disease affecting millions of people worldwide, and persistent HCV infection can lead to progressive liver disease with the development of liver cirrhosis and hepatocellular carcinoma. During treatment for hepatitis C, the occurrence of viral resistance is common. To reduce the occurrence of resistance, new viral treatments should target both viral and cellular factors. Many interactions occur between viral and host proteins during the HCV replication cycle and might be used for the development of new therapies against hepatitis C. Heat shock protein 90 (Hsp90) plays a role in the folding of cellular and viral proteins and also interacts with HCV proteins. In the present study, we knocked down the expression of the Hsp90 gene and inhibited viral replication using siRNA molecules. Reducing the expression of Hsp90 successfully decreased HCV replication. All siRNA molecules specific to the viral genome showed the efficient inhibition of viral replication, particularly siRNA targeted to the 5'UTR region. The combination of siRNAs targeting the viral genome and Hsp90 mRNA also successfully reduced HCV replication and reduced the occurrence of viral resistance. Moreover, these results suggest that an approach based on the combination of cellular and viral

siRNAs can be used as an effective alternative for hepatitis C viral suppression.

Keywords Hepatitis C · Hsp90 · RNA interference · siRNA · HCV · Heat shock protein

Introduction

Hepatitis C is caused by infection of the hepatitis C virus (HCV), and the precise number of individuals infected with HCV worldwide is unknown. However, it has been estimated that between 64 and 103 million people are chronically infected with HCV (Gower et al. 2014). Persistent HCV infection leads to progressive liver disease with the development of liver cirrhosis and hepatocellular carcinoma, potentially accounting for up to 0.5 million deaths annually (Lozano et al. 2012).

HCV is a member of the *Flaviviridae* family and is a relatively small enveloped virus with a positive-sense, single-stranded RNA genome (Giannini and Brechot 2003). The viral RNA encodes a polyprotein, which is cleaved by cellular and viral proteases to create structural (Core, E1, E2, and p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Bartenschlager et al. 2004; Giannini and Brechot 2003). The variability of viral RNA enables the classification of HCV into six genotypes (1 to 6) and several subtypes epidemiologically associated with risk factors and geographical areas (Simmonds 2013).

For many years, a combination of interferon and ribavirin has been used for the treatment of hepatitis C patients, but in addition to the side effects, this treatment displays low efficacy (Chou et al. 2013). Since 2011, direct acting antivirals (DAAs) have been made available for the treatment of chronically infected HCV individuals. These new drugs represented

Electronic supplementary material The online version of this article (doi:10.1007/s12192-016-0747-8) contains supplementary material, which is available to authorized users.

✉ Paula Rahal
prahal@ibilce.unesp.br

¹ Institute of Biosciences, Letters and Exact Sciences, UNESP, Rua Cristóvão Colombo, 2265, São José do Rio Preto, SP CEP: 15054-000, Brazil

² Institute of Exact and Natural Sciences, Mato Grosso Federal University, Rondonópolis, Brazil

a great breakthrough in HCV therapy, as patients treated with this drug can achieve sustained virologic response rates over 90 % for most viral genotypes (Sulkowski et al. 2014). However, in addition to the excessive costs of the DAA therapy, there are severe side effects, which are often the reason for the discontinuation of treatment before successful elimination of the virus (Imran et al. 2014). Moreover, pre-existing resistant HCV variants for these DAAs have been reported (Chen et al. 2016).

During viral replication, many cellular proteins are needed. Heat shock protein 90 (Hsp90/HSPC) plays a key role in folding and maintaining the conformational integrity of a wide range of cellular proteins (Kampinga et al. 2009; Taipale et al. 2010). In mammalian cells, there are two isoforms of Hsp90, including the stress-inducible isoform (Hsp90alpha/HSPC2) and the constitutively expressed isoform (Hsp90/HSPC3), which are encoded by different genes (Chen et al. 2005). It has been shown that many viruses use the host's chaperones in the viral replicative cycle, and these proteins might be involved in different stages of the viral cycle, including the entry, biogenesis, and assembly of viruses (Moriishi and Matsuura 2007; Tai et al. 2009). Other studies also have shown that Hsp90 forms a complex with NS5A HCV protein and FKBP8 (a folding and trafficking gene), which is essential for HCV replication (Okamoto et al. 2006). Notably, Nakagawa et al. (2007) demonstrated that the down-regulation of Hsp90 in Huh-7 cells expressing subgenomic replicon Con-1 (genotype 1) significantly reduced HCV replication and no cellular cytotoxicity or interference on cellular proliferation or apoptosis has been reported.

The post-transcriptional silencing mechanism of the RNAi pathway is a promising alternative for the inhibition of viral replication (Motavaf et al. 2012), and studies using siRNA against hepatitis C virus have shown promising results (Carneiro et al. 2015; Prabhu et al. 2005; Yokota et al. 2003). However, as a result of the high mutation rate of HCV RNA, siRNA molecules may become ineffective after longer treatments. One alternative to prevent the selection of RNAi resistant viral mutants would be the use of siRNA molecules directed to more conserved regions of the viral genome and the combination with siRNAs to host proteins involved in the viral replication cycle (Samreen et al. 2012). Based on this approach, Bian et al. (2012) showed that the use of siRNA molecules targeted to the hepatitis B viral genome combined with siRNA to Hsc70 (HSPA8), a member of the heat shock protein 70 gene family, successfully reduced viral replication.

Therefore, because heat shock protein Hsp90 plays an important role in HCV replication, this protein might be a good target for HCV drug development (Nakagawa et al. 2007). Moreover, combination therapies against both the virus and host genes that support viral replication are likely to represent a valid approach for the treatment of hepatitis C (Khaliq et al. 2010). Thus, in the present study, we examined Hsp90

inhibition in combination with siRNA directed to the HCV genome to reduce HCV replication.

Materials and methods

Cell culture and HCV replicon cells

The human hepatoma cell line Huh-7.5 was cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (Cultilab, Campinas, SP, Brazil), 1 % (v/v) non-essential amino acids (Gibco Life Technologies, USA), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen, Grand Island, NY, USA) at 37 °C in 5 % CO₂ atmosphere.

The replicons used in the present study were SGR-Feo-JFH-1, a bicistronic subgenomic replicon based on the JFH-1 sequence, which possesses the firefly luciferase gene fused to a neomycin resistance gene and non-structural HCV proteins (Wyles et al. 2009) and the JFH-1 replicon, a full HCV genome construct (Wakita et al. 2005).

After the linearization of plasmids using XbaI (NEB-New England Bio Labs, Ipswich, MA, USA) and treatment with mung bean nuclease (NEB-New England Bio Labs, Ipswich, MA, USA), the RNA was transcribed with the T7 RiboMAXTM Express Large-Scale RNA Production System (Promega, Madison, WI, USA). Huh-7.5 cells were electroporated with either SGR-Feo-JFH-1 or JFH-1 RNA. Cells expressing SGR-Feo-JFH-1 were selected for 21 days with 800 µg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA). The supernatant of the electroporated JFH-1 cells was collected at 17 days after electroporation and subsequently stored. For all experiments, the cells were plated at 24 h prior to the start of treatments at initial following cell densities: 1×10^5 cells/well for a 6-well plate and 5×10^3 cell/well for a 96-well plate.

RNAi molecules and transfection

For knocking down Hsp90 mRNA, a commercial siRNA was obtained (SC-35608, Santa Cruz Biotechnology, Dallas, TX, USA). This siRNA product consists of a pool of three to five target-specific 19–25 nt siRNAs designed to knockdown gene expression of HSP90alpha/HSPC2 and HSP90/HSPC3. For the inhibition of the viral genome, we generated four siRNA molecules using siDirect 2.0 software (Naito et al. 2009) for different regions of the HCV genome: siRNA 278 (5'UTR), siRNA 361 (5'UTR), siRNA 5010 (NS3), and siRNA 6652 (NS5A). All HCV siRNAs were synthesized at Sigma-Aldrich (St. Louis, MO, USA). As a control, we used a commercial negative control (siRNA#1-NC1) (Applied Biosystems, Foster City, CA, USA), with no specificity to any HCV sequence used or cellular mRNA in the present

study. Different siRNA concentrations (0.1, 1, 5, 15, or 25 nM) were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Cell viability

The cellular cytotoxicity of siRNA molecules was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA). Cytotoxicity was assessed in 96-well plates at 72 h after the cells were transfected with siRNAs. Shortly, after the appropriate incubation time, the supernatant was removed, and 100 μ L of 1 mg/mL of MTT reagent was dissolved in FBS-free DMEM culture medium and added to the cells. After 30 min at 37 °C, the MTT solution was removed, and 100 μ L of DMSO was added (Sigma-Aldrich, St. Louis, MO, USA). The absorption measurements were performed at 562 nm on a spectrophotometric plate reader (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE)

RNA isolation and qPCR

Total RNA from siRNA-transfected cells was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and 2 μ g of extracted RNA was used for cDNA synthesis with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The gene expression levels and HCV replication rate were determined by real-time PCR using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, CA, USA). The primers for Hsp90 α , Hsp90 β (Yao et al. 2010), and HCV were used, and GAPDH gene expression was analyzed as an endogenous control. The sequences are described in the supplementary material. The value of the relative expression was obtained after calculating $\Delta\Delta C_t$.

Luciferase assay

To assess the HCV replication level, we evaluated the luciferase activity of stably transfected Huh-7.5/SGR-Feo-JFH-1 cells. After siRNA treatment for 72 h, the cells were disrupted with 30 μ L of passive lysis buffer (Promega, Madison, WI, USA), and the luciferase substrate (Promega, Madison, WI, USA) was automatically added to each plate well. Luciferase activity was measured using a luminometer (FLUOstar Omega/BMG LABTECH, Offenburg, BW, DE), and a BCATM protein assay kit (Thermo-Scientific, Rockford, IL, USA) was used to normalize the protein concentrations.

Western blot analysis

The proteins were extracted from siRNA-treated cells using Cellytic MT Lysis reagent (Sigma-Aldrich, St. Louis, MO,

USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total protein extract was quantified using a BCATM protein assay kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. Proteins (6 μ g) were subjected to electrophoresis on 10 % denaturing polyacrylamide gels (SDS-PAGE) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After blocking with 5 % (w/v) non-fat milk diluted in TBS-T, the membranes were incubated with the following primary antibodies: anti-NS3 at a 1:3000 dilution (ab65407, Abcam, Cambridge, MA, USA) and anti-GAPDH at a 1:5000 dilution (ab8245, Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by further incubation with a secondary rabbit polyclonal antibody to mouse IgG (H&L; HRP) at a 1:2000 dilution (ab6728, Abcam, Cambridge, MA, USA). The membrane was finally incubated with the Pierce ECL Western Blotting Substrate (Thermo-Scientific-Pierce, Rockford, IL, USA), and luminescence was captured using a Chemi-Doc System (Bio-Rad, Philadelphia, PA, USA). The band intensity was analyzed using ImageLab 4.1 software.

Long-term siRNA treatment and resistance assay

First, we evaluated the effect of a single transfection using the selected siRNA for 17 days. To this end, Huh-7.5SGR-Feo-JFH-1 cells cultured in 2 % FBS DMEM were seeded (5×10^5) onto a 6-well plate (day 0) and after 24 h were transfected with siRNA (day 1). On day 3, selection began, using the antibiotic G418 (1 mg/mL) in the culture media, which were replaced every 2 days. Upon reaching confluence (day 6), the cells were sub-cultured at 1:4 and maintained under selection until the 17th day. The cells were fixed with 10 % formaldehyde and stained with 0.01 % crystal violet.

Subsequently, we evaluated the effect of repeated treatments on viral resistance. To this end, Huh-7.5/SGR-Feo-JFH-1 cells cultured in 2 % FBS DMEM were seeded (1×10^5) onto 6-well plates (day 0). The cells were transfected with siRNA on days 1, 6, and 9. From day 3, the cells were treated with G418 (1 mg/mL) and subsequently fixed and stained on day 17 as previously described.

Statistical analysis

Three independent experiments were performed in triplicate. The results of the inhibition of SGR-Feo-JFH-1, JFH-1, and MTT were calculated as a percentage of the mock (medium with Lipofectamine reagent only). All statistical analyses were performed using one-way ANOVA, followed by Tukey's post-test using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). A *P* value <0.05 was considered statistically significant.

Results

siRNA silencing

In the present study, four different siRNA molecules were designed to knockdown the HCV coding sequence. The RNAi were developed based on the JFH-1 sequence. The first molecule (siRNA 278) was directed to the 5' untranslated region (5'UTR) of the virus, corresponding to nucleotides 278–296. This siRNA is complementary to six genotypes (1–6) of hepatitis C virus. The second (siRNA 361), also located in the 5'UTR at positions 361–379, was complementary with genotypes 2a and 3. The siRNA 5010, targeting the region 5010–5028, was specific to genotypes 1 and 2a, in which the coding sequence of the NS3 protein is inserted. The siRNA 6652 is specific for the regions 6652–6670 in NS5A protein, and this molecule is exclusive to genotype 2a (Fig. 1). The siRNA sequences are described in the supplementary material 1.

To knockdown the genes encoding Hsp90 protein isoforms, Huh7.5 cells were transfected with a commercial pool of siRNAs designed to knockdown gene expression simultaneously. The efficiency of siRNA Hsp90 was evaluated after 72 h based on qPCR analysis of the mRNA expression levels of Hsp90 α and Hsp90 β genes. In the presence of siRNA Hsp90, the gene expression of Hsp90 α and Hsp90 β was reduced 3 Log₂ at a 15 nM concentration (Fig. 2).

SGR-Feo-JFH-1 expression and MTT assay

The siRNA molecules were subsequently examined for anti-HCV activity using a luciferase assay. All four siRNA molecules targeting the virus and Hsp90 were effective in reducing SGR-FeoJFH-1 replication (Fig. 3). To confirm this result, the expression of NS3 protein was evaluated by Western blotting using a monoclonal anti-NS3 antibody. All siRNAs, including the Hsp90 siRNA, reduced NS3 expression at least 50 % at a 1 nM concentration, and the levels of inhibition increased in a dose-dependent manner.

The siRNA 361 efficiently inhibited HCV replication, reducing the luciferase activity more than 95 % at a 1 nM

concentration. The siRNA 278 inhibited approximately 80 % at 1 nM and exhibited more than 90 % inhibition at higher concentrations. Transfection with siRNA 5010 inhibited HCV replication 30 % at 1 nM, and 70 % inhibition was observed at a 5 nM concentration; siRNA 6652 showed similar results.

To evaluate the cytotoxicity of the RNAi molecules, the cells were transfected with the four siRNAs to HCV, Hsp90 siRNA, and a negative control at the same concentrations for the luciferase assay. After 72 h, no significant cellular cytotoxicity was observed for any siRNA molecule.

JFH-1 expression

To determine whether the siRNA molecules are also efficient in the context of a full-length virus, Huh-7.5 cells were transfected with siRNAs under the same conditions used for the sub-genomic replicon assay. The cells were transfected with siRNA molecules, and after 24 h, the cells were infected with a JFH-1 containing supernatant at an MOI of 0.1. The analysis of the levels of intracellular viral RNA content obtained using qPCR showed better inhibition rates compared with the subgenomic virus. The siRNA 278 and siRNA 361 showed the most satisfactory results, inhibiting viral replication at the lowest concentration tested (20 and 23 %, respectively) (Fig. 4). The results of Western blotting were consistent with HCV expression in the presence of siRNA molecules.

Combination of siRNA molecules

To verify whether the siRNAs are also efficient in combination, we transfected either SGR-Feo-JFH-1 stable or JFH-1-infected cells with Hsp90 siRNA and one siRNA specific to viral RNA. Based on the higher efficiency observed in the previous assays, siRNA 278 and siRNA 361 were used at 1 nM in these experiments. The results of luciferase and Western blot assay using SGR-Feo-JFH-1 cells demonstrated that the combined transfection of siRNAs 361 and Hsp90 was efficient (7.6 % of RLU) compared with the single transfection with siRNA Hsp90 only (48 %). The results with JFH-1 infected cells showed that siRNA 361 in combination with

Fig. 1 Target sites for the siRNA molecules on replicon subgenomic SGR-Feo-JFH-1 and full JFH-1 system

Fig. 2 Hsp90 α and Hsp90 β relative gene expression based on qPCR analysis after treating Huh-7.5 cells with siRNA Hsp90. The samples were normalized with mock-transfected cells (Lipofectamine reagent only), and the results are represented in Log₂

siRNA Hsp90 (7.8 % of HCV expression) was more efficient than the use of molecules singly (9 % to siRNA 361 and 54 % to siRNA Hsp90). Furthermore, we also observed a reduction in viral replication in the combination of molecules siRNA 278 with siRNA HSP90 (11 %) (Fig. 5).

Long-term siRNA treatment

We further evaluated the selection of resistant cells treated with siRNA molecules after treatment with G418, which facilitates the selection of cells containing the replicon HCV

Fig. 3 Luciferase and MTT assays. HCV expression levels in Huh7.5 cells stably harboring SGR-FEO-JFH-1 and treated with siRNAs targeting the viral genome, siRNA Hsp90, and NC1. The values are expressed in luciferase activity (% RLU-relative light units) normalized to mock-transfected cells (represented as circles). The cell viability was

determined using an MTT assay, and the values obtained are expressed as a percentage of control mock cells (represented as squares). *** P 0.0001 vs Mock. The results of the Western blot assays, showing band intensities at the same siRNA concentrations, are shown under the graphics

(SGR-FEO-JFH-1). The continuous use of drugs for hepatitis C treatment might favor the selection of resistant clones, and to assess the effect of siRNAs, we performed repeated treatments. The results of long-term tests using three consecutive transfections showed no differences between siRNA 278 alone (21 % of viral inhibition) and the combination of siRNA 278/ Hsp90 (24 %). However, the use of siRNA 361 combined with siRNA Hsp90 (74 %) suppressed the occurrence of resistant colonies compared with isolated siRNA 361 (59 %) (Fig. 6). Finally, the combination of two viral siRNA reduced virus replication less efficiently, when compared to siRNA Hsp90/361 mix (supplementary material 2).

Discussion

Hepatitis C is a viral disease affecting millions of people worldwide, and it is the major disease leading to liver

transplantation. This virus develops resistant mutations, thereby inducing drug resistance to antivirals targeting viral proteins; thus, these adaptive mutations might be selected in the presence of compounds directed against viral replication. These adaptive mutations might also occur in treatments with RNA interference, but studies have shown that the combined use of RNAi molecules was efficient in preventing viral escape (Konishi et al. 2006).

Another strategy to avoid viral resistance is to focus on therapies targeting the host. Many studies have demonstrated interactions between viral and host proteins during the HCV replication cycle, and these interactions can be used for the development of new therapies for the treatment of hepatitis C (Imran et al. 2014). In the present study, we analyzed the interference of HCV replication by RNAi molecules (siRNA) targeted to viral regions and a host gene from the chaperone family (Hsp90), which is important in viral replication.

Fig. 4 Relative levels of intracellular HCV RNA expression in Huh-7.5 cells infected with JFH-1 and treated with siRNA targeting the viral genome, Hsp90, and NC1 siRNAs. The values are expressed as a percentage of HCV intracellular RNA relative to the viral RNA of mock-

transfected cells. The samples were normalized to the endogenous GAPDH gene. *** $P < 0.0001$ vs Mock. The results of the Western blot assay showing the band intensities at the same siRNA concentrations are shown under the graphic

Fig. 5 The treatment of the siRNA combination in Huh-7.5 cells stably expressing SGR-Feo-JFH-1 and Huh-7.5 cells infected with JFH-1. The values are expressed in luciferase activity (% RLU-relative light units) or JFH-1 replication (% of control), normalized with Mock cells. The results of the Western blot assay for the same experiment are shown under the graphic

The cell viability assay showed that siRNA molecules targeting the viral genome demonstrated no cytotoxic effect until 25 nM, showing that gene silencing and inhibition by siRNA are a safe mechanism. Ansar et al. (2011) and Xing et al. (2012) reported similar results, showing the use of siRNA concentrations of up to 100 nM.

The reduced HCV expression observed in the RNAi negative control NC1 at concentrations higher than 5 nM might reflect an off-target effect, as this molecule has no specificity to the JFH-1 sequence or any other human mRNA. Although the cell viability assay showed no cytotoxicity at this concentration, this result might reflect the fact that siRNA can silence other non-specific genes. This off-target effect can be diminished using siRNA concentrations as low as 1 nM (Caffrey et al. 2011), and we used this concentration to assess the efficiency of the siRNA molecules used in the present study.

The inhibition of Hsp90 α and Hsp90 β genes by siRNA was confirmed using qPCR. Geldanamycin (GA) and its derivatives also exhibit Hsp90 inhibition activity (Li et al. 2014; Stebbins et al. 1997), but we selected siRNA molecules because of the silencing specificity of these molecules and to avoid the side effects typically resulting from treatment with the drug. Generally, Hsp90 is highly expressed in carcinoma cells and plays an important role in malignancy for the maintenance of the stability and activation states of oncogenic client proteins (Haupt et al. 2012; Workman et al. 2007).

However, it should be noted that Hsp90 participates in many essential cellular processes and its inhibition could cause serious side effects (Prodromou 2016; Taipale et al. 2010). An alternative to reducing these effects would be the use of nanocarriers that can be targeted to specific target cells. Some studies have shown the use of nanoparticles in the delivery of RNAi for HCV (Duan et al. 2016; Torrecilla et al. 2016).

The knockdown Hsp90 reduced viral replication (SGR-Feo-JFH-1 and JFH-1), and other studies have also demonstrated this finding using siRNA for Hsp90 α and Hsp90 β or chemical compounds, such as GA (Nakagawa et al. 2007; Ujino et al. 2009). The interference of viral replication might reflect Hsp90 physically interacting with the NS2 and NS3 viral proteins, preventing the degradation of NS3 through the proteosomal pathway (Ujino et al. 2009; Waxman et al. 2001). Furthermore, HSP90 interacts with several other oncogenic viruses, such as hepatitis B virus (HBV), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpes virus (KSHV), demonstrating the importance of this protein in viral processes (Beck and Nassal 2003; Sun et al. 2010; Wen and Damania 2010).

Many studies have demonstrated that HCV can be efficiently inhibited by RNAi, and in the present study, the use of siRNA molecules directed against the viral genome effectively suppressed HCV replication, particularly siRNA 361 targeting the 5'UTR. The target site of this siRNA is

Fig. 6 Long-term siRNA treatment in Huh7.5/SGR-Feo-JFH-1 cells cultured in DMEM containing 2 % FBS. The cells were treated three times with siRNA molecules, followed by selection with G418 and staining after 17 days. The densitometry of the wells was performed using ImageJ software

conserved between genotypes 2a and 3 of HCV, which exhibit worldwide distribution (Gower et al. 2014). Based on the level of sequence conservation among different HCV genotypes, the IRES of the 5'UTR represents a promising RNAi target site (Brown et al. 2005). The relatively conserved nature of these regions suggests their functional importance in the viral life cycle, and point mutations in that region are less likely to occur (Radhakrishnan et al. 2004).

We observed the reduced activity of siRNA 5010 and siRNA 6652, likely reflecting the physical accessibility of certain mRNA structures as important determinants in the gene silencing of siRNAs (Gredell et al. 2008). The secondary structures of the target sites in mRNAs strongly reduce siRNA-mediated RNAi activity (Overhoff et al. 2005). This hypothesis may also explain why the combination of Hsp90 and 361 siRNAs had a better result in long-term assay when compared to Hsp90 and 278 siRNAs mix. Although all HCV RNA structures are incompletely characterized, many regions of the genome are highly structured, likely reflecting the differences observed in the efficiency of siRNA molecules (Li et al. 2015).

Still on the efficiency difference observed in long-term treatment with siRNAs Hsp90 and 361 or 278, although the viral molecules are directed to the 5'UTR region of the virus, a highly conserved part of the genome, this site is also subject to mutation (Araujo et al. 2008; Bukowska-Osko et al. 2015), and therefore, an siRNA targeted to specific mutated region could have its efficiency decreased. Consequently, a mutation in the target region of the molecule 278 may have impaired this siRNA in the long-term test.

Many antiviral drugs for HCV eventually become ineffective after a period of treatment by promoting the selection of resistant mutations. This effect has also been observed for siRNAs, and studies have demonstrated the occurrence of resistant mutants with the isolated use of siRNA; however, the use of two molecules together were effective in eliminating the development of resistance (Chandra et al. 2012). Because the mutation rates are lower in the host than in viruses, many studies have focused on cellular proteins to inhibit viral replication.

Thus, we combined siRNA molecules directed against the virus and siRNA to Hsp90. Although the qPCR results showed the individual high efficiencies of siRNA 278 and 361 when used alone, in combination with HSP90, the repeated transfection in long-term assays showed a reduced number of resistant cells. The siRNA 278 reduced viral replication, as shown by qPCR, but the continued use of this molecule was not efficient in suppressing the occurrence of resistance. The siRNA 361 also efficiently reduced the occurrence of resistant colonies. In combination with Hsp90 siRNA, this molecule also achieved the best results in viral suppression (qPCR and prolonged test). These results are consistent with the data from previous studies suggesting that the combination therapy of

cellular and viral targets is more effective in suppressing the occurrence of viral resistance. Bian et al. (2012) demonstrated the same approach for hepatitis B virus (HBV), showing that the use of siRNA molecules targeted to this virus in combination with siRNA directed against the heat shock protein Hsc70 was more effective than individualized treatment.

Conclusion

In the present study, we demonstrated that siRNA 361, a molecule targeted to the 5'UTR region, showed excellent levels of viral suppression in SGR-Feo-JFH-1 and JFH-1 models, and in combination with Hsp90 siRNA, this molecule reduced the amount of resistant clones, representing a powerful reduction of HCV replication. However, additional studies are needed to achieve a safe and effective therapy for hepatitis C.

Acknowledgments This study was financially supported by CAPES (Coordination for the Improvement of Higher Education Personnel) and FAPESP (São Paulo Research Foundation) grant number 2011/15786-4.

References

- Ansar M et al (2011) Inhibition of full length hepatitis C virus particles of 1a genotype through small interference RNA. *Virology* 8:203. doi:10.1186/1743-422X-8-203
- Araujo FM, Sonoda IV, Rodrigues NB, Teixeira R, Redondo RA, Oliveira GC (2008) Genetic variability in the 5' UTR and NS5A regions of hepatitis C virus RNA isolated from non-responding and responding patients with chronic HCV genotype 1 infection. *Memorias do Instituto Oswaldo Cruz* 103:611–614
- Bartenschlager R, Frese M, Pietschmann T (2004) Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* 63:71–180. doi:10.1016/S0065-3527(04)63002-8
- Beck J, Nassal M (2003) Efficient Hsp90-independent in vitro activation by Hsc70 and Hsp40 of duck hepatitis B virus reverse transcriptase, an assumed Hsp90 client protein. *J Biol Chem* 278:36128–36138. doi:10.1074/jbc.M301069200
- Bian Z et al (2012) Anti-HBV efficacy of combined siRNAs targeting viral gene and heat shock cognate 70. *Virology* 9:275. doi:10.1186/1743-422X-9-275
- Brown KM, Chu CY, Rana TM (2005) Target accessibility dictates the potency of human RISC. *Nat Struct Mol Biol* 12:469–470. doi:10.1038/nsmb931
- Bukowska-Osko I et al (2015) Genetic variability of hepatitis C virus (HCV) 5' untranslated region in HIV/HCV Coinfected patients treated with Pegylated interferon and ribavirin. *PLoS One* 10:e0125604. doi:10.1371/journal.pone.0125604
- Caffrey DR et al (2011) siRNA off-target effects can be reduced at concentrations that match their individual potency. *PLoS One* 6:e21503. doi:10.1371/journal.pone.0021503
- Carneiro B, Braga AC, Batista MN, Harris M, Rahal P (2015) Evaluation of canonical siRNA and dicer substrate RNA for inhibition of hepatitis C virus genome replication—a comparative study. *PLoS One* 10:e0117742. doi:10.1371/journal.pone.0117742
- Chandra PK, Kundu AK, Hazari S, Chandra S, Bao L, Ooms T, Morris GF, Wu T, Mandal TK, Dash S (2012) Inhibition of hepatitis c virus

- replication by intracellular delivery of multiple siRNAs by nanosomes. *Mol Ther* 20(9):1724–1736
- Chen B, Piel WH, Gui L, Bruford E, Monteiro A (2005) The HSP90 family of genes in the human genome: insights into their divergence and evolution. *Genomics* 86:627–637. doi:10.1016/j.ygeno.2005.08.012
- Chen ZW, Li H, Ren H, Hu P (2016) Global prevalence of pre-existing HCV variants resistant to direct-acting antiviral agents (DAAs): mining the GenBank HCV genome data. *Scientific reports* 6:20310. doi:10.1038/srep20310
- Chou R, Hartung D, Rahman B, Wasson N, Cottrell EB, Fu R (2013) Comparative effectiveness of antiviral treatment for hepatitis C virus infection in adults: a systematic review. *Ann Intern Med* 158:114–123
- Duan L, Yan Y, Liu J, Wang B, Li P, Hu Q, Chen W (2016) Target delivery of small interfering RNAs with vitamin E-coupled nanoparticles for treating hepatitis C. *Scientific reports* 6:24867. doi:10.1038/srep24867
- Giannini C, Brechot C (2003) Hepatitis C virus biology. *Cell Death Differ* 10(Suppl 1):S27–S38. doi:10.1038/sj.cdd.4401121
- Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H (2014) Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol* 61:S45–S57. doi:10.1016/j.jhep.2014.07.027
- Gredell JA, Berger AK, Walton SP (2008) Impact of target mRNA structure on siRNA silencing efficiency: a large-scale study. *Biotechnol Bioeng* 100:744–755. doi:10.1002/bit.21798
- Haupt A et al (2012) Hsp90 inhibition differentially destabilises MAP kinase and TGF-beta signalling components in cancer cells revealed by kinase-targeted chemoproteomics. *BMC Cancer* 12:38. doi:10.1186/1471-2407-12-38
- Imran M et al (2014) Current and future therapies for hepatitis C virus infection: from viral proteins to host targets. *Arch Virol* 159:831–846. doi:10.1007/s00705-013-1803-7
- Kampinga HH et al (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 14:105–111. doi:10.1007/s12192-008-0068-7
- Khaliq S et al (2010) RNAi as a new therapeutic strategy against HCV. *Biotechnol Adv* 28:27–34. doi:10.1016/j.biotechadv.2009.08.004
- Konishi M, Wu CH, Kaito M, Hayashi K, Watanabe S, Adachi Y, Wu GY (2006) siRNA-resistance in treated HCV replicon cells is correlated with the development of specific HCV mutations. *J Viral Hepat* 13:756–761. doi:10.1111/j.1365-2893.2006.00752.x
- Li Z et al (2014) Design, synthesis and biological evaluation of 17-arylmethylamine-17-demethoxygeldanamycin derivatives as potent Hsp90 inhibitors. *Eur J Med Chem* 85:359–370. doi:10.1016/j.ejmech.2014.07.101
- Li Y, Yamane D, Masaki T, Lemon SM (2015) The yin and yang of hepatitis C: synthesis and decay of hepatitis C virus RNA nature reviews. *Microbiology* 13:544–558. doi:10.1038/nrmicro3506
- Lozano R et al (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet* 380:2095–2128. doi:10.1016/S0140-6736(12)61728-0
- Moriishi K, Matsuura Y (2007) Host factors involved in the replication of hepatitis C virus. *Rev Med Virol* 17:343–354. doi:10.1002/rmv.542
- Motavaf M, Safari S, Alavian SM (2012) Therapeutic potential of RNA interference: a new molecular approach to antiviral treatment for hepatitis C. *J Viral Hepat* 19:757–765. doi:10.1111/jvh.12006
- Naito Y, Yoshimura J, Morishita S, Ui-Tei K (2009) siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. *BMC bioinformatics* 10:392. doi:10.1186/1471-2105-10-392
- Nakagawa S, Umehara T, Matsuda C, Kuge S, Sudoh M, Kohara M (2007) Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. *Biochem Biophys Res Commun* 353:882–888. doi:10.1016/j.bbrc.2006.12.117
- Okamoto T et al (2006) Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J* 25:5015–5025. doi:10.1038/sj.emboj.7601367
- Overhoff M, Alken M, Far RK, Lemaître M, Lebleu B, Sczakiel G, Robbins I (2005) Local RNA target structure influences siRNA efficacy: a systematic global analysis. *J Mol Biol* 348:871–881. doi:10.1016/j.jmb.2005.03.012
- Prabhu R, Vittal P, Yin Q, Flemington E, Garry R, Robichaux WH, Dash S (2005) Small interfering RNA effectively inhibits protein expression and negative strand RNA synthesis from a full-length hepatitis C virus clone. *J Med Virol* 76:511–519. doi:10.1002/jmv.20391
- Prodromou C (2016) Mechanisms of Hsp90 regulation. *The Biochemical journal* 473:2439–2452. doi:10.1042/BCJ20160005
- Radhakrishnan SK, Layden TJ, Garte AL (2004) RNA interference as a new strategy against viral hepatitis. *Virology* 323:173–181. doi:10.1016/j.virol.2004.02.021
- Samreen B et al (2012) Hepatitis C virus entry: role of host and viral factors. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 12:1699–1709. doi:10.1016/j.meegid.2012.07.010
- Simmonds P (2013) The origin of hepatitis C virus. *Curr Top Microbiol Immunol* 369:1–15. doi:10.1007/978-3-642-27340-7_1
- Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP (1997) Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell* 89:239–250
- Sulkowski MS, Jacobson IM, Nelson DR (2014) Daclatasvir plus sofosbuvir for HCV infection. *N Engl J Med* 370:1560–1561. doi:10.1056/NEJMc1401726
- Sun X et al (2010) Hsp90 inhibitors block outgrowth of EBV-infected malignant cells in vitro and in vivo through an EBNA1-dependent mechanism. *Proc Natl Acad Sci U S A* 107:3146–3151. doi:10.1073/pnas.0910717107
- Tai AW, Benita Y, Peng LF, Kim SS, Sakamoto N, Xavier RJ, Chung RT (2009) A functional genomic screen identifies cellular cofactors of hepatitis C virus replication. *Cell Host Microbe* 5:298–307. doi:10.1016/j.chom.2009.02.001
- Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nat Rev Mol Cell Biol* 11:515–528. doi:10.1038/nrm2918
- Torreccilla J et al (2016) Silencing of hepatitis C virus replication by a non-viral vector based on solid lipid nanoparticles containing a shRNA targeted to the internal ribosome entry site (IRES) colloids and surfaces B. *Biointerfaces* 146:808–817. doi:10.1016/j.colsurfb.2016.07.026
- Ujino S, Yamaguchi S, Shimotohno K, Takaku H (2009) Heat-shock protein 90 is essential for stabilization of the hepatitis C virus non-structural protein NS3. *J Biol Chem* 284:6841–6846. doi:10.1074/jbc.M806452200
- Wakita T et al (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11:791–796. doi:10.1038/nm1268
- Waxman L, Whitney M, Pollok BA, Kuo LC, Darke PL (2001) Host cell factor requirement for hepatitis C virus enzyme maturation. *Proc Natl Acad Sci U S A* 98:13931–13935. doi:10.1073/pnas.241510898
- Wen KW, Damania B (2010) Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. *Oncogene* 29:3532–3544. doi:10.1038/onc.2010.124
- Workman P, Burrows F, Neckers L, Rosen N (2007) Drugging the cancer chaperone HSP90: combinatorial therapeutic exploitation of oncogene addiction and tumor stress. *Ann N Y Acad Sci* 1113:202–216. doi:10.1196/annals.1391.012
- Wyles DL, Kaihara KA, Korba BE, Schooley RT, Beadle JR, Hostetler KY (2009) The octadecyloxyethyl Ester of (S)-9-[3-hydroxy-2-(phosphonomethoxy) propyl]adenine is a potent and selective inhibitor of hepatitis C virus replication in genotype 1A, 1B, and 2A

- replicons. *Antimicrob Agents Chemother* 53:2660–2662. doi:[10.1128/AAC.01546-08](https://doi.org/10.1128/AAC.01546-08)
- Xing XK, Li SJ, He JL, Chen Z (2012) Inhibition of hepatitis C virus replication by single and dual small interfering RNA using an HCV-infected cell model. *Biotechnol Lett* 34:295–301. doi:[10.1007/s10529-011-0761-y](https://doi.org/10.1007/s10529-011-0761-y)
- Yao JQ et al (2010) Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin inhibits the proliferation of ARPE-19 cells. *J Biomed Sci* 17:30. doi:[10.1186/1423-0127-17-30](https://doi.org/10.1186/1423-0127-17-30)
- Yokota T et al (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 4:602–608. doi:[10.1038/sj.embor.embor840](https://doi.org/10.1038/sj.embor.embor840)