Sequence-specific cleavage of hepatitis C virus RNA by DNAzymes: inhibition of viral RNA translation and replication

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DNAzyme (Dz) molecules have been shown to be highly efficient inhibitors of virus replication. Hepatitis C virus RNA translation is mediated by an internal ribosome entry site (IRES) element located mostly in the 5’ untranslated region (UTR), the mechanism of which is fundamentally different from cap-dependent translation of cellular mRNAs, and thus an attractive target for designing antiviral drugs. Inhibition of HCV IRES-mediated translation has drastic consequences for the replication of viral RNA as well. We have designed several Dzs, targeting different regions of HCV IRES specific for 1b and also sequences conserved across genotypes. The RNA cleavage and translation inhibitory activities of these molecules were tested in a cell-free system and in cell culture using transient transfections. The majority of Dzs efficiently inhibited HCV IRES-mediated translation. However, these Dz molecules did not show significant inhibition of coxsackievirus B3 IRES-mediated translation or cap-dependent translation of reporter gene, showing high level of specificity towards target RNA. Also, Northern blot hybridization analysis showed significant cleavage of HCV IRES by the Dz molecules in Huh7 cells transiently transfected with the HCV–FLuc monocistronic construct. Interestingly, one of the Dzs was more effective against genotype 1b, whereas the other showed significant inhibition of viral RNA replication in Huh7 cells harbouring a HCV 2a monocistronic replicon. As expected, mutant-Dz failed to cleave RNA and inhibit HCV RNA translation, showing the specificity of inhibition. Taken together, these findings suggest that the Dz molecule can be used as selective and effective inhibitor of HCV RNA replication, which can be explored further for development of a potent therapeutic agent against HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus, belonging to the family Flaviviridae. The viral RNA genome is approximately 9600 nt and encodes a single polyprotein of about 3000 amino acids. The long open reading frame is flanked by 5’ and 3’ untranslated regions (UTRs) that are highly conserved among the majority of HCV genotypes and contain elements that are essential for genome replication (Bartenschlager et al., 2004). The translation initiation of HCV RNA is mediated by the binding of the 40S ribosomal subunit at the internal ribosome entry site (IRES) located mostly in the 5’UTR region. It has been shown that the HCV IRES can directly bind to the 40S ribosomal subunit, even in the absence of any initiation factors, in a manner similar to prokaryotic translation initiation. Subsequently, several canonical and non-canonical trans-acting factors facilitate the formation of a functional initiation complex during internal initiation of translation (Hellen & Sarnow, 2001). Since this mechanism is fundamentally different from the ribosome assembly at the 5’ cap-binding complex in cap-dependent translation of host cell mRNA, it serves as an attractive target for antiviral agents (Dasgupta et al., 2004).

HCV causes a multitude of liver diseases in humans, including liver cirrhosis, and often leads to hepatocellular carcinoma if left untreated. Current treatment options involving interferon-α (INF-α) alone or in combination with ribavirin are not very effective. The majority of the patients do not respond well to this therapy because of the short half-life of interferon or degradation of the molecules. Failure to achieve a sustained virological response in majority of the patients has also been shown to be partly due to the varying genotypes of the infecting strain of the virus. HCV has six major genotypes with several subtypes. HCV genotype 1 has been shown to be more resistant to interferon therapy than genotype 3. Genotype 3 was found to be the most prevalent in India, followed by genotype 1 (Gupta et al., 2006). Thus
developing effective antiviral therapeutics using novel approaches is the need of the hour.

Several strategies are being explored to develop antiviral agents against HCV, targeting different viral processes. Recent availability of HCV subgenomic (Lohmann et al., 1999) and full-length (Blight et al., 2002) replicon systems has helped immensely to assay the inhibitory effect of antiviral candidates on HCV genome replication. Earlier, several studies have shown effective inhibition of viral RNA translation when viral enzymes were targeted. Since the translation of genomic RNA is the initial obligatory step, interference with this process will have direct consequence on the viral RNA replication. HCV RNA is translated by recruitment of the ribosome at the IRES element which comprises most of the 5’UTR sequences (except the first 40 nt) and extends to a short stretch of 30–40 nt downstream of the initiator AUG. Since the IRES-mediated translation is distinct from the cap-dependent translation of host cell mRNA, this could be exploited by different approaches to achieve selective inhibition of HCV gene expression.

Currently, nucleic-acid-based antiviral approaches, which include ribozyme (Rz), DNAzyme (Dz), short hairpin RNA (shRNA) and small interfering RNA (siRNA), are being used for inhibiting the gene expression of several target RNAs (Jarczak et al., 2005; Goila & Banerjea, 2004). Among these, catalytic Dzs with 10–23 catalytic motifs are increasingly being exploited over Rzs because they either match or exceed the catalytic efficiencies of the known Rzs. Deoxyribozymes or DNAzymes or DNA-enzymes (Dzs), as originally described (Santoro & Joyce, 1997), are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner (Silverman, 2005; Dash & Banerjea, 2004; Joyce, 2004). Dzs are synthetic single-stranded DNA molecules which have three domains: a catalytic domain consisting of 15 nt flanked by two substrate-recognition domains which bind the target RNA through Watson–Crick base pairing. In Dzs a single nucleotide change in the 10–23 catalytic motif completely abrogates the sequence-specific cleavage activity; for example, G14C completely abolishes the catalytic cleavage (Goila & Banerjea, 2001). In some instances efficient inhibition of gene expression was achieved with 10–23 Dz (Ackermann et al., 2005). Based on sequence recognition of the binding arms, Dzs can be synthesized to cleave a target gene in a sequence-specific manner similar to that of Rzs (Asahina et al., 1998; Goila & Banerjea, 1998).

Compared with synthetic Rzs, Dzs are easier to prepare, less sensitive to chemical and enzymic degradation and, more importantly, easier to deliver into cells (Santoro & Joyce, 1997). Over the years, several kinds of Dzs with unique catalytic motifs have been described, but Dzs possessing the 10–23 catalytic motif have been exploited more extensively by several investigators (Banerjea et al., 2004). Various studies suggest that all the target sites are not available for cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal Watson–Crick base pairing with Rzs or Dzs. More than one site is usually selected in the target RNA to get maximum cleavage by catalytic nucleic acids. 10–23 DNA-enzyme cleaves the RNA sequence at a phosphodiester bond between an unpaired purine and a paired pyrimidine residue (5’-AU-3’ most efficiently cleaved). This results in the formation of 5’ and 3’ products, which contain a 2’-3’ cyclic phosphate and 5’ hydroxyl terminus, respectively (Santoro & Joyce, 1998).

**METHODS**

*Deoxyribozyme synthesis.* All the oligodeoxynucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys. The conserved 15 nt (5’-GGCTAGCTACACGGA-3’) 10–23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. Mutant-Dz was also assembled using a 10–23 catalytic motif that possessed a single nucleotide substitution (G to C) in the 10–23 catalytic motif. This change is known to render the Dz catalytically inactive (Goila & Banerjea, 2001).

We have initially designed five DNA-enzymes, namely Dz88, Dz219, Dz305, Dz327 and Dz336, targeting different regions of HCV 5’UTR IRES (Table 1). Additionally, mutant-Dz219, possessing a point mutation as stated above, was designed, which is termed the ‘mutant Dz’. These molecules were specific only for HCV genotype 1b. Later we designed another four 10–23 catalytic motif-containing Dzs, namely Dz161, Dz165, Dz285 and Dz288, that were targeted to cleave 5’UTR regions of all the currently known HCV genotypes (Table 2). The location of cleavage for each Dz is shown by arrows in the predicted 5’UTR IRES (Fig. 1).

**Cell culture and plasmid.** Human hepatocellular carcinoma cells (Huh-7 and Huh-7.5 cells) monolayers (Blight et al., 2002) were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37˚C in 5% CO₂ atmosphere. For cells supporting the HCV full-length replicon (genotype 1b), 0.8 mg G418 sulfate ml⁻¹ (Sigma-Aldrich) was added to the culture medium and for cells bearing the HCV monocistronic replicon (genotype 2a), 25 μg hygromycin B ml⁻¹ was added to the culture medium. Replicon 1b carries the 1b genotype HCV 5’UTR followed by a neoycin resistance gene (neo), EMCV IRES and NS2–NS5 and the 3’UTR sequence. The replicon 2a carries the 2a genotype HCV 5’UTR followed by a hygromycin resistance gene (hyg), a ubiquitin gene (ubi) and NS3–NS5 and the 3’UTR. The HCV–FLuc monocistronic plasmid construct pCD (HCV–IRES–FLuc) construct carrying HCV IRES (nt 18–383) was

<table>
<thead>
<tr>
<th>DNAzyme</th>
<th>Sequence 5’–3’</th>
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<tbody>
<tr>
<td>Dz88</td>
<td>AACGCCAGGCTAGCTACAACGGGCTAGAC</td>
</tr>
<tr>
<td>Dz305</td>
<td>GCAAGCGAGGCTAGCTACAACGGCAGTATCAG</td>
</tr>
<tr>
<td>Dz219</td>
<td>CACGGCAGGCTAGCTACAACGATGACCGG</td>
</tr>
<tr>
<td>Dz327</td>
<td>CTACGAGGCTAGCTACAACGACCTCCGG</td>
</tr>
<tr>
<td>Dz336</td>
<td>TGCAGCGGCTAGCTACAACCGACTACGAG</td>
</tr>
<tr>
<td>mutDz219</td>
<td>CCAGGCAGGCTAGCTACAACGATGACCGG</td>
</tr>
</tbody>
</table>

The mutated residue in the mutant mutDz2-219 is highlighted in bold.
upstream of the firefly luciferase reporter gene. The pCDCVB3-IRES-FLuc construct contains the CVB3 5' and the coxsackievirus B3 (CVB3)–FLuc monocistronic plasmid (Pudi et al., 2003). The pCDFLuc construct contains the luciferase reporter gene using the T7 RNA polymerase (Promega) in the presence of [α-32P]UTP, following the manufacturer’s protocol. The extra nucleotides in the labelled transcript (387 nt) came from the region between the T7 promoter and upstream of the HCV sequence (cloned in polylinker). Equimolar amounts of unlabelled Dz and labelled substrate RNA (100 pmol each) were allowed to interact in a final volume of 10 μl in a buffer containing 50 mM Tris.HCl, pH 7.5 and 10 mM MgCl2 (standard conditions) as described earlier (Santoro & Joyce, 1997) for 2 h at 37 °C. The cleaved products were resolved by electrophoresis and cleavage efficiency was determined as described earlier (Goila & Banerjea, 2001).

Transfections and reporter assay. Monolayers (60–70% confluent) of Huh7 cells in 35 mm dishes were co-transfected with HCV monocistronic plasmid pCDHCV–FLuc or pCDCVB3–FLuc or pCDFLuc. Dzs and pSV-β-gal plasmid were used for normalizing transfection efficiency using Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection the cells were harvested using passive lysis buffer (Promega) and FLuc activity was analysed using a luciferase assay system (Promega) in a TD 20/20 luminometer (Turner Designs).

Semiquantitative RT-PCR. HCV full-length and subgenomic replicon-bearing cells were transfected with 0.4 and 0.8 μM Dzs and, 24 h post-transfection, total cellular RNAs were extracted using TRI-reagent (Sigma-Aldrich). Semiquantitative RT-PCR was performed for the HCV-IRES positive strand and actin as described earlier (Dhar et al., 2007). In brief, 5 μg total RNA was reverse transcribed with the HCV 5’UTR and actin primers by annealing at 65 °C and extending at 42 °C for 50 min. After cDNA was synthesized, PCR reaction was performed using both 5’ and 3’ primers specific for HCV 5’UTR to amplify and quantify HCV RNA. The PCR products were run in 1% agarose gel and densitometric analysis was done using MultiGauge software (Fujifilm) and the values were expressed as ratio of HCV IRES to actin.

Northern blot analysis. Total cellular RNA (20 μg) was isolated from Huh7 cells transfected with HCV–FLuc monocistronic constructs with or without Dzs and resolved on formaldehyde-agarose gel (0.8%) under denaturing conditions. RNA were transferred and cross-linked to a nylon membrane (Sigma-Aldrich) and probed with a [α-32P]-labelled firefly luciferase antisense probe, followed by autoradiography. Densitometric analysis was done and the ratio of HCV-IRES to 18S rRNA was expressed graphically.

RESULTS

Sequence-specific cleavage of HCV IRES RNA by various DNAzymes

Since the mechanism of HCV IRES-mediated translation is novel and fundamentally different from cap-dependent translation of host cell mRNA, we have designed several Dzs to target the IRES element for selective inhibition of HCV RNA translation. Furthermore, the sequence-specific cleavage in this region will consequently block viral RNA replication and therefore we designed a number of Dzs that were targeted to the predicted single-stranded loop regions within the HCV IRES element (Fig. 1, Tables 1 and 2). In order to evaluate the cleavage efficiency of the Dzs, in vitro cleavage reaction were performed. Out of five Dz molecules only three, Dz219, Dz305, and Dz327, have shown significant cleavage activity in vitro in cell-free conditions (Fig. 2a and 2b). Interestingly, the three active Dzs were found to cleave the target RNA in a sequence-specific manner with varying efficiencies (Fig. 2b, lanes 3, 4 and 5). Dz219 showed maximum cleavage activity under standard conditions.

Table 2. Sequence of DNAzymes for HCV IRES based on conserved regions

<table>
<thead>
<tr>
<th>DNAzyme</th>
<th>Sequence 5’–3’</th>
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<tr>
<td>Dz161</td>
<td>GTACTCAGGCTAGCTACAACGACGGTCC</td>
</tr>
<tr>
<td>Dz165</td>
<td>CGGTGTAAGCTAGCTACAACGATACCGG</td>
</tr>
<tr>
<td>Dz285</td>
<td>CAGTACCAGGCTAGCTACAACGAAAGGCT</td>
</tr>
<tr>
<td>Dz288</td>
<td>GGCAGTAGGCTAGCTACAACGACAACAGGG</td>
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![Fig. 1. Schematic diagram of HCV IRES (adopted from Brown et al., 1992), showing the Dz targets. The numbers in bold are specific for genotype 1b and those in italics are conserved for all major genotypes.](http://vir.sgmjournals.org)
**Effect of Dz on HCV IRES-mediated translation ex vivo**

In order to evaluate the intracellular cleavage efficiencies of the Dz molecules, transient co-transfection experiments were performed using plasmid HCV–FLuc monocistronic constructs and the Dz molecules in human hepatocellular carcinoma cells (Huh7). The monocistronic RNA generated ex vivo from the HCV–FLuc monocistronic plasmid encodes the HCV IRES element upstream of the firefly luciferase reporter gene (Pudi et al., 2003). Although three Dz molecules specific for genotype 1b showed significant cleavage activity in vitro, only one of them, Dz219, showed impressive inhibition (81%) of HCV IRES-mediated translation. However, the mutant-Dz219 with a single substitution in the catalytic domain of Dz219 failed to inhibit HCV IRES-mediated translation, suggesting high specificity of the approach (Fig. 3a). Interestingly, two other Dzs (305 and 327) showed significant in vitro cleavage activity, but failed to interfere with the HCV translation (Fig. 3a). When conserved Dzs were tested for inhibition of HCV IRES function, Dz285 and Dz288 showed 38 and 35% inhibition, respectively, whereas Dz161 showed only 30% inhibition (Fig. 3b).

To investigate the cleavage of the HCV–FLuc monocistronic RNA by the Dzs in ex vivo conditions, Northern blot hybridization was performed. For this purpose, Huh7 cells were transiently transfected with the monocistronic DNA constructs and different Dz molecules (Fig. 4a). Total RNAs were isolated 24 h post-transfection and used for Northern assay. Dz molecules used in the assay included Dz219 and Dz285, which showed maximum activity ex vivo, and Dz305, Dz327 and mutant-Dz219, that did not exhibit any ex vivo activity. Results showed significant cleavage activity of Dz219 and Dz285; however, Dz305 and Dz327 failed to cleave HCV–FLuc RNA ex vivo (Fig. 4a), which is consistent with our reporter gene (luciferase) assay (Fig. 2b). The mutant-Dz219 didn’t show any cleavage activity ex vivo, as expected. For clarity we have quantified the band intensity corresponding to the HCV–FLuc RNA and normalized it with that of the loading control band (18S rRNA). The densitometric analysis of the ratio of HCV–FLuc monocistronic RNA to the 18S rRNA (Fig. 4b), clearly demonstrated that cleavage activity of Dz219 as 48% and that of Dz285 as 25%, respectively. However, Dz305, Dz327 or the mutant-Dz219 did not exhibit any significant cleavage activity. Interestingly, the translation inhibitory activity corresponding to Dz219 and Dz285 was found to be slightly higher (Fig. 3) than the RNA cleavage activity (Northern analysis, Fig. 4), which could be due to a higher sensitivity of the luciferase assay.

To further investigate the specificity of the Dz activity, the Dz molecules were tested against other viral IRES as well as cap-dependent translation. For this purpose the pCDFLuc DNA construct was transiently transfected with representative Dz molecules (Dz219, 285, 288 and mutant-Dz219) and the luciferase reporter gene was assayed 24 h
Results suggest that none of the Dz molecules inhibited the cap-dependent translation of the luciferase reporter gene (Fig. 5a).

Similarly, CVB3 FLuc monocistronic DNA (pCDCVB3-FLuc) was cotransfected with the above set of Dz molecules and the luciferase assay was performed 24 h post-transfection. Results suggest no significant change in luciferase activity in presence of the above Dz molecules (Fig. 5b). Taken together, the results suggest the target specificity of the Dz molecules and the high levels of selectivity of this approach.

Effect of Dzs on the HCV RNA replication

Finally, we have analysed the inhibitory effect of the Dzs in Huh7.5 cells harbouring HCV1b replicon (Fig. 6a) (Blight et al., 2002). Increasing concentration (0.4 and 0.8 μM) of Dz219 was transiently transfected into replicon-containing cell line using Lipofectamine 2000 (Invitrogen). After 24 h, total RNA was isolated and the HCV positive-strand RNA corresponding to the 5’UTR was detected by semiquantitative RT-PCR. Results suggest approximately 70% inhibition of the HCV1b genotype replicon RNA synthesis when 0.8 μM Dz219 was used. However, the same concentration of Dz219 failed to inhibit the HCV-RNA synthesis in Huh7 cells harbouring HCV2a genotype replicon (Lohmann et al., 1999) (Fig. 6b and 6c). Upon inspection we found that the Dz219 target sequence was designed on the basis of HCV 1b sequences, which is not fully conserved in HCV2a sequence. The result also proved that bio-efficacy of Dz219 was sequence-specific. Furthermore, when the conserved Dzs (0.4 μM) were transfected into cells containing HCV replicon 1b (Fig. 6d), significant inhibition of RNA synthesis was observed with Dz285 and Dz288 (30 and 50%, respectively). However, the inhibitory effect was relatively more pronounced (60% for Dz285 and 70% for Dz288) on HCV replicon 2a cell line.

DISCUSSION

A couple of studies have demonstrated previously the use of DNAzyme molecules to cleave HCV RNA ex vivo (Trepanier et al., 2006), but this study constitutes the first report on the effect of Dzs on HCV replication in cell lines harbouring HCV subgenomic or full-length replicons.
Although we have designed several Dzs targeting different regions of HCV IRES and tested their activities in vitro as well as ex vivo in cell lines harbouring HCV replicon, only a couple of them were found to be more effective in the in vitro and ex vivo assays. Interestingly, when all the Dzs used in the study were mapped to the target sequences/structures within HCV IRES (Fig. 1), it appears that the Dz285 and Dz288, targeting HCV SLIIId loop, and Dz219, targeting SL IIIb, achieved maximum inhibition, perhaps due to the importance of the target site in ribosome assembly during internal initiation of translation. This could be also due to the fact that all target sites are not available for efficient cleavage by a single kind of catalytic nucleic acid molecule, most probably because the secondary and tertiary structures in the target RNA prevent optimal base pairing. Base pairing and cleavage activity also depend on the arm length of the RNA-binding site of the Dzs. Enzymes with longer arms sometimes showed higher cleavage activity compared with enzymes with shorter arms (Oketani et al., 1999). Modifications in the 5' and 3' termini of these molecules help in preventing nuclease degradation without affecting its catalytic activity (Oketani et al., 1999). Interestingly, it has been demonstrated earlier that the efficiency of some Dz molecules can be enhanced by using them in combination with some oligodeoxynucleotides (ODNs) which would hybridize the target RNA near the Dz cleavage site to facilitate the cleavage reaction (Sood et al., 2007). Thus, it would be interesting to explore whether the apparently inactive/inefficient Dz molecules in our study could also be used in combination with ODNs to potentiate catalytic efficiency for the RNA cleavage.

It appears that, if required, Dz molecules can be used at higher concentration to achieve maximum inhibition of viral protein synthesis with minimum effect on host cell RNA translation (data not shown). It is also possible that the effective concentration required to achieve 50% inhibition of viral RNAs could be lowered to a large extent by using a cocktail of Dz molecules in the line of combination therapy.

Dz molecules have been shown to be a more stable antiviral agent compared with Rz or siRNA (Santoro & Joyce, 1997). Unlike siRNA or shRNAs, Dz molecules are not expected to activate double-stranded RNA-activated protein kinase.
(PKR) and result in attenuation of host cell RNA translation due to phosphorylation of eIF2 by PKR (Gil & Esteban, 2000). It is also possible to make more stable derivative of the Dzs molecules such as morpholino- or phosphorothio- derivatives etc. Remarkable stability was also achieved by modifying (inverting) the first and last nucleotide residues, especially at the 3’-end of the Dz, which will have serum stability enhanced tenfold (Sun et al., 1999). In this connection, it has been shown also that efficient uptake of macrophage tropic-anti-HIV-1 Dz by human macrophages in the complete absence of charged lipid molecules can be enhanced by attaching ten G residues at the 3’-end of a 10–23 catalytic motif-containing Dz. G residues form G quartet-like structures that are recognized by the scavenger receptor present on macrophages (Unwalla & Banerjea, 2001).

Taken together, these results provide proof of the concept that the HCV IRES could be an effective and selective target using conserved DNA-enzyme molecules to develop novel antiviral therapeutics against hepatitis C virus infection. It would be interesting to couple this with organ-specific delivery approaches. Liver-specific delivery of Dz molecules using Sendai virus virosome- (Ramani et al., 1997) or lentivirus- (Kusunoki et al., 2003) based vectors would be ideal for developing Dz-based antiviral therapeutics against hepatitis C virus infection.

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