Publications

PUBLICATIONS


5. Aalia Shahr Bano, **Nidhi Gupta**, Vikas Sood and Akhil C Banerjea; Vpr from HIV-1 subtype B and C exhibits significant differences in their ability transactivate LTR mediated gene expression and also in their ability to promote apoptotic DNA ladder formation. *AIDS*. 2007; 21(13):1832-4.


7. Vikas Sood, **Nidhi Gupta** and Akhil C.Banerjea; DNA-enzyme mediated cleavage of HIV-1 Gag RNA is significantly augmented by antisense-DNA molecules targeted to hybridize close to the cleavage site. *Oligonucleotides*. 2007; 17,113-121.

8. Vikas Sood, **Nidhi Gupta**, Vijay Shankar, Aalia Shahr Bano, and Akhil C.Banerjea; Basal RANTES promoter activity differs considerably among


Potent Knockdown of the X RNA of Hepatitis B by a Novel Chimeric siRNA-Ribozyme Construct and Modulation of Intracellular Target RNA by Selectively Disabled Mutants

Nidhi Gupta, Aalia S. Bano, Yogeshwar Sharma, and Akhil C. Banerjea

A multitarget approach is needed for effective gene silencing that combines more than one antiviral strategy. With this in mind, we designed a wild-type (wt) and selectively disabled chimeric mutant (mt) constructs that consisted of small hairpin siRNA joined by a short intracellular cleavable linker to a known hammerhead ribozyme, both targeted against the full-length X RNA of hepatitis B. These chimeric RNAs possessed the ability to cleave the target RNA under in vitro conditions and were efficiently processed at the cleavable site. When this wt chimeric RNA construct was introduced into a liver-specific mammalian cell line, HepG2, along with the HBx substrate encoding DNA, very significant (-70%) intracellular downregulation in the levels of target RNA was observed. When the siRNA portion of this chimeric construct was mutated, keeping the ribozyme (Rz) region unchanged, it caused only ~25% intracellular reduction. On the contrary, when only the Rz was made catalytically inactive, about 55% reduction in the target RNA was observed. Construct possessing mt Rz and mt siRNA caused only 10% reduction. This wt chimeric construct also resulted in almost complete knockdown of intracellular HBx protein production, and the mt versions were less effective. The intracellular reduction of target RNA with either wt or mt constructs also interfered with the known functions of HBx protein with varying efficiencies. Thus, in this proof of concept study we show that the levels of the target RNA were reduced potently by the wt chimeric siRNA-Rz construct, which could be modulated with mt versions of the same.

Introduction

Small interfering technology allows one to interfere strongly with the gene expression of variety of genes and is currently favored over any other RNA-based approaches, as it is known to be effective in the presence of very small amounts (Rossi, 2006). It is an evolutionary conserved mechanism and its role has been identified in many cellular and developmental programs (Scherer and Rossi, 2004; Martin and Caplein, 2007). In the present study we focused our attention on the X gene product (HBx) of hepatitis B virus, which is known to activate several cellular and viral promoters, including the HIV-1 long-terminal reporter (LTR) promoter (Robinson, 1994; Gomez-Ganzalo et al., 2001; Gupta et al., 2007). Although it is strongly associated with the formation of hepatocellular carcinoma (HCC), the exact role in HBx protein is not well understood. It is very likely that the intracellular levels of target protein may be critically important for causing cytopathogenesis in this case, and the same may be true for other diseases/disorders. In order to correlate more meaningfully the extent of pathogenesis with the levels of target gene expression, it is important to device novel approaches that allow the downregulation of target gene expression in a controlled manner. Several nucleic acids–based antiviral approaches are currently available to achieve gene-specific suppression. They include ribozymes (Rzs), DNA enzymes (Dzs), antisense RNA or DNA, aptamers, and decoy RNAs (Akkina et al., 2003; Kurreck, 2003; Joyce, 2004; Banerjea et al., 2005; Schubert and Kurreck, 2006; Bhindi et al., 2007). Although catalytic nucleic acids (Rzs and Dzs) have been used extensively to knock out a target gene expression individually (Unwalla et al., 2006), we showed for the first time that it was possible to obtain both Rz- and Dz-specific cleavage under same in vitro conditions, and they acted synergistically to knock down the intracellular target gene expression (Sood et al., 2007; Gupta et al., 2008). Very often Rz, Dz, or siRNAs target sites are not accessible for hybridization with target RNA owing to secondary structures (Cairns et al., 1999; Schubert et al., 2004). It is becoming...
increasingly clear that some sequences may be more prone to cleavage with either Rz, Dz, or siRNAs, which needs to be experimentally determined (Goila and Banerjea 1998; Kurreck et al., 2002). The cleavage of HBx RNA by either Rz or Dz was recently shown by our group to be significantly augmented with oligodeoxynucleotides that were designed to hybridize immediately next to the hybridizing arms of either Rz or Dz (Gupta et al., 2008). Earlier constructs with antisense RNA and multimeric Rzs were used against HIV-1 (Ramezani et al., 2006). Very recently siRNA against HIV-1 gene along with anti-gp120 aptamer was shown to have potent anti-HIV-1 activity (Zhou et al., 2008).

Several mono- (Martinez et al., 2002) and bispecific siRNA constructs were described earlier that targeted CD4, CXCR4, CCR5 genes (Anderson et al., 2003; Anderson and Akkina, 2005). The unique feature of this bispecific siRNA construct is that the two small-hairpin siRNAs were joined together by an intracellular cleavable linker as originally described by Leirdal and Sioud (2002). Using this approach impressive inhibition of HIV-1 replication was observed. Here, for the first time, we designed and tested constructs that combined an earlier described potent hammerhead ribozyme against the X gene of hepatitis B (Goila and Banerjea, 2004) virus with a small hairpin siRNA using the same short cleavable linker [henceforth called wild-type (wt) chimeric construct]. We show that this and mutant (mt) constructs were processed correctly in the presence of mammalian cell cytoplasmic extracts. The wt construct interfered strongly with the expression of target gene expression both at RNA and protein levels. Furthermore, by selectively inactivating either Rz or siRNA portion of this chimeric construct, it was possible to modulate the intracellular target gene expression.

Materials and Methods

Cloning of HBx gene and in vitro transcription

Plasmid pSG5.HBX encoding the X gene of HBV (Kumar et al., 1996) was a kind gift from Vijay Kumar, ICGEB, New Delhi, India. The entire gene was placed under SV40 and T7 promoters. The former promoter was used for intracellular expression and the latter was used for obtaining in vitro transcripts using the transcription kit from Promega Biotech, as described earlier (Banerjea and Joklik, 1990). After linearization with appropriate restriction enzyme at the 3'end, full-length (465 nt) X RNA was synthesized.

Construction and cloning of HBx-Rz-170 targeted against X gene

The construction of Rz-170 (Fig. 1A) has been described by one of us in detail previously (Goila and Banerjea, 2004). It possessed the hammerhead catalytic motif and was targeted against the GUC sequence (C residue is 170th nt in the target RNA; Kumar et al., 1996). EcoRI and BamHI restriction sites were engineered at the ends of the hybridizing arms of the Rz that facilitated cloning it into pCDNA3 expression vector (Promega). This placed the expression of Rz under T7 and CMV promoter. Mg++ dependent sequence-specific cleavage of the target RNA is expected in the presence of Rz.

Construction of chimeric siRNA-Rz constructs against HBx gene

The detail methodology for making chimeric constructs has been described by one of us in detail previously (Anderson et al., 2003). In brief, an oligonucleotide was synthesized and the desired RNA was synthesized in vitro using T7 RNA polymerase as described earlier (Donze and Picard, 2002; Anderson et al., 2003) using the transcription kit provided by Promega Biotech. A four-point mt version of the siRNA was made, which retained the normal sequences of the Rz (Fig. 2B, shown in italics—henceforth referred to as mt-siRNA-wt-Rz construct). The other chimeric construct retained the wt siRNA portion but the Rz was disabled by changing a single nucleotide (G to U—henceforth referred to as wt-siRNA-nt-Rz) in the hammerhead catalytic motif. Finally, we made a construct that consisted of mutant siRNA and mutant Rz (mt-siRNA-nt-Rz). In vitro synthesis of these RNAs indicated that they were of correct size when compared with RNA control markers (data not shown).

In vitro cleavage of target RNA with Rz-170 and chimeric constructs

In vitro transcription of the linearized plasmid DNA encoding X gene was carried out in the presence of labeled UTP using T7 RNA polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmoles each) of the labeled target RNA and unlabeled Rz in a reaction buffer containing 50 mM Tris/HCl, pH 7.5, in a volume of 10 µL. The reaction mixture was heated briefly at 94°C and the cleavage reaction was initiated by adding MgCl2 (final concentration 10 mM) at 37°C for 2 hours. The cleaved RNA fragments were subjected to gel analysis as described before (Shahi et al., 2001). The radioactive RNA bands on the X-Ray film were quantitated by densitometry (GS-710 Calibrated Imaging Densitometer, Bio-Rad, Hercules, CA, USA).

Cleavage of wt chimeric RNA in the presence of mammalian cell extracts

Cytoplasmic protein extract was prepared from 293 cells. Briefly, the cells were washed twice with PBS and lysed at 4°C with lysis buffer (0.2% NP40 in PBS + protease inhibitors). After 20 minutes of incubation, cell lysate was centrifuged for 5 minutes at 10,000 rpm and the supernatant was collected and used as cytoplasmic protein extract for the cleavage of chimeric RNAs. Uniformly labeled wt-chimeric RNA (50 ng) was incubated in the presence of this extract (20 µg) for 2 minutes and subjected to gel analysis as described before by Leirdal and Sioud (2002).

Intracellular decrease in HBx RNA by WT and chimeric constructs

The intracellular decrease in HBx-specific RNA was studied after cotransfection with substrate encoding
**HEPATITIS X GENE INHIBITION BY CHIMERIC siRNA-Rz RNA**

DNA (pSG5.HBx) + indicated amounts of chimeric RNA constructs into HepG2 cells, and the levels of target and control RNAs were monitored by RT-PCR based assays. Rz-170 RNA, which was placed under T7 promoter (Goila and Banerjea, 2004), was in vitro synthesized using T7 RNA polymerase-based transcription kit and used for comparison with chimeric RNA constructs. Cells that received HBx encoding DNA (control) also received equivalent amounts of unrelated chimeric RNA. To ensure uniform transfection efficiency, we always transfected equal amounts of reporter gene containing plasmid DNA (pSV-β-gal, Promega, WI, USA) using lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and repeatedly obtained about 70-80% transfection efficiency. All the plasmid DNAs were Qiagen column (Qiagen, Hilden, Germany) purified and the in vitro synthesized RNAs showed no cellular toxicity under our experimental conditions. The cell lysates were prepared 48 hours after transfection with lipofectin. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using TRIZOL reagent (GIBCO-BRL, MD, USA) following the procedure described by the manufacturer and divided into two equal sets. One set was used for estimating the levels of full-length X RNA using HBx1 and HBx2 primers, and the second set was used for estimating the levels of the housekeeping gene, human glyceraldehyde phosphodehydrogenase (HuGAPDH), as described earlier (Paik et al., 1997). The following primers were used for estimating the intracellular levels of full-length X RNA.

(1) Forward (HBx1): 5'-TTAGGCAGAGGTGAAAAAGTG CAGTCTGCTCG (2) Reverse (HBx2): 5'- ATGGCTGCTAGGCTGTACTGC CAACTGGATCCTTCG

**Northern blot analysis**

Total RNA was isolated from HEK 293 cells with Trizol (Invitrogen) that were transfected with 1 μg HBx encoding plasmid DNA (along with 2 μg of unrelated RNA) or HBx + 2 μg of various RNA constructs in 1 mL volume using lipofectamine for 48 hours. RNA was quantitated and equal amounts (20 μg) were loaded in each lane and resolved on a 1% formaldehyde-agarose gel under denaturing conditions. RNA was transferred onto a nylon membrane (Amersham),

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**FIG. 1.** Construction of Rz-170 and cleavage of HBx RNA: Panel A depicts the sequence of the target RNA that was used to design a hammerhead Rz. Eight nt-long hybridizing arms were synthesized to provide specificity. When the substrate RNA (HBx) is mixed with equimolar amounts of cold Rz-170, specific cleavage products are expected that are 170 and 295 nt long. Panel B: Lane 1 shows the synthesis of 465-nt-long HBx transcript along with some prematurely truncated transcripts, and when mixed with equimolar amounts (100 pmoles) of unlabeled Rz-170, specific cleavage products (295 and 170-nt-long RNA fragments) were observed (lane 2). Mutant Rz-170 failed to show any cleavage activity (lane 3).
·lipofectin (Invitrogen). Cell lysates were synthesized that consisted of either mutant siRNA or inactive an oligonucleotide is synthesized that encodes the chimeric construct is synthesized using synthesis of this chimeric RNA using FIG. 2.

UV cross-linked, and probed with 32P-labeled HBx antisense probe (full-length), followed by autoradiography.

Western blot analysis

About 1 × 10^6 HepG2 cells were grown to 80% confluence in a six-well plate. They were transfected with 1 μg of HBx encoding DNA (pSGS.HBx) (along with 1 μg of unrelated RNA of similar length) or in combination with 1 μg of the wt chimeric construct in a final volume of 1 mL for 48 hours using lipofectin (Invitrogen). Cell lysates were prepared and equivalent amounts of proteins were loaded in each lane as described earlier (Golia and Banerjea, 2001, 2004). They were divided into two equal parts; one was used for estimating the levels of HBx protein and the other was used for the control actin protein. They were subjected to gel analysis and transferred onto a nitrocellulose membrane. Rabbit polyclonal antibody to HBx (Biovendor, NS, USA) was used as a primary antibody in 1:500 dilution in PBS pH 7.2 containing 0.1% Tween 20. The blot was developed with HRP conjugated secondary anti-rabbit IgG antibody (Santa Cruz, CA, USA) followed by NBT/BCIP treatment (Promega Biotech). Antibody to actin (mouse-antiactin monoclonal antibody—Calbiochem, San Diego, CA, USA) was used to determine the levels of this control protein.

Results

Sequence-specific cleavage of HBx RNA with Rz-170

When equimolar (100 pmol) amounts of the full-length target RNA (465 nt long) were subjected to cleavage with Rz-170 in the presence of Mg++, specific cleavage products (170 and 295 nt long fragments) were observed (Fig. 1B, lane 2). Lane 1 shows the synthesis of the full-length 465 nt long target RNA (HBx) as expected along with minor prematurely truncated transcripts (lane 1). Rz-170, which was created by introducing a single change in the catalytic motif, failed to show any cleavage (lane 3). No cleavage was observed in the absence of Mg++ (data not shown).

In vitro cleavage of the HBx RNA with chimeric siRNA-Rz constructs

Equivalent amounts (100 pmol each) of labeled HBx RNA (Fig. 3, lane 1) were subjected to cleavage with various constructs as indicated on top of each lane. Incubation of labeled HBx RNA with Rz-170 (lane 2) or chimeric constructs containing wt Rz component (lanes 3 and 4) showed specific cleavage products, and as expected, chimeric constructs that possessed mt Rz component failed to cleave the target RNA (lanes 5 and 6). We conclude that chimeric constructs, despite their large size, are still capable of cleaving the target RNA specifically using their Rz component efficiently.

Processing of chimeric constructs with cytoplasmic constructs

Uniformly 32P-labeled 110 nt-long wt chimeric RNA (Fig. 4, lane 1) when treated with equal amounts of cytoplasmic extracts yielded two cleavage products (52 and 40 nt long) (lane 2); lanes 3 and 4 represent the cleavage obtained with mt-siRNA-wt-Rz and wt-siRNA-mt-Rz, respectively. We
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FIG. 3. HBx RNA cleavage by wt and chimeric siRNA-Rz mutants. The nature of the experiment is same as described for Figure 1B. Lane 1 shows the synthesis of full-length HBx RNA (labeled substrate RNA). Specific cleavage products were observed with Rz-170 (lane 2), wt-siRNA-wt-Rz (lane 3), and mt-siRNA-wt-Rz. Chimeric constructs containing inactive Rz, namely wt-siRNA-mt-Rz (lane 5) and mt-siRNA-mt-Rz (lane 6), failed to generate any Rz-specific cleavage products.

conclude that wt and the two mt constructs were processed correctly by cellular nucleases with equal efficiencies.

Modulation of intracellular target gene expression by wild-type and mutant constructs

Either HEK 293 or HepG2 (1 x 10^6) cells were transfected with 1 µg of HBx encoding DNA (along with 1 µg of unrelated RNA of same length) in the final volume of 1 mL. A prominent HBx-specific gene expression was observed in these cells (Fig. 5A, lane 2) and taken as 100% for comparison (panel B and D). Wt chimeric construct transfected cells showed about 70% reduction (compare lanes 1 and 2). Mt siRNA-wt-Rz caused about 50% and siRNA-mt-Rz about 45% (relative intensities of the corresponding lanes are shown in panel B). Cells co-transfected with 1 µg each of Rz-170 RNA + HBx encoding DNA caused only about 30% reduction (Fig. 5C and D, lane 3). When 3 µg of Rz-170 was used for transfection, about 60% reduction was observed (data not shown). On the contrary, 1 µg of mt-siRNA-mt-Rz showed only 10% reduction (lane 4) and 1 µg of wt-siRNA-wt-Rz showed about 70% reduction (lane 5), as observed earlier (Fig. 5A and B, lane 1). The relative intensity of the amplified DNA bands are shown in panels B and D. These are representative results from three independent experiments. We conclude from this experiment that wt construct was significantly more effective in reducing the target gene expression when compared with chimeric mts or Rz-170 alone. The levels of the control RNA (huGAPDH) remained same in all the corresponding lanes, which suggest that chimeric constructs downregulated the target gene expression in a specific manner. PCR carried out in the absence of RT showed no HBx-specific amplification (data not shown).

The efficacy of various RNA constructs was determined by Northern blot analysis also, and the results are shown in panel E along with the quantitation of the target RNA (lower panel). Cells transfected with HBx encoding plasmid showed a prominent HBx RNA (lane 1). Wt-siRNA-wt-Rz construct showed 70% reduction (compare lanes 1 and 2). The two selectively disabled mts showed 28–34% reduction (lanes 3 and 4), and the double mt exhibited about 15% reduction (lane 5) (representative of three experiments). The ribosomal RNAs (control) isolated simultaneously showed no changes in all the corresponding lanes.

Inhibition of protein synthesis by chimeric constructs

When HepG2 cells were transfected with HBx encoding DNA (along with an equivalent amount of unrelated RNA), a prominent 16.5 KDa immuno-reactive X protein band was observed (Fig. 6, lane 1) by western blot analysis. In cells that were cotransfected with wt-siRNA-wt-Rz along with equal amounts of HBx encoding DNA (final concentration 1 µg/mL), potent (>70%) knockdown of the expression of X protein was observed (Fig. 6A and B, lane 4, shown by an arrow). Cells that received no DNA (cell control) failed to show any HBx protein-specific band (data not shown). Cells that received mi-siRNA-wt-Rz reduced the expression by 20% (lane 2) and wt-siRNA-mt-Rz caused about 45% reduction (lane 3). Chimeric construct mt-siRNA-mt-Rz showed only about 5% reduction when compared with control (lane 5). This reduction of target gene is specific because the levels of control actin protein in all the corresponding lanes remained constant (panel B). These are representative data from three independent experiments.

Wild-type and mutant constructs interfere with the X protein-mediated transactivation function in a differential manner

The HBx protein is known to activate a number of cellular promoters, including the HIV-1 LTR promoter (Robinson,
1994; Gomez-Gonzalo et al., 2001), and in the presence of wt or mt chimeric constructs against the X gene, reduced activation is expected because of either cleavage of the HBx RNA with Rz or selective degradation by siRNA-mediated pathway. We cotransfected 100 ng each of HIV-1 LTR-driven LTR-β-luciferase reporter plasmid DNA (reporter DNA) and either wt or mt chimeric constructs in 1 mL of a six-well plate (1 × 10⁶) of HEK 293 cells for 48 hours, and determined luciferase activity as described earlier (Goila and Banerjee, 2001). Representative results from three independent experiments are shown in Figure 7. The cell control (lane 1), HBx encoding DNA + unrelated RNA (lane 2) and wt construct (lane 3) treated cells showed basal levels of reporter gene activity (all less than 50 units) as expected. Cells that received LTR-β-luciferase reporter DNA (henceforth called Reporter DNA) showed activity that was arbitrarily assigned 1 for comparison (lane 4). HBx + Reporter DNA showed >6.5-fold more activation as expected (lane 5). When the same experiment was carried out in presence of the wt chimeric construct (lane 6) more than twofold drop was observed (compare lanes 6 and 5). On the contrary, the mt constructs caused less than 1.5-fold reduction (lanes 7 and 8). The wt-siRNA-mt-Rz showed almost no reduction in reporter gene activation (compare lanes 5 and 9). We conclude that wt-chimeric construct interfered very significantly with the known function of HBx and that this inhibition can be modulated using mt constructs.

Discussion

In the present work, we made a novel chimeric construct with the idea that it will interfere with the intracellular target gene expression more powerfully, and by using selectively disabled mt versions of it, it may be possible to modulate the level of target gene expression. In order to achieve this objective it was important to establish that this novel unprocessed chimeric RNA constructs with active Rz component possessed the ability to cleave the target RNA and that these chimeric RNAs were processed correctly by nucleases present in the cytoplasmic extracts of a mammalian cell. Our in vitro cleavage data with siRNA-Rz chimeric constructs suggest that they possessed the ability to cleave the target RNA specifically. Thus even the unprocessed chimeric constructs may contribute toward intracellular reduction of the target RNA. Furthermore, our in vitro data suggests that the wt and two mt constructs were processed by intracellular nucleases with equal efficiency. Thus, the varying efficacy of the wt and the two mt constructs was not due to different in vitro processing activities.

It is quite conceivable that the full-length wt chimeric RNA (110 nt long) will acquire additional secondary structures that may decrease the cleavage efficiency. Indeed efficiency of Rz-170 mediated cleavage of HBx RNA was about 10–15% better than any of the chimeric constructs that possessed wt Rz component (Fig. 3). It was earlier observed by several investigators that although Rz may show excellent in vitro cleavage activity it may not be effective in causing intracellular reduction of the target RNA. This may be largely due to poor accessibility of the target sites inside the mammalian cells besides other reasons (Schubert et al., 2004). The fact that most of the in vitro synthesized wt and mt chimeric RNAs were cleaved specifically in the linker region in less than 2 minutes in the presence of cytoplasmic extracts strongly suggests that the same may be happening inside the mammalian cells also in a rapid manner. As expected, our wt chimeric construct interfered with the target gene expression very powerfully based on our transient transfection data. The selectively disabled chimeric mts allowed us to conclude that siRNA component played a predominant role toward the inhibition of gene expression.

It is noteworthy that we selected the siRNA target site that involved the AUG of the target RNA, which is usually accessible toward siRNA-mediated downregulation. The potency of our approach could also be explained on the basis that once the target RNA is cleaved by either of the two antiviral approaches (siRNA or Rz), it may open up secondary structures at the target sites. This may facilitate more efficient hybridization between the target RNA and Rz or siRNA. Alternatively, the cleaved target RNA fragments are known to become increasingly more susceptible to degradation by nucleases. The potency of our wt chimeric construct was not only established at the target RNA level but
FIG. 5. Inhibition of intracellular target RNA with wt and mutant chimeric constructs: Panel A: Indicated amounts of HBx encoding DNA in presence and absence of wt or selectively inactivated chimeric constructs were transfected into HepG2, and total RNA was isolated as described in the text. Target RNA and control RNA were estimated using RT-PCR based assays. Panel B: The levels of the target RNA was evaluated by quantitating the intensity of the PCR-amplified bands in lane 2 of panel A as 100. Panel C and D: Cells were transfected with Rz-170 RNA (lane 3), wt-siRNA-wt-Rz (lane 4), and mutant-siRNA-wt-Rz (lane 5). Lane 2 is same as lane 2 of Figure 5, panel A. Northern blot analysis (panel E): HEK 293 cells transfected with HBx encoding DNA + unrelated RNA (lane 1); HBx + wt-siRNA-wt-Rz (lane 2); HBx + mutant-siRNA-wt-Rz (lane 3); HBx + mutant-siRNA-wt-Rz (lane 4); and HBx + mutant-siRNA-mt-Rz (lane 5). 32P-labeled full-length HBx gene was used as a probe. Control ribosomal RNA and quantitation of the intensity of the HBx-specific bands are shown in corresponding lanes.
almost a complete knockdown of protein production was observed by western blot analysis. On the other hand the two selectively disabled mts were less effective in causing the downregulation of target gene expression. Completely disabled mt construct (mt-siRNA-mt-Rz) failed to show any significant intracellular reduction of either target RNA or at the protein expression level. Antisense components present in the design of Rz and siRNA in our constructs may account for the residual (5-10%) inhibitory activity present in siRNA-Rz chimeric RNA. These chimeric constructs can be delivered to the desired cell via lentiviral vectors or other vectors for achieving specific gene suppression (Banerjea et al., 2004). Selectively disabled mt versions may allow us to modulate the extent of gene suppression, which can be exploited for variety of studies, especially for genes that are temporally regulated.

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up to two-thirds of its C-terminal portion, exists as two major tetramolecular species that differ in electrophoretic mobility. Virology 179, 460–462.


Address reprint requests to:
Dr. Akhil C. Banerjea
Chief Lab.- Virology
National Institute of Immunology
JNU Campus, Aruna Asaf Ali Marg
New Delhi-110067, India

E-mail: akhil@nii.res.in

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Potent Intracellular Knock Down of Hepatitis B Virus X RNA by Catalytic Hammerhead Ribozymes or DNA-Enzymes with Antisense DNA-Oligonucleotides or 10-23 DNA-Enzymes that Powerfully Augment In Vitro Sequence-Specific Cleavage Activities

Nidhi Gupta, Aalia S. Bano, Yogeshwar Sharma, Vikas Sood and Akhil C. Banerjea*

National Institute of Immunology, Department of Virology, New Delhi-110067, India

Abstract: Novel antiviral approaches are needed to control Hepatitis B virus infection worldwide. X protein of this virus activates various promoters and is strongly associated with hepatocellular carcinoma. Although several groups, including ours, reported sequence-specific cleavage of X RNA by either ribozymes (Rzs) or DNA-enzymes (Dzs) earlier, but none of these studies reported 100% in vitro cleavage of the full-length X RNA. We reasoned that by melting the secondary structures near the Rz/Dz cleavage site with specific antisense DNA oligonucleotides (ODNs) or 10-23 Dzs, it may be possible to achieve this objective. Hammerhead motif containing Rz-170 specific for X RNA was constructed by recombinant techniques and Dz-237 was synthesized using the 10-23 catalytic motif. When specific ODNs or 10-23 Dzs were included in the cleavage reaction with either Rz-170 or Dz-237, increased cleavage was observed in a dose-dependent manner which often resulted in almost complete in vitro cleavage of the target RNA. Rz-170 in combination with specific ODNs caused potent intracellular reduction of HBx RNA. Thus, the cleavage activity of catalytic nucleic acids (Rzs or Dzs) can be increased significantly by specific ODNs or Dzs and this treatment also results in potent intracellular target RNA reduction. These findings have important therapeutic implications.

INTRODUCTION

Ribozymes (Rzs) are short catalytic RNA molecules that possess the ability to cleave the target RNA in a sequence-specific manner and control splicing reactions [Cech, 1987] [1,2,3,4]. Although several types of catalytic motifs have been described, the hammerhead Rz being the smallest and with minimal target sequence requirement, has been exploited extensively for variety of purposes including therapeutic applications [5,6,7,8]. On the other hand, Deoxyribozymes or DNA-enzymes (Dzs), as originally [9] are short DNA molecules that can be designed to cleave any target RNA in a sequence-specific and catalytic manner [10,11,12]. Over the years several kinds of Dzs with unique catalytic motifs have been described but Dz possessing the 10-23 catalytic motif has been exploited extensively by several investigators [11]. This Dz possesses a conserved 15 nucleotide long 10-23 catalytic motif and is able to cleave any target RNA between purine and pyrimidine under in vitro and in vivo conditions. Their ability to cleave any target RNA has been exploited for knocking down gene expression against variety of target genes including HIV-1 genes [11,13,14] with varying outcomes. In some instances very efficient intracellular gene inhibition was observed with 10-23 Dz [15]. We also reported earlier that 10-23 Dz was more effective in cleaving the full-length CCR5 (HIV-1 coreceptor) RNA compared to a hammerhead ribozyme [16,17] carried out a comparative study between hammerhead ribozymes (Rzs) and Dzs that were targeted against the same sequences and concluded that some sequences were cleaved better by Rzs and some by Dzs. There are several other nucleic-acid based approaches that have been used for specific inhibition of target genes. The catalytic ribozymes, aptamers, antisense DNA or RNA or small interfering RNAs (siRNAs) have been used for inhibiting the expression of foreign genes including HIV-1 replication [8]. Physiologically relevant RNAs are usually long and consist of multiple stem-loop structures and up to 90% putative cleavage sites were earlier shown to be in inaccessible to either Rz or Dz mediated cleavage [18]. Earlier, unwinding activity of an RNA helicase was used to increase the cleavage potential of Rzs [19]. Collectively, all these studies suggest that not all the target sites are 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. Introduction of locked nucleic-acids in antisense design resulted in enhancing its stability [20] and when incorporated in the substrate recognition arms of a Dz, it improved the catalytic efficiency [21].

Earlier few nucleic-acid based approaches were used to modulate the cleavage activity of the Dzs and Rzs with moderate success but none of them were tested for bio-efficacy. Oligonucleotide effectors (regulators) that bind to both enzyme and substrate were used to regulate the catalytic activity of Rzs & Dzs [22, 23]. Whether oligodeoxynucleotides (ODNs) that hybridize specifically to the target RNA alone could modulate in vitro and in vivo the catalytic activity of Rzs or Dzs have not been earlier attempted. [24] reported enhancement of ribozyme catalytic activity by a contiguous oligonucleotide (facilitator) and by 2'-O-methylation. Oligonucleotide facilitators have been used to either enhance

*Address correspondence to this author at the National Institute of Immunology, Department of Virology, New Delhi-110067, India; E-mail: akhil@nii.res.in

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[25] or even inhibit catalytic activity of Rzs [26]. We recently reported Dz mediated augmentation of in vitro cleavage of a truncated HIV-1 Gag gene by specific antisense molecules [27]. Oligonucleotide facilitators have earlier been used to enhance hammerhead ribozyme mediated cleavage of long RNA substrates with multiple-turnover activity [28]. Although all the above approaches resulted in enhanced cleavage of the target RNA under in vitro conditions, it was not known if combinations of ribozymes or DNA-enzymes with facilitators could be exploited to down regulate intracellular levels of the target RNAs.

In the present study, we have identified and characterized those ODNs and Dzs that significantly enhanced the Rz and Dz mediated cleavage of full-length X RNA (HBx RNA) of hepatitis B virus (HBV) on one hand and inhibit intracellular expression of the target RNA in a liver specific cell line, HepG2 on the other. The Hepatitis B virus X protein (HBx) acts as a powerful transactivator for several genes including HBV genes and is known to be strongly associated with hepatocellular carcinoma [29, 30]. These results are potentially important for therapeutic purposes for the treatment of HBV infections against which there are no effective antiviral treatment available.

METHODOLOGY
Cloning of HBx Gene and In Vitro Transcription

Plasmid pSG5.HBx encoding the X gene of HBV [31] was a kind gift from Vijay Kumar, ICGEB, New Delhi, India. The entire gene is placed under SV40 and T7 promoters (Fig. 1). The former promoter is used for intracellular expression and the latter was used for obtaining in vitro transcripts using the transcription kit from Promega Biotech., as described earlier [32]. After linearization with appropriate enzyme at the 3’-end, full-length X RNA will be synthesized.

Construction and Cloning of HBx-Rz-170 Targeted Against X Gene

The construction of Rz-170 (Fig. 2A) has been described by us in detail previously [33]. It possessed the hammerhead motif and targeted against the GUC sequence. EcoRI and BamH1 restriction sites were engineered at the ends of the hybridizing arms of the Rz which facilitated cloning it into pcDNA3 expression vector (Promega). This placed the Rz under T7 and CMV promoter.

In Vitro Cleavage of Target RNA with Rz

In vitro transcription of the linearized plasmid DNA was carried out in the presence of labeled UTP using T7 RNA polymerase. The cleavage reaction was initiated by adding equimolar amounts (100 pmols each) of the labeled target RNA and unlabeled Rz in a reaction buffer containing 50mM Tris.HCl, pH 7.5, in a volume of 10µl. The reaction mixture was heated briefly at 94°C and the cleavage reaction was initiated by adding MgCl2 (final concentration 10mM) at 37°C for 2 hours. The cleaved RNA fragments were subjected to gel analysis as described before [34]. The radioactive RNA bands on the X-Ray film were quantitated by densitometry (GS-710 Calibrated Imaging Densitometer, BioRad, Hercules, CA, USA).

Primers & DNA-Enzyme

All the 20nt long antisense oligonucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys (The Woodlands, TX). The conserved 15 nt long (5’-GGCTAGCTACAACGA) 10-23 catalytic motif was flanked on both sides by substrate-binding arms of the Dz that were made complementary to the target RNA. We have earlier described in detail the construction of Dz-237 that cleaved the X RNA specifically into two fragments (Fig. 2B) [35]. Dz-155, wild-type and mutant versions of Dz-192 were also assembled using 10-23 catalytic motif that possessed a single nucleotide substitution (G to C) in the 10-23 catalytic motif (Fig. 3B). This change is known to render the Dz catalytically inactive [35].

Dz-Mediated Cleavage

Equivalent amounts of unlabeled Dz and labeled substrate RNA (100 pmols each) were allowed to interact in a final volume of 10µl in a buffer containing 50mM Tris.HCl, pH 7.5 in presence of 10mM MgCl2 (standard conditions) as described earlier [9] for 2 hours at 37°C in the absence or in the presence of indicated amounts of ODNs. The cleaved products were resolved by gel analysis and cleavage efficiency was determined as described earlier [34, 35].

Fig. (1). Full-length HBx encoding DNA and in vitro transcription.

Plasmid pSG5.HBx (Kumar et al., 1996) contains the full length X gene of hepatitis B virus. It is placed downstream of the powerful SV40 and T7 promoter of the expression vector pSG5 (Clontech, Palo, Alto, CA, USA). After linearizing it with Bgl II restriction enzyme, a 465nt long transcript is generated by in vitro transcription using T7 RNA polymerase.

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The intracellular decrease in HBx-specific RNA after cotransfection into HepG2 cells with substrate encoding DNA (pSG5.HBx) + Rz in the presence or absence of ODNs were monitored by RT-PCR based assays. To ensure uniform transection efficiency, we always transfected equal amounts of reporter gene containing plasmid (pSV-β-gal, Promega). We repeatedly obtained about 70 – 80% transection efficiency. All the plasmid DNAs used was Qiagen column purified which showed no toxicity under our experimental conditions. Several dilutions were initially made to determine the linear range for PCR-amplified products. Total RNA was isolated using TRIZOL reagent (GIBCO-BRL) following the procedure described by the manufacture and divided into two equal sets. One set was used for estimating the levels of full-length X RNA using HBx1 and HBx2 primers and the second set was used for estimating the levels of the house keeping gene, human glyceraldehyde-phosphodehydrogenase (HuGAPDH) as described earlier. The following primers were used for estimating the intracellular levels of full-length X RNA.

(1) Forward (HBx1): 5'-TTAGGCGAGAGGTGAAAAAGTTGCATGGTGCTGG.
(2) Reverse (HBx2): 5'-TGGCTGCTAGGCTGACTGCCA

Fig. (2). Sequence of the target RNA against which Rz-170 and Dz-237 were designed are shown along with their specific cleavage products.

Intracellular Inhibition of the Expression of Target RNA by Rz + ODNs

For constructing Rz-170, eight bases long hybridizing arms are made complementary to the target RNA to provide specificity (Panel A). The hammerhead catalytic motif is same as described before (Shahi et al., 2001). The site of cleavage in the target RNA is shown by an arrow. When the full length HBx RNA is subjected to cleavage by Rz-170 in presence of MgCl₂, two specific RNA fragments are expected that are 170 and 295nt long. Panel B: 10-23 catalytic motif containing Dz-237 was chemically synthesized that targeted AU dinucleotide in the target RNA for cleavage. 7 bases long hybridizing arms were made complementary to the target RNA to provide specificity. The expected cleavage products generated due to the action of Dz are shown.

Western Blot Analysis

1 x 10⁶ HepG2 cells were grown to 80% confluence in a six well plate. They were transfected with either Rz-170 (1μg/ml) alone or in the presence of increasing concentrations of ODN #1 and # 2 in a final volume of 1ml for 48 hours along with 1μg of X gene encoding DNA, pSG5.HBx, using lipofectin (Invitrogen). Cell lysates were prepared and equivalent amounts of proteins were loaded in each lane as described earlier. They were subjected to gel analysis and transferred on to a nitrocellulose membrane. Rabbit polyclonal antibody to HBx (Biovendor, NJ, USA) was used as a primary antibody in 1:500 dilution in PBS pH 7.2 containing 0.1% Tween 20. For β-actin as control, mouse raised monoclonal antibody (Calbiochem, CA, USA) was used as primary with 1:10,000 dilution in PBS-T. The amounts of pSG5.HBx DNA was kept constant in each experimental lane at 1μg/ml. The absolute amounts of DNA introduced in each well was made equivalent by adding unrelated DNAs.

Inhibition of X Protein Mediated HIV-1 LTR Activation

HEK 293 or HepG2 cells were cotransfected with 100 ng of pBS-LTR-B-Luciferase (henceforth referred to as pLTR-B – the Luciferase reporter gene is placed down stream of the HIV-1 LTR-B promoter) (obtained from AIDS Research and Reference Reagent Program of NIH, MD, USA) plasmid
in the presence of indicated amounts of ODNs or Dzs in wells that received 100ng of pSG5-HBX plasmid DNA that was kept constant in all the wells. Lipofectin was used to introduce the mixture of various DNA combinations and cell lysates were prepared 24 hours later using 1x reporter lysis buffer (Promega). The extent of Luciferase activity was determined according to the manufacturer’s instructions (Promega). A control reporter plasmid (pSV-P-gal, Promega) was always included to ensure uniform transfection efficiency.

RESULTS

In Vitro Synthesis of Full-Length HBx RNA by T7 RNA Polymerase

Plasmid pSG5-HBX (Fig. 1) was linearized with Bgl II restriction enzyme and when subjected to in vitro transcription using T7 RNA polymerase, a 465nt long full-length HBx transcript is expected using the Riboprobe transcription kit (Promega Biotech., WI, USA) in the presence of 32P UTP. Transcripts were subjected to gel analysis and dried gels were exposed to X Ray.

Rz-170 Mediated Cleavage of HBx RNA in the Presence of Upstream and Downstream ODNs

Entire sequence of the Rz-170 along with the target RNA is shown in Fig. (2A). The target sequence was GC located at nucleotide position 170 in the HBx gene. The 22nt long hammerhead motif was flanked with eight nucleotides long hybridizing arms on either side of the target sequence that were made complementary to the target RNA to provide specificity. When an equimolar amount (100pmoles each) of Rz-170 is used for cleaving a full-length HBx RNA (465nt), specific RNA fragments (170 and 295 bases long) are expected (Fig. 2A). In order to increase the Rz-170 mediated cleaved products, 3 antisense ODNs, each 20nt long, were synthesized. Two of these ODNs (1 to 3) were designed to hybridize immediately adjacent to the two hybridizing arms of the hammerhead Rz-170. We designed another ODN (ODN#3) that was 33 nt upstream from the Rz-170 cleavage site (Fig. 3, panel A). In the similar manner, 10-23 catalytic motif containing Dzs were synthesized immediately upstream and downstream of Rz-170. A mutant (disabled) version of Dz-192 was synthesized by substituting a C residue in place of G in the catalytic motif.

Fig. (3). ODNs and Dzs designed to augment Rz-170 mediated cleavage products.

Panel A: Three 20 nucleotides long antisense oligodeoxynucleotides (ODNs) (1 to 3) were synthesized. Two of them (ODN #1 and 2) were targeted to hybridize immediately next to the hybridizing arms of the Rz-170 and their sequences are shown. ODN #3 was 33nt away from the Rz cleavage site and its sequence is also shown. All the oligonucleotides were chemically synthesized and obtained from Sigma Genosys. Panel B: Two Dzs (Dz-155 and 192) possessing 10-23 catalytic motif, were synthesized immediately upstream and downstream of Rz-170. A mutant (disabled) version of Dz-192 was synthesized by substituting a C residue in place of G in the catalytic motif.
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Fig. (4A). Augmentation of Rz-170 mediated cleavage by specific ODNs.

Lane 1 shows the synthesis of uniformly labeled 465nt long HBx RNA. When equimolar amounts (100pmoles each) of substrate and Rz-170 were mixed in presence of 10mM MgCl₂, specific cleavage products (295 and 170nt long RNA fragments) were observed (lane 2). When the same cleavage reactions were carried out in presence of increased amounts of ODN #1 (10 pmoles – lane 3; 50 pmoles – lane 4; 100 pmoles – lane 5) or ODN #2 (10 pmoles – lane 6; 50 pmoles – lane 7 and 100 pmoles – lane 8), a significant reduction in the input substrate RNA was observed with concomitant increase in the cleavage products.

Fig. (4B). ODN#3 fails to augment Rz-170 mediated cleavage products.

When the same substrate RNA (lane 1) was subjected to Rz-170 mediated cleavage in the presence of increasing amounts of ODN #3 (lane 3 -1pmole; lane 4 -10pmoles; lane 5 - 100pmoles and lane 6 -200pmoles), no significant increase in the cleavage products were observed when compared with Rz-170 mediated cleavage only (lane 2).

Augment the Rz-170 mediated cleavage and that ODN#2 was more effective than ODN#1.

**Antisense ODN #3 Targeted to Hybridize 33nt Away from the Rz-170 Cleavage Site Fails to Augment its Cleavage**

The nature of the experiment is same as described in Fig. (4A). Briefly, Rz-170 mediated cleavage of the target RNA (Fig. 4B, lane 1) was studied in the presence of increasing amounts of ODN#3 (Fig. 3A) and the results are shown in Fig. (4B). Lane 2 exhibits the cleavage obtained with equimolar amounts (100pmoles) of labeled substrate and Rz-170. When increasing amounts of ODN#3 (1pmole – lane 3; 10pmole – lane 4; 100pmoles – lane 5 and 200pmoles – lane 6) were included in the cleavage reaction, the extent of cleavage remained unchanged. We conclude that ODN that hybridized 33nt upstream from the Rz-170 cleavage site failed to augment Rz-170 mediated cleavage.
Only Downstream Dz (Dz-192) Significantly Enhanced Rz-170 Mediated Cleavage

Since we observed significant enhancement of Rz-170 mediated cleavage products by the two ODNs (#1 & #2) that hybridized immediately upstream and downstream to the hybridizing arms of the Rz, we wanted to find out if the two Dzs (Dz-155 and Dz-192) that were designed in a similar manner (Fig. 3B) could also enhance the Rz-170 mediated cleavage. The various cleavage products (including partially cleaved products) that are expected due to the combined actions of Rz + Dz-155 and Rz + Dz-192 are shown schematically in Fig. (5). The reaction conditions were same as described for Fig. (4A) and the results are shown in Fig. (6A). Lane 1 shows the labeled full-length in vitro synthesized HBx RNA as described earlier. Lane 2 shows sequence-specific cleavage of the target RNA when equimolar amounts of Rz were used. In presence of increasing amounts of Dz-155 (lane 3 -10pmole; lane 4 - 50pmoles; lane 5 - 100pmoles), no significant reduction in the amounts of input substrate RNA was observed. Lane 3 and 4 show no decrease but lane 5 shows 1.5 fold decrease when compared with lane 2. On the other hand when the cleavage reaction was carried out in presence of increasing amounts of Dz-192 (lane 6 - 10 pmoles; lane 7 - 50 pmoles and lane 8 - 100pmoles), a dose-dependent decrease in the input substrate RNA was observed with almost complete disappearance at 50 and 100pmoles of Dz-192. Lane 6 shows 4 folds, lane 7 shows 5 folds and lane 8 shows 5.5 folds decrease when compared with input substrate RNA present in lane2 (Fig. 6A). We conclude that only the downstream Dz-192 was able to significantly enhance the Rz-170 mediated cleavage.

Wild-Type & Mutant Dz-192 Augment Rz-170 Mediated Cleavage

The purpose of this experiment was to compare augmentation of the Rz-170 mediated cleavage between the wildtype and mutant-Dz-192 (disabled). As reported earlier, this mutant Dz [35] failed to cleave the target RNA completely and served as an important antisense control for the wildtype Dz. Exactly same amounts of wild-type (Dz-192) and mutant-Dz-192 were added to the cleavage reaction which contained equimolar amounts of Rz-170 and Substrate RNA. The results of this experiment are shown in Fig. (6B). Lane 1 is the HBx transcript and when cleavage reaction was carried out with Rz-170 alone (lane 2), two specific fragments were seen as described before. When the Rz-170 mediated cleavage reaction was carried out in the presence of increasing amounts of Dz-192 (lane 3 -1pmole; lane 4 -10pmoles and lane 5 -100pmoles), a dose-dependent decrease in the substrate RNA was observed along with multiple cleaved fragments. These fragments match the predicted pattern of cleavage described earlier (Fig. 5, steps C and D). The same experiment was carried out with increasing amounts of Mutant-Dz-192 under identical conditions of cleavage (lane 6 -1pmole; lane 7 -10pmoles and lane 8 -100pmoles). In this case also a dose-dependent decrease in the input substrate RNA was observed. In both cases, up to 8 - 10 fold decrease in the input labeled substrate RNA was observed in the presence of highest amounts of Dz used (compare the extent of uncleaved input substrate RNA in lanes 1, 5 and 8). Since this mutant-Dz is catalytically inactive, no additional cleaved products were observed. The extent of reduction in the input RNA with either wild-type (WT) or mutant-Dz in corre-

Fig. (5). Modulation of Rz-170 mediated cleavage products with Dzs.

Full-length HBx RNA was used as target RNA for cleavage with Rz-170 in the presence of upstream (Dz-155) and downstream Dz (Dz-192). The expected cleavage pattern generated by cleavage with Rz, Dz and Rz + Dz is shown schematically in Fig. (6) (steps marked A to D).
HBX RNA Cleavage by Rz, Dz and ODNs

Fig. (6A). Downstream Dz (Dz-192) augments Rz-170 mediated cleavage very significantly.

Rz-170 mediated cleavage reaction was performed in the presence of Dz-155 and 192 in increasing concentrations. Lane 1 shows the HBx RNA (465nt). As expected, Rz-170 reaction generated two specific fragments (lane 2). When increasing concentration of Dz-155 (lane 3 - 10pmoles; lane 4 -50pmoles; lane 5 -100pmoles) was used in the reaction, no significant enhancement of cleavage was observed (lanes 3 -5). On the contrary increasing amounts of Dz-192 (lane 6 -10pmoles; lane 7 -50pmoles and lane 8 -100pmoles) was very effective in cleaving the target RNA (as evident by the reduced amounts of input RNA remaining in lane 7 and 8).

Fig. (6B). Both wild-type and mutant Dz-192 show increased cleavage of the target RNA.

The purpose of this experiment was to compare the ability of catalytically active and inactive Dz-192 in their ability to augment Rz-170 cleavage of the target RNA (lane 1) under exactly identical conditions. Lane 2 depicts the extent of cleavage obtained with Rz-170 alone. When increasing amounts of Dz-192 (lane 3 -1pmole; lane 4- 10pmoles; lane 5 -100pmoles) was added to the reaction mixture, a dose-dependent increase in the cleavage products was observed that matched the predicted pattern as shown in Fig. (6D). In the same manner, in presence of increasing amounts of mutant-Dz-192 (lane 6- 1pmole; lane 7-10pmoles; lane 8 -100pmoles), also resulted in dose-dependent cleavage of the substrate RNA.

HBX-Dz-237 Mediated Cleavage is Enhanced Moderately by Upstream but Powerfully by Downstream ODN

All the earlier experiments described above were designed to increase the cleavage of Rz-170 and we next wanted to know if the earlier identified Dz against HBx RNA, Dz-237 [35] could also be modulated by using 20nt long upstream or downstream ODNs (Fig. 7A). The cleavage results of this experiment are shown in Fig. (7B). Lane 1 is the input substrate HBx RNA and lane 2 shows the Dz-237 mediated specific cleavage products (237 & 228nt long RNA fragments). In the presence of increasing amounts of upstream ODN (ODN#4) (lane 3 -10pmoles; lane 4 -100pmoles; lane 5 -200pmoles), only about 2 fold decrease in the amounts of uncleaved substrate RNA was observed with 200pmoles of ODN# 4 (compare lane 4 with lane 2). When the same reaction was carried out in the presence of increasing amounts of downstream ODN (ODN# 5) (lane 6 -10 pmoles; lane 7 -100pmoles; and lane 8 -200pmoles), significant reduction in the substrate RNA was observed (> 20 fold reduction) with almost complete disappearance in the presence of 200pmoles of ODN# 5 lane 8. We conclude that downstream ODN is significantly more effective in enhancing the Dz-237 mediated cleavage reaction.
Fig. (7A). ODNs designed for augmenting Dz-237 mediated cleavage.

Dz-237 was used to cleave the target RNA in the presence of either ODN #4 or 5. The sequences of the two ODNs flanking the hybridizing arms of the Dz-237 are also shown along with the expected cleaved fragments. Panel 7B: Lane 1 is the substrate RNA and lane 2 exhibits the cleavage products due to Dz-237 cleavage. In the presence of increasing concentrations of ODN#4 (lane 3 - 10 pmol; lane 4 - 100 pmol; lane 5 - 200 pmol), a dose-dependent decrease in the input substrate RNA was observed. Similar observations were made with ODN# 5 but it was more effective in decreasing the input substrate RNA when used in increasing amounts (lane 6 - 10 pmol; lane 7 - 100 pmol and lane 8 - 200 pmol).

Rz-170 + ODNs Treatment Results in Potent Intracellular Reduction of Target RNA

HepG2 cells were cotransfected with 1μg each of pSG5.HBx DNA and Rz-170 construct in one ml for 48 hours using 10μl of Lipofectin (Invitrogen). This dose was predetermined to give about 10% intracellular reduction in the levels of target RNA (Fig. 8, lane 4) when compared with cells transfected with pSG5.HBx DNA alone along with
equal amounts of unrelated Rz (lane 3). Total RNA was isolated using TRIzol and target RNA and control RNA were estimated simultaneously by RT-PCR technique. When 4µg of ODN #2 was added to the Rz-170 construct + pSG5.HBx.DNA mixture (lane 5), approximately 70% reduction was observed (compare lane 5 with lane 3). When the same amount of ODN #1 was used, about 20% reduction was observed (lane 6). When half the above amount of ODN #1 & #2 (2µg each) were used, about 50% reduction in the target RNA was observed (compare lane 7 with lane 3). RT-PCR carried out with cells only (lane 2) and RT-PCR carried out in the absence of RT (data not shown), as expected, showed no HBx RNA specific amplification. The extent of house keeping gene (HuGAPDH) in all the corresponding lanes remained essentially unchanged. We conclude that Rz-170 + downstream ODN combination was more effective in causing decrease in the levels of intracellular target RNA.

Fig. (8). Intracellular reduction of target RNA in the presence of Rz + ODNs.

Estimation of target RNA (HBx RNA) in HepG2 cells treated with various combinations of Rz and Rz + ODN #1 and #2 by RT-PCR techniques. The levels of full-length X RNA and control RNA (HuGAPDH) were determined simultaneously by RT-PCR as described in materials and methods. Wells that received 1 µg of pSG5.HBx DNA + equivalent amounts of unrelated ODN of similar length, showed a prominent X gene-specific band. This amount was kept constant in all the wells. Cells were cotransfected with 1µg each of Rz-170 + pSG5.HBx DNA (lane 4); pSG5.HBx +1µg Rz + 4µg of ODN #2 (lane 5); pSG5.HBx +1µg Rz + 4µg of ODN #1 (lane 6); pSG5.HBx +1µg Rz + 2µg of ODN #1 + 2µg of ODN #2 (lane 7).

Western Blot Analysis

HepG2 cells cotransfected with Rz + ODNs were processed for total protein and subjected to western blot analysis as described before [35,33] and the results are shown in Fig. (9). Lane 1 represents control cells only. A prominent immunoreactive HBx protein was observed (lane 2) when transfected with 1µg each of pSG5.HBx plasmid DNA. About 2-fold reduction in the HBx protein band was observed when cells were cotransfected with 1µg each of pSG5.HBx plasmid DNA and Rz-170 DNA (lane 3). When ODN #1 and #2 (4µg in one ml) were also included, a complete knock down of the HBx protein was observed (lanes 4 & 5). This is not due to unequal amounts of cell lysates that were analyzed because equivalent amounts of cells containing same amounts of total protein were loaded (as determined by colorimetric reaction with BCA reagent from Pierce). We conclude that both the ODNs in combination with Rz-170 caused complete intracellular reduction in the levels of X protein.

Potent Knock Down of HBx Mediated HIV-1 LTR Reporter Gene Activation by Rz-170 + ODNs or 10-23 Dz

HepG2 cells were cotransfected with pSG5.HBx + p-LTR-B plasmid DNA (100 ng each in one ml for 1X 10^6 cells) along with indicated amounts of ODNs or Dz and the reporter gene activity (luciferase) (mean +/- SD) obtained from three independent experiments are shown in Fig. (10). As expected HBx encoding DNA showed significant LTR-promoter activity (compare lane 4 with lane 3). This activity diminished more than 2.4-fold when 100ng of Rz-170 was added (lane 5). Upon addition of ODN#1, it dropped 8.4 fold (lane 6), with ODN#2 about 5.8 fold (lane 7) and in combination of both about 8.4-fold (lane 8). Control cells and pSG5.HBx treated cells showed no significant reporter gene activity as expected (lanes 1 & 2). Thus, an additional 3 - 4 fold decrease (when compared with Rz-170 alone) in X gene mediated reporter gene activity with ODN #1 is observed. More than 17-fold reduction in reporter gene activity was observed with Dz-192 under identical conditions (lane 9) and about 12.6-fold reduction with the mutant version of Dz-192 (lane 10). We conclude that ODNs (#1 & #2), wild-type and mutant Dz-192 treated cells showed impressive functional inhibition of HBx protein. As expected Dz-192 (lane 11) and mutant Dz-192 (lane 12) showed significant reduction in reporter gene activity.

Fig. (9). Intracellular reduction in the levels of X protein by western blot analysis.

HepG2 cells were transfected with pSG5.HBx plasmid alone (lane 2); cotransfected with pSG5Hbx + Rz-170 construct (lane 3); pSG5Hbx + Rz-170 construct + ODN #1 (lane 4); pSG5Hbx + Rz-170 construct + ODN #2 (lane 5) with indicated amounts (see result section). The total amount of DNA used in each well was kept constant by adding unrelated or unrelated ODNs of similar length. Cell lysates were prepared from equivalent amount of cells and subjected to western blot analysis as described before.
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The inhibition of X protein mediated HIV-1 LTR reporter gene activation with Rz + ODNs.

293 or HepG2 cells were grown to 80% confluency in 24 well plate and then transfected with various combinations of plasmid DNAs as indicated + ODNs or 10-23 Dzs as shown in the bottom of the figure using 2.5μl of Lipofectin (Invitrogen) per well. 24 hours post transfection equivalent amounts of cells were subjected to lysis using 1 x reporter lysis buffer (Promega) and the extent of luciferase activity (light units on Y axis) was determined as described earlier (Goila & Banerjea, 2001).

DISCUSSION

Efficient cleavage of a target RNA by Rz or Dz is important for several reasons including biochemical and gene therapy approaches. Although there are several potential Dz or Rz target sites in a target RNA, most of them are cleaved poorly or not at all when tested on longer and physiologically relevant RNAs. We report that ODNs that were targeted to hybridize directly adjacent to the Rz or Dz target sites were able to significantly augment Rz or Dz mediated cleavage activity in dose-dependent manner and that the downstream Dz or ODNs usually worked better than the upstream Dz. In both the cases (Rz + ODNs or Dz + ODNs) almost complete cleavage of the target RNA was achieved under defined set of conditions. Showing close to 100% in vitro cleavage of the target RNA with Rz or Dz in the presence of specific ODNs or Dz on one hand and potent intracellular reduction in a mammalian liver-specific cell line on the other are the major findings of the present work. It must be emphasized that all the previous studies that employed either Rz or Dz alone, complete in vitro disappearance of the input substrate RNA was never achieved under standard conditions of cleavage. This data assumes more significance because ODNs and Dzs are currently being exploited for therapeutic purposes [6, 11]. The most likely explanation for enhanced cleavage by Rz or Dz with specific ODNs is their ability to melt the secondary structure near the Rz and Dz target sites which facilitated efficient hybridization between Rz-170 or Dz-237 with HBx RNA. This ODN mediated enhancement of cleavage was not observed with unrelated ODN of similar length (data not shown) or with ODN that hybridized 33nt upstream of the target RNA. This is most probably because long RNAs are known to exist in separate stem-loop structures or independent domains. Results with upstream and downstream (wild-type and mutant Dz-192) are somewhat surprising but not totally unexpected. The upstream Dz-155 not only failed to increase the cleavage activity of Rz-170 but also was catalytically inactive as no cleavage products were seen. It is likely that Dz-155 failed to hybridize with the target RNA because of the secondary structure constraints. In this connection it is important to note that the ability of ODNs to successfully hybridize to the target RNA (as measured by RNAse H based assay) is essential but not sufficient for affording catalytic activity to Dz [37]. It is noteworthy that the hybridization arms of the Dz or Rz span only 14 or 16 nt (with one gap in the design) in the target RNA whereas the ODNs are uninterrupted 20nt long, which is likely to melt secondary structures better. The advantage of using downstream Dz would obviously be its ability to cleave the target RNA in a catalytic manner. A comparative Rz-170 mediated study between wild-type and mutant Dz-192, suggests that the enhancement of Rz-170 cleavage is largely due to the antisense effects because the amounts of
input substrate RNA after various treatments remained the same. Similar intracellular level of reduction of target RNA was also observed with Dz-237 + ODN#5 (data not shown).

Although very few studies on enhanced cleavage by nucleic acid approaches have earlier been reported, we report, for the first time, that a combination of Rz-170 and specific ODNs or 10-23 Dz lead to not only complete cleavage of the target RNA but can also cause very substantial intracellular reduction of X RNA. The ability of ODNs to augment in vivo cleavage by Rz-170 correlated with their ability to interfere with the intracellular expression of the target gene.

In summary, we show that both Rz and Dz mediated cleavage of the full-length HBx RNA can be powerfully augmented by specific ODNs and 10-23 Dzs. They not only enhanced the Rz & Dz mediated in vitro cleavage products, but also reduced very significantly the intracellular target gene expression in a mammalian liver specific cell line - HepG2. The intracellular effectiveness of these ODNs or Dzs could be substantially improved by using chemically modified (stabilized) nucleotide [37]. These observations are of immediate therapeutic importance and should be applicable to down regulating other target genes also.

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ABBREVIATIONS

ODNs = Oligodeoxynucleotides
Dz = DNA-enzyme
Rz = Ribozymes
RT = Reverse transcriptase
HBV = Hepatitis B virus
LTR = Long terminal repeat

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

Swagata Roy, Nidhi Gupta, Nithya Subramanian, Tanmoy Mondal, Akhil Chandra Banerjee and Saumitra Das

1Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore-560012, India
2National Institute of Immunology, Department of Virology, New Delhi-110067, India

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 Dzas 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

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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; Golla & Banerjea, 2004). Among these, catalytic Dzs with 10–23 catalytic motifs are increasingly being explored 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 (Golla & 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; Golla & 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 explored 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 oligodeoxyribonucleotides (ODNs) were synthesized chemically and obtained from Sigma Genosys. The conserved 15 nt (5'-GGCTAGCTACAAGCA-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 (Golla & 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 neomycin 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 (hgy), 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 selected.

### Table 1. Sequence of DNAzymes for subtype 1b

The mutated residue in the mutant mutDz219 is highlighted in bold.

<table>
<thead>
<tr>
<th>DNAzyme</th>
<th>Sequence 5'–3'</th>
</tr>
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<tbody>
<tr>
<td>Dz88</td>
<td>AACGCCAGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
<tr>
<td>Dz305</td>
<td>GCAAGCCAGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
<tr>
<td>Dz219</td>
<td>CGCGCCAGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
<tr>
<td>Dz327</td>
<td>CTACGGGAGGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
<tr>
<td>Dz336</td>
<td>TGGACGGGCGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
<tr>
<td>mutDz219</td>
<td>CGAGGCAGGCTAGCTACAAGCGCGTAGAC</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>DNAzyme</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dz161</td>
<td>GTACTCAGGCTAGCTACAAACGCAGGTTCC</td>
</tr>
<tr>
<td>Dz165</td>
<td>CCGTCTAGCTGAAGTACAGCAAGAAGG</td>
</tr>
<tr>
<td>Dz285</td>
<td>CAGTACAGCTAGCTACAAACGAAAGGCTT</td>
</tr>
<tr>
<td>Dz288</td>
<td>GGCAGTACAGCTAGCTACAAACGACACAAGG</td>
</tr>
</tbody>
</table>

Cloned upstream of the gene for the firefly luciferase (Pudi et al., 2003). The pCDFLuc construct contains the luciferase reporter gene and the coxsackievirus B3 (CVB3)-FLuc monocistronic plasmid construct (pDCVB3-IRES-FLuc) contains the CVB3 5'UTR cloned upstream of the gene for the firefly luciferase reporter gene.

In vitro cleavage of target RNA with Dz. In vitro run off transcripts of HCV IRES RNA (387 nt) was made from linearized HCV-FLuc monocistronic construct DNA (containing nt 18-383 of HCV IRES) 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 (Golla & Banerjee, 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 pDCVB3-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

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in vivo cleavage conditions (Fig. 2b). However, Dz288 and Dz336 failed to show detectable cleavage activity.

Further, to investigate the inhibitory effect of Dzs that would target conserved sequences in all six major genotypes of HCV, several conserved Dz molecules (including Dz161, Dz165, Dz285 and Dz288) were synthesized (Table 1 and Fig. 2a). For this purpose, HCV 5’UTR sequences of all six genotypes were aligned using the CLUSTAL W program (data not shown). The designing of Dzs was based on the secondary structures of RNA for all the strains, obtained by the mfold program (data not shown). All four conserved Dzs were first tested for RNA cleavage activity (genotypes of Dz336 failed to show detectable cleavage activity. Further, to investigate the inhibitory effect of Dzs that target conserved sequences across the different genotypes across the different genotypes are indicated in italics. (b) Equimolar concentrations (100 pmol) of the labeled HCV IRES RNA and respective Dz were used and the cleavage products were analysed on 8% PAGE. (c) In vitro cell-free cleavage products of HCV IRES by conserved Dzs were analysed on 8% PAGE. The asterisks show the cleavage products as indicated in (a) and their respective sizes are shown on the right.

Effect of Dz on HCV IRES-mediated translation ex vivo

In order to evaluate the intracellular cleavage efficiencies of the Dz molecules, transient cotransfection 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 Dzs 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 pCDIFLuc DNA construct was transiently transfected with representative Dz molecules (Dz219, 285, 288 and mutant-Dz219) and the luciferase reporter gene was assayed 24 h
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 HCV1b 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 

**in 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 SLIIIId 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.

**Fig. 5.** Effect of Dz molecules on cap-dependent translation and CVB3 IRES-mediated translation. (a) Dz219, Dz285, Dz288 or mDz219 (0.4 μM) were co-transfected with 1 μg pCDFluc DNA construct and β-gal DNA construct in Huh7 cells. Luciferase assay was performed 24 h post-transfection. The transfection efficiency was normalized with β-gal activity and the normalized luciferase activity was plotted taking the control as 100%. (b) Similarly, 1 μg CVB3Fluc DNA construct was cotransfected with the above Dzs and β-gal construct and the normalized luciferase values were plotted taking the control as 100%. The results shown represent the average of three independent experiments done in duplicate.

**Fig. 6.** Effect of Dz molecules **ex vivo** in replicon cell line. (a) Schematic representation of the HCV replicon genotype 1a and 1b. (b and c) Increasing concentrations (0.4 and 0.8 μM) of Dz219 were transfected either in Huh7.5 cells harbouring replicon 1b genotype (b) or Huh7 cells harbouring replicon 2a genotype (c). RNA was isolated using TRizol reagent 24 h post-transfection, and semiquantitative RT-PCR was performed using HCV-5′UTR-specific primers or the actin primers. The products were analysed on 1% agarose gel. (d and e) Similarly, the effects of Dz285 and Dz288 (0.4 μM) were analysed with HCV replicon 1b genotype (d) and HCV replicon 2a genotype (e).
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 & Banerjee, 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 virome- (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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REFERENCES


X Protein of hepatitis B virus potently activates HIV-1 subtype C long terminal repeat promoter: implications for faster spread of HIV-1 subtype C

Several genetic subtypes of HIV have been identified throughout the world, but it is predominantly subtype C that is responsible for causing the epidemic in India, some regions of South Asia and Africa. Besides HIV-1 infection, individuals are also co-infected with other pathogens such as hepatitis B virus (HBV), and other bacterial and yeast pathogens. HBV encodes the X gene (HBx) whose product is known to activate several heterologous promoters, including the HIV-1 long terminal repeat (LTR) promoter [1-4]. HIV-1 gene expression is controlled by the LTR promoter, which is rich in various transcription factor binding sites [5]. The HIV-1 LTR sequence of genetic subtype B is significantly different than subtype C, and it is therefore reasonable to assume that HBx may have different effects on promoter activity [5].

In order to study the possible impact of HBx protein on HIV-1 subtype-specific LTR-mediated activation, we co-transfected HIV-1 LTR-B and C reporter constructs (pBlue-3'-LTR-B and C-Luc) along with HBx encoding DNA, pSG5.HBx (465 nucleotide long X gene is placed under SV40 promoter that allows intracellular expression) [6] into human 293 cells using lipofectin (Invitrogen, Carlsbad, California, USA) for 48 h. Cell lysates were prepared and the amounts of luciferase were determined according to the manufacturer's instructions (Promega Biotech, Madison, Wisconsin, USA); the results are shown in Fig. 1a (mean of three independent experiments). To ensure uniform transfection efficiency we included an internal reporter control plasmid (pSV-β-gal; Promega). Control cells or cells transfected with 100 ng pSG5.HBx alone showed background luciferase activity. LTR-B and LTR-C reporter plasmids showed basal promoter activity, and the latter showed approximately twofold more basal activity. When co-transfected with 100 ng of either LTR-B or LTR-C plasmids along with 100 ng pSG5.HBx construct, the latter combination (LTR-C plus HBx) showed more than sevenfold more activation when compared with basal activity, whereas it was less than twofold with LTR-B plus HBx combination. When 100 ng of each LTR and 100 ng of pSG5.HBx DNA were used for transfection, the pattern and the extent of activation remained unchanged. When co-transfected with HBx plus B-Tat (cytomegalovirus promoter driven Tat derived from pNL4-3-DNA) plus reporter construct (LTR-B-luciferase), a strong synergistic effect was observed. It is noteworthy that the level of LTR-B promoter activation by the HBx protein is

![Fig. 1. Amounts of luciferase determined from prepared cell lysates according to the manufacturer’s instructions.](image-url)

(a) Human 293 cells were grown in Dulbecco's modified essential medium plus 10% fetal bovine serum, grown to 80% confluence in a 12-well plate. They were co-transfected with the indicated amounts of pSG5.HBx [6] and HIV-1 subtype B and C specific long terminal repeat (LTR)-luciferase constructs using lipofectin as described by the manufacturer. Twenty-four hours after transfection, cell lysates were prepared and the amounts of luciferase from equivalent fractions were determined from prepared cell lysates according to the manufacturer's instructions. (b) Various combinations of plasmid DNA as indicated (100 ng each) were co-transfected and luciferase activity was determined as described in (a).
equivalent to what we observed with LTR plus B-TAT interaction (mean ± SD from three experiments, Fig. 1b).

This is the first study that suggests that the HBx protein might play an important role in upregulating HIV-1 subtype-C LTR-mediated gene expression compared with subtype-LTR-B-driven expression. These observations have strong implications for the increased replication of subtype C virus.

Acknowledgements

HIV-1 subtype-B and C-LTR-luciferase vectors and pNL4-3 DNA plasmid DNA were obtained from the AIDS Research and Reference Reagent Program of National Institutes of Health, Bethesda, Maryland, USA. Plasmid pSG5.HBx was a gift from Vijay Kumar, ICGEB, New Delhi, India.

Nidhi Gupta, Vikas Sood, Aalia S. Bano and Akhil C. Banerjea, National Institute of Immunology, JNU Campus, New Delhi 110067, India.

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The first reported case and management of multicentric Castleman’s disease associated with Kaposi’s sarcoma in an HIV-2-infected patient

The multicentric variant of plasma-cell Castleman’s disease (MCD) often coexists with Kaposi’s sarcoma (KS) in HIV-1-infected patients, and this association could be explained by the possible involvement of human herpes virus 8 (HHV-8) in their pathogenesis. MCD has never been described in an HIV-2-infected patient. We report the first case of MCD associated with HIV type 2 infection and its successful management with rituximab.

A 46-year-old man was referred for asthenia and anorexia with important loss of weight. This homosexual patient originating from Cape Verde had no past medical history. A few days before his admission, seropositivity for HIV-2 was discovered. Physical examination revealed enlargement of all lymph nodes, mild hepatomegaly and palpable spleen. The initial laboratory evaluation included the following: moderate normocytic regenerative anaemia and positive direct Coombs tests without haemolysis, hypergammaglobulinemia, elevated C-reactive protein (45 mg/l), and a high ferritin level with low serum iron level. HIV-2 was confirmed, with a high viral load (93 100 copies/ml) and low CD4 lymphocyte count (133 cells/µl). The HHV-8 viral load was detectable (3231 copies/150 000 cells).

Histological examination of the biopsy specimen from axillary lymph nodes confirmed the diagnosis of Castleman’s disease, and staining for HHV-8 (Fig. 1) underlined HHV-8-positive cells in the mantle zone, large HHV-8-positive cells dispersed in interfollicular areas and foci of KS with HHV-8 and latent nuclear antigen type 1 stain being present in fusiform-shaped cells. The patient was treated with two cycles of vinblastine, with a dramatic improvement, but symptoms relapsed within 2 weeks. At this time, bone marrow analysis showed evidence of toxic aplasia and a large amount of cytomegalovirus (290 000 copies/ml) in spite of cytomegalovirus viraemia negativity. HAART combining tenofovir, emtricitabine, lopinavir and ritonavir was initiated one week after the beginning of vinblastine; it was interrupted a few weeks later because of an episode of massive bloody diarrhoea with important loss of weight. Histological analysis of a colic biopsy sample showed non-specific ulcerated colitis with marked nuclear dystrophy, evocating a viral disease, but immunohistochemical methods failed to isolate any

References