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Activity of Core-Modified 10–23 DNAzymes against HCV

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The highly conserved untranslated regions of the hepatitis C virus (HCV) play a fundamental role in viral translation and replication and are therefore attractive targets for drug development. A set of modified DNAzymes carrying (2′*R*)-, (2′*S*)-2′-deoxy-2′-*C*-methyl- and -2′-*O*-methylnucleosides at various positions of the catalytic core were assayed against the 5′-internal ribosome entry site element (5′-IRES) region of HCV. Intracellular stability studies showed that the highest stabilization effects were obtained when the DNAzymes' cores were jointly modified with 2′-*C*-methyl- and 2′-*O*-methylnucleosides, yielding an increase by up to fivefold in the total DNAzyme accumulation within the cell milieu within 48 h of transfection. Different regions of the HCV IRES were explored with unmodified 10–23 DNAzymes for accessibility. A subset of these positions was tested for DNAzyme activity using an HCV IRES-firefly luciferase translation-dependent RNA (IRES-FLuc) transcript, in the rabbit reticulocyte lysate system and in the Huh-7 human hepatocarcinoma cell line. Inhibition of IRES-dependent translation by up to 65% was observed for DNAzymes targeting its 285 position, and it was also shown that the modified DNAzymes are as active as the unmodified one.

Introduction

Hepatitis C virus (HCV) affects more than 175 million people worldwide. The majority (70%) of them develop the infection chronically, with a potential cirrhosis prognosis and a consequent need for liver transplantation.[1] The mortality rate of this disease is ranges from 2–5% of the infected population, and until now no effective vaccine is available.[2] Once the sickness is diagnosed the traditional treatment consists of the joint administration of α-interferon and ribavirin.[3] Unfortunately, the response to this approach shows variable results mainly depending on the virus genotype.

The HCV carries a 9600-nucleotide-long single-strand RNA with two highly conserved untranslated regions (5′- and 3′-UTRs), which play a fundamental role in viral translation and replication.[4] HCV translation starts with cofactor recruitment at the internal ribosome entry site element (IRES) in the 5′-UTR.[5] This region is very different from the host cell UTRs, and consequently is interesting as a target for anti-HCV drugs.[6] In this sense, different strategies using oligonucleotides have been directed against the IRES, including a catalytic RNA cleaving domain with an aptamer in the 3′-edge,[6] aptamers against domains II and III–IV,[7] bifunctional molecules containing antisense DNA or RNA attached to functional groups performing RNA hydrolysis,[8] siRNA,[9] and 10–23 DNAzymes.[10–12]

In particular, DNAzymes, which are oligonucleotide catalysts, have been assessed as antiviral agents against other targets such as respiratory syncytial virus,[13] hepatitis B virus,[14] or HIV.[15] DNAzymes, like other oligonucleotide based strategies, have the common drawback of their poor stability in biological fluids. To overcome this limitation, some modified DNAzymes have been reported with increased stability against degradation by nucleases. Most of the modifications, such as inverted nucleotides,[16] phosphorothioates,[17] LNA analogues,[18] or 2′-*O*-methylnucleosides,[19] have been placed in the recognition arms. For the particular case of HCV, as far as we know, only one report dealing with a DNAzyme carrying four 2′-*O*-methylnucleotides consecutively located on the distal ends of the annealing arms is known.[20]

On the other hand, due to the sensitive structure–activity relationship the catalytic core presents, there are only a few examples of chemical modifications in the 10–23 DNAzyme catalytic core, using for example 2′-*O*-methylnucleosides,[18] phosphorothioates,[21] amino-acid-like bases,[22] or abasic phosphoramidites.[23]

In this sense, the 2′-*C*-methyl-2′-deoxynucleosides[24] constitutes an interesting nucleoside class, because they show differential preferred sugar conformations depending on the absolute configuration at the 2′-carbon. The (2′*S*)-2′-deoxy-2′-*C*-methylnucleosides mainly adopt the C3′-*endo* puckering whereas the 2′*R* isomers prefer the C2′-*endo* state. However, they are flexible enough to change their puckering when forming part of single or double-stranded structures.[25] Using these

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modified nucleosides we have recently reported the use of core modified 2′-*C*-methyl DNAzymes for the inhibition of Stat3 expression and decrease in cell proliferation using the human breast cancer cell line T47D.[26]

Taking into account these facts, we present in this work an activity study of 2′-*C*-methylnucleoside triple modified 10–23 DNAzymes targeting the HCV IRES region in vitro and in cultured Huh-7 cells. We have also analyzed the intracellular stability of the modified and unmodified DNAzymes in the same human cells. Our results indicate a decrease of up to 65% in the HCV IRES-FLuc signal relative to control scrambled oligonucleotides when the 2′-*C*-methyl and 2′-*O*-methylnucleoside DNAzymes were assayed.

Results and Discussion

Modified 10–23 DNAzymes

The 10–23 DNAzymes,[27] consist of a 15-nucleotide core (Dz-XXX, Table 1) flanked by two recognition arms (S_1 and S_2, Table 1). We have previously shown that it is possible to modify the deoxyribozyme core with three 2′-*C*-methylnucleosides conserving hydrolytic activity close to that of the unmodified controls,[26] obtaining the best results with two sets of modifications. The first group (Dz-XXX-A, Table 1), consists of the introduction of (2′*S*)-2′-deoxy-2′-*C*-methyluridine, (2′*S*)-2′-deoxy-2′-*C*-methylcytidine, and (2′*R*)-2′-deoxy-2′-*C*-methyluridine at positions 4, 7, and 8, respectively, of the catalytic core of the 10–23 DNAzyme (Table 1). The second family of 2′-*C*-methyl-modified DNAzymes (Dz-XXX-B, Table 1), results from the incorporation of (2′*S*)-2′-deoxy-2′-*C*-methyluridine at positions 4 and 8, and (2′*R*)-2′-deoxy-2′-*C*-methylcytidine at position 7 of the catalytic core.

Additionally, it has been previously reported that the incorporation of 2′-*O*-methylnucleosides at positions 2, 11, 14, and 15 of the catalytic core (Dz-OMe, Table 1) improves the stability of 10–23 DNAzymes in biological fluids.[19] With this aim, we have combined the 2′-*C*-methyl and 2′-*O*-methyl modifications in the catalytic core, preparing Dz-XXX-AOMe and Dz-XXX-BOMe families (Table 1), with the aforementioned 2′-*C*-methyl modifications plus the 2′-*O*-methyl modifications at positions 2, 11, 14, and 15. Regarding the recognition arms (S_1, S_2, Table 1), both have been modified with two phosphorothioate at the 5′ and 3′ edges (Table 1) to increase resistance against exonucleases.

A set of unmodified sequences have been designed to explore the accessibility to the HCV IRES target at positions 143, 173, 190, 219, 285, and 288 (Table 1, Figure 1). Positions 219, 285, and 288 had been previously reported as suitable for DNAzyme activity.[10–12]

In activity experiments an unmodified scrambled sequence (Sc, Table 1) with the same nucleotide content as the DNAzyme 219 and a modified scrambled sequence with 2′-*C*-methyl, 2′-*O*-methyl and phosphorothioate modifications (Sc-mod, Table 1) as in 219 modified DNAzymes were used as negative controls. We have also prepared mutated 10–23 DNAzymes for each IRES studied target (Dz-XXX-M, Table 1) with an inactive catalytic core, to differentiate between the antisense and the catalytic effects.

The 2′-deoxy-2′-*C*-methyluridine and cytidine were prepared as previously reported,[26] and all the oligonucleotides were synthesized using normal phosphoramidite chemistry, increasing the coupling time for the modified positions. After C_{18} column purification, oligonucleotide purity was verified by HPLC.

Table 1. Synthesized DNAzyme sequences.

Name	Core sequence	
Dz-XXX[a]	5′-G_1 G_2 C_3 T_4 A_5 G_6 C_7 T_8 A_9 C_{10} A_{11} A_{12} C_{13} G_{14} A_{15}-3′	
Dz-XXX-M	5′-G_1 G_2 C_3 T_4 A_5 G_6 C_7 T_8 A_9 C_{10} A_{11} A_{12} C_{13} C_{14} A_{15}-3′[b]	
Dz-XXX-A	5′-G_1 G_2 C_3 dU_{4S} A_5 G_6 C_{7S} dU_{8R} A_9 C_{10} A_{11} A_{12} C_{13} G_{14} A_{15}-3′[c]	
Dz-XXX-B	5′-G_1 G_2 C_3 dU_{4S} A_5 G_6 C_{7R} dU_{8S} A_9 C_{10} A_{11} A_{12} C_{13} G_{14} A_{15}-3′[c]	
Dz-XXX-AOMe	5′-G_1 $G_{2OMe}$$C_3$ dU_{4S} A_5 G_6 C_{7S} dU_{8R} A_9 C_{10} A_{11OMe} A_{12} C_{13} G_{14OMe} A_{15OMe}-3′[c,d]	
Dz-XXX-BOMe	5′-G_1 G_{2OMe} C_3 dU_{4S} A_5 G_6 C_{7R} dU_{8S} A_9 C_{10} A_{11OMe} A_{12} C_{13} G_{14OMe} A_{15OMe}-3′	
Dz-OMe	5′-G_1 G_{2OMe} C_3 T_4 A_5 G_6 C_7 T_8 A_9 C_{10} A_{11OMe} A_{12} C_{13} G_{14OMe} A_{15OMe}-3′[c,d]	
Controls	**Sequence**	
Sc	5′-CATAGAGCGAACGCCGGCACGATGGCAGT-3′[a]	
Scr-Mod	5′-CA dU_{4S} AG A_{OMe} GC G_{OMe} AACG C_{7R} CG G_{OMe} C A_{OMe} CGA dU_S GGCAGT-3′[c–e]	
Dz-XXX	**Recognition arm sequence**	
	S_1	S_2
Dz-143[e]	5′-**CA**GACCA-3′	5′-TATGG**CT**-3′
Dz-173	5′-**CT**GGCAA-3′	5′-TCCGG**TG**-3′
Dz-190	5′-**CC**AGGCA-3′	5′-TGAGC**GG**-3′
Dz-219	5′-**CC**AGGCA-3′	5′-TGAGC**GG**-3′
Dz-285	5′-**AG**TACCA-3′	5′-AAGGC**CT**-3′
Dz-288	5′-**GG**CAGTA-3′	5′-CACAA**GG**-3′

[a] XXX in the DNAzyme name indicates the sites of hydrolysis. [b] G_{14} was mutated to C_{14}. [c] dU_S: (2′*S*)-2′-deoxy-2′-*C*-methyluridine; dU_R: (2′*R*)-2′-deoxy-2′-*C*-methyluridine; C_S: (2′*S*)-2′-deoxy-2′-*C*-methylcytidine; C_R: (2′*R*)-2′-deoxy-2′-*C*-methylcytidine. [d] G_{OMe}: 2′-*O*-methylguanosine; A_{OMe}: 2′-*O*-methyladenosine. [e] Bold upper case indicates phosphorothioate modifications.

10–23 DNAzymes: stability in Huh-7 cells

To study the DNAzyme intracellular stability, a set of experiments were performed using Dz-219 and Huh-7 cells as the model system. Huh-7 cells were transfected with the unmodified and modified Dz-219 family (Dz-219, Dz-219A, Dz-219-B, Dz-219-AOMe, Dz-219-BOMe, Table 1) using TransFectin, and after 18 and 48 h, total RNA and DNAzyme extraction was performed. The extraction mixtures were separated by PAGE under denaturing conditions and subsequently transferred to a nylon membrane. To localize the full-length DNAzyme a radioactively labeled full-length probe was used. A 37-nt-long probe directed to HSU6RNA was used to normalize the quantification.

The results of the stability experiments are shown in Figure 2. When the full-length DNAzyme was measured, it could be observed that all tested modifications confer im-

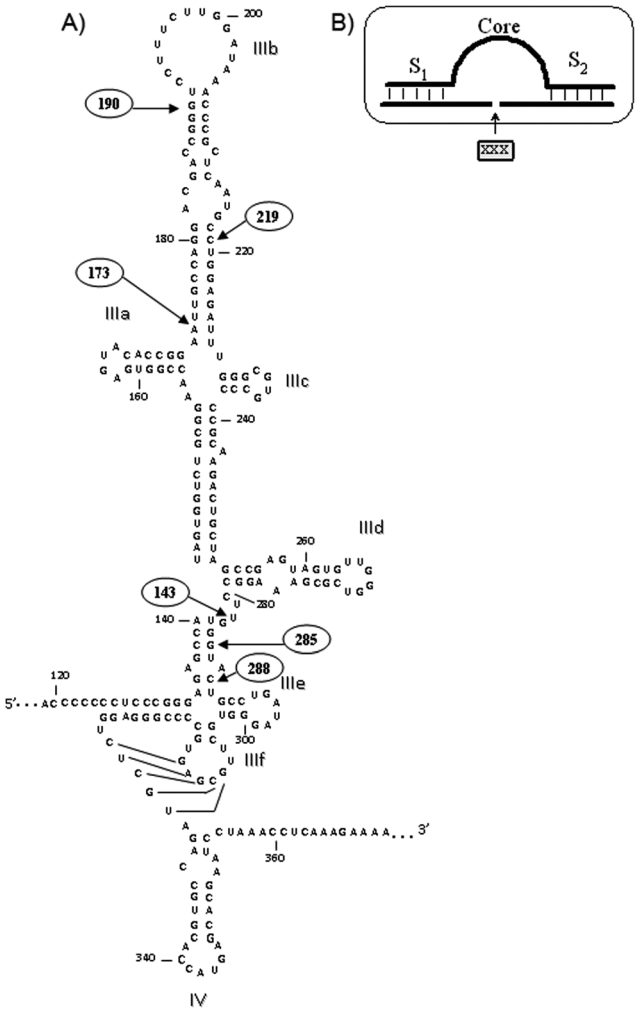

Figure 1. A) Representation of the secondary structure of domains III and IV of the HCV IRES. Potential HCV IRES target sites of 10–23 DNAzymes are indicated. B) Description of unmodified, modified DNAzymes, and scrambled control sequences used.

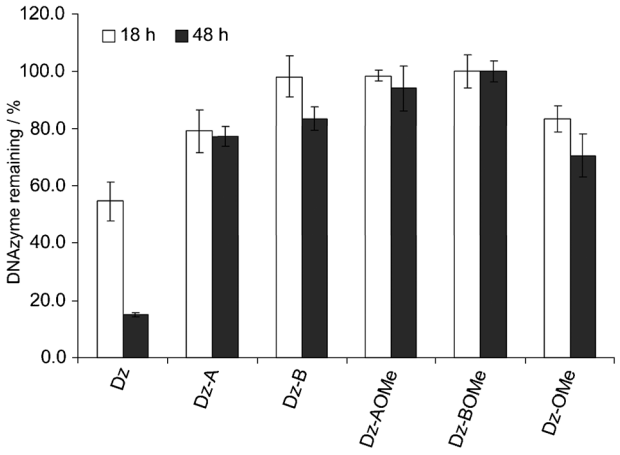

Figure 2. Dz-219 series stability in Huh-7 cells at 18 and 48 h. Data are the mean ± SD of three independent experiments.

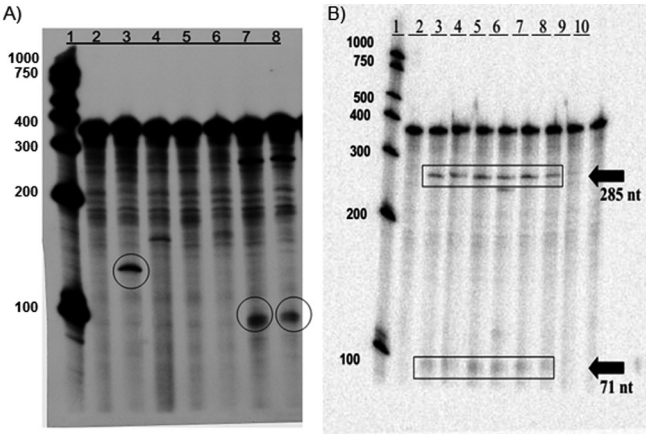

Figure 3. Reaction products of the 5′ HCV-356 transcript. A) Lanes 1: marker [nt], 2: 5′ HCV-356, 3: Dz-143, 4: Dz-173, 5: Dz-190, 6: Dz-219, 7: Dz-285, 8: Dz-288. B) Lanes 1: marker [nt], 2: 5′ HCV-356, 3: Dz-285, 4: Dz-285-A, 5: Dz-285-AOMe, 6: Dz-285-B, 7: Dz-285-BOMe, 8: Dz-285-OMe, 9: Dz-285-M, 10: 5′ HCV-356.

proved relative biological stability compared with unmodified DNAzyme at both 18 and 48 h. This increase in stability in the cellular milieu was higher at 48 h than at 18 h, and it is dependent on the increased number of modified positions and on the 2′-*C*-methyl and 2′-*O*-methyl combined modifications, reaching up to a fivefold increase in the DNAzyme accumulation with respect to the unmodified Dz-219. These data are in accordance with our previous results,[24,26] and encouraged us to explore the combined 2′-*C*-methyl and 2′-*O*-methyl modifications in lysate and cell systems.

DNAzyme catalytic activity using HCV transcripts

To explore the accessibility and activity of unmodified and modified DNAzymes against the HCV RNA target, a 5′ HCV-356 transcript, which corresponds to the first 356 nucleotides of the 5′-UTR region of HCV was used. In all cases the reactions were performed using excess of DNAzyme, in the presence of Mg^{2+}, at 37 °C and with a 5′-end ^{32}P-radiolabeled substrate (see the Experimental Section below). The reaction products were separated and analyzed by PAGE.

Firstly, a set of positions in the 5′ HCV-356 were explored for accessibility using unmodified DNAzymes (Dz-143, Dz-173, Dz-190, Dz-219, Dz-285, Dz-288, Figure 3 A). In the case of Dz-143, Dz-285, and Dz-288, at least one of the cleavage products could be clearly observed. For the rest of the positions it was not possible to determine the exact fragments, probably as a consequence of unspecific RNA degradation.

To evaluate whether there was a substantial difference in activity between modified and unmodified deoxyribozymes, the activity of a set of modified DNAzymes (Dz-285A, Dz-285-AOMe, Dz-285-B, Dz-285-BOMe, Figure 3 B) was analyzed, using Dz-285 as a positive control and Dz-285-M as a negative control. It can be observed in Figure 3 B that all modified DNAzymes showed a similar activity to Dz-285, suggesting that the modifications introduced in the DNAzyme core did not significantly affect the hydrolytic activity.

DNAzymes inhibitory effect using the rabbit reticulocyte lysate system

To study the potential HCV IRES-inhibition function of each modified DNAzyme, in vitro translation assays using the rabbit reticulocyte lysate system were carried out. For this purpose a HCV IRES-firefly luciferase translation-dependent RNA (IRES-FLuc, see Experimental Section) was used. A mRNA CAP-dependent *Renilla* luciferase RNA was also prepared and used as a normalizing control. Then, a set of modified DNAzymes directed against positions 143, 173, 190, 219, 285, and 288 of HCV IRES were assayed (Figure 4) and luciferase activity was determined by luminescence measurement using a dual-luciferase reporter assay kit (Experimental Section). Unmodified and inactive DNAzymes and a scrambled sequence were used as controls.

No significant difference could be found when comparing the modified DNAzymes with the corresponding unmodified DNAzymes (Figure 4). Again, these results suggest that the modifications did not compromise catalytic activity. As the activity was evaluated after a short time, no differential stability effects could be observed. Differences between groups were observed, which could be related to their specific target site. Among the modified DNAzymes, inhibition of up to 60% was observed for the 285 and 288 sets (Dz-285A, Dz-285-AOMe, Dz-285-B, Dz-285-BOMe, Dz-285-OMe, Dz-288A, Dz-288-AOMe, Dz-288-B, Dz-285-OMe, Figure 4). Surprisingly, an inhibitory effect was not observed for Dz-288-BOMe (similar to Dz-288 M). As expected, the scrambled sequence did not inhibit IRES-FLuc translation (Figure 4). The mutated DNAzyme (Dz-M, Figure 4) yielded 20% translation inhibition that could be attributed to a small antisense effect.

DNAzyme IRES-FLuc inhibition in Huh-7 cells

The intracellular toxicity of the 2′-*C*-methyl modifications was evaluated by co-transfecting Huh-7 cells with variable amounts of the scrambled sequence (Sc or Sc-mod, Table 1), IRES-FLuc mRNA, and R-Luc mRNA as control reporter. Then firefly and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega). No differences were found between fluorescence values obtained from Sc and Sc-mod treated cells, which indicates that no additional toxicity originated from the 2′-*C*-methylnucleotides (data not shown). Then the optimal DNAzyme concentration was explored. For this purpose, Huh-7 cells were co-transfected with constant amounts of IRES-FLuc and CAP-RLuc and variable quantities of the DNAzyme. A negative control using a scrambled sequence was also performed. Results showed that 4.5 μg of DNAzyme was adequate for the experiments (data not shown). Then, Huh-7 cells were co-transfected using a mixture of IRES-FLuc, CAP-RLuc, and the modified DNAzymes, using TransFectin as carrier. After 18 h IRES-FLuc and CAP-RLuc luminescence was measured with a dual-luciferase reporter assay. Each IRES-FLuc experiment was normalized by CAP-RLuc and all the values were referred to the scrambled values which were considered as 100% of translation.

The assay was conducted with Dz-285 and Dz-288 families which showed good inhibitory activity in the reticulocyte system. Figure 5 shows the results for IRES-FLuc RNA inhibition. As in the case of the cell lysate experiments, the best results were achieved using the Dz-285 series, which yielded inhibition of the IRES-FLuc translation up to 65% of the value obtained with the scrambled sequences (Figure 5). Core mutated DNAzymes (Dz-M, Figure 5), again generated a small antisense

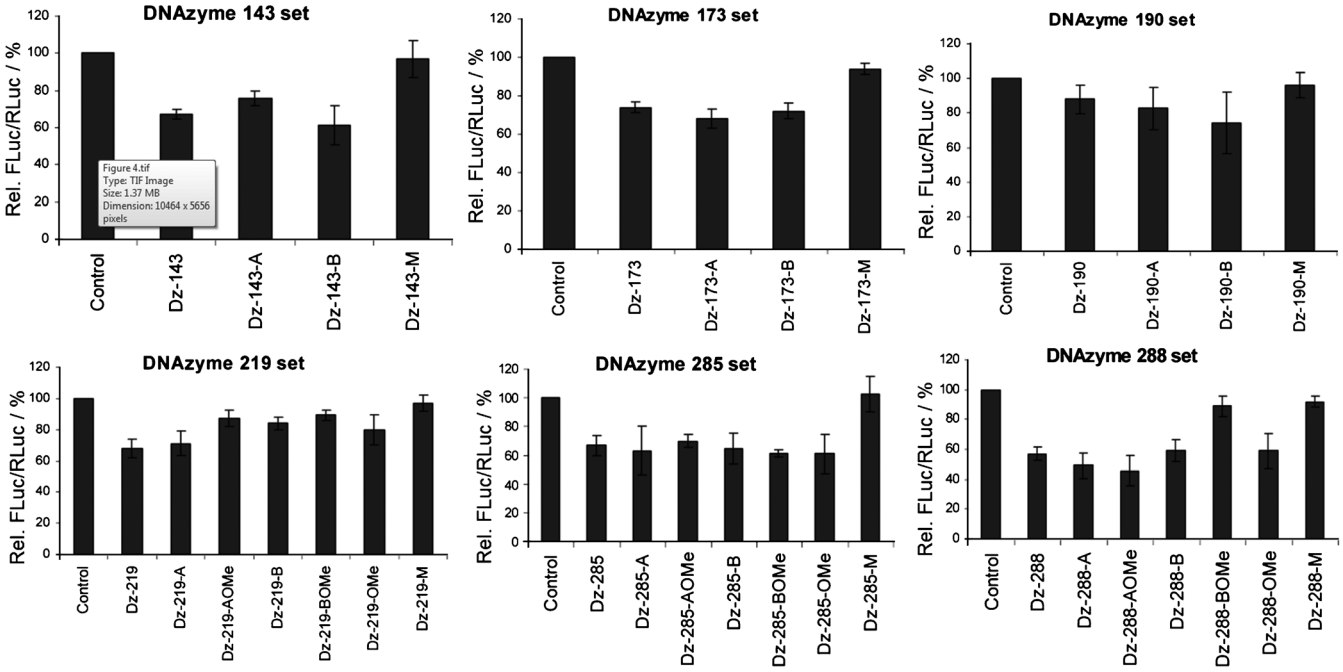

Figure 4. Inhibitory effect of DNAzymes 143, 173, 190, 219, 285, and 288 on IRES-FLuc transcript using the rabbit reticulocyte lysate system. Data are the mean ± SD of three independent experiments.

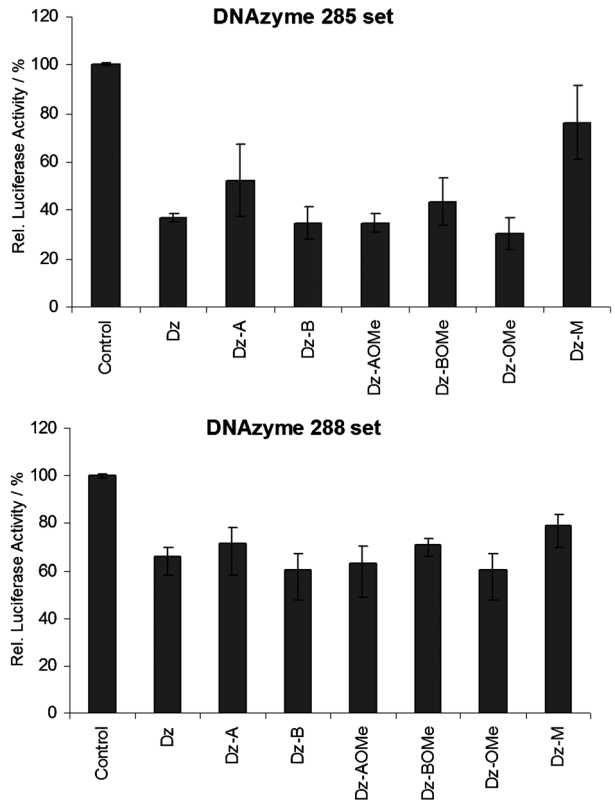

Figure 5. RNA IRES-FLuc inhibition in Huh-7 cells using DNAzymes 285 and 288, and their modified analogues. Data are the mean ± SD of three independent experiments.

effect when compared with the scrambled sequence. All of the Dz-288 series were active, even Dz-288-BOMe which presented low activity in the reticulocyte system. Differences between modified and unmodified DNAzymes were not observed.

Conclusions

A set of 2′-*C*-methyl and 2′-*O*-methyl-modified 10–23 DNAzymes have been assayed against the 5′-IRES region of HCV showing that position 285 was clearly and specifically hydrolyzed. Intracellular stability of modified deoxyribozymes was studied in Huh-7 cells and it was found that Dz-BOMe modifications were stable up to 48 h. Natural and modified DNAzymes were assayed using a HCV IRES-firefly luciferase-translation-dependent RNA, in a cell lysate and with Huh-7 human cells. In this last system, modified DNAzymes directed against position 285 of 5′-IRES diminished IRES-FLuc translation up to 65%.

Experimental Section

Syntheses

Modified phosphoramidite synthesis: The synthesis of (2′*S*)- and (2′*R*)-2′-deoxy-2′-*C*-methyluridine and cytidine phosphoramidites was carried out according to previously reported procedures.[24,26]

Oligonucleotide synthesis: For oligonucleotide synthesis, the following solvents and reagents were used: triethylamine (Sintorgan), glacial acetic acid (Sintorgan), acetonitrile (HPLC grade, J. T. Baker, < 13 ppm water), THF (J. T. Baker), Pyridine (J. T. Baker), dichloromethane (J. T. Baker), 2,6-lutidine (Aldrich), acetic anhydride (Aldrich), and iodine (Aldrich). Commercial amidites were from ChemGenes (USA).

Modified and unmodified DNAzymes were synthesized using an ABI392-DNA synthesizer (Applied Biosystem, USA) or a MerMade 6 synthesizer (BioAutomation USA), at 40 nmol or 50 nmol scale, respectively. All reagents and solvents were high purity grade and the syntheses were performed according to the manufacturer's recommendations. The coupling time for modified positions was increased to improve reaction efficiency. DMTr-ON oligonucleotides were purified on a C18 chromatography column. The purity of DNAzymes was confirmed by RP-HPLC (Gilson).

DNA templates and RNA synthesis: DNA templates, and 5′ HCV-356,[28] IRES-FLuc, and CAP-RLuc RNAs were obtained and purified according to previously reported procedures.[29]

Biological methods

Cell culture and transfection of oligonucleotides: Human hepatocarcinoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 37 °C incubator with 5% CO_2. The day before transfection, cells were loaded into 24 well plates (90 000 cells/well) and incubated overnight. Next, cells were transfected with the corresponding oligonucleotide using TransFectin (Bio-Rad).

Stability of the modified DNAzymes in Huh-7 cells: Cells were transfected by DNAzymes (4.5 μg) targeted to position 219 (Dz-219) using TransFectin (Bio-Rad). At 18 h and 48 h after transfection cells were collected and total RNA and the remaining Dz-219 were extracted using the Trizol reagent (Invitrogen) following the manufacturer's instructions without a DNase treatment step.

Full-length DNAzyme and RNA were resolved by 12% denaturing polyacrylamide gels, transferred to a nylon membrane using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, 30 min 300 mA). Nylon membranes were fixed with UV light (15 min 70 000 μJ cm^{-2}) and hybridized with a radioactive probe specific to the DNAzyme in PerfectHyb Plus Hybridization Buffer (SIGMA) for 20 min at 65 °C and then at 40 °C overnight, for detection and quantitation of remaining full-length DNAzyme. The 37-nt-long probe (5′-GAA TTT GCG TGT CAT CCT TGC GCA GGG GCC ATG CTA A-3′) against the human small nuclear U6RNA, was used as control to normalize the nucleic acids levels. Dry membranes were scanned in a PhosphorImager (Storm 820, GE Healthcare) and quantified with Image Quant 5.2 software (GE Healthcare).

RNA transcript cleavage assays: DNAzymes and an internally ^{32}P-labeled 5′ HCV-356 were incubated for 1 h in 10 mM $MgCl_2$, 50 mM Tris·HCl buffer (pH 7.5). The reaction was carried out under single turnover conditions using an enzyme/substrate molar ratio of 10:1. The cleavage products were resolved by 4% denaturing polyacrylamide gels. Gels were dried and scanned in a PhosphorImager.

Intracellular activity of DNAzymes in Huh-7 cells: The human hepatocarcinoma cell line Huh-7 was transfected with a mixture containing IRES-FLuc RNA (1 μg), CAP-RLuc mRNA (0.5 μg), and DNAzyme or scrambled (4.5 μg) sequences using transfectin. After 18 h post-transfection, cells were harvested, and firefly and *Renilla* luciferase

activities were determined using the dual-luciferase reporter assay system (Promega).

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