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An engineered inhibitor RNA that efficiently interferes with hepatitis C virus translation and replication

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ABSTRACT

Hepatitis C virus (HCV) translation is mediated by a highly conserved internal ribosome entry site (IRES), mainly located at the 5′untranslatable region (5′UTR) of the viral genome. Viral protein synthesis clearly differs from that used by most cellular mRNAs, rendering the IRES an attractive target for novel antiviral compounds. The engineering of RNA compounds is an effective strategy for targeting conserved functional regions in viral RNA genomes. The present work analyses the anti-HCV potential of HH363-24, an *in vitro* selected molecule composed of a catalytic RNA cleaving domain with an extension at the 3′ end that acts as aptamer for the viral 5′UTR. The engineered HH363-24 efficiently cleaved the HCV genome and bound to the essential IIId domain of the IRES region. This action interfered with the proper assembly of the translationally active ribosomal particles 48S and 80S, likely leading to effective inhibition of the IRES function in a hepatic cell line. HH363-24 also efficiently reduced HCV RNA levels up to 70% in a subgenomic replicon system. These findings provide new insights into the development of potential therapeutic strategies based on RNA molecules targeting genomic RNA structural domains and highlight the feasibility of generating novel engineered RNAs as potent antiviral agents.

1. Introduction

Hepatitis C virus (HCV) is the sole member of *Hepacivirus*, a genus belonging to the family Flaviviridae. It is responsible for chronic liver disease that frequently leads to cirrhosis and occasionally hepatocellular carcinoma (Hoofnagle, 1997). Current therapeutic strategies based on the use of alpha interferon (α-IFN) and ribavirin only achieve the complete clearance of viral particles in a limited number of patients. Moreover, resistance to anti-HCV compounds can develop quickly and expand among viral pools. Novel antiviral options are therefore required.

The direct targeting of functional genomic domains is a promising option for the control of infection (Romero-López et al., 2006). This might be undertaken alone or in combination with generic antiviral drugs. The HCV genome is a 9600 nt-long, positive, single-stranded RNA molecule that exists as a ribonucleoprotein particle covered by a lipidic envelope (Ishida et al., 2001). It encodes a unique open reading frame (ORF) flanked by two highly conserved untranslatable regions (5′ and 3′UTRs) (Choo et al., 1989; Kato et al., 1990; Takamizawa et al., 1991); these are extensively folded domains with essential roles in viral protein synthesis and replication (Friebe and Bartenschlager, 2002; Friebe et al., 2001; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The initiation of HCV translation, a crucial step in early infection, is governed by an internal ribosome entry site element (IRES) mostly located at the 5′UTR but spanning 40 nt of the coding sequence (Fig. 1A); (Reynolds et al., 1995; Tsukiyama-Kohara et al., 1992; Wang et al., 1993). Operating in a cap-independent manner it bypasses most of the control pathways involved in the translational regulation of cellular mRNAs. The initiation of viral protein synthesis is mainly influenced by the three-dimensional folding of the IRES element (Fig. 1A); (Collier et al., 2002; Kieft et al., 2002; Lukavsky et al., 2000; Odreman-Macchioli et al., 2000) and involves the direct binding of the ribosomal subunit 40S to domain IIId (Fig. 1A); (Babaylova et al., 2009; Ji et al., 2004; Kolupaeva et al., 2000; Lytle et al., 2002; Otto and Puglisi, 2004). This functional genomic element is a highly conserved domain in the HCV IRES consisting of a short stem with an internal E loop and an apical loop folded into a U-turn motif (Fig. 1A) (Jubin et al., 2000; Klinck et al., 2000; Lukavsky et al., 2000; Smith et al., 1995). Domain IIId has specific structural features that determine its plasticity and predisposition to interact with both proteins and nucleic acids to achieve its most stable conformation (Babaylova et al., 2009; Kolupaeva et al., 2000; Lafuente et al., 2002; Lukavsky et al., 2000; Romero-López and Berzal-Herranz, 2009; Shimoike et al., 1999). Along with its crucial role in viral translation, the 5′UTR also participates in HCV replication (Friebe

Abbreviations: α-IFN, alpha interferon; HCV, hepatitis C virus; IRES, internal ribosome entry site; UTR, untranslatable region.

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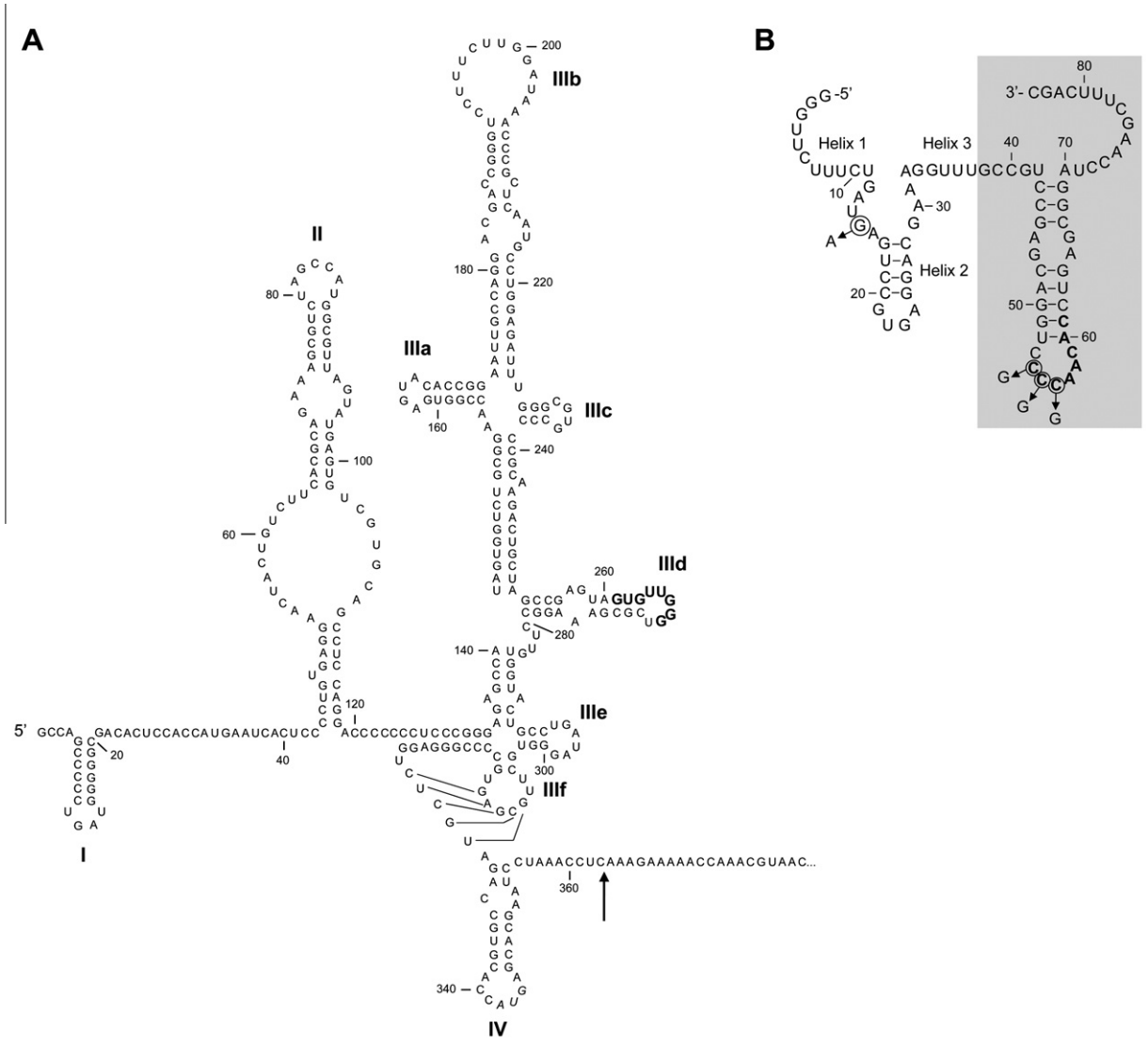

Fig. 1. The HCV-IRES domain and HH363-24. (A) Representation of the secondary structure of the HCV IRES. Nucleotides involved in the interaction with HH363-24 are marked in bold. The translation start codon is shown in italics. The cleavage site for HH363 is indicated by an arrow. Numbering corresponds to nucleotide positions of the HCV Con1 isolate, genotype 1b. (B) The proposed secondary structure of HH363-24 as determined by Mfold software analysis and using experimental constraints. Helix 1 and Helix 3 denote the interacting residues of the catalytic domain with its target sequence in the RNA substrate. The aptamer domain is boxed. Residues involved in the association with domain IIId of the HCV IRES are indicated in bold. Encircled nucleotides were mutated as indicated to generate the respective inactive variants.

et al., 2001). All these items make the IRES, and particularly domain IIId, outstanding candidates for HCV targeting.

The use of nucleic acids – particularly RNA molecules – as anti-HCV agents has been extensively reported (Romero-López et al., 2006, and references therein). The use of aptamers as such is an attractive option since their mechanism of action is based on the direct recognition of primary, secondary and even tertiary structure. The functioning of antisense and siRNAs, in contrast, relies on sequence complementarity. The structural recognition of conserved genomic elements is important for engineering new therapeutic tools that reduce the appearance of resistant viral mutants, a major goal in the search for long-term treatments. The combination of several inhibitors with different specificities might also help hold back the generation of escape variants (Jarczak et al., 2005; Macejak et al., 2001).

A hammerhead ribozyme that targets positions 357–369 of the HCV genome and cleaves it at 3′ of the nucleotide C_{363} (HH363; Lieber et al., 1996) was extended at its 3′ end with a random sequence. A two-step *in vitro* selection method (Romero-López et al., 2005) was then used to isolate RNA compounds with double activity: a common catalytic core fused to different aptamers for the viral 5′UTR. Such engineered hammerhead ribozymes may interfere with HCV IRES function (Romero-López et al., 2005). Tests on the antiviral role of one of these molecules, HH363-24 (Fig. 1B), showed it to efficiently cleave the HCV genome as well as bind and block the function of the targeted IIId domain of the IRES. In a human liver-derived cell line it prevented both translation and replication steps of the viral cycle.

2. Materials and methods

2.1. Cell lines and culture conditions

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1 mM sodium pyruvate (Sigma, St. Louis, MO), at 37 °C in a 5% CO_2 atmosphere. Cells harbouring the bicistronic,

subgenomic HCV replicon Huh-7 NS3-3′ (Larrea et al., 2006; Romero-López et al., 2009) were grown in DMEM supplemented with 20% heat-inactivated FBS, 1 mM sodium pyruvate and 0.5 mg/ml G-418.

2.2. DNA templates and RNA synthesis

The RNA molecules used in *in vitro* analysis were all produced and purified as previously described (Barroso-delJesus et al., 1999). For *ex vivo* inhibition assays, RNA synthesis was performed using the RiboMAX™-T7 large-scale RNA production system (Promega, Madison, WI) (Romero-López et al., 2007, 2009).

DNA templates for the RNAs 5′HCV-356, 5′HCV-691, 5′HCV-691gg, RNA80, IRES-FLuc and cap-Rluc were obtained as previously described (Romero-López et al., 2005, 2007, 2009). The molecule RNA334 used in RNA probing assays was generated by *in vitro* transcription from the pBSSK plasmid digested with the restriction enzyme *Pvu*II.

The dsDNAs encoding HH363-24, HH363m-24, HH363-24m and HH363m-24m were generated by annealing and extension of specific oligonucleotides, as described in (Barroso-delJesus et al., 2005): 5′HH363is (5′-TATGAATTCTAATACGACTCACTATAGGGTTCT TTCTGATGAGTCCGTgaggacgaaaggttt-3′) and asHH363-24 (5′- GCT GAAAGCTTGGATCCGCTCAGGTGTTGGGGACCTGCTCGGACGGCaaac cHH363-24; 5′T7pHH363 m (5′-TAATACGACTCACTATAGGGTTCTT TCTGATAAGT CCGTgaggacgaaaggttt-3′) and asHH363-24 were used to generate HH363m-24; 5′HH363is was used with asHH363-24m (5′-GCTGAAAGCTTGGATCCGCTCAGGTGTACCCGAC CTGCTCGGACGGCaaacctttcgtcctc-3′) to generate HH363-24m; 5′T7pHH363m was used with asHH363-24m to generate HH363m-24m. The T7 promoter sequence is underlined; lower case letters indicate the complementary residues.

2.3. Binding reactions

The evaluation of the dissociation constant (K_d) for each inhibitor RNA was essentially performed as previously reported (Romero-López et al., 2005, 2009). The resulting inhibitory RNA-target complexes were resolved, analyzed and quantified as previously described (Romero-López et al., 2005, 2009; Romero-López and Berzal-Herranz, 2009). The K_d values were calculated from the equation $y = (B_{max}{\cdot}X)/(K_d + X)$ (Puerta-Fernández et al., 2005), where y is the percentage of complexed inhibitor RNA, B_{max} corresponds to the amplitude of the reaction, and X represents the concentration of the substrate RNA 5′HCV-691gg.

2.4. Cleavage assays

The catalytic activity of HH363-24 was determined as previously described (Barroso-delJesus et al., 1999; Romero-López et al., 2009). Cleavage products were resolved and identified in 4% denaturing polyacrylamide gels. Data were fitted to a nonlinear equation, $y = y_0 + a(1 - e^{-k_{obs}t})$, where y represents the percentage of cleavage, a is the final extent of the cleavage reaction, t corresponds to time, and k_{obs} is the rate constant.

2.5. RNA–RNA interaction probing assays

RNA probing assays of the HH363-24:5′UTR complex were essentially performed as described previously (Romero-López et al., 2007, 2009). Briefly, 50 fmol of the ^{32}P 5′ end labelled RNA molecule (∼200 cps) under study were incubated with a molar excess (10 pmol) of the non-labelled partner (5′HCV-356 for HH363-24 probing or the chimeric inhibitor for 5′UTR probing). Control reactions were performed in the presence of an equal amount of a non-related RNA (RNA334 or tRNA, respectively). Complexes were constructed as described above. Degradation reactions were initiated by the addition of RNase T1 (0.1 U), RNase A (0.2 ng) or Pb^{2+} acetate (30 mM). They were allowed to proceed at 37 °C for 5, 2 and 5 min, respectively. Two micrograms of tRNA were added to avoid massive degradation. Cleavage was halted by the addition of 50 mM EDTA, followed by phenol extraction and ethanol precipitation. The resulting products were analyzed in high-resolution denaturing polyacrylamide gels (6%). These were dried, scanned and analyzed as above.

2.6. Ribosome-IRES complex assembly

The identification of 48S and 80S ribosomal particles was essentially performed as previously described (Romero-López et al., 2007, 2009). Briefly, 40 nM of ^{32}P-internally labelled IRES-FLuc RNA was incubated with 5 μM of each inhibitor RNA and 7 μl of the translation mix containing 60% cell extract (Flexi® rabbit reticulocyte lysate system; Promega, Madison, WI). Ribosomal assembly was achieved by incubation at 30 °C for 20 min. Translational complexes were loaded onto a linear continuous sucrose gradient (10–30%) and then resolved by ultracentrifugation at 30,000 rpm for 4 h. Fractions of 0.5 ml were collected from the top of the gradient and their radioactivity measured in a QuickCount QC-4000/XER Benchtop Radioisotope Counter (Bioscan, Inc., Washington DC).

2.7. RNA transfection

Cell lines Huh-7 and Huh-7 NS3-3′ were transfected basically as previously reported (Romero-López and Berzal-Herranz, 2012; Romero-López et al., 2007, 2009). Briefly, 24 h before transfection, 90,000 cells were seeded onto a 24-well plate and allowed to reach 80–90% confluency. For the analysis of IRES-dependent translation inhibition, a mixture containing 1.5 μg of the RNA reporter constructs IRES-FLuc and cap-RLuc was supplemented with 5 μg (∼180 pmol) of each inhibitor molecule or the non-related RNA80. This was employed for the transfection of Huh-7 cells using the TransFectin™ reagent (Bio-Rad, Hercules, CA) following the Manufacturer's instructions. Luciferase activity was detected 17 h after transfection using the Dual-Luciferase™ reporter assay system (Promega).

To examine the interference of the chimeric RNAs on HCV replication, cells stably harboring the bicistronic subgenomic HCV replicon construct Huh-7 NS3-3′ were cultured as above and transfected with 5 μg of inhibitor or control RNA molecules, using TransFectin™. They were harvested 18 h later for subsequent analysis.

2.8. Relative quantification of HCV replicons

Intracellular HCV replicon positive-strand RNA levels were measured as previously described (Romero-López et al., 2009) and normalized with those obtained for the internal mRNA reference molecule encoding GAPDH (Human GAPDH Endogenous Control Kit, Applied Biosystems, Carlsbad, CA). PCR was performed using an ABI PRISM 7000 Sequence Detector System and the results analyzed with an ABI PRISM 7000 SDS software v.1.1 (both from Applied Biosystems).

3. Results

3.1. HH363-24 effectively binds and cleaves the HCV IRES region

HH363-24 is a hammerhead ribozyme that cleaves the HCV RNA genome at nucleotide 363, and which has a 47 nt-long extension at its 3′ end that acts as an aptamer for domain IIId of IRES.

This extension contains a complementary sequence motif for the apical loop of domain IIId (Romero-López et al., 2005). Binding and cleavage assays were performed on the target molecule to further assess the catalytic and binding properties of the chimeric molecule.

The cleavage capacity of HH363-24 was tested on a molecule containing the first 691 nucleotides of the viral RNA 5′HCV-691 (Romero-López et al., 2005). Reactions were performed under single-turnover conditions to calculate the k_{obs} value. These results showed HH363-24 to efficiently cleave the substrate RNA, returning a k_{obs} of 0.042 ± 0.006 min^{-1} (Table 1).

The binding affinity of HH363-24 was subsequently analyzed using the 5′HCV-691gg construct as the target RNA (Romero-López et al., 2005). This molecule contains the HCV genomic sequence from nucleotides 1 to 691 plus two point mutations, $U_{362}G$ and $C_{363}G$, which impede cleavage by the catalytic core. Two nanomoles of the inhibitor molecule were assayed against increasing amounts of the substrate RNA. Analysis of the titration curve (see Table 1 and data not shown) indicated that HH363-24 bound to 5′HCV-691gg with high affinity, returning a K_d value of 12.28 ± 1.31 nM.

These results show that the engineered inhibitor HH363-24 has both catalytic and binding activities.

3.2. The essential IIId domain of the IRES region is the target for HH363-24

In earlier work we identified the sequence motif $C_{54}CCAACAC_{61}$ in the apical loop of the aptamer domain (Fig. 1B; Romero-López et al., 2005). These residues are complementary to the apical loop of the highly conserved IIId domain in the viral IRES region, i.e., $G_{261}UGUUGGG_{268}$ (Fig. 1A; Jubin et al., 2000). RNA probing assays were performed on both interacting molecules to confirm their interaction.

The secondary structure of HH363-24 was first analyzed to map the nucleotides involved in the association with the IRES region. The inhibitor was ^{32}P 5′ end labelled and subjected to partial degradation with nucleases (RNase T1 and A) or lead (Fig. 2A). These reagents preferentially cleave single-stranded RNA, at G residues for RNase T1, U or C residues for RNase A, and at any nucleotide for lead. Reactions proceeded in the presence of a molar excess of the non-related molecule RNA334. The data extracted from these assays fitted perfectly with the theoretical secondary structure predicted by MFold software (Zuker, 2003) (Fig. 1B; and data not shown), in which the catalytic and aptamer domains appeared as two well-defined secondary structural elements (Figs. 1B and 2A). The aptamer region adopted a typical stem-loop secondary structure with an internal purine-rich loop and an apical loop containing the sequence motif $C_{54}CCAAC_{59}$ (Figs. 1B and 2A). This was partially resistant to degradation by RNase A and lead in the presence of the 5′UTR (5′HCV-356; (Romero-López et al., 2005) Fig. 2A). This confirms the involvement of the apical loop of the aptamer element in the interaction with the HCV IRES.

The identification of the interacting residues in the IRES region was then analyzed. For this purpose, the RNA construct 5′HCV-356 was ^{32}P 5′ end labelled and partially digested with specific endonucleases and lead, as described above. Native folding of the IRES was confirmed with the degradation pattern, which firmly resembled that previously reported by other authors (Kieft et al., 1999); (Fig. 1A, 2B, and data not shown). In the presence of HH363-24, the cleavage map reproducibly showed a significant reduction in the accessibility of the apical loop of domain IIId (Fig. 2B). This reflects a critical role of this element in the recruitment of HH363-24.

Table 1
Binding and cleavage constants of the inhibitor RNA HH363-24.

Binding		Cleavage	
K_d (nM)	B_{max} (%)	k_{obs} (min^{-1})	a (%)
12.28 ± 1.31	91.88 ± 2.34	0.042 ± 0.006	71.43 ± 3.81

Values are the means of four independent trials ± the standard deviation. K_d, dissociation constant; B_{max}, final amplitude of the binding formation; k_{obs}, rate constant of the cleavage reaction; a, cleavage reaction amplitude.

3.3. Inhibition of the 80S ribosomal particle assembly

Domain IIId is a functional element with essential roles in IRES-dependent translation. It directly recruits the ribosomal subunit 40S (Barria et al., 2009; Ji et al., 2004; Kolupaeva et al., 2000; Lytle et al., 2002; Otto and Puglisi, 2004) and contributes to the proper positioning of the IRES to constitute the productive translational complex (Babaylova et al., 2009). We have previously shown moderate inhibitory activity of HH363-24 in *in vitro* translation assays (Romero-López et al., 2007). The effect of HH363-24 on the 80S ribosomal particle assembly was therefore investigated. *In vitro* translation assays were performed with an internally ^{32}P-labelled RNA target encoding the IRES region fused to the FLuc coding sequence. The 48S and 80S particles were resolved by sucrose density gradient ultracentrifugation and identified as two well-defined peaks after fractioning (Fig. 3). In the presence of HH363-24, the formation of the translational complexes 48S and 80S was clearly reduced (Fig. 3), suggesting that the chimeric inhibitor interferes with the early steps of IRES-dependent initiation of protein synthesis.

The contribution of both catalytic and aptamer activities to this effect was further investigated. Inactive mutants of HH363-24 for each functional domain were constructed and subsequently tested for their ability to interfere with the ribosomal complex assembly (Fig. 1B). The catalytically inactive variant HH363m-24 contains the point mutation $G_{15}A$ that inhibits the cleavage activity of the hammerhead ribozyme, while the non-functional aptamer molecule HH363-24m has three nucleotide changes that abolish its aptamer binding ability (data not shown). Finally, the completely null variant, HH363m-24m, combines both modifications to render a potentially inactive chimeric inhibitor. While the mutant in the catalytic core was still active, inactivation of the aptamer domain completely abolished the inhibitory effect yielding a similar sedimentation profile to that detected in the absence of HH363-24 (Fig. 3). This result suggests a major contribution of the aptamer to the interference with ribosome recruitment shown by HH363-24. The same behavior was observed for the fully inactive construct HH363m-24m (Fig. 3).

Together, these results suggest a potential inhibitory role of HH363-24 in HCV IRES-dependent translation via interference with the recruitment of ribosomal subunits. This is likely mediated by the interaction with domain IIId.

3.4. Inhibition of the HCV translation and replication in cell culture by HH363-24

The engineered molecule HH363-24 has been shown to exert a moderate inhibitory effect on IRES function *in vitro* (Romero-López et al., 2007). This might be explained by its interaction with IRES domain IIId, as well as the cleavage activity performed by the catalytic core. Thus, viral translation and replication were analyzed in a human cell line to further investigate the antiviral role of HH363-24.

The RNA molecule ICU (Romero-López and Berzal-Herranz, 2012), which contains the *fluc* gene flanked by both the HCV IRES and the whole 3′ end of the viral genome (including the CRE region plus the 3′UTR), was used to examine viral protein synthesis. It has been reported that the CRE element regulates viral translation via the establishment of long range RNA–RNA interactions with

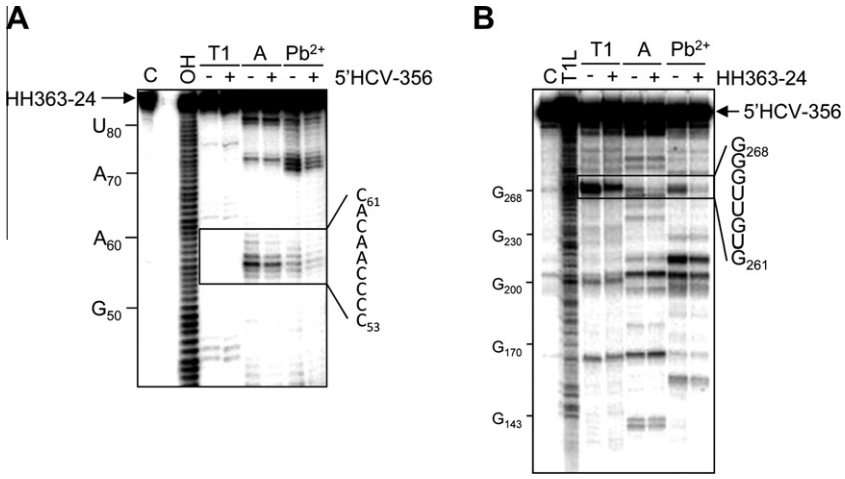

Fig. 2. Probing assays of the IRES:HH363-24 complex. (A) The figure shows an autoradiograph of the probing assay for HH363-24 in the absence (−) or presence (+) of the target RNA 5′HCV-356. Digestion reactions were initiated by the addition of hydrolytic reagents (RNase T1, RNase A or Pb^{2+}). Specific cleavage products were resolved in 6% high resolution denaturing polyacrylamide gels. Residues involved in the interaction with the HCV-IRES are boxed. (B) Identification of the targeted nucleotides in the substrate RNA 5′HCV-356. Trace amounts of the ^{32}P 5′ end-labelled 5′HCV-356 construct were partially digested with RNase T1, RNase A or Pb^{2+}, either in the absence (−) or presence (+) of HH363-24. Nucleotides resistant to degradation are boxed. C, control reaction without hydrolytic reagents. OH, alkaline ladder. T1L, T1 cleavage ladder.

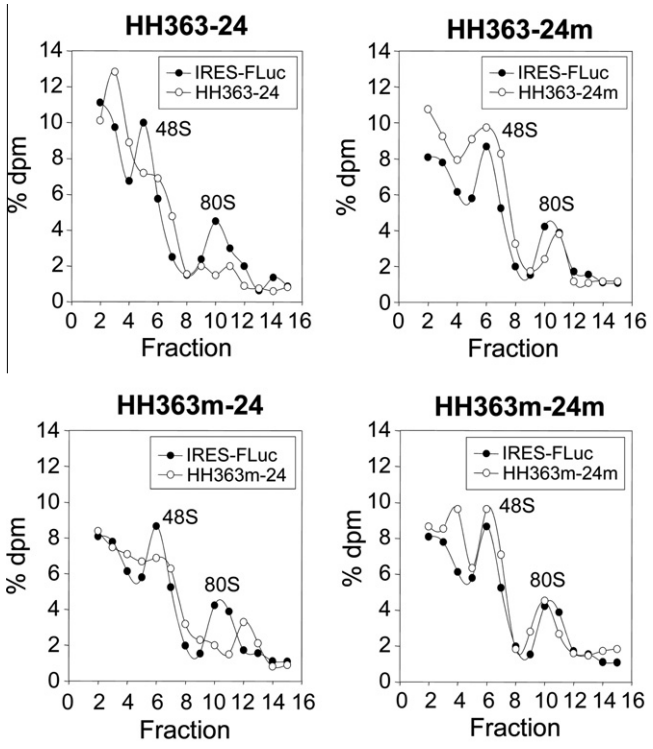

Fig. 3. The aptamer domain prevents the formation of the 48S and 80S translational particles. Sucrose gradient sedimentation profiles of ^{32}P-internally labelled IRES-FLuc mRNA incubated in rabbit reticulocyte lysates in the absence and presence of a molar excess of the RNA molecules HH363-24, HH363m-24, HH363-24m or HH363m-24m. Filled circles, control reaction profile; open circles, sedimentation profile with the inhibitor. In all cases, the percentage disintegrations are represented against the corresponding gradient fraction number. The 48S and 80S complexes are indicated. Fractions were collected from the top downwards.

domains essential for IRES activity (Romero-López and Berzal-Herranz, 2012). This prompted attempts to use ICU as a surrogate of the molecular and conformational environment of the HCV genome 5′ end. A human hepatic cell line, Huh-7, was co-transfected with a mixture containing the ICU reporter and the mRNA encoding the RLuc protein, which acted as an internal control. A molar excess of the chimeric inhibitor was added and the resulting luciferase levels compared with those obtained in the presence of a non-related RNA (RNA80). A significant reduction of around 80% of Fluc protein activity was detected in the presence of HH363-24 (Fig. 4A). No significant changes were seen in RLuc protein synthesis (<5%; data not shown). This shows that the inhibitory activity of HH363-24 does not affect cap-dependent translation. Tests were then performed to determine whether the catalytic module and its aptamer region remained active in the engineered molecule *ex vivo*. Inactivation of either element failed to significantly influence its inhibitory activity (Fig. 4A). No significant changes in the relative luciferase levels were seen for the null variant HH363m-24m with respect to the control reaction (Fig. 4A). These data show that both domains preserve their inhibitory function in cell culture and remain active in the chimeric compound.

The effect of HH363-24 on HCV RNA synthesis was investigated in cell culture. Huh-7 cells harbouring stable and autonomous subgenomic RNA replicons derived from HCV-1b (Huh-7 NS3-3′; (Larrea et al., 2006; Romero-López et al., 2009) were transiently transfected with the chimeric inhibitor or the non-related RNA80. Real time RT-PCR was used for relative quantification of the intracellular HCV RNA levels. The cellular mRNA coding for GAPDH protein was selected as an internal standard. The synthesis of the viral positive strand was clearly reduced to around 70% in the presence of the chimeric inhibitor HH363-24 (Fig. 4B). Importantly, mutations in either the hammerhead or the aptamer regions partially impaired this activity, with inhibition levels close to 40% (Fig. 4B). The transfection of the completely inactive variant, HH363m-24m, yielded no significant reduction in HCV RNA levels (Fig. 4B). This shows that the extension of the hammerhead ribozyme with an aptamer module for domain IIId provides the most efficient antiviral activity.

4. Discussion

The finding of novel therapeutic strategies for fighting multiple viral diseases is a major research goal. Targeting conserved structural and functional elements of viral genomes with nucleic acids, particularly RNA, has been shown an effective and promising alternative to non-specific antiviral treatments (Dausse et al., 2009; Puerta-Fernández et al., 2003; Romero-López et al., 2006). The design and engineering of therapeutic tools based on RNA might also

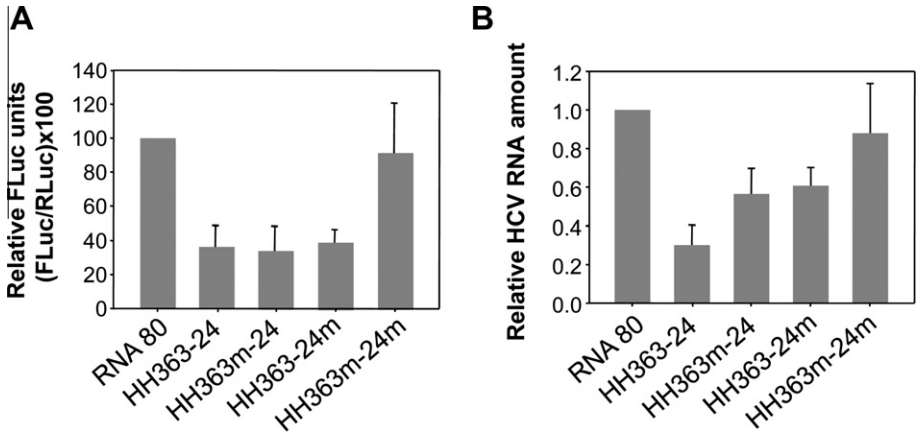

Fig. 4. HH363-24 inhibits IRES-dependent translation and HCV replication in a hepatoma cell line. (A) Huh-7 cells were co-transfected with different amounts of inhibitory RNAs and 1.5 μg of a mixture containing the transcripts IRES-FLuc and RLuc. IRES function was measured as the activity of FLuc protein and normalized to that obtained for RLuc. Luciferase activity in the control reactions was established as 100%. Data points are the mean of at least three independent experiments. (B) Inhibitory RNAs were transfected into Huh-7 cells stably supporting viral replication, Huh-7 NS3-3′. Intracellular total RNA was isolated 18 h after transfection and used for real time RT-PCR to measure viral RNA levels, which were referred to those detected for GAPDH mRNA. The results were analyzed using ABI PRISM SDS Software 1.1 (Applied Biosystems).

provide multi-target compounds with different antiviral functions that could inhibit the emergence of escape variants (Jarczak et al., 2005; Macejak et al., 2001). We previously proposed the feasibility of this approach for the inactivation of essential steps of the HCV cycle, such as protein synthesis and replication (Romero-López et al., 2005, 2007, 2009). This manuscript analyzes the mechanism of action of the potential HCV inhibitor, HH363-24. It is composed of a ribozyme core (which cleaves the viral genome at position 363) attached to an aptamer element for the conserved structural domain IIId in the IRES region. Both activities remain *in vitro* and *ex vivo* in the engineered molecule and their combination is a requisite for the strongest antiviral effect (Table 1, Fig. 4), suggesting that they cooperate in the overall conformation and functionality of HH363-24. This shows the potential of the method used (Romero-López et al., 2005) to select these compounds. Unfortunately, neither the present nor earlier data show whether the cleavage and domain IIId binding activities are undertaken by the same molecules. Indeed, previous data suggest that the inhibitory function of these chimeras cannot be explained by an additive effect of their independent modules (Barroso-delJesus et al., 2005; Kikuchi et al., 2009; Puerta-Fernández et al., 2005; Romero-López et al., 2005, 2007, 2009).

The results show that the IIId domain in the IRES is the main anchoring site of HH363-24 (Fig. 2B). The isolation of aptamers that bind to domain IIId has previously been reported (Kikuchi et al., 2005). This 'selection convergence' could be determined by the structural features of domain IIId, which is especially prone to interact with other viral genomic elements and proteins (Babaylova et al., 2009; Kolupaeva et al., 2000; Lafuente et al., 2002; Romero-López and Berzal-Herranz, 2009; Shimoike et al., 1999). The three-dimensional structure of domain IIId, resolved by NMR, reveals the formation of a stem with non-canonical pairings, closed by a highly disordered apical hexaloop (Klinck et al., 2000; Lukavsky et al., 2000). This adopts the so-called U-turn motif (Babaylova et al., 2009; Jubin et al., 2000; Klinck et al., 2000; Kolupaeva et al., 2000; Lukavsky et al., 2000), a widespread element employed by many RNA molecules to efficiently interact with other functional RNA domains or proteins (Brunel et al., 2002). Therefore, selection convergence may be influenced by the specific structural properties of domain IIId. This entails the recognition of a three-dimensional architecture by the aptamer, which provides increased specificity compared to antisense or siRNA molecules (Darfeuille et al., 2006). Moreover, HH363-24 interacts with the apical loop of domain IIId through a sequence motif located at an apical loop of the aptamer region. Apical loop-apical loop contact is a very efficient and commonly used mechanism for the regulation of biological processes (Brunel et al., 2002). This strategy could be successfully exploited to engineer effective inhibitory RNA molecules (Dausse et al., 2009; Puerta-Fernández et al., 2005; Romero-López et al., 2007, 2009).

The present work shows that targeting domain IIId efficiently interferes with the assembly of translational particles 48S and 80S (Fig. 3). We propose that HH363-24 inhibits the formation of active ribosomal complexes by the direct apical loop–apical loop interaction between the aptamer region and the domain IIId, leading to the efficient inhibition of IRES-dependent protein synthesis (Fig. 3 and 4A). Moreover, the catalytic activity on its own significantly affects to HCV translation and replication (Fig. 4) by directly cleaving the viral genome. Interestingly, the presence of the aptamer element on its own not only reduced IRES function but also HCV RNA levels (Fig. 4). This could be related to the fact that domain IIId has been shown to interact with the stem-loop 5BSL3.2 (Romero-López and Berzal-Herranz, 2009), which is an essential partner in viral replication (Lee et al., 2004; You et al., 2004). This contact regulates HCV IRES function (Romero-López and Berzal-Herranz, 2012), but no information exists regarding its involvement in viral replication. Thus, these data are insufficient to propose a detailed mechanism that could explain the inhibitory effect observed for HH363m-24. The present results show that targeting domain IIId is an effective way of interfering with both viral protein and RNA synthesis. To our knowledge, this is the first report describing this effect. Hence, the chimeric inhibitor HH363-24 is a potential tool for the investigation of additional roles of domain IIId in the HCV cycle.

In this work we have accomplished the investigation of the mechanism of action for the molecule HH363-24, which is a relevant issue that must be considered in the design of therapeutic approaches combining different inhibitor RNA molecules. The reported data point to domain IIId as the key target element in the antiviral activity for HH363-24, in contrast to previously isolated inhibitor RNAs HH363-10 and HH363-50, which target the highly conserved domains IIIf (Romero-López et al., 2007) and IV (Romero-López et al., 2009), respectively. Domain IIIf participates in the formation of a pseudoknot structure (Berry et al., 2011), an essential partner in the formation of translationally active complexes (Wang et al., 1995), while domain IV efficiently positions

the translation start codon at the P site (Ji et al., 2004; Lytle et al., 2002). Thus, the effect these molecules, HH363-10 and HH363-50, on ribosomal complex assembly and viral replication considerably diverges from that observed for HH363-24, resulting in different inhibitory effects for both the wild-type and the different mutant constructs tested. This argues for the importance of understanding the pathways that lead to the observed inhibitory effect. Moreover, the combination of inhibitor RNA molecules that interfere with different steps of essential routes for the viral cycle may provide novel efficient anti-HCV strategies.

Taken together, the present results confirm the effectiveness of adding an aptamer module to the 3′ end of a plain hammerhead ribozyme for the efficient targeting of genomic domains in viral RNAs (Barroso-delJesus et al., 2005; Puerta-Fernández et al., 2005; Romero-López et al., 2005, 2007, 2009). The investigations of the mechanism of action by which HH363-24 exerts its activity may contribute to a further rational design of novel anti-HCV agents with improved inhibitory properties. The engineering of molecules with dual specificity and activity could help prevent the appearance of escape variants and encourage strong interference of viral propagation.

5. Conclusions

The engineering of RNA compounds with different specificities and activities is a promising alternative strategy to generic antiviral treatments. They would provide strong inhibitory effects and could restrain the emergence of resistant virus variants. The selected molecule HH363-24 efficiently inhibits both HCV translation and replication, showing a potential antiviral activity. This calls for future investigations in the development of RNA conjugates with high internalization rates and intracellular stability. Finally, HH363-24 has great potential as a novel tool to study the functional contribution of domain IIId to the HCV cycle.

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