Multivalent engineered RNA molecules that interfere with hepatitis C virus translation and replication

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MULTIVALENT ENGINEERED RNA MOLECULES THAT INTERFERE WITH HEPATITIS C VIRUS TRANSLATION AND REPLICATION

Graphical abstract

**HCV IRES**

Translation ↓↓ 80%

Replication ↓↓ 80%
Abstract: The design of novel and efficient compounds fighting against the highly variable RNA viruses, such as hepatitis C virus (HCV), is a major goal. Engineering different antiviral RNAs into a single molecule yields the so-called multivalent compounds, which are promising candidates for the development of new therapeutic strategies. In this work, the previously developed chimeric inhibitor RNA HH363-10 was used as archetype for the development of improved anti-HCV inhibitors. HH363-10 consists of a hammerhead ribozyme domain, targeting the essential internal ribosome entry site (IRES) region; and an aptamer RNA molecule, directed against the highly conserved IIIf domain of the IRES. Following to the application of an in vitro selection process, new multivalent optimized chimeric anti-HCV RNA molecules derived from HH363-10 were isolated. The aptamer RNA domain was evolved to contain two binding sites: the one mapping the IIIf domain, and a newly acquired targeting site, either to the IRES domain IV (which contains the translation start codon) or the essential linker region between the IRES domains I and II. These chimeric molecules efficiently and specifically interfered with HCV IRES-dependent translation in vitro (with IC$_{50}$ values in the low µM range). They also inhibited both viral translation and replication in cell culture. These findings highlight the feasibility of using in vitro selection strategies for obtaining improved, multivalent RNA molecules with potential clinical applications.

Keywords: RNA aptamer; hepatitis C virus; IRES; RNA targeting
THERAPEUTIC NUCLEIC ACIDS

The structural flexibility of nucleic acids, particularly RNA, results in their great functional versatility. This has led to propose the use and engineering of nucleic acids as new therapeutic drugs targeting the genetic information of viruses. Moreover, advances in chemical synthesis techniques have improved the possibilities of developing therapeutic RNA molecules.

**RIBOZYMES**
Small catalytic RNAs are all naturally involved in the replication process of RNA genomes in which they are contained. Potentially therapeutic ribozymes includes the hammerhead and the hairpin ribozyme.

**APTAMERS**
Aptamers are oligonucleotides able to efficiently bind to a wide variety of ligands. Aptamers are isolated by a SELEX (Systematic Evolution of Ligands by Exponential enrichment) process, which consists on iterative cycles of synthesis, binding, positive selection and amplification steps over a randomized oligonucleotide pool.

The combination of different RNA molecules with proven antiviral activity renders the so-called multivalent compounds, chimeric entities with enhanced therapeutic properties.
The isolation of RNA molecules directed against different targets of the hepatitis C virus (HCV) genome has been largely described. The HCV genome is a (+)ssRNA molecule encoding a single open reading frame (ORF) flanked by untranslated regions (UTRs), which are essential for viral replication, translation and infectivity. Viral translation initiation is mediated by a highly structured motif mainly located at the 5'UTR, which acts as an internal ribosome entry site (IRES). The presence of structural elements at the 3' end of the viral genome may also modulate the initiation and elongation steps involved in HCV translation. This might be mediated via the HCV genomic RNA, assuming a circular topology resembling the closed-loop structure adopted by cellular cap-mRNAs. Such circular formations depend on the existence of a direct, long-range RNA-RNA interaction, involving subdomain IIIId of the IRES element and the essential stem-loop 5BSL3.2 of the CRE (cis-acting replication element) region at the 3' end of the viral protein coding sequence.
THE HCV IRES REGION AND
THE PARENTAL HH363-10 RNA INHIBITOR

The minimum HCV IRES element required for translation initiation folds into a well-defined structure composed of two major domains (II and III) and a short stem-loop (domain IV) containing the initiation codon (enlarge lettering in the figure). Domains II and III are aligned at both sides of a complex double pseudoknot structure allowing the correct positioning of the start codon (PK1 and PK2). Importantly, many of the crucial structural regions required for IRES-dependent translation are located in the highly branched domain III. The cleavage site of the HH363-10 inhibitor RNA is marked by an arrow. The nucleotides that interact with the aptamer domain of HH363-10 are shown in red.

The RNA inhibitor HH363-10 was isolated by an in vitro selection method. The catalytic hammerhead domain, HH363, is shadowed. Tertiary contacts are indicated by dotted lines. Residues in the aptamer domain responsible for the interaction with domain IIIf of the IRES are shown in red.
IN VITRO SELECTION OF ANTI-HCV MULTIVALENT COMPOUNDS AGAINST THE IRES REGION OF THE HCV GENOME

The chimeric inhibitor HH363-10 was subjected to an in vitro selection procedure, with the aim of identifying inhibitory RNA molecules that act against the IRES region of the HCV genome, and that showed improved aptamer and catalytic properties. For this, an RNA pool was designed, based on the HH363-10 inhibitor, adhering to the following criteria: i) the maintenance of the sequence motif responsible for the aptamer’s interaction with the domain IIIf of the IRES; ii) randomization of the nucleotides flanking the interacting sequence with the aim of isolating RNA molecules bearing secondary anchoring sites targeting additional sites of the HCV IRES region besides the primary domain IIIf.
ISOLATION OF MULTIVALENT INHIBITORY RNAs

After seven selection rounds, 10 RNA variants (HH-11, HH-13, HH-15, HH-17, HH-22, HH-24, HH-26, HH-28, HH-33 and HH-43) were isolated. Sequence analysis using the RNAup software tool showed new theoretical interaction sites to map to the conserved domain IV, and to the interdomain I-II regions. This showed the selection strategy to have worked properly.

The sequences of the 10 selected chimeric inhibitory RNAs after seven rounds of selection are boxed. Residues in grey denote the conserved catalytic domain of the inhibitory RNA. The sequence motif involved in the interaction with the domain IIIf is indicated in red. The nucleotides theoretically targeting domain IV and the linker sequence between domains I and II within the IRES are pictured in blue and green, respectively. The unique sequence in HH-26 that binds to the apical loop of IRES domain IV is also underlined.
INHIBITION OF HCV IRES-DEPENDENT TRANSLATION IN VITRO BY THE SELECTED CHIMERIC RNAs

The activity of the different chimeric inhibitors was examined in in vitro translation assays involving two monocistronic RNA molecules (used as templates) in rabbit reticulocyte lysates. One of these monocistronic RNAs, IRES-FLuc, encompasses the first 698 nts of the HCV genome, containing the IRES region, which directs the synthesis of the reporter Fluc protein; the other, cap-RLuc, codes for the RLuc enzyme (which is translated in a cap-dependent manner). Each inhibitor was assayed at a concentration of 5 µM. HH-11, HH-13, HH-26, HH-28 and HH-43 efficiently interfered with IRES function, showing inhibition values close to 97%. HH-15, HH-17, HH-22, HH-24 and HH-33 also promoted a reduction in Fluc synthesis, but to a lesser extent (20-60%). Cap-dependent translation was not affected for any of the assayed chimeric inhibitory RNAs at the concentration tested, demonstrating the specific nature of the inhibition of IRES function.
INHIBITION OF HCV IRES-DEPENDENT TRANSLATION IN VITRO
BY THE SELECTED CHIMERIC RNAs

The potential of the five most efficient chimeric inhibitor RNAs - HH-11, HH-13, HH-26, HH-28 and HH-43 - as IRES interfering agents was then further characterised using different concentrations of each, ranging from an IRES-FLuc:inhibitor RNA molar ratio of 1:1 to 8:1. Potent dose-dependent inhibition was recorded for HH-11, with an IC\textsubscript{50} value of 170 ± 20 nM and a maximum inhibition of close to 100%. These results are better than those reported with the parental chimeric inhibitor HH363-10, which showed a maximum inhibition of 90%. More importantly, maximum inhibition with HH-11 was attained at 1 µM, whereas HH363-10 required a concentration of 2.5 µM to achieve the 90% inhibition. Inhibitor RNAs HH-13, HH-26, HH-28 and HH-43 also affected the function of the HCV IRES in a dose-dependent manner, with IC\textsubscript{50} values in the low µM range and maximum inhibition values of 90-95%.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC\textsubscript{50} (µM)\textsuperscript{a}</th>
<th>Relative Fluc synthesis\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH363-10</td>
<td>0.15 ± 0.04</td>
<td>11.22 ± 2.77</td>
</tr>
<tr>
<td>HH-11</td>
<td>0.17 ± 0.02</td>
<td>0.01 ± 1.10</td>
</tr>
<tr>
<td>HH-13</td>
<td>0.91 ± 0.31</td>
<td>8.78 ± 2.21</td>
</tr>
<tr>
<td>HH-26</td>
<td>1.46 ± 0.24</td>
<td>9.25 ± 1.47</td>
</tr>
<tr>
<td>HH-28</td>
<td>0.44 ± 0.06</td>
<td>2.56 ± 1.34</td>
</tr>
<tr>
<td>HH-43</td>
<td>1.02 ± 0.15</td>
<td>1.00 ± 0.40</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} values of the different inhibitory RNAs derived from the equation $y = \frac{100}{1+10^{\log(\text{IC}_{50})}}$.

\textsuperscript{b} Data correspond to the highest concentration of inhibitor tested. Values are the mean of three independent assays ± standard deviation.
ANTIVIRAL SPECIFICITY OF THE MULTIVALENT RNAs

To further investigate the specificity of the inhibitors against the HCV IRES region, *in vitro* translation assays were performed with the construct GBV-B IRES-FLuc, which bears the IRES region of the closely related hepacivirus GBV-B fused to the coding sequence for the FLuc protein. Interestingly, only the chimeric inhibitor HH-11 significantly affected GBV-B IRES activity, while the rest of the tested inhibitory RNAs showed marked specificity for the HCV IRES region. The notable conservation of the 5'UTR among hepacivirus suggests that HH-11 might target a common key structural motif, thus resulting in the inhibition of GBV-B IRES activity. It should be noted that although HH-11 interfered with GBV-B IRES-dependent translation, it did not affect cap-mediated RLuc synthesis. This shows that HH-11 is not a generic translation inhibitor, but rather that it is specific for sequence and/or structural motifs in the hepacivirus IRES.

![Inhibition of GBV-B IRES function in vitro](image)
The effect of the HH-11, HH-13, HH-26, HH-28 and HH-43 chimeric inhibitors on HCV IRES-dependent translation was also evaluated in cell culture. Human hepatoma cell line Huh-7 was co-transfected with a mixture containing the RNA constructs IRES-FLuc and cap-RLuc and a molar excess of an inhibitory RNA (25:1). The resulting data were compared with those obtained in the presence of a non-related RNA molecule, RNA80. The five tested molecules provoked a significant reduction (60-80%) in HCV-IRES activity. HH-11 appeared as a potent inhibitory RNA, in good agreement with the results of the in vitro translation assays. HH-26 and HH-28 also induced significant reductions (nearly 80%) in IRES activity, showing them to be improved anti-HCV agents.
THE CHIMERIC INHIBITOR RNAs INHIBIT HCV REPLICATION IN CELL CULTURE

Huh-7 cells bearing a subgenomic replicative RNA molecule derived from HCV (Huh-7 NS3-3’) were transiently transfected with 175 nM of inhibitory RNA or the non-related RNA80 molecule. The amount of replicative HCV RNA was determined 24 h post-transfection by RT-qPCR, and normalized to that of the intracellular mRNA encoding for GAPDH protein. The selected chimeric inhibitors significantly interfered with HCV replication, promoting a 60-80% reduction in viral RNA positive strands with respect to the control assay. Interestingly, these data contrast with those obtained for HH363-10, which affected HCV replication much more weakly (~25% reduction in HCV RNA levels). This result confirms the improved inhibitory ability of the selected chimeric inhibitor RNAs over the parental HH363-10.
**IN SILICO FOLDING ANALYSIS OF THE CHIMERIC RNAs**

RNAstructure software was used for in silico folding analysis of the chimeric compounds. These studies revealed that the catalytic hammerhead domain was preserved and the aptamer domain folded as a single stem-loop, suggesting a structural and functional convergence during the selection procedure. The newly acquired sequence motif (in blue or green) could be used as a secondary anchorage after the initial interaction with domain IIIf, which fits well with the hypothesis that both anchoring sites could be used by a single, multivalent compound. This agrees with the potent inhibitory effect promoted by the chimeric inhibitory RNAs.

**Results and Discussion**
CONCLUSIONS

An *in vitro* selection method was performed to isolate novel chimeric inhibitory RNA molecules based on the previously designed HH363-10 compound.

After seven selection rounds, ten different RNA molecules were isolated.

Sequence analysis using the RNAup software tool showed new theoretical interaction sites that map at the conserved domain IV, and to the interdomain I-II regions.

Highly efficient inhibition *in vitro* of the HCV IRES function was assessed for five of the ten selected chimeric RNA molecules, showing inhibition values closed to 97%. Importantly, a potent dose-dependent inhibition was recorded for HH-11, with an IC$_{50}$ value of 170 nM.

The five tested molecules provoked a significant reduction (60-80%) of HCV-IRES activity in cell culture. HH-11 appeared as a potent inhibitory RNA, in good agreement with the results of the *in vitro* translation assays.

The selected chimeric inhibitors significantly interfered with HCV replication, promoting a 60-80% reduction in viral RNA positive strand synthesis. These data contrast with those obtained for HH363-10, which affected HCV replication much more weakly (~25% reduction in HCV RNA levels).

The results confirms the improved inhibitory ability of the selected chimeric inhibitor RNAs over the parental HH363-10.
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