Application of RNA aptamers to the control of the hepatitis C virus-CRE region function

Alba Fernández-Sanlés †, Beatriz Berzal-Herranz †, Rodrigo González-Matamala, Pablo Ríos-Marco, Alfredo Berzal-Herranz * and Cristina Romero-López *

† Equal contribution

Instituto de Parasitología y Biomedicina López-Neyra, IPBLN-CSIC. Av. Conocimiento s/n. PTS Granada, 18016. Armilla (Granada), Spain

* Corresponding author: aberzialh@ipb.csic.es; cristina_romero@ipb.csic.es
APPLICATION OF RNA APTAMERS TO THE CONTROL OF THE HEPATITIS C VIRUS-CRE REGION FUNCTION

HCV RNA genome

CRE

Replication >90%

3'X-tail

3'SLIII

3'SLII

3'SLI

3'UTR

IRES

ORF

AUG start codon

Stop codon

PK1

PK2

Alt

5BSL3.2

5BSL3.1

5BSL3.3

PolyU/UC

HV
Abstract:
Hepatitis C virus is an enveloped, ssRNA virus, which infects 3% of the world population. No fully
efficient therapy for treating hepatitis C exists. This is mainly due to the quasispecies structure of
the RNA genome population, which favors the emergence of resistant viral variants. Despite the
high variability rate, significant sequence and, more importantly, structure conservation can be
found in the so-called functional genomic RNA domains, many of them with unknown roles for the
consecution of the viral cycle. Such genomic domains are potential therapeutic targets. This study
validates the use of RNA-based inhibitors (aptamers) as molecular tools to control the activity of
the cis-acting replication element (CRE) within the HCV genome. The CRE is an essential partner
for viral replication. Also this structural domain is involved in the regulation of the protein synthesis.
A set of forty-four RNA aptamers was assayed for the ability to interfere with the viral RNA
synthesis in a subgenomic replicon system. Four aptamers emerged as potent inhibitors of HCV
replication by direct interaction with specific and well-defined functional RNA domains of the CRE,
yielding a decrease in the HCV genomic RNA levels higher than 90%. Concomitantly, one of them
also promoted a significant increase in viral translation (>50%), likely by its interaction with the
nucleotides surrounding the viral stop translation codon. The three remaining aptamers efficiently
competed with the binding of the NS5B protein to the CRE, thus explaining their antiviral activity.
Present findings confirm the potential of the CRE as an anti-HCV drugs target and support the use
of aptamers as molecular tools for challenging the functionality of RNA domains in viral genomes.

Keywords: RNA aptamers; antiHCV Aptamers; HCV genome; CRE; 5BSL3.2; functional RNA
domains.
Aptamers are oligonucleotides able to recruit a wide variety of ligands. Aptamers are isolated from 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 resulting population is enriched in those molecules able to bind to the desired target molecule. The highly dynamic folding of nucleic acids is the key to understand the specific and efficient interaction of aptamers to their cognate target, thus demonstrating the versatility and flexibility of nucleic acids.
The isolation of aptamers directed against different protein targets of the hepatitis C virus (HCV) 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. Conserved functional RNA domains have also been identified within the coding region, such as the cis-acting replication element (CRE), which is defined by three stem-loops, 5BSL3.1, 5BSL3.2 and 5BSL3.3. The 5BSL3.2 domain is critical for efficient HCV replication and the regulation of viral protein synthesis. These functional features depend on the establishment of long-distance RNA-RNA interactions with other genomic RNA domains. In addition, the 5BSL3.2 domain interacts with viral and host protein factors.

NS5B, viral RNA-dependent RNA polymerase; EWSR1, Ewing sarcoma RNA-binding protein 1
ANTI-HCV RNA APTAMER SELECTION AGAINST THE CRE REGION

Introduction

PBS, primer binding site; R, randomized sequence; T7p, T7 promoter
Anti-HCV RNA Aptamer Selection against the CRE Region

After six rounds of selection, a set containing forty-four different aptamers isolated for their binding ability to the CRE was assayed for their capacity to interfere with the CRE functionality: replication, translation and binding to the viral RNA-dependent RNA polymerase (NS5B). All of them bear sequence motifs (the so-called consensus motifs, indicated as groups 1-5) complementary to different conserved elements within the HCV CRE.
INHIBITION OF HCV REPLICATION BY A COLLECTION OF RNA APTAMERS TARGETING THE HCV-CRE REGION

Huh-7 cells stably bearing a dicistronic selectable subgenomic replication construct derived from HCV-1b genotype (Huh-7 NS3-3’ET) were transfected with the different aptamers independently. Total RNA was extracted 18 h post-transfection and the relative amount of the subgenomic HCV RNA was monitored by quantitative RT-PCR. Transfection with a non-related small RNA molecule, RNA80 was used as control experiment for data calibration. From this functional screening, four aptamers – P6-89, P6-96, P6-103 and P7-49 – emerged as potential tools for HCV detection and inhibition.
Aptamers-mediated inhibition is directly related to their biochemical features, such as their three-dimensional folding and binding affinity to their target. *In silico* structural analysis of the RNA molecules P6-89, P6-96, P6-103, and P7-49 was performed with the aim of identifying common structural motifs that could define a functional domain within the inhibitor RNA. The TurboFold tool was employed for that goal. The use of this strategy reported a common secondary structure for the tested inhibitors, in which the constant sequences, corresponding to the ones used as primer binding site (PBS) during the selection process, appeared as single-stranded tails flanking the stem-loop containing the selected consensus motifs. These nucleotide motifs locate, at least partially, exposed in the apical loop. This folding gives the idea that the functional unit in the aptamer molecules is restricted to the stem-loop, which is used to efficiently interact with the target site in the CRE, in a similar way to that previously described for other regulatory RNA molecules.

**Results and discussion**
EFFICIENT BINDING OF THE SELECTED APTAMERS TO THE CRE REGION

Binding affinity was analyzed by incubating a constant concentration of each $^{32}$P-internally labeled aptamer (~2 nM) with increasing amounts of the unlabeled CRE. The titration curve showed differential interaction efficiency for the different aptamers under study. The results suggest that the selected aptamers P6-96, P6-103, and P7-49 may exert their anti-HCV activity by directly interacting with the CRE region.

Results and discussion

| Aptamer | $K_d$ (nM) ± SD | $B_{max}$ ± SD |
|---------|----------------|-----------------|
| P6-89   | 1706.34 ± 230.15 | n.d.           |
| P6-96   | 62.67 ± 0.74    | 1.15 ± 0.05    |
| P6-103  | 9.47 ± 3.49     | 1.04 ± 0.14    |
| P7-49   | 43.63 ± 16.22   | 1.07 ± 0.07    |
**Aptamers Targeting the 5BSL3.2 Domain Compete with the Recruitment of the Viral RNA-Dependent RNA Polymerase by the CRE Region**

The 5BSL3.2 domain binds to the viral NS5B protein. Since some of the inhibitors harbor complementary sequence motifs to the 5BSL3.2 domain, it seemed reasonable to monitor whether the aptamers P6-89, P6-96, P6-103 and P7-49 could compete with the viral polymerase recruitment, as replication inhibition mechanism. For that purpose, the aptamers were subjected to *in vitro* binding assays with the CRE RNA in the presence of the recombinant viral polymerase NS5BΔ21. Increasing concentrations of the aptamers under study were employed and the EC$_{50}$ value was calculated. The results showed that molecules P6-89, P6-96 and P6-103 efficiently interfered with the binding of the NS5B protein to the CRE in a concentration-dependent manner with EC$_{50}$ values in the nM range. Interestingly, the aptamer P7-49, which theroretically targets the stem-loop containing the stop codon, barely showed a slight competitor activity.

\[
y = \frac{100}{1 + 10^{(\log\text{EC}_{50} - x)}}
\]

| Aptamer | EC$_{50}$ (nM) | CRE:NS5BΔ21 Complex (%) |
|---------|----------------|-------------------------|
| P6-89   | 14.59 ± 1.11   | 5.09 ± 2.04             |
| P6-96   | 38.68 ± 5.63   | 0.00 ± 4.15             |
| P6-103  | 8.57 ± 0.58    | 11.98 ± 1.46            |
| P7-49   | Non determined | 75.37 ± 3.05            |

Results and discussion
**EFFECT OF THE APTAMERS ON HCV TRANSLATION**

Aptamers P6-89, P6-96, P6-103 and P7-49 contain sequence motifs complementary to functional RNA domains that are involved in the regulation of HCV translation, such as the 5BSL3.2 and the stem-loop containing the stop codon. This observation led us to study their role on viral protein synthesis in *ex vivo* translation assays. With that aim, a mixture containing the so-called transcripts cap-Rluc (for correcting potential different transfection yields), Rep-FLuc and a 20-fold molar excess of the aptamers or the non-related RNA80 was used to transfect Huh-7.5 cells. This strategy allows for measuring IRES activity in the early post-transfection period (4-20 h). The obtained results suggest that the tested inhibitors P6-96 and P6-103 specifically interfere with the HCV cycle mainly by blocking the replication step, while the molecules P6-89 and P7-49 exert a double role as replication interfering agents and HCV translational enhancers.

---

**Results and discussion**
The role of P7-49 as IRES-mediated translation inductor could be derived from its interaction with other essential domains of the CRE. By using the folding softwares RNAcofold and RNAup, it was detected a major interacting site in the apical loop of the 5BSL3.4 domain, involving the stop codon. This newly predicted targeted motif differs slightly from that previously defined as that of the group five from the initial sequence and clustering analysis. By modifying the conformation of the environment surrounding the stop codon, the ribosomal recycling could be enhanced to improve the IRES-dependent translation rate. Work in this area is currently being accomplished in our laboratory, since this idea opens new fields to evaluate the real role of the stem-loop containing the stop codon in the viral cycle, and for that purpose, P7-49 could be used as a novel molecular tool.

Theoretical model for the interaction between the 5BSL4 domain and the P7-49 RNA aptamer. Residues proposed to initiate the kissing-loop interaction are boxed. Nucleotides belonging to groups 1 and 2 are colored in gray and blue, respectively. PBS, primer binding site.
Four aptamers have emerged as potent inhibitors of HCV replication by direct interaction with functional RNA domains of the CRE region, yielding a decrease in the HCV RNA levels higher than 90%.

One of the selected aptamers, P7-49, also induced a significant increase in viral translation (>50%) by its direct interaction with the stem-loop containing the stop codon, 5BSL3.4.

The three remaining aptamers, P6-89, P6-96 and P6-103, efficiently competed with the binding of the NS5B protein to the CRE.

Our data confirm the potential of using the 5BS3.2 domain as antiviral target and support its function in viral translation and in the recruitment of the viral RNA-dependent RNA polymerase.

The results also point to the domain 5BSL3.4 as a novel efficient antiviral target and open a new field to investigate the role of this element in the consecution of the viral cycle.

The use of aptamers as molecular tools is a feasible strategy for investigating the functionality of RNA domains in viral genomes.
ACKNOWLEDGEMENTS

Dr. Alicia Barroso-delJesus
Vicente Augustín-Vacas

Dr. Esteban Domingo

Dr. Rafael Aldabe