RNA APTAMERS SPECIFIC FOR THE HCV NS3 PROTEASE AND HELICASE DOMAINS

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Abstract

The hepatitis C virus (HCV) non-structural protein 3 (NS3) is a multifunctional enzyme with protease and helicase activities. It is essential for HCV replication and proliferation and is therefore a target for anti-HCV drugs. To obtain efficient RNA aptamers specific for HCV NS3, and to develop inhibitors of HCV replication, we performed *in vitro* selection for the NS3 protease and helicase domains, respectively. *In vitro* selection, namely, SELEX (systematic evolution of ligands by exponential enrichment) is a useful strategy for isolating nucleic acid sequences that have a high affinity for a target molecule from a randomized oligonucleotide pool. Isolated RNA aptamers showed effective inhibition against either the protease or helicase activity of NS3 *in vitro*. In addition, new type of RNA ligands, which displayed dual-inhibitory functions targeting both NS3 protease and helicase activities were constructed. Furthermore, NS3 protease aptamers effectively inhibited NS3 protease activity in living cells.

Keywords: HCV, NS3, protease, helicase, *in vitro* selection, SELEX, aptamer

Introduction

The hepatitis C virus (HCV) is the major etiological agent of non-A, non-B hepatitis, with a worldwide carriage rate of 3%. Most patients develop chronic hepatitis, and persistent infection often leads to liver cirrhosis or hepatocellular carcinoma. Recently, the most effective treatment for HCV has been found to be a combination of interferon and ribavirin. However, not all subtypes respond to treatment and the drugs can cause serious side effects [1]. The development of anti-HCV drugs with greater safety and efficacy is therefore a priority.

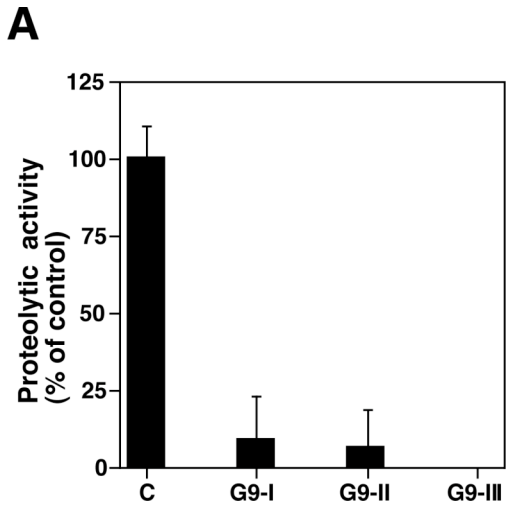
HCV is a single-stranded RNA virus that belongs to the Flaviviridae family [2]. The ~9.6-kb genome of positive polarity encodes a precursor polyprotein (~3,010 amino acids), which is processed to structural (core protein C, and envelope glycoproteins E1 and E2) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins by a host signal peptidase and two viral proteases, NS2-3 and NS3 [3]. The NS3 protein is a multi-functional enzyme with a trypsin-like protease within the amino (N)-terminal one-third, and an NTP-dependent RNA/DNA helicase in the remaining two-thirds [4–6]. It is generally accepted that the protease domain cleaves the junctions between the non-structural proteins, together with cofactor NS4A [7]. The helicase domain unwinds the double-stranded RNA generated by the RNA-dependent RNA polymerase NS5B during genome replication [8, 9]. As NS3 is essential for HCV replication and proliferation, new anti-HCV drugs could potentially be directed against this protein [10, 11]. One source of potential highly selective NS3 inhibitors might be RNA ligands, called **RNA aptamers**. Several RNA aptamers specific for HCV NS3 have been identified using an *in vitro* selection method (namely, systematic evolution of ligands by exponential enrichment [SELEX]) in our laboratory [12–16].

SELEX is a protocol for isolating rare nucleic acid sequences that have high affinity for a target molecule from a randomized oligonucleotide pool [17, 18]. *In vitro* selection has been used to isolate high affinity DNA or RNA ligands for target molecules including proteins, organic dyes, and amino acids [19]. Like antibodies, aptamers fold on the basis of nucleic acid sequence into three-dimensional structures that bind to their targets. Thus, aptamer binding to its target is a highly specific interaction,

discriminating between related proteins that share common sets of structural domains. Therefore, aptamers are an attractive class of molecules for use as therapeutic compounds. This review describes the isolation and characterization of RNA aptamers that bind with high specificity to the HCV NS3 protease and helicase domains, respectively. Furthermore, application of NS3 protease aptamers *in vivo* as antiviral compounds against HCV is also evaluated.

RNA aptamers specific for the HCV NS3 protease domain

Since the NS3 protease of HCV is essential for processing the viral polyprotein, its inhibition blocks viral replication and proliferation. Thus, there much interest in the possibility of using an inhibitor of the NS3 protease as an anti-HCV drug. To inhibit the HCV NS3 protease activity, RNA aptamers specific for the NS3 protease domain (ΔNS3) were selected by *in vitro* selection using a synthetic 30N random RNA pool [12]. Highly efficient RNA aptamers G9-I, -II, and -III (Table 1) were selected that contain a conserved loop sequence [5'- GA(A/U)UGGGAC -3'] and were efficient inhibitors of the NS3 protease *in vitro*. The NS3 protease activity toward a synthetic substrate was almost completely inhibited by a fivefold molar excess of the selected RNA aptamer (Fig. 1A and 1B). Furthermore, even in the presence of P41 peptide from NS4A, which is a stimulating cofactor for NS3 protease, 70% of the protease activity was inhibited by the selected RNA aptamers (Fig. 1C). In addition, these aptamers are all specific to ΔNS3 and do not bind to any other member of trypsin-like serine proteases, such as trypsin, chymotrypsin, α-thrombin, and activated protein C. In the case of the G9-I aptamer, both K_d and K_i values were in the nM range and mutational analysis demonstrated that the conserved loop sequence is crucial for the protease inhibition.



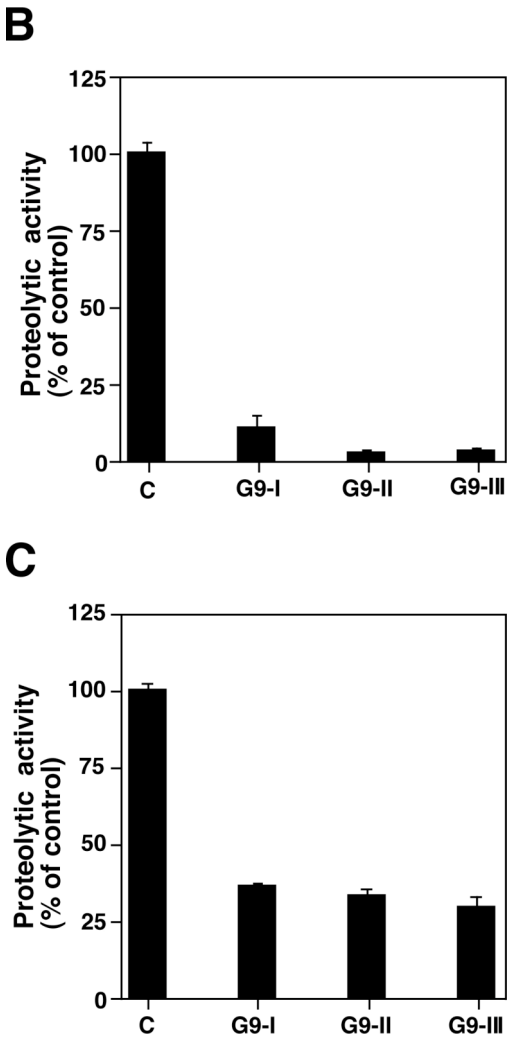


Fig. 1. Inhibition of the NS3 protease with RNA aptamers. The substrate S1 (86 μM) was mixed with (A) ΔNS3 (0.75 μM) and RNA aptamer (3.6 μM), (B) MBP-NS3 (2.0 μM) and RNA aptamer (10.0 μM) or (C) MBP-NS3 (2.3 μM), RNA aptamer (11.3 μM) and NS4A peptide (P41; 22.5 μM). After incubation, the reaction mixture was analyzed on a reverse phase HPLC and relative protease activity was calculated by normalizing to the activity in the reaction containing only ΔNS3 or MBP-NS3 (C, control).

The RNA aptamer-binding site of HCV NS3 protease

Further kinetic analysis of G9-I aptamer (an NS3 protease aptamer) indicated that G9-I aptamer is noncompetitive (i.e., does not compete with the substrate at the catalytic pocket of the protease) [12]. To elucidate the binding site of G9-I aptamer in NS3 protease domain, we carried out alanine scanning mutagenesis at positive charged residues on the surface of ΔNS3 [20]. The result of binding analysis by surface plasmon resonance measurements and protease inhibition assay clarified that Arg161 as well as

Arg130 of ΔNS3 are essential for interaction with G9-I aptamer.

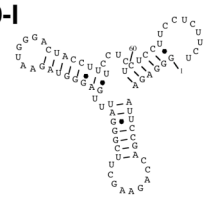
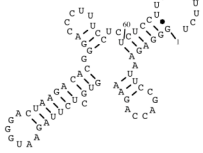
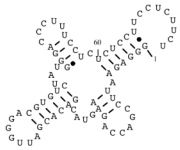
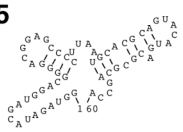
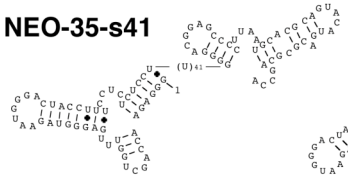
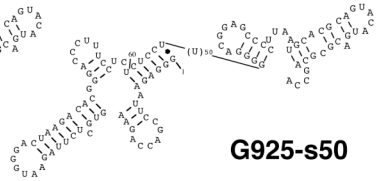
It is speculated that Arg161 and Lys165 in NS3 protease are located near the P6 position, which is the site of the conserved acidic amino acid that binds to the substrate [21]. X-ray structural analysis of entire NS3 protein [22] suggests that both local and global conformational changes occur during processing of HCV polyprotein. The polyprotein substrate itself may promote structural changes by forming a weak solvent-exposed salt linkage between the P6 acidic residue of the substrate and the basic (positive) amino acids, Arg161 and Lys165, in NS3. These residues may play a critical role in substrate recognition. Thus, this region appears to be a potential targeting site for anti-HCV drugs.

RNA aptamers specific for the HCV NS3 helicase domain

To obtain efficient RNA aptamers specific for the helicase domain of HCV NS3, and to develop an inhibitor of replication of HCV mRNA, we performed *in vitro* selection for the NS3 helicase domain using an RNA library containing N30 random core sequences [13]. Aptamers were obtained after eight rounds of selection using the competitor (poly U), which inhibited the helicase activity of NS3. Most RNA aptamers possessed 5' extended single-stranded regions and the conserved sequence [5'-GGA(U/C)GGAGCC-3'] in the stem-loop regions. Among them, aptamer #5 (Table 1) showed the highest inhibition activity ($IC_{50} \approx 50$ nM) against full-length NS3. Deletion and mutagenesis analysis of aptamer #5 showed that the conserved stem-loop is important and that the whole structure is needed for helicase inhibition.

Characterization of aptamer #5 led to the proposed mechanism of HCV replication. During the virus life cycle, the 3'(+/-)UTR of HCV is essential for replication and facilitates formation of the RNA polymerase complex containing non-structural viral proteins. NS3 has strong affinity for HCV 3'(+)UTR whose secondary structure is composed of three unique domains: a variable region (30-40 nt) and a poly (U) or poly (U/UC) tract (20-200 nt) followed by the highly conserved 3'X region [23]. There is some configurational similarity between 3'(+)UTR and RNA aptamers against NS3 helicase but no sequence similarity. Comparison of the helicase inhibition activity of aptamer #5 and HCV 3'(+)UTR led to the speculation that the variable region in 3'(+)UTR may regulate the NS3 helicase activity. Considering the expected involvement of this region in the regulation of NS3, the poly (U/UC) tract might provide an assembly site for NS3 to form the replication complex with other proteins. Therefore the

Table 1. The secondary structures of NS3 protease and helicase aptamers.

Target	Aptamer	Reference
NS3 protease	G9-I  G9-II  G9-III 	[12]
NS3 helicase	#5 	[13]
NS3 protease & helicase	NEO-III-14U 	[14]
NS3 protease & helicase	NEO-35-s41   G925-s50	[15]

strength of helicase inhibition might be affected by the efficiency of replication.

An RNA ligand inhibits HCV NS3 protease and helicase activities

NS3 helicase has been reported to bind preferentially to the poly(U) sequence, which is located in the 3′ untranslated region (UTR) of the HCV genome [23] and is necessary for viral replication [24]. The substrate-binding site is located along the cleft between the first two domains of the helicase and the third domain of the tertiary structure, complexed with a deoxyuridine octamer (dU8) [25]. As mentioned above, we obtained RNA aptamers that specifically and efficiently inhibited NS3 protease activity (G9 aptamers). To add helicase-inhibition capability, we attached (U)14 to the 3′-terminal end of a minimized G9 aptamer, ΔNEO-III [14]. The resulting NEO-III-14U (Table 1) was shown to inhibit NS3 protease activity more efficiently than the original aptamer and, furthermore, to efficiently inhibit the unwinding reaction by NS3 helicase. In addition, NEO-III-14U had the potential to suppress specific interaction between NS3 and the 3′ UTR of HCV-positive and -negative strands.

Members of the Flaviviridae family, such as the yellow fever virus, Dengue virus, Japanese encephalitis virus, and bovine viral diarrhea virus 1, are also known to have an NS3 protein with both protease and helicase domains [26]. Thus, the new strategy of designing an anti-viral RNA inhibitor by fusing a specific protease aptamer to a short single-stranded oligonucleotide should be applicable to other viral NS3 proteins.

Rational design of dual-functional aptamers that inhibit the protease and helicase activities of HCV NS3

To develop a novel aptamer with enhanced dual-inhibitory activity against the protease and helicase functions of NS3, advanced dual-functional (ADD) aptamers were created by fusing previous aptamers with known

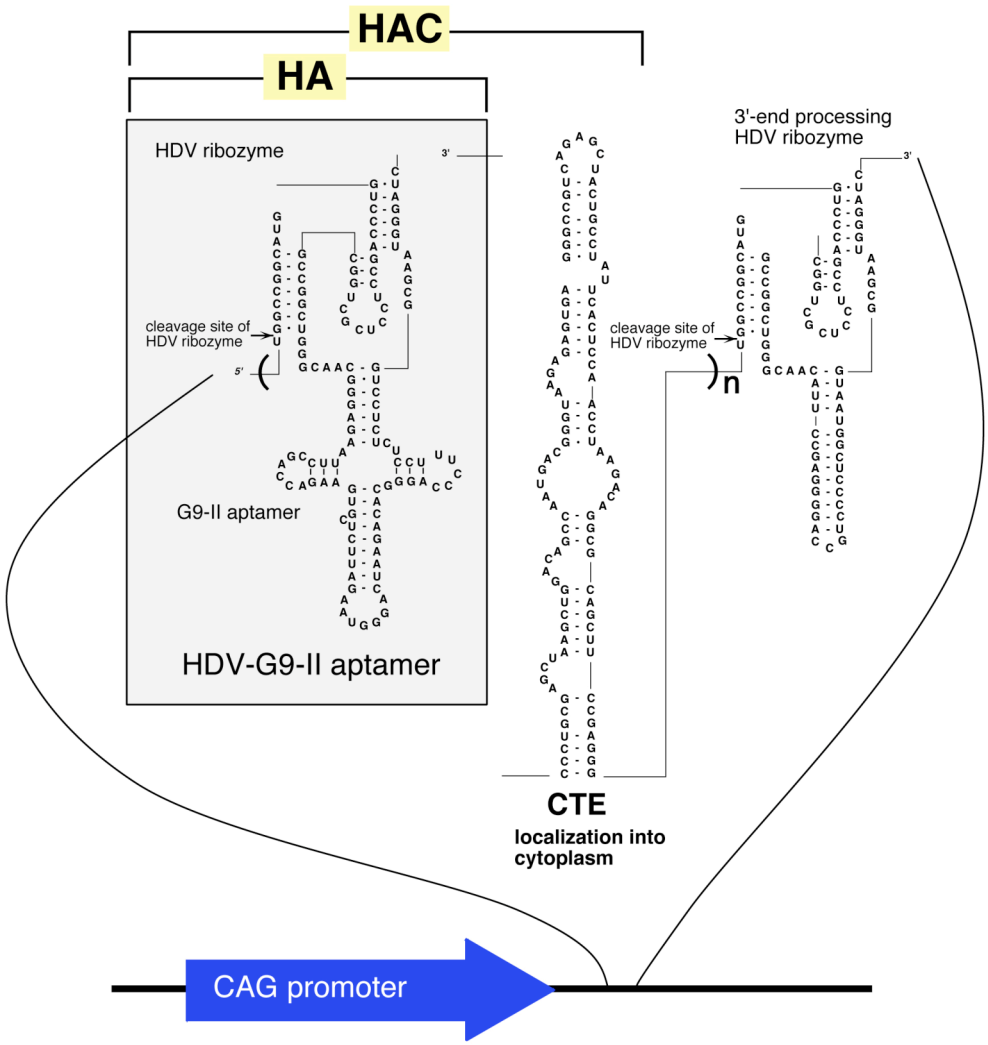


Fig. 2. Schematic diagram of an aptamer expression plasmid. The HDV-G9-II aptamer (HA) conjugated with CTE M45 (HAC) is designed to be transcribed under the control of the CAG promoter (Chicken ß-actin promoter + cytomegalovirus enhancer, indicated by CAG). The arrow indicates the self-cleavage site of the *cis*-acting HDV ribozyme. Protease inhibition activity of the tandem chimeric aptamer, (HAC)n, is related to the increase in the number (n) from 1 to 4 [16].

anti-protease and anti-helicase activities [15]. The structural domain of the helicase aptamer, #5Δ, was conjugated *via* an oligo(U) tract to the 3'-end of the dual functional aptamer NEO-III-14U or the protease aptamer G9-II. The spacer length was optimized to obtain two ADD aptamers, NEO-35-s41 and G925-s50 (Table 1); both were more effective inhibitors of NS3 protease/helicase activity *in vitro*, especially against the helicase activity with a four- to fivefold increase in inhibition compared to #5 and NEO-III-14U. Furthermore, G925-s50 effectively inhibited NS3 protease activity during HCV replication *in vitro*. Development of other aptamer conjugates will increase the range of their biological application.

Inhibition of HCV NS3 protease by RNA aptamers in cells

The inhibitory activity of aptamers within living cells was investigated by transfecting HeLa cells with various aptamers. The HeLa cells transiently expressed NS3 and a substrate protein (two types of GFP derivatives linked by a NS3 cleavage site). Using this system, FRET could easily detect inhibition of NS3 protease activity [27] and showed that NEO-III-14U, NEO-35-s41, and G925-s50 inhibited around 50% of NS3 proteolysis *in vivo* [14, 15].

Furthermore, we constructed the G9-II aptamer expression system in cultured cells using CAG promoter (cytomegarovirus enhancer + chicken ß-actin globin promoter) [16]. We designed a chimeric HDV ribozyme-G9-II aptamer (HA) conjugate, which was used to produce stable RNA *in vivo* and tandem repeats of the functional unit (Fig. 2). In addition, to facilitate the localization of transcribed RNA aptamers in cytoplasm, the minimal mutant of CTE (Constitutive Transport Element), derived from the type D retrovirus, was conjugated at the 3'-end of HA (HAC) (Fig. 2). Transcript RNAs, (HA)n and (HAC)n, were processed into the proper units containing the G9-II aptamer by the *cis*-acting HDV ribozyme, which was confirmed both *in vitro* and *in vivo*. The efficient protease inhibition activity of the HDV ribozyme-G9-II aptamer expression plasmid was demonstrated in HeLa cells. Protease inhibition activity of the tandem chimeric aptamer, (HA)n and (HAC)n, increased in relation to the number (n) from 1 to 4. This tandem system involving HDV ribozyme will be useful for controlling the expression of functional RNAs in cultured cells.

These lines of research have the potential to lead to the development of NS3 protease and helicase aptamers as anti-HCV compounds.

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