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Short peptide nucleic acids (PNA) inhibit hepatitis C virus internal ribosome entry site (IRES) dependent translation *in vitro*

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ABSTRACT

The internal ribosome entry site (IRES) of hepatitis C virus (HCV) which governs the initiation of protein synthesis from viral RNA represents an ideal target for antisense approaches. Using an original bicistronic plasmid, we first established that sequence and translational activity of HCV IRESs cloned from six patients, whether responders or not to combination therapy, were conserved. We then tested the hypothesis that antisense molecules, i.e. short peptide nucleic acids (PNA), could inhibit HCV translation by binding to the highly conserved IIId or IV loop regions of the IRES. Five 6–10 mer PNAs were designed. They strongly inhibit HCV IRES-driven translation in a rabbit reticulocyte lysate assay. This inhibition was highly specific since corresponding PNAs with only one mismatch were inactive. Short phosphorothioate oligonucleotides of same sequence were unable to inhibit HCV translation. PNA molecule was shown to have anti-HCV activity in Huh-7.5 cells when electroporated with a full-length HCV genome construct. Using oligonucleotide as carrier, PNA was also transfected in HCV replicon-harboring cells and in JFH1 infected Huh-7.5 cells.

1. Introduction

Worldwide, 170 million people are infected by hepatitis C virus (HCV) and in more than 70% of the cases the virus causes persistent infection that predisposes carriers to the development of liver diseases, cirrhosis and hepatocellular carcinoma (Moradpour et al., 2007). The average sustained viral response (SVR) to treatment based on the association of pegylated alpha interferon 2a or 2b and ribavirin is 50–60% (Chevaliez and Pawlotsky, 2007). However, this therapy is often poorly tolerated because of significant side effects (Manns et al., 2006). Thus, numerous efforts to develop new therapies are in progress to overcome the failure of current treatments (De Clercq, 2007; Manns et al., 2007).

HCV, a member of the *Flaviriridae* family, is a single-strand positive-sense RNA virus (Choo et al., 1989). The virus genome is 9,600 nucleotides long and encodes a polyprotein of approximately 3000 residues and an alternate reading frame that overlaps with the core protein gene (Moradpour et al., 2007). Initiation of translation depends on a cap-independent mechanism, mediated by an internal ribosome entry site (IRES) located in the 5′UTR region of the viral RNA (Tsukiyama-Kohara et al., 1992). Despite an overall high genetic variability of HCV (Smith et al., 1995), it has been shown that the IRES sequence is highly conserved. It was possible to define a single predicted RNA secondary structure of the IRES which contains three stem-loops (II–IV) and a pseudoknot (Fig. 1A) (Honda et al., 1999, 1996) as a necessary condition to maintain its activity. Therefore, it is an attractive drug target for antisense-based therapeutical approaches.

Several groups have already tested antisense oligonucleotides that inhibit HCV RNA and polyprotein synthesis both *in vitro* and in mice models (Alt et al., 1997; Brown-Driver et al., 1999; Hanecak et al., 1996; Lima et al., 1997; Mizutani et al., 1995; Seki and Honda, 1995; Vidalin et al., 1996; Wakita and Wands, 1994; Zhang et al., 1999). Phase I/II clinical trials based on a phosphorothioate (PS-ODN) molecule (ISIS14803) were also initiated but were stopped for reasons of lack of efficacy (McHutchison et al., 2006). More generally, antisense oligonucleotides used until recently as first-generation-oligonucleotides that were generally sensitive to nuclease degradation, showed low affinity profiles towards their target and side effects when used *in vivo* (Crooke, 2004).

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Second generation antisense oligonucleotides may improve the efficacy of such approaches. They possess modifications both on the sugar and the backbone and have already shown *in vitro* activity against HCV (McCaffrey et al., 2003; Tallet-Lopez et al., 2003). Nevertheless, the development of effective new therapeutical agents to control HCV infection is required and may be provided by another class of antisense molecules, i.e. peptide nucleic acids (PNA). PNA are nucleic acid analogues containing natural nucleoside bases on a pseudo-peptide backbone (Nielsen et al., 1991; Nielsen and Haaima, 1997). They can bind efficiently to DNA and RNA sequences and were shown to efficiently inhibit mRNA translation by binding to the entry site for the ribosomal small subunit (Doyle et al., 2001).

Here we tested the hypothesis that short PNAs of 7–10 nucleotides in length targeted at the HCV IRES IIId stem-loop region or to the IV loop region that contains the AUG start codon could inhibit *in vitro* HCV translation both in a rabbit reticulocyte lysate (RRL) assay and in cultured Huh-7.5 cells transfected with full length HCV genome RNA transcripts.

2. Materials and methods

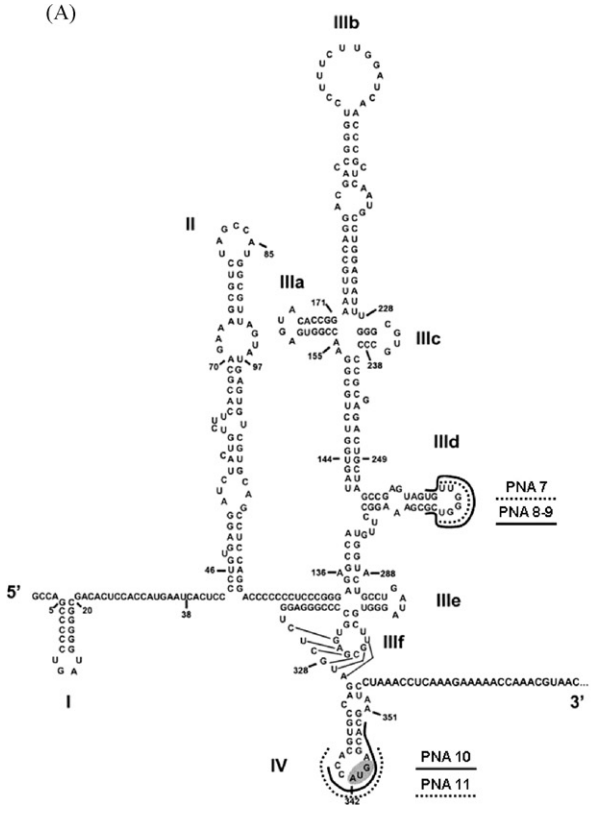
2.1. Plasmid construction

The bicistronic plasmid termed pFl-hRl is based on a pCDNA3.1(+) backbone (Invitrogen). Sequences coding for Firefly Luciferase (Fluc) were obtained from pGL3 basic (Promega). A HindIII–EagI fragment (containing the Fluc coding region) was PCR-amplified using specific primers (HindIII-Fluc_Fwd: CCCAAGCTTATGGAAGACGCCAAAAACATA and EagI-Fluc_Rv: ATAGTTTAGCGGCCGCTTACACGGCGATCTTTCCGCCCTTC). After restriction with HindIII and EagI as recommended by the Manufacturers (Promega and New England Biolabs), the amplification product was inserted between the HindIII and NotI sites of pCDNA 3.1(+), downstream of CMV and T7 promoters, thereby generating the intermediate construct pFl. Next, an XbaI–ApaI fragment (containing the humanized Renilla Luciferase coding region, hRluc) was obtained by PCR amplification of phRLuc-SV40 (Promega) using specific primers (XbaI-hRluc_Fwd: GCTCTAGAATGGCTTCCAAGGTCTACGACC and ApaI-hRluc_Rv: GCTGGGCCCTTACTGCTCGTTCTTCAGCAC). After restriction with XbaI and ApaI as recommended by the Manufacturer (Promega), the amplification product was inserted downstream from the Fluc sequence, thereby generating the construct pFl-hRl (Fig. 1C). The junction between the end of the Fluc sequence and the beginning of the hRluc sequence was analysed among the different clones (using primers vc_Fwd: TGTGGACGAAGTACCGAAAGGTC and vc_Rv: ATGGCTGGCAACTAGAAGGCA) by direct and reverse sequencing of the region (Genome Express, France). Ligation reactions were carried out using T4 DNA ligase (Promega). The *E. Coli* DH5α strain was used for plasmid transformation and propagation.

2.2. Patients

Six patients chronically infected by HCV, treated with a combination of interferon α-2b and ribavirin, were retrospectively included in the study. Three patients were sustained responders

(A)



(B)

Name	Length	Sequence (from 5' to 3')	Position (nt)
PNA-7	6 mer-K	ACCCAA-K	264 to 269 (IIId)
PNA-8	10 mer	CGACCCAACG	262 to 271 (IIId)
PNA-8 mm	10 mer	CGACACAACG	262 to 271 (IIId)
PNA-9	10 mer-K	CGACCCAACG-K	262 to 271 (IIId)
PNA-10	10 mer-K	GTGCTCATGG-K	340 to 349 (IV)
PNA-10 mm	10 mer-K	GTGCTCTTGG-K	340 to 349 (IV)
PNA-10 scramble	10 mer	ATCTGGTCGG	340 to 349 (IV)
PNA-10 fluoresceine	10 mer	ATCTGGTCGG-fluo	340 to 349 (IV)
PNA-1 1	7 mer-K	TCATGGT-K	339 to 345 (IV)
PNA control	4 mer	Z(A,G)-2-Arg-OMe	control
PS-7	6 mer	A*C*CC*A*A	264 to 269 (IIId)
PS-8/9	10 mer	C*G*ACCCAA*C*G	262 to 271 (IIId)
PS-8 mm	10 mer	C*G*ACACAA*C*G	262 to 271 (IIId)
PS-10	10 mer	G*T*GCTCAT*G*C	340 to 349 (IV)
PS-10 mm	10 mer	G*T*GCTCTT*G*G	340 to 349 (IV)
PS-11	7 mer	T*C*ATG*G*T	339 to 345 (IV)

(C)

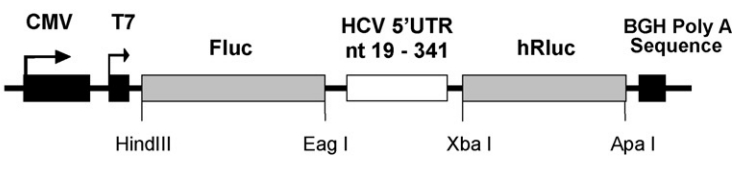


Fig. 1. (A) HCV 5′UTR secondary structure and PNA target sites. Major structural domains I to IV are indicated. The AUG start codon in the domain IV is highlighted. Target sites of the PNAs are indicated by straight or dashed lines. PNA-9 has the same sequence than PNA-8 with an addition of a lysine amino acid in 3′ end. Adapted from Honda et al. (1999). (B) Peptide nucleic acids and phosphorothioates used in this study. K represents lysine amino acids added at the 3′ end of some PNAs (*) represents phosphorothioates linkages. (C) Schematic representation of the bicistronic reporter cassette from plasmid pFL-hRL expressing both firefly and renilla luciferase. Transcription is initiated under the control of a composite CMV-T7 promoter. The upstream cistron encodes Firefly luciferase and is translated by a cap-dependent mechanism in transfected cells, while a downstream cistron encoding humanized Renilla luciferase is translated under the control of the HCV IRES. The HCV sequence within the intercistronic space represents the region from nucleotide 19 to 341 of the HCV genome of a genotype 1b virus fused in frame to Firefly luciferase.

and three patients were non-responders. Sustained response was defined as a decrease of HCV RNA titers below the detection limit with a normalization of ALT levels, which were maintained after the end of the treatment. Non-response was defined as no decrease of HCV RNA titers or a decrease of less than one $\log_{10}$ copy/ml, during and after treatment without a fall of ALT to the normal range. For sustained responder patients, a sample in the first month of treatment, before disappearance of RNA, was used for RNA extraction. In the case of non-responder patients, RNA from one serum sample at month 6 was studied. All patients were infected by genotype 1 HCV.

2.3. RNA extraction, amplification of HCV IRES and preparation of bicistronic plasmids

HCV RNA was extracted from the sera using the QIAmp viral RNA Kit (Qiagen), according to the mManufacturer's instructions. The 5′UTR was reverse transcribed and amplified using Qiagen OneStep RT-PCR Kit (Qiagen) and the set of primers determined from the IRES region (primer IRESfwd 5′ ggcggccgGCGACACTCC(G/A)CCATGAAT 3′ containing an EagI cloning site shown in lower-case letters and IRESrv 5′ cgtctagaAGGATTCGTGCTCATGGTGC 3′ containing an XbaI cloning site). These primers amplified fragments from nt 19 of the 5′UTR to nt 341. The RT reaction was performed for 30 min at 60 °C followed by 15 min at 95 °C. PCR was carried out for 40 cycles (94 °C, 30 s; 55 °C, 45 s; 72 °C, 30 s). RT-PCR reactions were performed on I-Cycler (BioRad). The final PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed under UV light. The PCR products were purified using QIAquick PCR purification kit (Qiagen) as specified by the Manufacturer and quantified by UV spectrophotometry. The fragments and the bicistronic plasmid were digested with EagI and XbaI. Ligation was performed overnight using T4 DNA ligase (Promega) as specified by the Manufacturer. XL2 bacteria were transformed with ligation products. Plasmids were labelled pFl-hRl SR or NR, respectively, for sustained responders and non-responders. sequencing was performed (Genome Express, France) after preparation of the plasmids to ensure that the integrity of the sequence was conserved.

2.4. In vitro coupled transcription and translation assay

To assay IRES activity, we used TnT Quick Coupled Transcription/Translation System (Promega). Briefly, instead of performing *in vitro* RNA synthesis using T7 polymerase followed by *in vitro* translation in a rabbit reticulocyte lysate (RRL), we added 1 μg of circular bicistronic plasmid in 25 μl of TnT T7 Quick Master Mix (includes RRL, Buffer, T7 RNA polymerase, aa mixture met−, RNasin) supplemented with 1 μl of methionine and incubated for 90 min at 30 °C. The synthesized proteins were analyzed by luciferase assay.

2.5. Cell culture and transfection

To assay the construct in cell culture, Huh-7 cells (Nakabayashi et al., 1982) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS). Subconfluent monolayers of cells in 24-well plates (15 mm diameter) were transfected with 1 μg of bicistronic plasmid DNA in 500 μl Opti-MEM containing 3 μl FuGene-6 (Roche). Four replicate wells were transfected with each construct and each experiment was performed at least twice. After 2 h of incubation at 37 °C, 1 ml of growth medium was added to the wells. The cells were harvested 24 h later and cell lysates were assayed for luciferase activity as described below. Electroporation of RNA transcripts was performed in Huh-7.5 cells, an Huh-7 derivative that is highly permissive for HCV replication (Blight et al., 2002). HCV RNA was transcribed *in vitro* from replicon I389/NS3-3′/lucUbiNeo-ET (kindly provided by Prof. Ralf Bartenschlager) (Krönke et al., 2004) or the HCV luc-JFH1 full-length genome construction (Wakita et al., 2005) and electroporated into cells as described previously (Blight et al., 2002). In brief, RNA transcripts (5 μg) were mixed with 2×10^6 washed cells in 0.2 ml in a 2-mm gap cuvette and immediately pulsed (820 V, 99-μs pulse length, five pulses at 1.1 s intervals) using a BTX ECM 830 square wave electroporation system (Genetronics, San Diego, CA). Electroporation was performed with RNA alone or RNA mixed with a range of PNA-10 or PNA control of 6.5, 13, 20 and 26 nM. The cells were seeded into 24-well plates and were harvested at 24, 48 and 72 h after plating. Cell lysates were assayed for luciferase activity as described below. Experiments were performed twice in duplicates.

Huh-7.5 cells were infected with virus particles (JFH1 strain) produced in cell culture as described (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). After three passages, cells were transfected with PNA-oligonucleotide complex and then fixed in methanol-acetone (1:1, v/v) for 20 min at −20 °C, 72 h post-transfection. Cells were then incubated in immunofluorescence assay buffer (PBS 1×, 5% bovine serum albumin). Mouse monoclonal anti-HCV core (C7.50, Abcam, Cambridge, MA) was added at 1:250 dilution in immunofluorescence buffer. After incubation for 8 h at room temperature, cells were washed, followed by incubation with anti-mouse IgG antibody at 1:300 during 2 h (Alexa Fluor 555; Invitrogen). Cells were examined by fluorescence microscopy and the number of HCV-positive foci counted in three separate wells (12 wells plate) per assay.

2.6. Luciferase assay

The enzymatic activities of Fluc and hRluc were measured using the Dual Luciferase Reporter Assay System (Promega). For RRL or TnT *in vitro* assays, 2.5 μl of reticulocyte lysate were analyzed for each experiment with sequentially programmed addition of 50 μl of luciferase assay reagent 2 and 50 μl of Stop and Glo. The ratio of hRluc activity to Fluc activity was presented in arbitrary units as the relative luciferase activities. Light emission was integrated over a period of 1 s in a luminoskan ascent, with 1 s of lag time between readings. Similar quantifications were performed after transfection of bicistronic plasmids in cell culture or electroporation of HCV RNA from the subgenomic luciferase replicon or the Luc-JFH1 construct. In these cases, after removing the growth medium from the cultured cells and washing the surface of the culture vessel with PBS, the minimum volume of passive lysis buffer (PLB) recommended by the Manufacturer (Promega) was dispensed into each culture well. For cultures performed in 24-well culture plates, 125 μl of PLB was added. After 15 min at room temperature, the lysate was transferred to a tube and 10 μl were analyzed in the conditions described above.

2.7. Peptide nucleic acid and phosphorothioate oligonucleotide synthesis

All PNAs (except PNA-10-scramble (PNA-10scr) and PNA-10-fluorescent) used in this study were synthesized using the standard synthesis protocol (Christensen et al., 1995). Phosphorothioate oligonucleotides and PNA-10 scramble were purchased from Eurogentec (Angers, France). All molecules were purified and analyzed by high-performance liquid chromatography (HPLC). Their targets on the predicted HCV IRES secondary structure (Honda et al., 1999) are indicated in Fig. 1A. Fig. 1B summarizes the sequences of the different PNAs.

2.8. Lipid-mediated transfection of PNA:oligonucleotide duplex

Oligonucleotides for transfection of PNAs were chosen to form PNA:DNA complexes in the melting temperatures of 65–75 °C as described by (Nulf and Corey, 2004). Briefly, four 14 mers oligonucleotides with a 5′-AAAA overhang per PNA were synthesized and empirically tested for cellular penetration using fluoresceine labelled selected PNA (PNA-10). Oligonucleotide duplex mixtures were annealed in the thin-walled PCR tubes in a thermocycler. Reductions in temperature occurred for 1 min the times indicated: 95 °C, 5 min; 85 °C, 1 min; 75 °C, 1 min; 65 °C, 5 min; 55 °C, 1 min; 45 °C, 1 min; 35 °C, 5 min; 25 °C, 1 min; 15 °C, 1 min; hold 15 °C as described (Nulf and Corey, 2004). Annealed PNA:oligonucleotides duplexes were prepared for transfection by using lipofectAMINE 2000 (Invitrogen). In a separate tube, 2 μl of lipofectamine were mixed with 198 μl lipofectAMINE 2000 for 15 s followed by equilibration at room temperature for 5 min. The diluted PNA:oligonucleotide duplex in Opti-MEM (Invitrogen) and lipofectAMINE (200 μl each) were mixed together and incubated for 30 min at room temperature. This solution was serially diluted to the final working concentrations in the culture medium.

3. Results

3.1. Comparison of the translational efficiencies of 5′UTRs in vitro and in cultured cells

To analyze IRES activities both after *in vitro* translation and in Huh-7 transfected cells, a novel bicistronic plasmid allowing easy cloning and measurement of the biological activity of IRES in HCV isolates obtained from infected patients was constructed. The constructs used include the sequence from nt 19 of the 5′UTR to nt 15 of the core protein (nt 356 of the HCV genome). pFl-hRl, represented in Fig. 1C, has a bicistronic translational unit controlled by a composite CMV/T7 promoter. Rather than the classic *Renilla luciferase* gene, a humanized Renilla sequence was used as a reporter gene for the monitoring of HCV IRES activity because of its supposedly improved translational activity levels (Manufacturer's data). In the rabbit reticulocyte lysate (RRL) system, using a construct with an HCV-H77 reference strain IRES (genotype 1a), whereas the ratio of Renilla luciferase to Firefly lucificerase activity was about 0.4 when the humanized Renilla gene was used, it was only about 0.1 with the unmodified gene (data not shown).

The IRESs from the selected patients (three sustained responders and three non-responders, all infected with HCV genotype 1) were isolated and cloned into the bicistronic pFl-hRl and all constructs were sequenced. Very few differences between the sequences were observed (Table 1). When positioned onto the predicted IRES secondary structure, two were found to lie in unpaired regions of the IRES (C204A and C204U). The importance of maintaining the secondary structure of the IRES is shown by the other substitutions located in paired regions improving base pairing (U78C and G107A). No differences were observed in the IIId and IV loop regions.

The IRES activities of these different isolates were analyzed both *in vitro* in RRL and after transfection in Huh-7 cells. Results are summarized in Table 1. In both cases, no significant difference of IRES activity was observed between the different constructs. Taken together, these results confirmed that the IRES of HCV is a highly conserved region at the nucleotide level and does not present inter-individual variations in activity.

3.2. Inhibition of IRES dependent translation in vitro by peptide nucleic acids

The capacities of PNAs to inhibit the translation of HCV RNA were evaluated in the *in vitro* TnT coupled transcription/translation system using the construct pFl-hRl NR2. The IRES derived from NR2 patient was identified as being genotype 1a HCV. Non-specific inhibitory effects were evaluated with the same construct by measuring the IRES-independent translation of Firefly luciferase.

Among the five PNAs studied, four showed a dose-dependent inhibitory effect. Inhibitions obtained using PNAs targeted to both regions, the IIId stem-loop region (PNA 7, 8 and 9) and the IV stem-loop region (PNA-10 and 11), are shown in Fig. 2A. PNA-7 did not show an inhibitory effect at the tested concentrations. PNA-8, 9 and 11 exhibited a specific inhibitory effect with IC_{50}s calculated, respectively, at 100, 100 and 250 nM. PNA-10 was the most effective with an IC_{50} estimated at 54 nM and an IC_{90} at 100 nM. Concentrations exceeding 1 μM led to non-specific inhibition as indicated by significant effects on Firefly luciferase expression. As a control, a scrambled PNA was also used and showed no inhibitory activity. As PNA is able to target RNA as well as DNA, we sought to determine whether the effects observed were associated in our experimental conditions to an inhibitory activity of the PNA on the RNA template. We therefore performed an analysis using *in vitro* transcribed RNA from pFl–hRl NR1 in standard RRL. Similar results were obtained with similar IC_{50} values (data not shown). We also performed northern blotting on the TnT lysate after performing *in vitro* coupled translation and transcription. No modification of size or amounts of the transcripts was observed (data not shown).

Table 1
Analysis of IRES from HCV infected patients

Patient	Genotype	IRES activity in RRL[a] (%)	IRES activity in HuH7 cells[a] (%)	Mutations in IRES sequences[b]	Mutations[c]
SR1	1a	102	92	2/338	U78C C204A
SR2	1b	94	107	0/338	
SR3	1b	77	95	1/338	C204U
NR1	1b	70	107	0/338	
NR2	1a	81	72	0/338	
NR3	1a	62	99	1/338	G107A

Patients are named according to their response to ribavirine and interferon therapy: SR is for sustained responders and NR for non-responders. HCV genotype is indicated for each patient.

[a] Bicistronic plasmids corresponding to the indicated patients were used to perform *in vitro* coupled transcription–translation in TnT rabbit reticulocyte lysate system or after transfection in HuH7 cells. Relative IRES directed translation efficiency was assessed by measuring the ratio of hRluc to Fluc by using the dual luciferase kit assay. Results shown are percentage of ratio for a construct compared with ratio obtained with a construct with an H77 IRES.

[b] Sequencing was performed and compared with the reference strain (HCV-H77 for genotype 1a and HCV-J for genotype 1b). The number of mutations in IRES sequences, from nt 19 to nt 356 (338 nt), is indicated.

[c] Mutations observed and numbered according to the reference strains. C204U and C204A are located in unpaired regions. U78C and G107A results in improvement in base pairing.

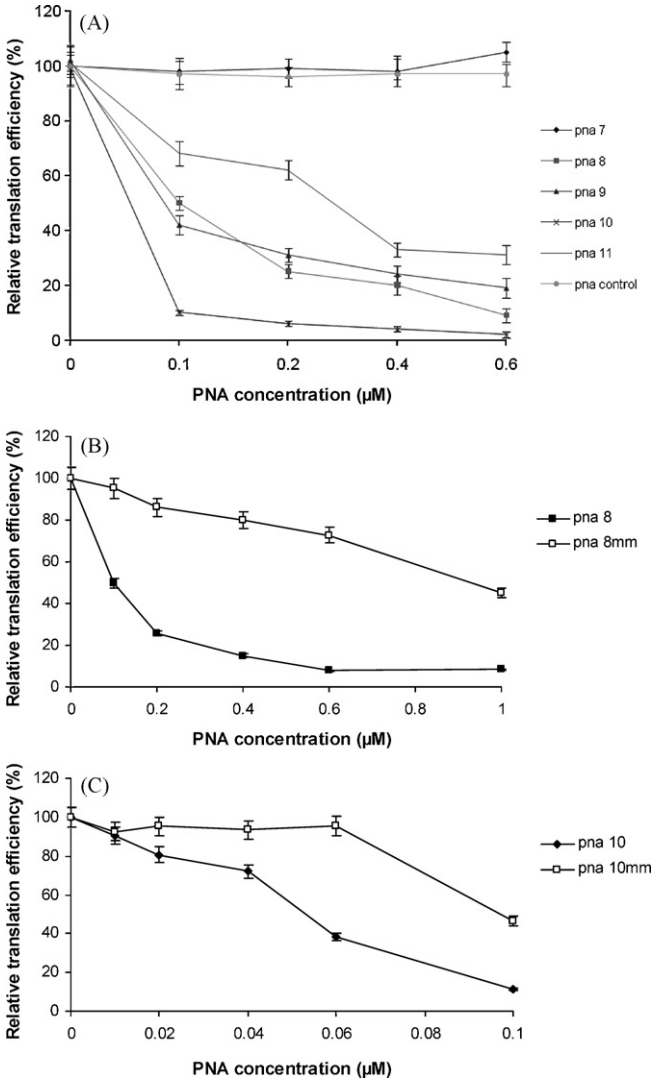


Fig. 2. *In vitro* inhibition of HCV IRES dependent translation by PNA. Bicistronic plasmid with IRES sequence of patient NR2 was used to perform *in vitro* coupled transcription–translation in TNT rabbit reticulocyte lysate system. Relative IRES directed translation efficiency was assessed by measuring the ratio of hRluc to Fluc, using the dual luciferase kit assay in presence of the different PNA (A). The effect on the inhibitory activity of one mismatch nucleotide on PNA-8 (B) and 10 (C) was tested. Values are the mean of three independent experiments ± standard deviations (S.D.).

The influence of mismatched nucleotides was then analyzed. We introduced only one mismatch nucleotide into the sequences of PNA-8, targeting the IIId loop region (GGG triplet) and PNA-10, targeting the IV loop region (AUG start codon). Fig. 2B shows a 10-fold reduction of efficiency for mismatched PNA-8 compared to control PNA (IC_{50} values increasing from 100 to 1 μM). For PNA-10 (Fig. 2C), only one mismatch in the sequence pairing with the AUG start codon led to a clear decrease of inhibitory activity (IC_{50} values increasing from 54 to 100 nM).

Lastly, we compared PNAs to PS-ODNs of the same sequence and length. Only one PS-ODN, corresponding to the most effective PNA-10, showed an inhibitory effect with an IC_{50} value of 4 μM (Fig. 3). This represents a 70-fold increase of IC_{50} compared with PNA-10. Other PS-ODNs did not have any effect, consistent with other work showing that PS-ODNs must have a length of at least 20 nt and be used at higher concentrations to be effective.

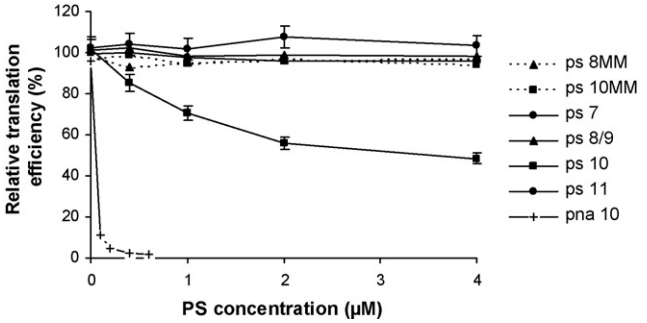


Fig. 3. Comparison of *in vitro* inhibition of HCV IRES dependent translation by phosphorothioates and PNA-10. Bicistronic plasmid with IRES sequence of patient NR2 were used to perform *in vitro* coupled transcription–translation in TNT rabbit reticulocyte lysate system. Relative IRES directed translation efficiency was assessed by measuring the ratio of hRluc to Fluc using the dual luciferase kit assay. Values are the mean of three independent experiments.

3.3. Inhibition of IRES dependent translation in cultured cells

The question of whether these short PNAs could have an effect in cellular models was then asked. Huh-7 cells were first transfected with pFl-hRL-NR1 and then PNA-10 was transfected while the cells were still transiently expressing Firefly and hRenilla luciferases. Results were normalized against hRluc activity. However, no inhibition was observed (data not shown). We then performed assays with a fluoresceine-labelled PNA-10. It appeared that neither transfection with cationic lipids nor electroporation of PNA alone or with the bicistronic plasmid allowed penetration of PNA into cultured cells (data not shown). We therefore performed electroporation of PNA mixed with an RNA transcript from an *in vitro*-transcribed full length HCV genome construct Luc-JFH1 (Wakita et al., 2005). Four different concentrations of PNA-10, i.e. 6.5, 13, 20 and 26 nM, corresponding to a ratio PNA/HCV-RNA of 65, 125 and 250, respectively, were used. In this experiment (Fig. 4), PNA-10 showed a dose-dependent inhibition in the establishment of the JHF1 complete replication cycle, up to 65% relatively to control at a concentration of 26 nM. By comparison, a scrambled PNA-10 did not show any significant effect. These results strongly suggest that the tested PNA associated with HCV single stranded RNA molecule harboring a complementary sequence is able to enter the cell and to inhibit the establishment of luc-JFH1 replication. To check this finding, PNA delivery was performed using lipid-mediated transfection of PNA-oligonucleotide hybrids (Nulf and Corey, 2004) in a luc-HCV replicon cell line (Fig. 5). A dose-dependent inhibition of the HCV replication directed luciferase activity by PNA-10 was observed while PNA-10scr was inactive in the range of tested concentration.

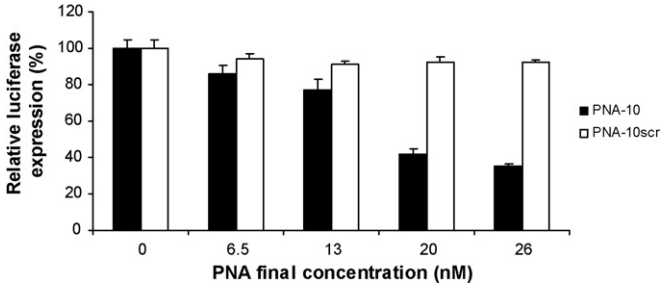


Fig. 4. Inhibition in establishment of luc-JFH1 replication in Huh-7.5 cells after electroporation of RNA transcripts with PNA-10. HCV IRES directed luciferase synthesis was assessed after electroporation of Huh-7.5 cells with luc-JFH1 construct RNA in presence of a range of 0, 6.5, 13, 20 and 26 nM of PNA-10 (black bar) or PNA-10 scramble (white bar). Forty-eight hours after incubation, cell lysates were subjected to a luciferase assay. The data shown are averages of two independent assays ± S.D.

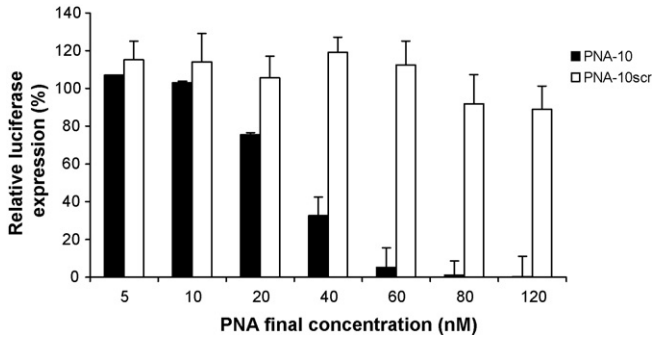


Fig. 5. Inhibition of replication in replicon I389/NS3-3′/lucUbiNeo-ET after transfection of complex oligonucleotide:PNA-10 and PNA-10scr. HCV replication directed luciferase synthesis was assessed after transfection of HCV-replicon cell line with 0, 5, 10, 20, 40, 60, 80 and 120 nM of PNA-10 (black bar) or PNA-10 scramble (white bar) coupled with oligonucleotide. Seventy-two hours after incubation, cell lysates were subjected to a luciferase assay. The data shown are averages of three independent assays ± S.D.

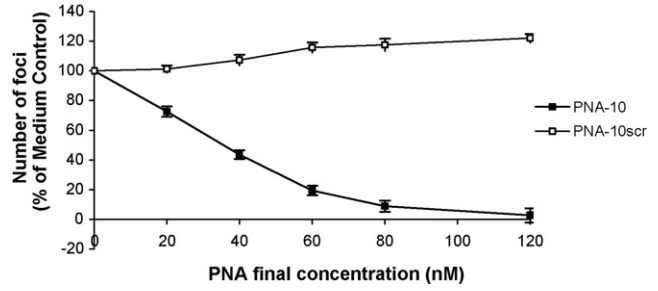


Fig. 6. Inhibition of infection in infected JFH1 Huh-7.5 cells after transfection of oligonucleotide-PNA-10 or PNA-10scr complex. HCV (JFH1 strain) infected Huh7.5 cells were transfected with PNA-10 (black bar) or PNA-10 scramble (white bar) coupled with oligonucleotide at indicated concencentration. Seventy-two hours after incubation, the infection was monitored by detection of the HCV core by immunofluorescence, and the number of HCV-positive foci observed in wells was counted in triplicate. The error bars represents the S.D. from two independent assays.

In a second experiment, Huh-7.5 cells were infected with cell-culture produced JFH1 virus. After three passages, cultures showed around 90% of infected cells by immunofluorescence analysis and were transfected with PNA-oligonucleotide duplex at the indicated concentrations (Fig. 6). Efficiency of infection was monitored by immunofluorescence detection of HCV core and counting positive foci. Again a clear decrease in the number of infected cells was observed when cells were transfected with PNA-10 (Fig. 6). This inhibition is dose-dependent with an IC_{50} between 20 and 40 nM of PNA and specific, since control PNA (PNA-10 scramble) was clearly inactive.

4. Discussion

While waiting for the clinical use of new anti-HCV compounds which are now in the late development stage, the design of original and complementary antiviral approaches seems important, especially in the perspective of combating and preventing the emergence of drug-resistant mutants. Indeed, new anti-HCV agents that target the viral polymerase or protease (De Francesco and Migliaccio, 2005) induce a rapid emergence of drug-resistant mutants, both *in vitro* in the replicon system and in vivo in the first phase I/II clinical trials (Dutartre et al., 2006; Le Pogam et al., 2006; Mo et al., 2005). In this context, the IRES of HCV represents an interesting target, since it is the most conserved region among all HCV genotypes and the least susceptible to mutation.

In this study, we first analyzed the sequence and translational activity of IRES obtained from different clinical isolates. Very low sequence variability and low variation of translational activity was observed. These observations are in agreement with other studies that showed that sequence variability of IRES does not appear to correlate with any difference in levels of HCV replication *in vitro* and in cell culture (Thelu et al., 2004) and does not depend on the response status of the patients (Soler et al., 2002). This further confirms the potential of an antisense-based strategy targeted against IRES as an alternative approach for the treatment of non-responder patients, either to IFN-based therapy or to future combination therapy including polymerase or protease inhibitors.

Previous studies have demonstrated that the HCV IRES could be inhibited *in vitro* by a wide range of antisense-based molecules, notably ribozymes, siRNAs and oligonucleotides (Alt et al., 1999; Brown-Driver et al., 1999; Hanecak et al., 1996; Lima et al., 1997; Mizutani et al., 1995; Seki and Honda, 1995; Vidalin et al., 1996; Wakita and Wands, 1994; Zhang et al., 1999). The ability of such molecules to inhibit HCV replication in cell culture or in vivo was also demonstrated, although with poor results and selectivity (Crooke, 2004; McHutchison et al., 2006). Second generation antisense oligonucleotides may improve the efficacy of such approaches. Some have already shown *in vitro* activity against HCV (McCaffrey et al., 2003; Tallet-Lopez et al., 2003). Nevertheless, the development of effective new therapeutic agents to control HCV infection is required and may provide another very promising class of antisense molecules, i.e. peptide nucleic acids (PNA). They have an improved selectivity and affinity to DNA or RNA and lower affinity to proteins compared to phosphorothioate oligonucleotides (PS-ODN) and are highly resistant to nuclease and peptidase degradation (Demidov et al., 1994). They have been used as antisense molecules against various viruses such as HIV (Depecker et al., 2004), HBV (Robaczewska et al., 2005) and HCV (Nulf and Corey, 2004). Here, we have analyzed the inhibitory effect of short PNAs (6–10 mers) for their ability to specifically inhibit HCV IRES-driven translation by binding to the IIId or IV loop regions of IRES.

Among the six PNAs designed, five were found to be effective in inhibiting the HCV IRES-dependent translation. PNA-10 was the best molecule tested with an IC_{50} of 54 nM in RRL. This inhibition appeared highly specific since PNA molecules with only one mismatch lost their inhibitory activity. Furthermore, no inhibition of Firefly luciferase activity was observed in the range of concentrations that effectively inhibited Renilla luciferase activity. In our constructions, Firefly luciferase translation is cap-dependent and not IRES-dependent, and any inhibition would reflect non-specific side effects. Moreover, we found that phosphorothioate analogues of the same length and sequence are generally inefficient. Only the PS-ODN corresponding to the most efficient PNA was found to be effective, with an IC_{50} of 4 μM that is seventy times higher than the IC_{50} obtained with PNA-10. Results obtained by electroporating PNA-10 with luc-JFH1 RNA in Huh-7.5 cells showed an inhibition of the establishment of the HCV replication cycle in a dose-dependent manner that is also specific since a scrambled PNA was ineffective. We cannot exclude that PNA-10 enters the cell and exhibits an inhibitory activity when electroporated with the target RNA because it enters bound to the RNA while the scrambled PNA-10, which is unable to bind to the RNA, may not be able to enter the cell. To resolve this issue, we use the methodology described by (Nulf and Corey, 2004) allowing lipid-mediated transfection of PNA after formation of duplex with selected oligonucleotides. In both HCV replicon-harboring cells and JFH1 strain infected Huh-7.5, PNA-10 appears as an efficient and selective inhibitor of HCV replication.

Our demonstration of short PNA activity opens new perspectives and further demonstrates the potency of such molecules. PNA

length appears critical since all compounds less than 10 mer that we have tested are far less efficient. It has been calculated that lengths under 17 nt can statistically match with more than one target in cellular genes (Branch, 1998). Operationally, long PS-ODNs were used because of the duplex Tm that was inferior to natural double-stranded DNA and thus needed to be longer than 17–20 nt to bind efficiently to RNA targets (Crooke, 1993). With PNA, such thermodynamic limitations disappear since they bind more efficiently DNA and RNA (Lundin et al., 2006). Efficient inhibition of HCV IRES directed translation with PNA molecules (16–23 mer) longer than those that we have used and targeting the same regions has been already described (Nulf and Corey, 2004). Numerous ways of coupling PNA with cell-penetrating peptides have been proposed but led to molecules with a very high molecular weight (Rasmussen et al., 2006). The use of short PNAs, with the limitations listed above, can help in the creation of smaller molecules with enhanced abilities to enter the cell. Furthermore, as we have demonstrated elsewhere (Caldarelli et al., 2005), small PNAs permit cyclization of the molecules, thus mimicking highly stable loop–loop complexes. Such modifications, although they still needed to be optimized, could lead to highly selective molecules as described for RNA aptamers by Toulmé and co-workers (Aldaz-Carroll et al., 2002). It is possible that PNA molecules directed against the HCV IRES shorter than 17-mers can be used without interfering with cellular sequences since what they are targeting is not just a short sequence but a short sequence that occupies an important position in a far larger complex structure.

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