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Inhibition of hepatitis C virus RNA replicons by peptide aptamers

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Abstract

Background/aims: Hepatitis C virus infection is a major worldwide health problem, causing chronic hepatitis, cirrhosis and primary liver cancer. In addition to its role in the viral polyprotein-processing, the viral NS3 serine protease has been implicated in interactions with various cell constituents resulting in phenotypic changes including malignant transformation. NS3 is currently regarded a prime target for anti-viral drugs thus specific inhibitors of its activities should be important. With the aim of inhibiting NS3 protease activity as a means to inhibit HCV replication we used a novel bacterial genetic screen to isolate NS3-inhibiting peptide aptamers.

Methods: We have isolated and characterized seven NS3-inhibiting peptide aptamers. We investigated the phenotypic changes that SEAP-secreting subgenomic RNA replicons undergo upon intracellular expression of these peptide aptamers, assayed by real-time RT-PCR and inhibition of SEAP secretion by transfected replicon cells.

Results and conclusions: The peptide aptamers inhibited NS3 protease activity *in vitro* with an IC_{50} in the low micromolar range. Upon transfection, aptamers inhibited the replication of SEAP-secreting genotype 1b subgenomic RNA replicons. Aptamer-based intracellular immunization may emerge as a promising antiviral approach to interfere with the life cycle and pathogenicity of HCV.

Keywords: HCV; NS3 serine protease; Liver diseases; Peptide aptamers; RNA replicons

1. Introduction

Hepatitis C virus (HCV) is an RNA virus that causes acute or chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Poynard et al., 2003; Purcell, 1997). Despite recent advances in the therapy of HCV, even the most recent combination of PEGylated alpha-interferon and ribavirin fails to eliminate infection in nearly 50% of those infected (Manns et al., 2001; Pawlotsky, 2000). This high frequency of treatment failure points to the need for more specific, less toxic and more active antiviral therapies for HCV (Molla and Kohlbrenner, 2003; Pawlotsky, 2000). HCV is a single-stranded RNA virus whose 9.6 kb genome is organized to contain a single, large translational open-reading frame that encodes a large polyprotein precursor of 3010–3033 amino acids (Bréchot, 1996; Purcell, 1997). Most precursor proteolytic processing events are directed by the virally encoded NS3 serine protease that requires the adjacent NS4A cofactor for efficient cleavage activity. NS3 directs the proteolytic cleavages at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions and is thus essential for replication of the virus (Failla et al., 1994; Suzuki et al., 1999). In addition, expression of NS3 has been found to interfere with signal transduction pathways, promote cell proliferation and cause cell transformation (Borowski et al., 1999a,b; Ishido and Hotta, 1998; Zemel et al., 2001). Due to its essential role in viral replication and its effects on the physiology of the infected cell, NS3 represents a prime target for antiviral therapy.

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To date, several types of rationally designed NS3 serine protease inhibitors have been discovered. These included substrate-based peptide inhibitors targeting the active site, strong electrophiles located at the position of the scissile amide bond, phenanthrenequinones, thiazolidines, benzoylamides, small-interfering RNAs (siRNA) (De Francesco et al., 2003; Kakiuchi et al., 1998; Randall and Rice, 2004; Steinkühler et al., 1998), and RNA aptamers (Umehara et al., 2005). In spite of the difficulties posed by the lack of readily available laboratory models of viral infection, a few investigational compounds have just started to show promising results in early-phase clinical trials (De Francesco and Migliaccio, 2005; Malcolm et al., 2006; Perni et al., 2006; Reiser et al., 2005).

Because of the difficulties of rational design of efficient non-toxic small molecule NS3 inhibitors, several groups have turned to high throughput screening of chemical and natural product libraries to search for novel lead molecules. However, in most cases the tested compounds also inhibited human serine proteases such as chymotrypsin and elastase making them inappropriate candidates for clinical use (Johansson et al., 2002; Kwong et al., 1998; Sperandio et al., 2002; Wyss et al., 2004). Some groups have turned to screening libraries of human pancreatic secretory trypsin inhibitor and minibodies (Dimasi et al., 1997), of camelized antibody VH domains (Martin et al., 1997) and antibodies (Kasai et al., 2001; Ueno et al., 2000) for NS3 protease inhibitors. These studies used the isolated molecules as leads toward developing small-molecule inhibitors (Martin et al., 1999; Tsumoto et al., 2002). Recently we reported the development of a novel bacterial genetic screen for the isolation of NS3 protease inhibitors and its application for the isolation of NS3-inhibiting single-chain antibodies (scFvs). The genetic screen was bases on concerted co-expression of NS3, an engineered, NS3 cleavable β-galactosidase as reporter and of potential inhibitors in the same bacterial cell. Once isolated, these scFv inhibitors were validated as specific NS3 binders and as NS3 serine protease inhibitors by an *in vitro* catalysis assay. We further showed that upon expression as cytoplasmic intracellular antibodies (intrabodies) in NS3-expressing mammalian cells, these scFvs inhibited NS3-mediated cell proliferation (Gal-Tanamy et al., 2005). Finally, these intrabodies inhibited subgenomic HCV RNA replicons (Gal-Tanamy et al., unpublished results).

In the study described herein we sought to evaluate the potential of an additional class of molecules to act as NS3 protease inhibitors. Peptide aptamers are combinatorial protein reagents which are usually selected from randomized expression libraries by virtue of their ability to bind to a given target protein under intracellular conditions. Typically, peptide aptamers consist of a short variable binding domain which is presented in the context of a supporting protein scaffold (Baines and Colas, 2006; Borghouts et al., 2005; Geyer and Brent, 2000; Hoppe-Seyler et al., 2004). Peptide aptamers can disrupt specific protein interactions and thus represent a useful method for manipulating protein function *in vivo*. It has been shown that peptide aptamers can specifically bind to and block the intracellular activities of a wide range of proteins, including cell cycle regulators (Cohen et al., 1998; Colas et al., 1996, 2000; Fabbrizio et al., 1999; Kolonin and Finley, 1998), oncoproteins (Butz et al., 2000; Xu and Luo, 2002), transcription factors (Chattopadhyay et al., 2006; Nagel-Wolfrum et al., 2004), signal transducers (Cui et al., 2005; Norman et al., 1999), membrane receptors (Buerger et al., 2003), and structural virus proteins (Butz et al., 2001). Peptide aptamers have thus emerged as valuable new tools to study intracellular protein–protein interactions and to constitute recognition modules that allow the creation of a molecular tool kit for the intracellular analysis of protein function. With regard to the application of peptide aptamers that inhibit viral proteins, it was recently shown that intracellular targeting of the HPV16 E6 oncoprotein by E6-binding peptide aptamers resulted in the apoptotic elimination of HPV16-positive cancer cells (Butz et al., 2000). More recently, peptide aptamers that bind to Geminivirus replication protein were applied to inhibit the replication of the virus within plant cells (Lopez-Ochoa et al., 2006).

Here we present peptide aptamers that were isolated from a library in *Escherichia coli* (*E. coli*). The library (of 10^8 clones) consisted of random peptide octamers displayed as C-terminal fusions with the *E. coli* maltose-binding protein (MBP). The aptamers were used by applying a unique bacterial genetic screen for HCV NS3 inhibitors we recently used to isolated NS3-inhibiting single-chain antibodies (Gal-Tanamy et al., 2005). We show that the aptamers can inhibit the NS3 protease activity *in vitro*, and upon introduction by gene transfection into Huh7 cells that support HCV-N subgenomic RNA replicons (Bourne et al., 2005), inhibit RNA replication and SEAP secretion that is directed by these replicons. Though not conclusive, these experiments support the hypothesis that blocking the replicon-encoded NS3 protease activity is the mechanism responsible for this inhibition. Our results suggest that peptide aptamers may possess therapeutic potential, both as lead structures for drug design and as a basis for the development of protein drugs.

2. Materials and methods

General Molecular Biology techniques and reagents were as described (Berdichevsky et al., 2003).

2.1. Bacterial strains

The following *E. coli* strains were used: TG-1 (*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB hsdSM*)*5* ($r_K^- m_K^-$) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]) (Stratagene, La Jolla, CA) for plasmid propagation and BL-21 (DE-3) (F^-, *omp*T, *hsd*S$_B$($r_\beta^- m_\beta^+$), *dcm*, *gal*, (DE3) *ton*A) (Novagen, Madison, WI) for the *tac* promoter driven aptamers expression. MC1061 (F^- *ara*D139 Δ(*ara-leu*)7696 *gal*E15 *gal*K16 Δ(*lac*)X74 *rps*L (Str^R) *hsd*R2 ($r_k^- m_k^+$) *mcr*A *mcr*B1) cells were used library construction and screening.

2.2. Expression vectors

2.2.1. For production of recombinant NS3 protease

Recombinant single-chain NS4A/NS3 (scNS3) where the NS3 protease is derived from the HCV BK strain (genotype 1b) was expressed from plasmid pMALc-NN-scNS3 and purified as described (Gal-Tanamy et al., 2005). This plasmid codes

Table 1
Oligonucleotide primers

Name of primer	Primer sequence
MBP-NS5A/B′-for	5′AGTGCCAAGCTTAACAGCAGACGACATCCTCGCTAGC 3′
MBP-FLAG-for	5′AGTGCCAAGCTTACTTGTCATCGTCGTCCTTGTAGTCCGGTACCGAGCTCGAATTAGTC 3′
H6-MBP-back	5′GATATACATATGCACCATCACCATCACCATTCCGGCAAAACTGAAGAAGGTAAACTG 3′
MBP-NNS8-HindIII-for	5′AGTGCCAAGCTTASNNSNNSNNSNNSNNSNNSNNCGGTACCGAGCTCGAATTAGTC 3′[a]
MalE-133-for	5′ CAACGGCAAGCTGATTGC 3′
pMALc-NHNN-XbaI-REV	5′ TTTAAATCTAGACGGCCAGTGCCAAGCTTA 3′
MBP-RT-AS primer	5′ GTCGATGTTGGACCATGCC 3′
MBP-RT-S primer	5′ AGGCGAAACAGCGATGACC 3′

[a] N represents A/C/T/G; S represents a 1:1 C/G mix.

for MBP-NS4A-NS3-His tag, where the NS3 protease is cloned between NcoI and NotI sites. A similar plasmid that codes for NS3 protease of the 1a genotype was constructed by using plasmid pVax-NS3/4a (Frelin et al., 2006) as template for the NS3 protease sequence and replacing it with the 1b protease of the pMALc-NN-scNS3 plasmid.

2.2.2. For expression of peptide aptamers by transfection

For evaluation as cytoplasmic peptide aptamers via gene delivery, we expressed them from a vector, pCMV/H6myc/Cyto-MBP, initially constructed for expression of fusion-stabilized, MBP-scFvs (Gal-Tanamy et al., 2005; Shaki-Loewenstein et al., 2005). The aptamer coding DNA was obtained by using the corresponding pMALc-NHNN-aptamer plasmid as template with primers MalE133-seq-for and pMALc-NHNN-XbaI-Rev (Table 1). The resulting PCR product was digested by BglII and XbaI and ligated with a vector fragment that was obtained by digesting pCMV/H6myc/Cyto-MBP with the same enzymes.

2.2.3. Construction of control aptamers and NNS8 library

Our design for expression of fusion-stabilized peptides (peptide aptamers) was based on fusion of peptide coding sequence to the C′-terminus of the *E. coli* maltose-binding protein (MBP). Control aptamers were constructed by PCR using pMALc-NN-EGFP (Bach et al., 2001) as template in a PCR reaction with primers H6-MBP-back and MBP-NS5A/B′-for or MBP-FLAG-for (Table 1) to fuse NS5A/B′ (EASEDVVCC) or FLAG (DYKDDDDK) peptides respectively. H6-MBP-back primer encodes for His-tag that allow purification of aptamers on Talon resin (as described below). The PCR product was digested by NdeI and HindIII restriction enzymes and re-cloned into the backbone of plasmid pMALc-2x (New England Biolabs, Ipswich, MA) digested by the same enzymes. The resulting plasmids, pMALc-NHNN-NS5A/B′ and pMALc-NHNN-FLAG, respectively, were introduced into *E .coli* MC-1061 strain and plated on X-gal containing indicator LB-Agar plates to validate the expected phenotypes.

The NNS8 library was constructed essentially as described above for aptamer controls with the following modification: the MBP-NNS8-HindIII-for degenerate primer (Table 1) was used as the 3′ end primer to fuse random octapeptides to the C-terminus of MBP. The pMALc-NHNN backbone was digested by BglI restriction enzyme and then by NdeI and HindIII restriction enzymes to reduce self-ligation. After ligation, the library was introduced to competent *E.* coli MC1061 bacteria by electroporation.

2.3. Applying the bacterial genetic screen to isolate NS3-inhibitory aptamers

The library encoding DNA was recovered from NNS8 library pool and introduced into MC1061 bacteria carrying the pMGT14 plasmid. This plasmid carries the enzyme and substrate components of our bacterial genetic screen; NS3 protease under the control of the arabinose-inducible *araBAD* promoter and an engineered *lacZ* gene encoding for the NS3-cleavable β-galactosidase reporter gene under the control of a weak constitutive *trpR* promoter (Gal-Tanamy et al., 2005).

The enrichment of cells that express NS3-inhibiting peptide aptamers by virtue of faster growth rate of NS3-inhibited clones on lactose as a sole carbon source was carried out as follows: MC1061 bacteria were used instead of the TG-1 strain that was used originally in the genetic screen since MC1061 cells cannot use arabinose as a carbon source because they carry a deletion of the arabinose utilization operon. The transformants (10^9 cells) were used to inoculate 1 liter of lactose M9 minimal medium (Miller, 1992), incubated at 30 °C for 24 h. In pMALc-NHNN, expression of peptide aptamers is under the control of an IPTG-inducible *tac* promoter and the plasmid carries an ampicillin-resistance cassette and the *col*E1 origin of replication, and is thus compatible with pMGT14 (kanamycin resistance, p15A replicon). The culture containing enriched NNS8 library clones was pooled and plated to yield individual colonies on 2 × YT agar plates supplemented with 0.004% (w/v) X-gal, 100 mg/ml of ampicillin, 50 mg/ml of kanamycin, 0.2% (w/v) arabinose and 0.05 mM IPTG. The plates were screened and blue colonies were picked. pMALc-NHNN-aptamer DNA were recovered from them and re-introduced into pMGT14 expressing cells to validate the results of the initial screen.

2.4. NS3 catalysis inhibition assay

Selected aptamers were expressed and purified from the soluble fraction of IPTG-induced plasmid carrying *E. coli* BL-21 cells using Talon resin (BD Biosciences) chromatography as recommended by the supplier.

An *in vitro* fluorometric assay for the measurement of NS3 protease catalysis inhibition by the purified aptamers was carried out essentially as described (Gal-Tanamy et al., 2005). Both peptide aptamers (MBP-peptide) and synthetic free peptides were evaluated for inhibition of NS3 catalysis. Synthetic peptides were purchased from Peptron Inc. (South Korea).

2.5. Immunoblot

An immunoblot was carried out to verify the expression of the bacterial genetic screen components in isolated colonies with "blue" phenotype. The isolated clones were grown in LB media supplemented with 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, to $A_{600\,nm} = 0.6$. The cells were induced with 0.2% (w/v) arabinose and 0.5 mM IPTG for 4 h at 30 °C and harvested by centrifugation. The cells were disrupted by a brief sonication on ice and the insoluble fraction was removed by centrifugation at 20,000 × *g* for 10 min at 30 °C. Next, 20 μg protein of the total soluble fractions were separated by SDS/PAGE and transferred onto nitrocellulose membrane as described (Berdichevsky et al., 2003). The membranes were blocked and reacted with mouse polyclonal anti-MBP-scNS3 mouse serum (Gal-Tanamy et al., 2005) and detected by HRP-conjugated goat anti-mouse antibodies (Jackson Immunoresearch Laboratories, Westgrove, PA). The membrane was developed using ECL reagents as described (Berdichevsky et al., 2003).

2.6. Cells and transfections

For HCV replication inhibition studies, we used the HCV genotype 1b strain N subgenomic replicon cell lines (Bourne et al., 2005; Yi et al., 2002). The control En5-3 cells are a clonal cell line derived from Huh7 cells by stable transformation with the plasmid pLTR-SEAP (Yi et al., 2002). These cells were cultured in DMEM supplemented with 10% fetal calf serum, 2 μg/ml blasticidin (Invitrogen, USA), penicillin, and streptomycin. Following transfection with Ntat2ANeo replicon RNAs, cells supporting replicon amplification were selected and maintained in the above media containing in addition 400 μg/ml G418 (geneticin). For inhibition assay, replicon cell were transfected with peptide aptamer-coding and control plasmids using the FuGENE reagent. About 10^5 replicon cells/0.5 ml medium were plated into each well of a 24-well plate 1 day prior to transfection. For each transfection, up to 0.5 μg of DNA was mixed with 1.5 μl of FuGENE reagent (Boehringer Mannheim, Germany) diluted in 25 μl of Optimem (Gibco BRL, Carlsbad, CA) and incubated for 20 min at room temperature. The DNA–FuGENE complex was than added directly to the cells. The culture medium was replaced every 24 h post-transfection and the secreted alkaline phosphatase (SEAP) activity was measured in these fluids as described below, reflecting the daily production of SEAP by the cells.

2.7. Alkaline phosphatase assay

SEAP activity was measured in supernatant culture fluids using the Phospha-Light Chemiluminescent Reporter Assay (Tropix, Bedford, Mass.) with the manufacturer's suggested protocol was reduced 3× in scale as follows: 10 μl-aliquots of the supernatant fluids from transfected cells were mixed in a tube with 30 μl of 5× dilution buffer diluted 1:1 in sterile water. The tubes were heated to 65 °C for 30 min; 35 μl of the samples were transferred to Luminometer tubes and 35 μl of assay buffer were added to the tubes and incubated for 5 min at room temperature. 35 μl of CSPD substrate diluted 20× in reaction buffer was added to the tubes and the tubes were incubated for 20 min at room temperature. The luminescent signal was read using a TD-20/20 Luminometer (Turner Designs, Inc.).

2.8. Real-time quantitative RT-PCR analysis of HCV RNA

Quantitative RT-PCR assays were carried out using SYBR Green I dye chemistry (Applied Biosystems, Foster City, CA) on a PRISM 7000 instrument (ABI). For detection and quantitation of HCV RNA, we used primers complementary to the 5′ non-translated region of HCV as previously described (Takeuchi et al., 1999). Results were normalized to the estimated transfection efficacy which was determined by the abundance of plasmid vector mRNA detected in a similar real-time RT-PCR assay using primers MBP-RT-AS and MBP-RT-S (Table 1) directed to the MBP gene encoded on the peptide aptamer vector. The primers were designed using the Primer Express software (Applied Biosystems). Briefly, Total RNA was isolated from transfected replicon cells 48-h post-transfection, and 1 μg of total RNA was converted to cDNA using random primers in a total volume of 20 μl. One microliter of the RT reaction was used as template for the Real time PCR reaction. The reaction was carried out and analyzed as recommended by the Supplier (Applied Biosystems).

3. Results

3.1. Construction and verification of the peptide aptamer controls

We chose to use the *Escherichia coli* Maltose Binding Protein (MBP) as a scaffold for our peptide aptamers, which was not tried before. To verify whether our recently established bacterial genetic screen can be applied for isolation of peptide aptamers with MBP as a scaffold, we constructed two aptamers to be used as controls. To that end, two different peptides were fused to the C-terminus of MBP that served as an aptamer scaffold in this study. We have chosen to use MBP as an aptamer scaffold because of its extreme stability, solubility and high expression level in the bacterial cytoplasm (Bach et al., 2001). In addition, MBP had been used in the past as a scaffold for presenting peptides to the immune system (Coëffier et al., 2000; Martineau et al., 1992). The FLAG epitope (amino acids sequence DYKDDDDK) was used as negative control. As a positive control we used the NS5A/B′ peptide (amino acids sequence EASEDVVCC). This peptide is derived from the N′-terminal region of the NS3 serine protease recognition site between non-structural proteins NS5A and NS5B of the 1b genotype, and was previously reported to inhibit NS3 pro-

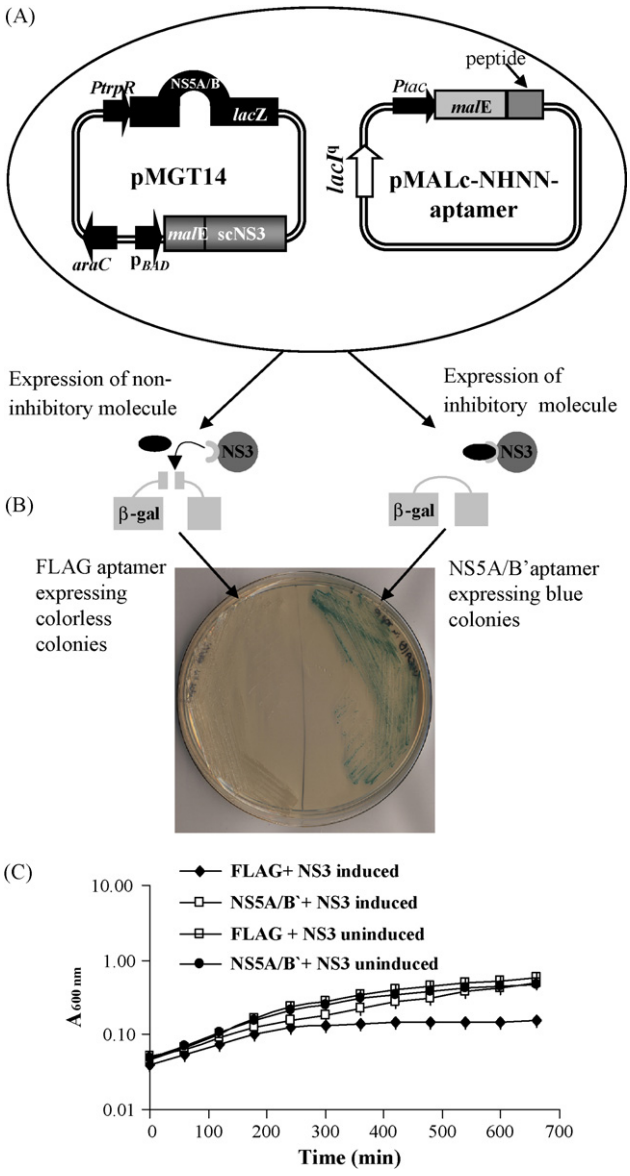

Fig. 1. Application of the bacterial genetic screen for validation of control aptamers phenotype. (A) Schematic representation of test bacteria containing two compatible plasmids: recombinant β-galactosidase and MBP-*sc*NS3 encoding pMGT14 plasmid and aptamer encoding pMALc-NHNN plasmid. Bacteria that express non-inhibiting FLAG aptamer formed colorless colonies when plated on X-gal supplemented plates (B, on the left) while bacteria expressing inhibitory NA5A/B′ aptamer grown in the same condition formed blue colonies (B on the right). (C) Twenty-four hours growth curves of FLAG and NS5A/B′ expressing test bacteria grown in M9 minimal media containing lactose as a sole carbon source with or without arabinose. Error bars represent the standard deviation of the data.

tease activity by the product-inhibition mechanism (Steinkühler et al., 1998). Our bacterial genetic screen is based on phenotypic changes that result from the concerted expression of enzyme, substrate and potential inhibitor in the same cell (Fig. 1A). An engineered derivative of β-galactosidase, which is cleaved by NS3 at the NS5A/B site that was inserted in a permissive region, when co-expressed with MBP-scNS3 enzyme and inhibitor in *E. coli*, yields blue colonies when plated on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) indicator plates (Gal-Tanamy et al., 2005). To confirm that our aptamers, when expressed in bacteria, carrying the MBP-scNS3 and recombinant β-galactosidase encoding plasmid yield an expected phenotype, aptamers encoding pMALc-NHNN plasmids were transformed into pMGT14 containing *E. coli* MC1061 cells and plated on X-gal indicative plates. As shown in Fig. 1B, both controls exhibited the expected phenotype: NS5A/B′ aptamer expressing bacteria grew as blue colonies on M9 minimal plates supplemented with 0.2% arabinose for induction of MBP-scNS3 protein and 1% lactose as inducer of aptamers and as the sole carbon source. In contrast, FLAG aptamer-expressing bacteria grown under the same conditions formed white (colorless) colonies.

To further verify the ability of control aptamers to interfere with the growth rate of the bacteria which is dependent on the activity of β-galactosidase, test bacteria carrying plasmid pMGT14 and one of the control aptamers, were grown in minimal media containing lactose as a sole carbon source in presence or absence of arabinose (as inducer of NS3) for 24 h. As shown in Fig. 1C, NS5A/B′ encoding bacteria retain the same growth rates in the presence and absence of arabinose in contrast to FLAG encoding bacteria that grew normally without arabinose but stopped to grow after 4 h when arabinose was added.

3.2. Construction and screening of the aptamers library

For the isolation of NS3 inhibitory peptide aptamers a large library, named NNS8, was constructed. The octapeptide encoding sequences were fused to the C-terminus of MBP using degenerated primers that were designed based on the NNS doping strategy (Barbas et al., 1992) to reduce the probability of stop codon in the undesirable position. The digested PCR products were ligated into pMALc-NHNN vector as described in Materials and Methods and introduced into *E. coli* MC1061 strain. The total library size was $>10^8$ individual clones. The aptamers encoding plasmids were recovered and transformed into *E. coli* MC1061 cells bearing the pMGT14 plasmid resulting in bacterial genetic screen bacteria. To partially enrich the library for NS3 inhibitors, we took advantage of the slower growth of cells where NS3 is active (as evident from Fig. 1C) on lactose. Thus, the library was subjected to one cycle of selective growth in M9 minimal media supplemented with lactose as sole carbon source. Under such conditions, clones that encode NS3-inhibiting aptamers should exhibit a faster growth rate. The enriched library was screened on X-gal indicator agar plates and 20 blue colonies were picked and analyzed firstly by PCR for the presence of aptamer and MBP-scNS3 encoding genes and later by an immunoblot for the expression of MBP-scNS3 and aptamer proteins (Fig. 2). The aptamer encoding plasmids of eight (seven inhibitors and one control) clones that were positive in both experiments were recovered and reintroduced into test bacteria to validate the results of the initial screen. All eight clones regained their "blue" phenotype (five of them are represented in Fig. 2C), confirming that the phenotypic change was indeed a direct result of aptamers expression as compared to aptamer recovered from the colorless colony. The aptamers were sequenced and seven different peptides were identified (Table 2).

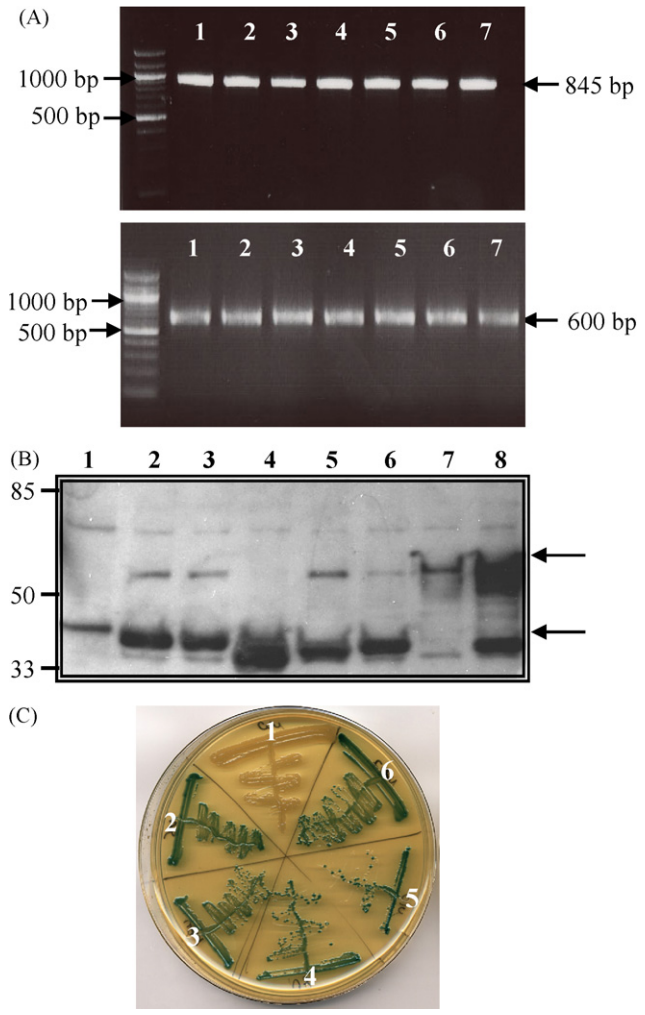

Fig. 2. Validation of the blue colonies isolated from the NNS8 library. (A) The presence of the aptamer (upper) and MBP-*sc*NS3 (bottom) encoding genes in the colonies with "blue" phenotype were validated by colony PCR using aptamer specific and MBP-scNS3 specific primers, respectively. The PCP products were separated using 1.5% agarose gel electrophoresis. Lanes 1–6 represent six different blue colonies and lane 7 represents a colorless colony isolated from the library. Arrows mark the positions of aptamer and MBP-scNS3, respectively. (B) Immunoblot: total soluble extract prepared from un-induced bacteria (1) and seven different induced blue colonies isolated from the library (2–8) were separated using SDS/PAGE, transferred onto nitrocellulose membrane and incubated with anti-MBP-scNS3 mouse polyclonal serum followed by HRP-conjugated goat anti-mouse secondary antibodies. The upper arrow marks the position of MBP-scNS3 and lower arrow marks the position of the peptide aptamer. (C) A sample of five of the clones N5, N7, N8, N10 and N21 (sections 2–8, respectively) and one colorless colony (Section 1), isolated from the NNS8 library, after the aptamer encoding plasmids were recovered from the original bacteria and reintroduced anew into test bacteria to validate their "blue" phenotype.

3.3. Validation of isolated aptamers as inhibitors of NS3 protease in vitro

To further confirm the inhibitory effect of aptamers on NS3 protease catalysis, aptamer-coding plasmids were introduced into an appropriate bacterial expression host for overproduction of the peptide-aptamers. The purified aptamers were characterized for NS3 catalysis inhibition by our *in vitro* fluorometric assay as described in Materials and Methods. As shown in Fig. 3A, all the tested aptamers inhibited NS3 catalysis in a dose-dependent manner with the IC_{50} in the low micromolar range, as compared to the control aptamer. Based on Fig. 3A, the IC_{50} values were about 10 μM for the N5, N7, N8 and N10 aptamers, and about 20 μM for the N21, N22, and N23 aptamers. However, their binding to NS3 could not be detected by ELISA (data not shown). Similar results were obtained when NS3 of the 1a genotype was used instead of the NS3 of 1b genotype that was used in the isolation of the aptamers (not shown).

Table 2
Deduced amino acids sequences of the peptide regions of the aptamers

Name of clone	Aptamer sequence
NNS8/N5	MBP: L G L A V E V R
NNS8/N7	MBP: F S R A E W F C
NNS8/N8	MBP: G A A L N T S S
NNS8/N10	MBP: A V L A G L G V[a]
NNS8/N21	MBP: F W G G A L W R
NNS8/N22	MBP: C C A W L S I R
NNS8/N23	MBP: L A A W F C L W

[a] The sequence was isolated twice.

To study the mechanism of inhibition, we carried out the catalysis assay using a fixed concentration of enzyme (100 nm) and varying concentration of substrate (10, 5, 2,5 and 1.25 μM). Peptide aptamer N5 was tested at (1 μM), which is about the IC_{50} for the 1a enzyme that was used in the kinetics experiment. The results were plotted as a Lineweaver–Burk plot (Fig. 3B). As shown, the presence of aptamer increases

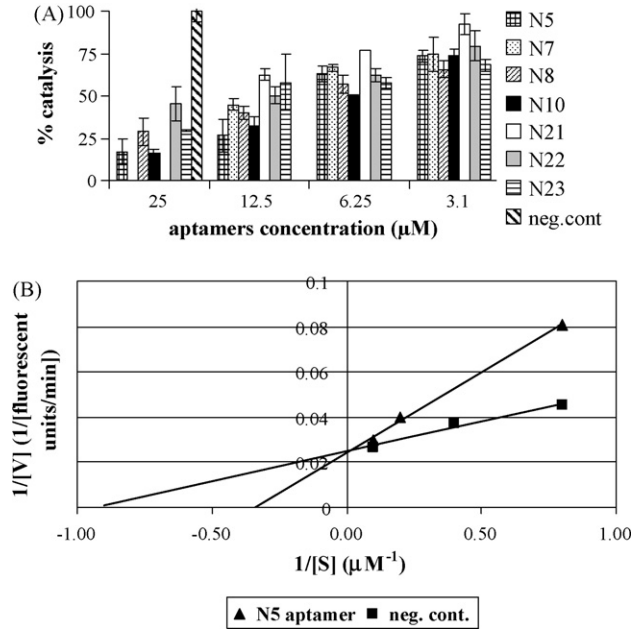

Fig. 3. Evaluation of the selected peptide aptamers from the NNS8 library inhibition of NS3 catalysis. (A) *In vitro* inhibition of NS3 catalysis at several concentrations of tested aptamers. Neg is a negative control peptide aptamer (isolated as a colorless colony). Error bars represent a standard deviation of the data (three independent experiments). (B) NS3 protease catalysis kinetics in the presence of 1 μM of peptide aptamers, Lineweaver–Burk plot.

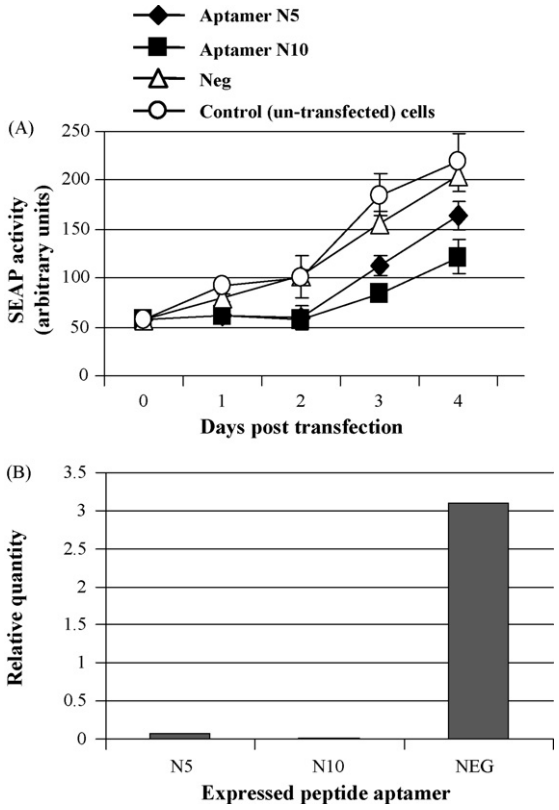

Fig. 4. Peptide aptamer-mediated inhibition of HCV RNA replicons. (A) Inhibition of SEAP secretion by RNA replicons. Activity of SEAP secreted from Huh7 cells supporting replication of HCV RNA replicons over successive 24-h intervals following transient transfection with peptide aptamers or control intrabodies (identified in the inset). Error bars represent the standard deviation of the data. (B) Real-time PCR analysis of replicon expression 48-h post-transfection. The results are shown as relative quantity of the HCV non-coding region normalized to level of aptamer expression. The experiments were repeated twice with triplicate RNA samples in each experiment.

the K_m of the enzyme, suggesting a competitive inhibition mechanism.

To study whether the free peptides derived from aptamers N5 and N10 (the most potent inhibitors) are inhibitory as well, we tested them in the catalysis assay using up to 100 μM. No catalysis inhibition could be observed with the free peptides (not shown).

3.4. Inhibition of HCV-RNA replicons by NS3 inhibitory peptide aptamers

The replication inhibition efficacy of our peptide aptamers was evaluated using the SEAP secreting replicon system (Yi et al., 2002). We introduced our most potent aptamers and a negative control into these replicon-bearing Huh7 cells by transient transfection of the pCMV-myc-cyto-MBP vectors. SEAP activity secreted from the cells was measured over successive 24-h intervals. As shown in Fig. 4A, SEAP secretion was inhibited in replicon cells transfected with the NS3-inhibiting peptide aptamer but not with the controls. Inhibition was well observed on the first and second days post-transfection, when peptide aptamer expression level was probably highest. On the third and fourth days post-transfection SEAP secretion increased, probably due to the decline in the expression of the transiently transfected aptamers.

To further evaluate the effect of the peptide aptamers on the HCV replicons, we carried out a real-time RT PCR analysis using primers that correspond to the non-coding region of the HCV RNA. The results (Fig. 4B) show that, 48 h post-transfection, replication of the RNA replicons was totally inhibited by both peptide aptamers. We tested the aptamers in control cells that contain all the components of the replicon system except for the replicon RNA itself. Those cells secreted a constant low level of basal SEAP activity from the reporter gene that was unaffected by the NS3-inhibiting or by the control aptamers (not shown). This suggests that the aptamers affect the replicon RNA itself in replicon-carrying cells, and that the inhibition of these replicons probably resulted from inhibition of the replicon-encoded NS3 protease activity which is required for the replicon RNA autonomous replication.

4. Discussion

The high prevalence of the disease caused by HCV and the limited efficacy of interferon-based therapies have stimulated the search for safer and more effective drugs. Our study of peptide aptamers as potential NS3 inhibitors was motivated by our recent studies with NS3-inhibiting intracellular antibodies (Gal-Tanamy et al., 2005; Zemel et al., 2004) that suggested that protein-based therapeutics merit further exploration. Peptide aptamers could provide protein-based therapeutics on the one hand, and leads for the identification of peptide-based drugs on the other.

Peptide aptamers are recombinant fusion proteins that have been selected for specific binding to a target protein in screens of combinatorial libraries (Hoppe-Seyler et al., 2004). They have been used to interfere with protein function in bacterial, yeast, and animal systems (Crawford et al., 2003). Their ability to interact with viral proteins and act as antiviral agents has been demonstrated in animal cells (Butz et al., 2001; Real et al., 2004). Our study is the first to employ a bacterial screen for peptide aptamers followed by their evaluation in mammalian cells.

In this report, we identified peptide aptamers that target the HCV NS3 protease and interfere with its function in vivo in an RNA replicon model system. Our research represents the first example of peptide aptamers that inhibit the replication of a model system for an RNA virus and supports the possibility of using aptamer technology to develop novel anti HCV therapeutics.

As a scaffold for our peptide aptamers we chose the *E. coli* MBP because of its robustness, ease of production and purification and its tolerance to peptide fusions or insertions at various permissive positions (Coëffier et al., 2000; Martineau et al., 1992; Vulliez-le Normand et al., 1997). Hence, ours is the first report of MBP as a scaffold for peptide aptamers, while in most reports, TrxA, GST, GFP and a catalytically inactive derivate of the staphylococcal nuclease were used as scaffolds (Hoppe-Seyler et al., 2004). We chose to evaluate C-terminally fused peptides rather than constrained ones as most current peptide-

based NS3 inhibitors were initially identified as linear (usually product-based) peptides (Kwong et al., 1998).

As a tool for aptamer discovery we used our bacterial genetic screen for NS3 inhibitors that we recently applied for the isolation of NS3-inhibiting single-chain antibodies (scFvs) (Gal-Tanamy et al., 2005), with a few modifications. A phenotypic screen such as ours is limited to several thousand clones, since several hundred colonies can be screened on each agar plate. Therefore, it is necessary to apply an enrichment step of the initial library (which was $>10^8$ clones) to reduce its size to a reasonable one. In our previous antibody study, we selected the initial library using NS3 as capturing antigen before applying the enriched population to the genetic screen. In the current study we were concerned that the binding affinity of the aptamers may be too low to allow affinity selection. Therefore, we took advantage of the faster growth rate of inhibitory-aptamer-expressing test bacteria in media where lactose is the sole carbon source that was evident from our control aptamers (Fig. 1C). The reason for the difference in growth rate is that, in bacteria where NS3 is un-inhibited, the NS3 protease cleaves the engineered β-galactosidase, limiting the cells' ability to utilize lactose. In contrast, in cells where NS3 is inhibited, β-galactosidase accumulates and allows more efficient growth on lactose.

Following the enrichment on lactose minimal medium we screened $\sim 10^4$ library clones on indicative plates. We picked 20 blue colonies for further analysis and validation by PCR, an immunoblot and re-cloning. Seven of the peptide aptamers survived the validation steps. They carry the coding DNA (Fig. 2A), they express NS3 and aptamer proteins (both are recognized by our anti MBP-scNS3 antibodies) (Fig. 2B), and they maintained the "blue" phenotype after re-cloning (Fig. 2C). Their sequences are listed in Table 2. These sequences bear no resemblance to known peptide or peptidomimetic inhibitors of NS3.

Next, the aptamer coding plasmids were used for overexpression, and purification of the aptamers was carried out using Talon metal chelate chromatography. The aptamers were evaluated for inhibition of NS3 catalysis using our *in vitro* fluorometric assay (Berdichevsky et al., 2003). As shown in Fig. 3A, the aptamers inhibited NS3 catalysis with IC_{50} values of 10–20 μM for the most potent ones, and >20 μM for others. These IC_{50} values are similar to those of known (mostly product-based) peptide inhibitors before they underwent massive engineering towards clinical development (De Francesco et al., 2003; Steinkühler et al., 2001). These values are about 10 fold higher than the IC_{50} values of the NS3-inhibiting scFvs we previously isolated using the same genetic screen. This is probably why we could not obtain significant binding signals when we evaluated the aptamers for NS3 binding in ELISA, since their binding affinity (would be around 10^{-5} M) is not sufficiently high for such analysis. The fact that we could identify such weak binders probably resulted from the fact the MBP and MBP fusion accumulate at high concentration when expressed in bacteria and also in mammalian cells (Bach et al., 2001; Shaki-Loewenstein et al., 2005).

Two of the most potent aptamers, N5 and N10 were chosen for further evaluation using an HCV RNA replicon. Until a few years ago, anti-HCV drug discovery efforts have been hampered by a number of factors including the lack of a fully permissive cell culture system allowing in vitro virus propagation. The development of replication-competent subgenomic replicon RNAs, in which most of the structural protein coding sequence is replaced by sequence encoding neomycin phosphotransferase (Lohmann et al., 1999) which was followed by other groups (reviewed in Bartenschlager et al., 2003; Lindenbach et al., 2005) has been a huge impetus for antiviral development. Recently, Lemon's group (Yi et al., 2002) described the construction of modified subgenomic genotype 1b HCV replicons. These are dicistronic subgenomic HCV replicons in which the upstream cistron was modified to express the human immunodeficiency virus (HIV) tat protein. When these replicons are stably transfected into Huh-7-derived cells that express secreted alkaline phosphatase (SEAP) under transcriptional control of the HIV long terminal repeat promoter, there is a strong correlation between intracellular HCV RNA abundance and the activity of SEAP secreted into the culture medium. Thus, active compounds are easily identified by direct enzymatic quantification of SEAP in the medium without post-assay processing (Bourne et al., 2005). These replicons were used to study the inhibition of HCV replication by various NS3 protease small-molecule inhibitors (Yi et al., 2006). We used these HCV-N replicons in the current study.

The results presented in Fig. 4 show that peptide aptamers N5 and N10 inhibited SEAP secretion by the replicons with varying efficiency. Examination of the N5 and N10 aptamer sequences reveal over representation of several amino acid residues, such as Leu, Ala and Gly. Interestingly, the two most potent peptide aptamers, N5 and N10 seem to share a sequence motif: N5, GLAV (residues 2–5) and N10, GLGV (residues 5–8). None of the aptamers we isolated bears any resemblance to a known peptide or peptide-based NS3 protease inhibitors. Therefore, at present, although we found that the inhibition is competitive, we cannot elaborate on the molecular details of the mechanism of inhibition. The modest IC_{50} values of even the most potent peptide aptamers preclude their consideration as potential therapeutic NS3 inhibitors. Rather, they should be considered as lead molecules that should be further improved.

When we tested the free synthetic peptides derived from aptamers N5 and N10, they did not exhibit any inhibition of NS3 protease activity up to 100 μM. This may not come as a total surprise, as there are many examples from the field of peptide phage display where peptides that are identified when displayed on the phage particle show reduced binding upon testing as free synthetic peptides (Ferrières et al., 2000; Galfrè et al., 1996; Zhu et al., 1999). A case-in point can be the recent description of B2.1, a peptide mimotope of anti HIV antibody B12. This peptide, isolated from a random peptide phage display was greatly reduced in affinity as a free peptide, and improved 60-fold by fusing it to the N terminus of MBP—the very scaffold we used for our peptide aptamers (Saphire et al., 2007). It is believed that in such cases, the supporting scaffold, be it the phage coat protein or the stabilizing protein scaffold, limit the degrees of freedom the peptide has in adopting 3D conformations and keeping it in a state more tuned to binding. An additional explanation may be some level of weak binding of the target protein by the supporting

protein scaffold. This interaction, which is undetectable by conventional immunoassays, adds to the weak binding affinity of the displayed peptide, which sums up to a significant binding signal. In such a case, the identified peptide aptamer should be considered as a primary "hit" in the drug discovery process, which should be considerable improved before it could be considered for further development.

The state of the art systems for studying HCV in culture are the so-called "infectious" HCV replicons such as the JFH1 system (Bartenschlager and Sparacio, 2007). Currently, none of the published HCV cell-culture system is of the 1b genotype. As a first step toward studying our peptide aptamers in such systems, we tested their ability to inhibit NS3 protease activity in vitro, when the enzyme is derived from the 1a genotype. We found that the 1a enzyme is inhibited by our aptamers similarly to the 1b isolate. This is quite encouraging since the 1b and 1a NS3 protease domains we used differ by 18 out of a 200 total residues (in the scNS3 configuration). In future studies we plan to test our aptamers using advanced HCV cell culture systems.

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