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Inhibition of protease-inhibitor-resistant hepatitis C virus replicons and infectious virus by intracellular intrabodies

Meital Gal-Tanamy[a,b,c], Romy Zemel[a], Larissa Bachmatov[a], Rohit K. Jangra[c], Assaf Shapira[b], Rodrigo A. Villanueva[c], MinKyung Yi[c], Stanley M. Lemon[c], Itai Benhar[b], Ran Tur-Kaspa[a,d,*]

[a] Molecular Hepatology Research Laboratory, Felsenstein Medical Research Center, Sackler School of Medicine, Tel-Aviv University, Petah Tikva, Israel
[b] Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat Aviv, Israel
[c] Center for Hepatitis Research, Institute for Human Infections and Immunity, The Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA
[d] Department of Medicine D and Liver Institute, Rabin Medical Center, Beilinson Campus, Petah Tikva, Israel

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ABSTRACT

Hepatitis C virus (HCV) infection is a common cause of chronic liver disease and a serious threat to human health. The HCV NS3/4A serine protease is necessary for viral replication and innate immune evasion, and represents a well-validated target for specific antiviral therapy. We previously reported the isolation of single-chain antibodies (scFvs) that inhibit NS3/4A protease activity in vitro. Expressed intracellularly (intrabodies), these scFvs blocked NS3-mediated proliferation of NS3-transfected cells. Here we show that anti-NS3 scFvs suppress HCV RNA replication when expressed intracellularly in Huh7 hepatoma cells bearing either subgenomic or genome-length HCV RNA replicons. The expression of intrabodies directed against NS3 inhibited the autonomous amplification of HCV replicons resistant to small-molecule inhibitors of the NS3/4A protease, and replicons derived from different HCV genotypes. The combination of intrabodies and interferon-α had an additive inhibitory effect on RNA replication in the replicon model. Intrabody expression also inhibited production of infectious HCV in a cell culture system. The NS3 protease activity was inhibited by the intrabodies in NS3-expressing cells. In contrast, cell-free synthesis of HCV RNA by preformed replicase complexes was not inhibited by intrabodies, suggesting that the major mode of inhibition of viral replication is inhibition of NS3/4A protease activity and subsequent suppression of viral polyprotein processing.

1. Introduction

Hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (De Francesco and Migliaccio, 2005). Despite recent advances in HCV therapy, even the best available therapy, namely a combination of PEGylated interferon-alpha and ribavirin, fails to eliminate infection in nearly 50% of patients (Feld and Hoofnagle, 2005). Such a high frequency of treatment failure points to the need for more specific, less toxic and more active antiviral therapies for HCV (Lindenbach and Rice, 2003).

The HCV NS3 protein plays a crucial role in viral replication and represents an ideal target for the development of antiviral agents (Lohmann et al., 1999). It is a component of the replicase complex along with the RNA-dependent RNA polymerase (RdRP) NS5B and other nonstructural proteins. However, its contribution to the role of the replicase complex in RNA synthesis is not understood (Zhang et al., 2005). Traditional drug discovery approaches, including small-molecule compounds active against the NS3-serine protease and RdRP, have been pursued for HCV. Despite the difficulties posed by the lack (until recently) of readily available laboratory models of viral infection, a number of investigational compounds have been developed and are in phase II clinical trials (De Francesco and Migliaccio, 2005; Malcolm et al., 2006; Perni et al., 2006; Reiser et al., 2005). However, the error-prone nature of the RdRP of HCV has resulted in the rapid emergence of drug resistance in patients treated with candidate antivirals, and, therefore, is likely to limit the efficacy of such therapies (Feld and Hoofnagle, 2005; Lin et al., 2004; Yi et al., 2006). Replicon systems provide a convenient cell culture system for studying HCV replication and candidate antiviral compounds targeting HCV. HCV replicons have also been used to

Abbreviations: FFU, focus forming unit; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NS, nonstructural protein; scFv, single-chain antibody; SEAP, secreted alkaline phosphatase.

* Corresponding author at: Department of Medicine D and Liver Institute, Rabin Medical Center, Beilinson Campus, Petah Tikva 49100, Israel. Tel.: +972 3 9376751/3; fax: +972 3 9220671.
E-mail address: rturkaspa@clalit.org.il (R. Tur-Kaspa).

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identify resistance mutations against candidate protease inhibitors (BILN 2061, VX950 and SCH6) (Lin et al., 2005; Yi et al., 2006). Resistance to these inhibitors is likely to be a major issue in treated patients.

A potential alternative antiviral approach for HCV could be the development of protein-based therapeutics. The rationale for such an approach is to inhibit the functions of key viral enzymes intracellularly. This can be achieved by intracellular expression of recombinant antibodies (so-called "intracellular immunization") targeting viral proteins (Cattaneo and Biocca, 1999; Marasco, 1997, 2001). Several attempts to inhibit HCV enzymatic activities with intrabodies have met with limited success (Heintges et al., 1999; Sullivan et al., 2002; zu Putlitz et al., 1999), while others have suggested the utility of this approach (Prabhu et al., 2004).

Previously we have described recombinant intrabodies that inhibit NS3-mediated cell transformation but do not inhibit the protease activity of NS3 (Zemel et al., 2004). More recently, we have isolated a panel of scFvs that inhibit NS3 protease activity in vitro (Gal-Tanamy et al., 2005). Here we show that these intrabodies inhibit replication of HCV RNA replicons in Huh7 cells when expressed by DNA transfection. Importantly, the intrabodies also inhibited replicons carrying mutations that confer resistance to small-molecule inhibitors of the NS3 protease (Yi et al., 2006). We also show that he intrabodies, initially directed against a genotype 1b NS3 protein inhibit replicons derived from multiple genotypes, and block production of infectious virus by an intergenotypic virus containing genotype 2a NS3 sequence. Our results suggest that, if problems of delivery can be overcome, the intracellular expression of antibodies that target HCV proteins and inhibit important viral functions may represent a promising new direction for therapy of HCV infection.

2. Methods

2.1. Cell lines

For HCV replication inhibition studies, we used an HCV genotype 1b strain N subgenomic replicon cell line (Bourne et al., 2005; Yi et al., 2002). The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 μg/ml blasticidin (Invitrogen), penicillin, streptomycin and 400 μg/ml G418 (geneticin). The full length NNeo/E1-5B replicon cell line was used for replicase assay and immunostaining and was cultured as described previously (Ikeda et al., 2002). Other subgenomic replicon cell lines included in these studies contained SEAP-secreting replicon cell lines with wt HCV-N NS3-NS5B sequence and three different mutant replicons: Sch6-pro-N2 (R109K mutant), Sch6-pro-N4 (A156T mutant) and Sch6-pro-N4-Re11 (A156T+P89L double mutant) (Yi et al., 2006; Yi et al., unpublished data) and replicon cell lines derived from genotypes 2a, 1a, and (Con1) 1b virus (Yi et al., unpublished data). For infectivity assays we used the FT3-7 cell line, a derivative of Huh7 cells (Yi et al., 2007). The Huh-7.5 cells (a gift from Charles Rice) were used for fluorescent focus forming unit (FFU) assays. Cells were cultured as described previously (Yi et al., 2007). For NS3 catalytic activity inhibition assay in cells, a tetracycline-inducible NS3 expression system was established. In this system, based on T-REx™ 293 cell line (Invitrogen), expression of a fusion between enhanced green fluorescent protein (EGFP) and the full length NS3 (protease/RNA helicase) followed by full length NS4A of the 1a HCV genotype (sequence was excised from the plasmid "NS3/4A-pVAX1" kindly provided by Prof. Matti Sällberg, Karolinska University Hospital, Sweden) was induced by addition of tetracycline (Tet) to the growth medium (Shapira et al., unpublished data).

2.2. Plasmids

To evaluate the cytoplasmic intrabodies, we expressed our scFv as fusion stabilized, MBP-scFv (Bach et al., 2001) cloned into the pCMV-myc-MBP-cyto-scFv plasmid. The construction of plasmid pCMV-myc-MBP-cyto-scFv used for cytoplasmic expression of fusion stabilized scFvs in transfected mammalian cells was described earlier (Gal-Tanamy et al., 2005; Shaki-Loewenstein et al., 2005). The NS3-inhibiting antibodies used here were previously described (Gal-Tanamy et al., 2005). However, the expression platform was modified as follows: the pCMV-myc-MBP-cyto-scFv mammalian expression vector was used as template for amplification of the scFv-coding DNA using primers 5′-AACAACCTCGGGTCCGAGAACCTCTACTTCCAGTCCATGGCCGAGGT-CCAGCTGCAGCAA-3′ and 5′-AGTGCCAAGCTTATGCGGCCCCATTCA-GATC-3′ by PCR. The PCR product was digested with restriction enzymes AvaI and HindIII and ligated with AvaI and HindIII digested fragment of the plasmid pMALc-2x (New England Biolabs) to generate pMALc-TNN-35. In this plasmid, the scFv-coding sequence was flanked with a TEV protease site (Kapust and Waugh, 2000) at the 5′-end and tandem His and myc tags at the 3′-end. The other scFvs were subcloned from the pMALc-NN plasmids (Gal-Tanamy et al., 2005) into the pMALc-TNN backbone as NcoI–NotI fragments.

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 (Gal-Tanamy et al., 2005). This plasmid codes for MBP-NS4A-NS3-His tag, where the NS3 protease is cloned between NcoI and NotI sites. For expression of NS3 protease derived from the HCV-N strain (also genotype 1b), its coding sequence was amplified by PCR using plasmid Ntat2ANeo (Yi et al., 2002) as template with primers 5′-agtaccatgGCGCCTATCGGCTCAGTAGTAATCGTAGGCAGAATCATCC-TGtccggccgtggtggcCCGATCACGGCCTACTCCCAACAG-3′ (that append a NcoI site followed by the coding sequence of the NS4A peptide to the 5′-end of the HCV-N NS3 protease coding sequence) and 5′-TGATGTgcggccgcTTACCGCATAGTGGTTTCC-3′ (that appends a stop codon followed by a NotI site to the 3′-end of HCV-N NS3 protease coding sequence). The resulting PCR product was digested with NcoI and NotI and replaced the corresponding NcoI–NotI fragment in the original pMALc-NN-scNS3 plasmid.

In order to monitor inhibition of specific NS3 proteolytic activity by intrabodies in tetracycline-induced cells, we constructed a plasmid that encodes for a modification of a previously described polypeptide which serves as a substrate for proteolysis by the NS3 protease (Berdichevsky et al., 2003). This plasmid, denoted "pCMV/MBP-EGFP-full 1b NS5AB-CBD", encodes for a fusion of maltose-binding protein (MBP), enhanced green fluorescence protein (EGFP), 18 amino acid NS3 cleavage sequence (P10–P8′) from HCV NS5A/B site derived from HCV genotype 1b and cellulose binding domain (CBD) (Shapira et al., unpublished data).

2.3. Virus

In vitro virus assays were carried out with an intergenotypic chimeric virus produced by replacing the core-NS2 segment of the JFH-1 virus genome with the comparable segment of the genotype 1a H77 virus (Yi et al., 2007). This chimeric virus, H-[NS2/NS3]-J/Y361H/Q1251L (hereinafter referred to as "HJ3-5"), contains two compensatory mutations that promote its growth in cell culture, one of which is within the E1 sequence (polyprotein residue 361) as shown (Yi et al., 2007). Virus stocks were produced in FT3-7 cells (Blight et al., 2002).

2.4. Replicon inhibition assay

Replicon cells (1×10^5) were transfected with 0.5 μg DNA of plasmid encoding NS3-neutralizing intrabodies using FuGENE reagent (Boehringer Mannheim, Germany) or Lipofectamine 2000™ (Invitrogen), according to the Manufacturer's protocol. G418 was removed from the cells before transfection with intrabodies. Transfection efficiency was determined by transfecting the cells with equivalent amounts of a GFP-coding plasmid and visualizing GFP fluorescence under a fluorescence microscope. Medium was replaced every 24 h post-transfection and the secreted alkaline phosphatase (SEAP) activity was measured as described (Yi et al., 2002). Results were normalized to 100% transfection efficiency.

2.5. Immunofluorescent staining

Replicon cells or FT3-7 cells infected with HJ3-5 chimeric virus were seeded into 8-well chamber slides (Nalge Nunc, Rochester, NY). At 24 h, cells were transiently transfected with intrabody-coding plasmids using Lipofectamine 2000™ (Invitrogen). Following 48 h of incubation, cells were fixed and stained with an anti-MBP monoclonal antibody alone or with mouse monoclonal antibody C7-50 specific for the core protein (1:300) and rabbit anti-MBP polyclonal antibody (1:100). This was followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (1:100) alone or with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes) (1:150) for double staining. Slides were examined using a Zeiss LSM 510 laser scanning confocal microscope.

2.6. Real-time RT-PCR

For quantitation of HCV RNA, we used primers complementary to the 5′ non-translated region of HCV as described previously (Takeuchi et al., 1999). Results were normalized to 18s RNA expression level and to the transfection efficiency determined by the abundance of plasmid vector mRNA in parallel by using the primers MBP-RT-AS and MBP-RT-S targeting the MBP gene that is fused to the scFvs genes as described previously (Trahtenherts et al., 2008). For quantitation of intrabody RNA level, we used primers MBP-RT-AS and MBP-RT-S and normalized the results to 18s RNA expression level. Briefly, total RNA was isolated from transfected replicon cells or infected cells at 48-h post-transfection and 1 μg of total RNA was used as template to synthesize cDNA by using random primers in a total volume of 20 μl. One microliter of the RT reaction was used as a template for the real-time PCR reaction. The reaction was carried out and analyzed as recommended by the supplier (Applied Biosystems).

2.7. Combination treatment with interferon-α

Replicon cells plated into a 24-well plate were transfected with 0.3 μg DNA of intrabody-coding plasmid. Before transfection, the medium was replaced with fresh medium containing various concentrations of recombinant IFN-α ranging from 0 to 100 units/ml. The medium was collected every 24 h for SEAP assay, and the cells were washed and re-fed with fresh IFN-α-containing medium.

2.8. Inhibition of infectious virus production

FT3-7 cells at 50% confluence were infected with HJ3-5 chimeric virus at a multiplicity of infection (moi) of 0.1. These cells were passaged until 100% of the cells were positive for HCV core antigen expression as tested by immunostaining. These cells were plated and transfected with intrabodies-coding plasmids using Lipofectamine 2000™ (Invitrogen), according to the Manufacturer's protocol. Medium was replaced at 48 h and collected 24 h later, and the infectious virus titer determined by focus forming unit (FFU) assay essentially as described previously (Yi et al., 2007).

2.9. ELISA

The NS3-inhibiting antibodies were expressed as fusion stabilized scFvs in the form of MBP-scFvs as previously described (Bach et al., 2001). MBP-scFvs were expressed and purified from the soluble fraction of IPTG-induced plasmid-carrying *E. coli* BL21 cells using amylose resin chromatography as previously described (Bach et al., 2001; Gal-Tanamy et al., 2005). As antigen in ELISA we used the MBP-scNS3 proteins that were also used in the NS3 catalysis assay (see below). ELISA plates were coated by diluting the protein to 4 μg/ml in 50 mM $NaHCO_3$ buffer pH 9.6. ELISAs were processed as described (Benhar and Reiter, 2002) using a mouse monoclonal anti-myc antibody (Sigma, Israel) followed by HRP-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories).

2.10. NS3 catalysis inhibition assays

An in vitro fluorometric assay for the measurement of NS3 protease catalysis inhibition by the purified scFvs was carried out as previously described (Berdichevsky et al., 2003; Gal-Tanamy et al., 2005) with the following modifications: the EFGP-NS5A/B-CBD substrate was immobilized onto cellulose prior to its exposure to enzyme and inhibitor. The reactions were carried out in 96-well plates in a volume of 100 μl containing 5 μM immobilized substrate, 100 nM MBP-scNS3 and 2.4 or 1.2 μM of tested MBP-scFv.

To evaluate the inhibition of specific NS3 proteolytic activity by intrabodies in cells we co-transfected 0.5 μg of the plasmids "pCMV MBP-EGFP-full 1b NS5AB-CBD" and 1.5 μg of intrabody encoding plasmids into T-REx 293 cells inducibly expressing EGFP-full NS3-full 4A (seeded 4×10^5 cells per well in 6-well Plate 24 h before transfection) using FuGENE 6 reagent (Roche, Germany), according to the manufacturer's instructions. The transfected cells were induced with 0, 10 or 1000 ng/ml tetracycline 24 h post-transfection. 24 h later the cells were washed with PBS, scraped and lysed in a buffer containing 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 10 mM Tris(HCl) pH 7.5, and protease inhibitors cocktail (Sigma, Israel). Following 30 min of incubation on ice, lysates were cleared by centrifugation at $20{,}000 \times g$ for 10 min, at 4 °C. For immunoblotting, protein samples were separated on 12% SDS/polyacrylamide gel, transferred to nitrocellulose and detected using rabbit polyclonal anti-CBD antibodies (kindly provided by Dr. Eli Morag) and anti-β actin for loading control, followed by goat anti-rabbit and goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories). Western blots were analyzed with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). For evaluation of EGFP-NS3 expression level following induction with different concentrations of tetracycline, T-REx 293 cells inducibly expressing EGFP-full NS3-full 4A (seeded 7×10^5 cells per well in 6-well Plate 24 h before addition of tetracycline) were supplemented with 3-fold dilutions of tetracycline (starting from 1000 ng/ml). Cells were lysed with RIPA buffer 48 h later and 30 ng of total protein were analyzed by immunoblotting with mouse anti-EGFP (Santa Cruz) (for the detection of EGFP-NS3) and mouse anti-actin antibodies (loading control) followed by HRP-conjugated secondary antibodies and ECL development.

2.11. In vitro HCV replicase assay

MBP-scFvs were expressed and purified from the soluble fraction of the IPTG-induced, plasmid-carrying *E. coli* BL21 cells using amylose resin chromatography as described earlier (Bach et al.,

2001; Gal-Tanamy et al., 2005). Replicon cells were trypsinized, washed twice with PBS and lysed in a hypotonic lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, and EDTA-free protease-inhibitor (Roche)] by passing 40 times through a 25-gauge needle at 4 °C over a period of 20 min. The cell lysate was spun at 1000 × *g* for 10 min (4 °C) to remove cell debris and nuclei. The post-nuclear fraction was centrifuged at 16,000 × *g* for 30 min (4 °C) to obtain a P16 fraction – the "heavy membrane" pellet that contains most of the HCV proteins. The pellet was re-suspended in a hypotonic lysis buffer and stored at 4 °C. Fresh P16 fractions were prepared for each experiment and used within the next 3–4 days. P16 isolated from naïve Huh7 cells was used as a negative control.

The P16 fraction (40 μg of total protein) that was isolated from replicon cells was incubated in 50-μl reactions with transcription mixture containing 50 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 15 μg/ml actinomycin D, 1 mM spermidine, 800 U/ml RNasin (Promega), 1 mM each of GTP, ATP, and UTP, 10 μM CTP, and 1 mCi/ml of α-^{32}P-CTP. The purified antibody/inhibitor was added to the mixture and the reactions were incubated at 37 °C for 3 h. Total RNA was isolated using an RNeasy Mini kit (Qiagen), ethanol precipitated and resolved on glyoxal containing 1% agarose gels (NorthernMax-Gly kit, Applied Biosystems). The gels were dried and imaged using a STORM 860 phosphorimager (Molecular Dynamics). The experiment was repeated 3 times starting with the isolation of fresh P16 fraction, with band intensities quantified using an ImageQuant TL (Amersham Biosciences) device.

3. Results

3.1. Inhibition of HCV RNA replicons by anti-NS3 intrabodies

We previously reported the isolation of seven different NS3-inhibiting scFvs that bind and inhibit subtype 1b BK strain NS3 protease (Gal-Tanamy et al., 2005). To evaluate the ability of these antibodies to inhibit viral RNA replication, we used replicons derived from the genotype 1b HCV-N strain virus (Yi et al., 2002). In this subgenomic HCV replicon system, sequence encoding the human immunodeficiency virus (HIV) tat protein is fused to the selectable marker Neo within the upstream cistron of these dicistronic replicons. Stably transformed Huh7 cells expressing SEAP under transcriptional control of the HIV LTR promoter actively secrete SEAP in response to tat expressed by these replicon RNAs (Yi et al., 2002). We expressed in replicon cells seven different intrabodies that we previously demonstrated to be capable of inhibiting the catalytic activity of the NS3/4A protease and, as a control, an intrabody (scFv 53Y) that binds NS3 but does not inhibit protease activity ("non-inhibitory" scFv) (Gal-Tanamy et al., 2005), as MBP-scFvs fusion proteins (Gal-Tanamy et al., 2005; Shaki-Loewenstein et al., 2005). Secreted SEAP activity was monitored every 24 h. For all transfections with intrabodies into replicon cells, transfection efficiency, as determined by immunostaining with anti-MBP antibody, was about 60%. We accounted for this in analyzing the subsequent SEAP results by normalizing changes in SEAP expression to a theoretic 100% transfection efficiency. As shown in Fig. 1A (left panel), SEAP secretion was reduced substantially by transfection of each of the seven intrabodies capable of inhibiting the protease, but not by expression of the control non-inhibitory anti-NS3 intrabody, 53Y. This inhibitory effect ranged between 70 and 100%. Inhibition was observed as early as the first day post-transfection, even though the SEAP reporter system lags 12–24 h behind changes in RNA abundance due to delays in tat-driven SEAP expression and secretion. Inhibition appeared to be maximal when intrabody expression was highest. By the third day post-transfection, SEAP secretion began to decrease, due to a decline in the expression of the transiently transfected intrabodies as shown by real-time RT-PCR analysis (Fig. S1B). Interestingly, scFvs 35 and 171 were the most potent inhibitors in these replicon assays, as they were also in our previous in vitro assays (Gal-Tanamy et al., 2005). Hence, these two intrabodies were selected for subsequent experiments.

To rule out the possibility that the inhibition of SEAP activity could have resulted from spurious inhibition of the functionality of one of the components of the SEAP reporter system present in these replicon cells, other than the NS3 protease, we carried out a control experiment. This was done using a control Huh7-cell line that constitutively expresses the tat2ANeo polyprotein under transcriptional control of a CMV promoter and translational control of the ECMV IRES (Yi et al., 2002) that constitutively directs the expression and secretion of SEAP by this control cell line. The intrabodies and additional control antibodies (anti-Tac, an anti IL-2R α subunit scFv (Kreitman and Pastan, 1994) and 225, an anti-EGFR scFv (Beerli et al., 1994)) were expressed by transfection as above. While SEAP secretion was inhibited in replicon cells after transient expression of NS3-inhibiting intrabodies 35 and 171 (Fig. 1B, left panel), there was no inhibition when the intrabodies were introduced into the control cells (right panel) or by the control intrabodies in either the replicon (left panel) or control cells (right panel). These results indicate that the inhibition of SEAP secretion results from intrabody-mediated inhibition of HCV RNA replication in the replicon cells and that the intrabodies do not cause non-specific cytotoxicity.

To further evaluate the intrabody-mediated inhibition of RNA replication, we carried out a real-time RT-PCR analysis using primers that targeted the 5′-non-coding region of the HCV RNA. In these experiments, to correct for intrabody transfection efficiency, the abundance of the HCV replicon RNA was normalized to the abundance of the intrabodies' coding RNA as determined by a similar real-time RT-PCR assay using primers specific for the MBP gene. The results (Fig. 1A, right panel) showed that, at 48 h post-transfection, the abundance of HCV replicon RNA was strongly reduced by expression of either intrabodies 35 or 171, relative to the replicon abundance observed in cells transfected with the control intrabody, 53Y. These results thus provide further confirmation of the inhibition observed in the SEAP assay (Fig. 1, left panel).

Next we utilized fluorescence microscopy to examine the impact of intrabody expression on viral protein expression in cells bearing a genome-length (genotype 1a, H77) replicon. Cells were transfected as above, then dually labeled for HCV core protein and intrabody expression by a two-color labeling protocol. As expected, core antigen labeling (Fig. 1C, green, left panels) was markedly reduced in cells expressing either intrabody 35 or 171 (Fig. 1C, red, middle panels). Cells lacking evidence of intrabody expression showed robust expression of the core protein. In contrast, dual-labeling of individual cells was observed in cultures transfected with the control 53Y intrabody (Fig. 1C, see merged images in the right panels). These results provide strong support for the intrabody-mediated inhibition of HCV RNA replicon observed in the normalized SEAP assay. The immunostaining also confirmed that the inhibitory and non-inhibitory intrabodies were expressed at comparable levels. This was also confirmed by immunoblot (data not shown).

3.2. Inhibition of protease-inhibitor-resistant HCV replicons by NS3-inhibitory intrabodies

Protease-inhibitor-resistant replicons were derived from the genotype 1b HCV-N replicons by selection against the peptidomimetic ketoamide inhibitor, SCH6 (Yi et al., 2006). These mutants carry mutations A156T and R109K in NS3 that variably confer resistance also to other protease inhibitors, including

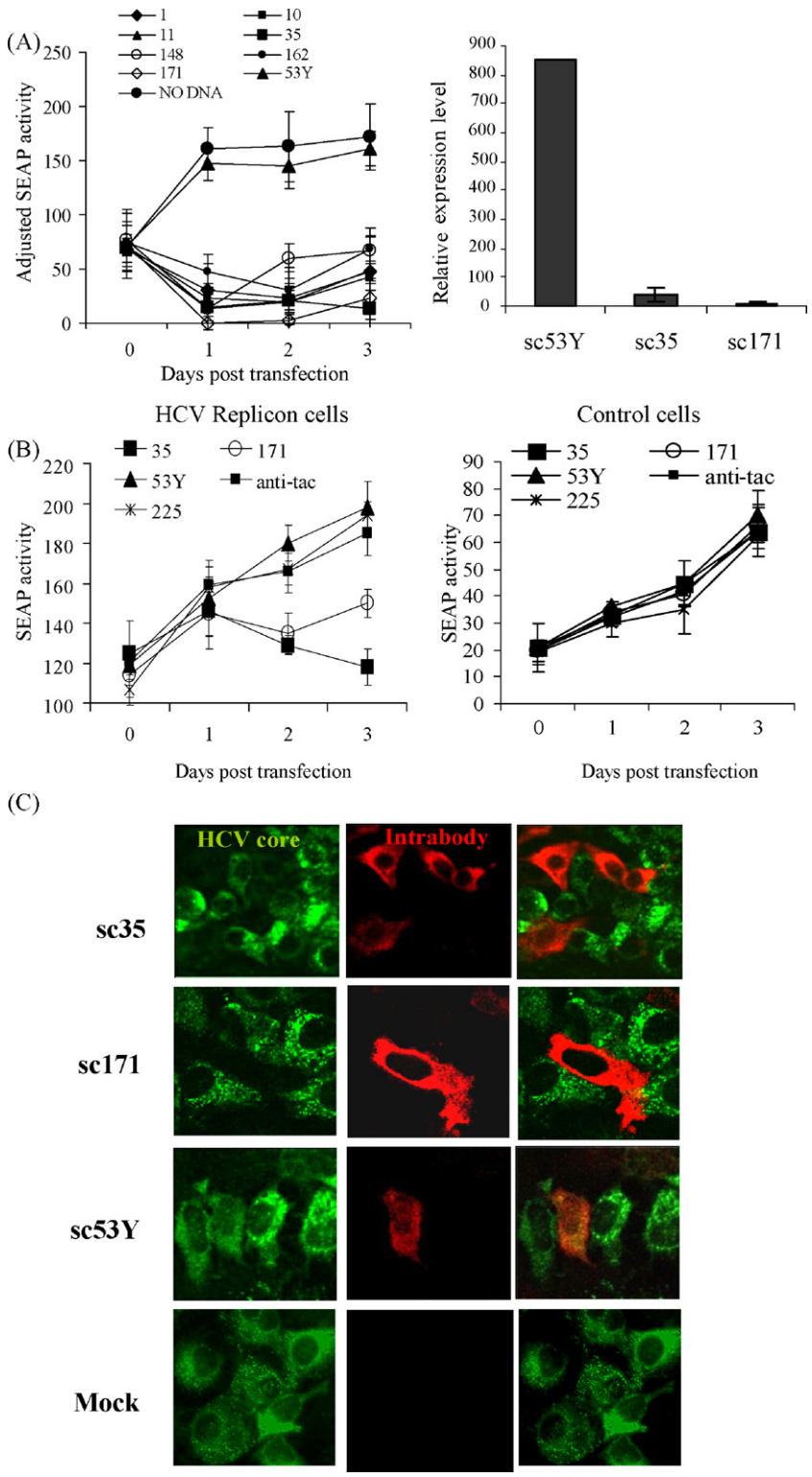

Fig. 1. Intrabody-mediated inhibition of HCV RNA replicons. (A) (Left panel) SEAP activity secreted from HCV RNA replicon cells every 24 h following transient transfection with seven NS3-inhibiting and one control non-inhibitory intrabodies. Results were corrected for DNA transfection efficiency as described in the text. Error bars represent the standard deviation of the data. (Right panel) Real-time PCR analysis of replicon expression at 48 h post-transfection. The results are shown as the relative quantity of the HCV non-coding region normalized to the level of intrabody expression. Error bars represent the standard deviation of the data. (B) Inhibition of HCV RNA replicon cells and control cells by two NS3-inhibiting and three control, non-inhibitory intrabodies. SEAP activities secreted from (left panel) HCV RNA replicon cells or (right panel) control Huh7/Et2A cells every 24 h following transient transfection with intrabodies. Error bars represent the standard deviation of the data. (C) Immunofluorescence detection of intrabody expression in replicon cells and related suppression of viral antigen expression. Replicon cells were transiently transfected with intrabodies 35, 171 or 53Y. Slides were fixed and stained with mouse mAb to core antigen (green) and rabbit polyclonal antibody to MBP antigen (red), followed by laser scanning confocal microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

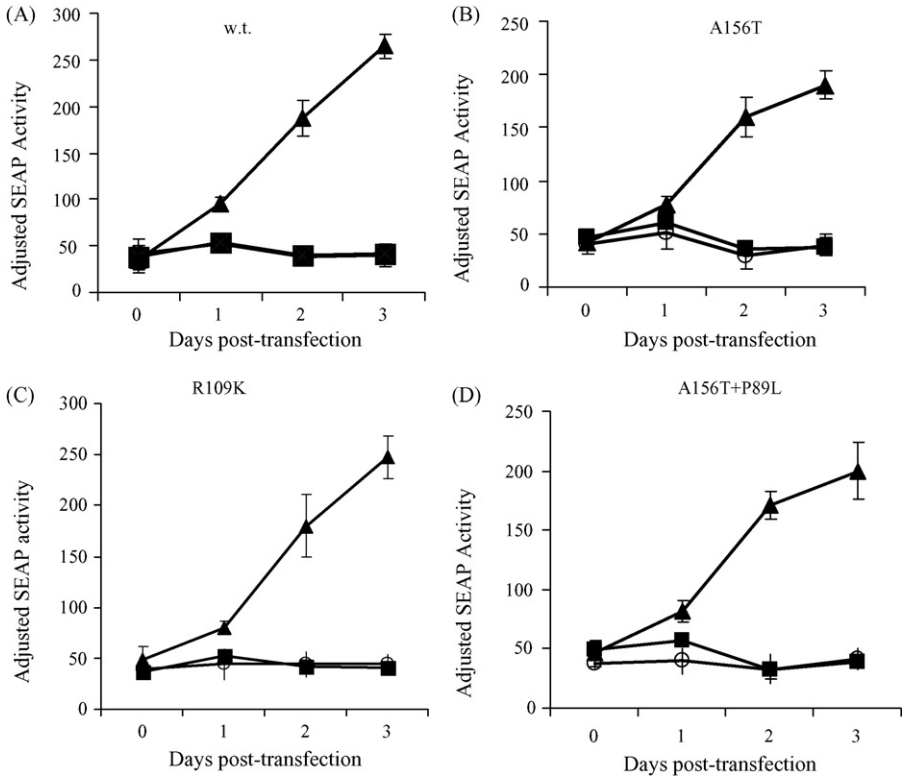

Fig. 2. Intrabody-mediated inhibition of protease-inhibitor-resistant HCV-N RNA replicons. Intrabodies 35 (filled squares) and 171 (open circles) were compared to the non-inhibitory (control) intrabody 53Y (filled triangle) as the control for their effect on SEAP activity secreted from four different replicon cell lines (A, B, C and D) with NS3 inhibitor-resistance; secreted SEAP activity was monitored every 24 h following transient transfection and normalized as in Fig. 1. Error bars represent the standard deviation of the data.

SCH503034, BILN 2061 and VX-950. To determine whether the replication of these protease-inhibitor-resistant mutants is inhibited by NS3 intrabodies, we expressed these intrabodies in replicon cell lines containing three different mutant replicons: Sch6-pro-N2 (R109K mutant), Sch6-pro-N4 (A156T mutant) and Sch6-pro-N4-Re11 (A156T+P89L double mutant) and monitored SEAP activity as described above. P89L is a compensatory mutation that restores replication fitness reduced by the A156T mutation without modifying the replicon's drug resistance (Yi et al., 2006). As shown in Fig. 2, amplification of the protease-inhibitor-resistant replicons was substantially inhibited by intrabodies 35 and 171, to the same extent as the wild-type (w.t.) replicon. There was no inhibition in cells transfected with the control intrabody, 53Y.

3.3. Cross-genotypic inhibition of HCV RNA replicons by the NS3-inhibiting intrabodies

The data shown in Fig. 1C indicate that the NS3 intrabodies are able to inhibit replication of genotype 1a as well as genotype 1b HCV replicons. To further evaluate the range of HCV genotypes that the intrabodies (selected against a genotype 1b protease) are capable of inhibiting, we utilized additional SEAP-secreting subgenomic replicons derived from genotype 1b (Con1), 1a (H77) and 2a (JFH1) virus sequences (M. Yi and S.M. Lemon, unpublished data). We expressed intrabodies 35, 171 and 53Y in these different replicon-bearing cells and monitored SEAP activity as described above. We observed efficient inhibition of the amplification of each of these replicon RNAs following transient expression of intrabodies 35 and 171, ranging from 90 to almost 100% (Fig. 3, results normalized according to transfection efficiency).

3.4. Inhibition of infectious virus production by anti-NS3 intrabodies

To evaluate the potential of the intrabodies to inhibit production of cell culture-infectious HCV, we expressed the intrabodies in cells infected with the intergenotypic HJ3-5 chimeric virus (Yi et al., 2007). The genome of this virus is comprised of genotype 1a sequence encoding the structural proteins, and genotype 2a proteins expressing the nonstructural proteins, including NS3. It is efficient for infection of Huh7 cells with subsequent cell-to-cell spread, resulting in 100% of FT3-7 cells being infected. Infected cells were transiently transfected with plasmids encoding intrabodies 35, 171 or 53Y as a control. Supernatant fluids were collected from the cell cultures 48 h post-transfection of intrabody DNA, and the titer of infectious virus was determined by FFU assay (see Section 2). Results were corrected according to the transfection efficiency. The results shown in Fig. 4A provide strong evidence for NS3 intrabody-mediated inhibition of infectious virus production. Intrabody 35 inhibited virus production by about 60%, and intrabody 171 by about 80%, compared to the titer of infectious virus released by cells expressing the control intrabody, 53Y. Consistently, intrabody 171 was the most potent inhibitor of virus production, as it was in previous in vitro protease and replicon inhibition assays (Fig. 1).

An efficient inhibition of HCV RNA production was observed also by a real-time RT-PCR analysis of infected cells transfected with intrabodies, using primers that targeted the 5′-non-coding region of the HCV RNA. To correct for intrabody transfection efficiency, the abundance of the HCV RNA was normalized to the abundance of the RNA encoding the intrabodies. The results (Fig. 4A, right panel) showed that, at 48 h post-transfection, the abundance of HCV RNA was strongly reduced by expression of either intrabodies 35 or 171,

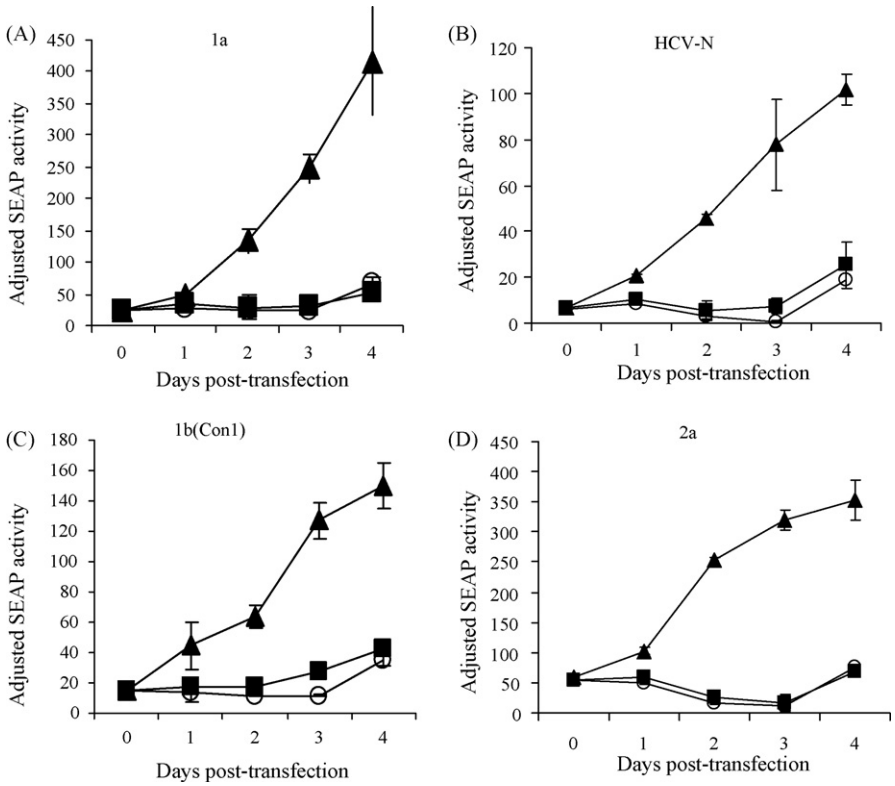

Fig. 3. Cross-genotypic intrabody-mediated inhibition of HCV RNA replicon amplification. Intrabodies 35 (filled squares) and 171 (open circles) were compared to the non-inhibitory (control) intrabody 53Y (filled triangle) as the control for their effect on SEAP activity secreted from HCV RNA replicon cells derived from genotypes (A) 1a (H77), (B) 1b (HCV-N), (C) 1b (Con1) and, (D) 2A (JFH1). SEAP activity in supernatant fluids was monitored every 24 h following transient expression of the intrabodies and normalized as in Fig. 1. Error bars represent the standard deviation of the data.

relative to the replicon abundance observed in cells transfected with the control intrabody, 53Y.

We confirmed these results using the dual-antibody immunostaining protocol described above. Infected cells were dually labeled with mouse mAb specific for the core protein and rabbit polyclonal antibody specific for MBP after being transfected with the intrabody-expressing DNAs. As shown in Fig. 4B, the infected cells transfected with intrabodies 35 and 171 that were stained with the anti-MBP antibody (red, middle panel), showed markedly reduced labeling with the anti-core antibody (green, left panel) (For interpretation of the references to color in this text, the reader is referred to the web version of the article.). These results indicate that virus replication and protein expression are inhibited within infected cells by the intrabodies, validating the data obtained with the FFU reduction assay.

3.5. Combination treatment with interferon-α

Since combination therapy with ribavirin and interferon-α is currently the most effective licensed therapy for chronic HCV infection (Okuse et al., 2005), it was of interest to determine if combination treatment with the NS3-inhibiting intrabodies and interferon-α would produce enhanced inhibition of replication in replicon cells. Replicon cells treated with 0, 10 or 100 units/ml interferon-α were transiently transfected with either intrabody-coding plasmid DNA (scFvs 35 or 171) alone, or co-transfected with both. We used half the amount of DNA for transfection in this assay compared to previous replicon inhibition assays to obtain 50% inhibition. Fresh medium containing interferon-α was added to the cell culture every 24 h. SEAP activity was measured at 48 h post-transfection. As shown in Fig. 5, SEAP activity was inhibited by treatment with interferon-α alone in a dose-dependent manner. An interferon-α concentration of 100 units/ml inhibited SEAP activity to about 30–35% over this relatively brief period of treatment. In the absence of interferon, the expression of the intrabodies resulted in an inhibition of about 50%. Importantly, at either concentration of interferon-α, there was a greater decrease in the SEAP activity in cells expressing intrabodies. In the presence of both intrabodies and interferon-α (100 units/u), the level of inhibition increased to almost 80%. Thus, the combination appeared to have an additive inhibitory effect on the replicons. Co-transfection with both scFvs 35 and 171 resulted in no significant increase in inhibition compared to that achieved by each scFv alone.

3.6. Effect of intrabodies on NS3 catalysis and cell-free HCV RNA synthesis

Previously, we have reported that the inhibitory antibodies bind and inhibit subtype 1b BK strain NS3 protease activity in vitro (to which the intrabodies were raised), with an IC_{50} of about 600 nM (Gal-Tanamy et al., 2005). The data shown in Fig. 1 indicate that the NS3 intrabodies inhibit the replication of replicons derived from the genotype 1b HCV-N strain. The HCV-N NS3 protein differs from the BK protease at 8 residues within the 179 residues of the protease domain. Here, we demonstrated that the antibodies bind and inhibit the NS3 protease of both genotype 1b viruses equally well (Fig. 6A–C).

To further verify that the intrabodies' mechanism of inhibition is mediated by disruption of HCV polyprotein processing, we monitored the cleavage of the substrate MBP-EGFP-full 1b NS5AB-CBD containing the NS3 cleavage site in the presence of NS3 and intrabodies in HEK293 cells that express NS3 upon tetracycline induction. Immunoblot analysis shows efficient cleavage of the substrate in cells transfected with the substrate-encoding

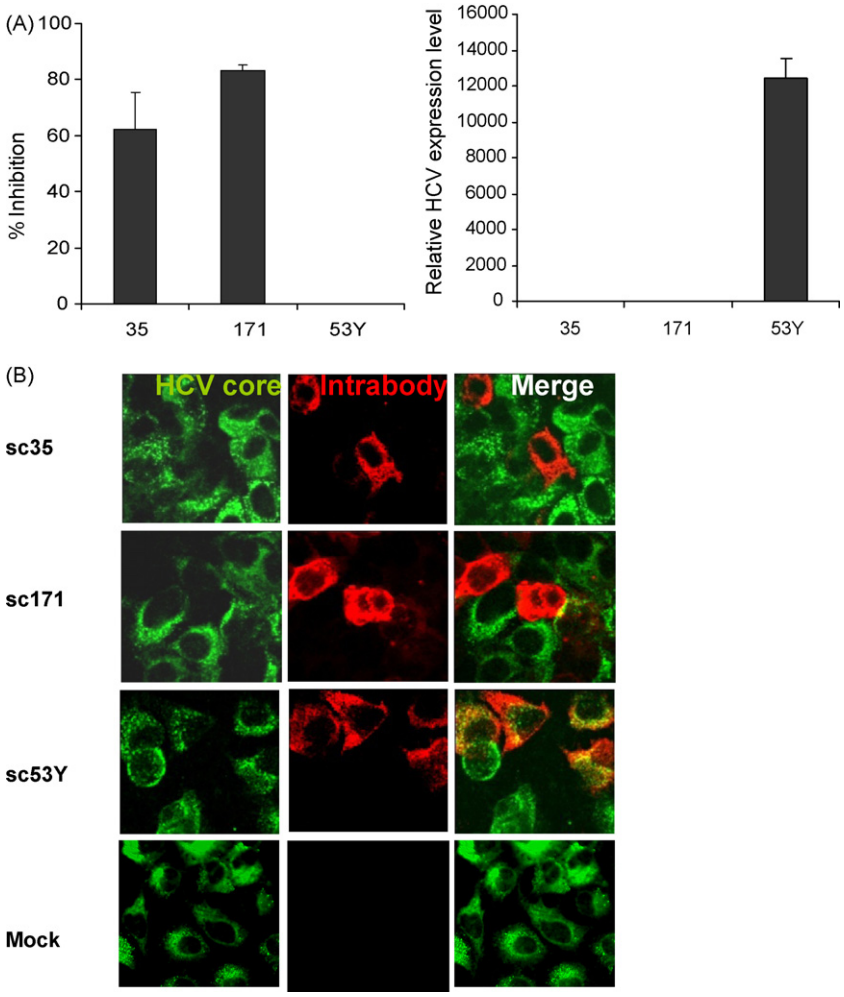

Fig. 4. Intrabody-mediated inhibition of infectious virus production. (A) (Left panel) Inhibition of HJ3-5 virus production, as determined by FFU assay of virus released into media by HJ3-5 virus-infected cells following transfection with intrabodies. Percent of inhibition was determined compared to virus released by non-transfected cells set as 100%. Error bars represent the standard deviation of the data (A, right panel). Real-time PCR analysis of HCV RNA expression level 48 h post-transfection with intrabodies. The results are shown as the relative quantity of the HCV non-coding region normalized to the level of intrabody expression. Error bars represent the standard deviation of the data. (B) Double label immunofluorescence detection of intrabodies and viral core protein in virus-infected cells and transiently transfected with plasmids encoding intrabodies 35, 171 or 53Y. Slides stained with mouse mAb to core antigen and rabbit polyclonal antibody to MBP antigen and analyzed by laser scanning confocal microscopy, as in Fig. 1C.

plasmid and induced for low or high NS3 expression by the addition of 10 or 1000 ng/ml of tetracycline, respectively (Fig. 6D). In contrast, inhibition of substrate cleavage was observed in NS3-expressing cells induced with either 10 or 1000 ng/ml tetracycline co-transfected with the substrate and intrabody 35-encoding plasmids (Fig. 6D).

The effect of anti-NS3 intrabodies on HCV RNA synthesis was examined in a cell-free HCV replicase assay incorporating membrane-bound HCV replication complexes isolated from subgenomic replicon cells in the presence or absence of purified bacterially expressed MBP-scFv 171 or control scFv proteins. As additional controls, we used a pyrimidine analog that is a potent active-site RdRP (NS5B) inhibitor (Summa et al., 2004; Tomei et al., 2004), and the ketoamide NS3/4A protease-inhibitor, SCH6. Radioactively labeled, in vitro HCV RNA products were extracted, and resolved through denaturing agarose gel electrophoresis. As shown in Fig. 6E, the NS5B inhibitor inhibited RNA synthesis in a dose-dependent manner, as expected (lanes 1–3). In contrast, the NS3 protease-inhibitor SCH6 did not reduce RNA synthesis (lanes 4–6). Similarly, neither the inhibitory anti-NS3 scFv-171 (Fig. 6E, lanes 15–19) nor the control non-inhibitory scFv (Fig. 6E, lanes 10–14) had much effect on RNA synthesis in this cell-free assay. Even at very high concentrations (800 μg/ml), the maximum inhibition of HCV RNA synthesis was generally <50% in replicate assays with the most potent inhibitory scFv, 171.

4. Discussion

The high prevalence of liver disease caused by HCV and the limited efficacy of interferon-based therapies have stimulated the search for less toxic, more effective treatment regimens. However, the replicative nature of HCV infections, coupled with the relatively high mutation rate of RNA viruses like HCV, has resulted in the rapid emergence of drug-resistant virus in patients treated with candidate small-molecule therapeutics. The emergence of such mutations has been reported in NS3 during the early weeks of treatment with protease inhibitors (Kieffer et al., 2007; Lin et al., 2005; Lu et al., 2004; Sarrazin et al., 2007; Yi et al., 2006), as well as in the RdRP, NS5B, during treatment with NS5B inhibitors (Kukolj et al., 2005; Mo et al., 2005; Nguyen et al., 2003). This may limit the clinical usefulness of these inhibitors, and enforce their use only in combination with other antivirals.

Our search for antibodies that could be therapeutically-useful NS3 inhibitors resulted in the isolation of 7 scFvs from an immune scFv library. Each of these is capable of inhibiting NS3 cat-

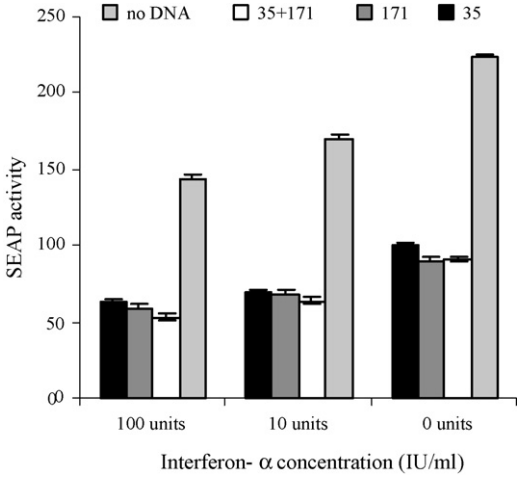

Fig. 5. Inhibition of HCV replicon amplification in combination with interferon α. SEAP activity secreted from HCV RNA replicon cells every 24 h following transient transfection with intrabodies. Following transfection, 0, 10 or 100 units/ml Interferon-α were added to the cells every 24 h. Results were not corrected for DNA transfection efficiency in this experiment. Error bars represent the standard deviation of the data.

alytic activity in vitro, and the NS3-mediated proliferation of NS3-transfected rat fibroblasts when expressed intracellularly (Gal-Tanamy et al., 2005). In the present study, we have further evaluated these intrabodies using SEAP-secreting HCV-N subgenomic RNA replicon. Previous studies have shown a strong correlation between intracellular HCV RNA abundance and the activity of SEAP secreted into the culture medium by these cells. Thus, active compounds that affect the autonomous replication of the replicon RNA are easily identified by direct enzymatic quantification of SEAP in cell culture medium without the need for post-assay processing of cells (Bourne et al., 2005). When we expressed anti-NS3 intrabodies by transient transfection of DNA into these replicon-carrying Huh-7 cells, we found that SEAP secretion from the replicons was inhibited (Fig. 1A, left panel). Consistent with this, viral RNA abundance was also reduced (Fig. 1A, right panel). Experiments with additional control intrabodies and control cell lines (Fig. 1B) and the use of a dual-labeling immunofluorescence approach (Fig. 1C) indicated that the inhibition resulted from specific suppression of the HCV RNA replicons.

We also characterized the ability of the intrabodies to inhibit replication of replicons with A156T and R109K substitutions that confer resistance to candidate antiviral compounds (Yi et al., 2006). Mutation at residue 156 of NS3 confers resistance to BILN2061, VX-950 (Telaprevir) (Lin et al., 2004; Lu et al., 2004), SCH6 (SCH446211) and SCH503034. In contrast, a Lys substitution at residue 109 resulted in resistance to SCH6 only (Yi et al., 2006). We found that protease-inhibitor-resistant replicons with A156T and R109K substitutions were inhibited by intrabodies 35 and 171 (the most potent of the set of seven intrabodies we studied), as effectively as with the protease sensitive w.t. replicon (Fig. 2). The development of intrabodies directed against NS3 was motivated by the premise that, because antibodies engage their target antigens through multiple contact residues and over a relatively large contact area, the emergence of escape mutants from antibody-mediated inhibition is likely to emerge more slowly than with small-molecule inhibitors of the protease. Indeed, the data presented here show that at least two drug resistance mutations have no effect on the inhibitory activity of intrabodies, despite the fact that the intrabodies tested inhibit the protease activity of NS3/4A and therefore might bind (near) to the protease active-site. Moreover, this data suggest that the intrabodies may be effective in combination with small NS3 inhibitors. We have also shown the potential of intrabodies to effectively inhibit replicons with NS3 sequences derived from diverse HCV genotypes. Intrabodies 35 and 171 demonstrated broad inhibitory activity against replicons derived from genotypes 2a, 1a and 1b (both HCV-N and Con1) (Fig. 3). The ability of intrabodies to function as inhibitors against different genotypes is demonstrated clearly by their ability to inhibit replication of the HJ3-5 virus (Fig. 4): the NS3 protease of this virus is derived from the genotype 2a JFH1 strain of HCV, while the intrabodies were raised to the protease of BK virus, a genotype 1b HCV. This observation, coupled with the ability of the intrabodies to inhibit mutant replicons resistant to protease inhibitors, suggest that these intrabodies show promise of overcoming challenges in HCV antiviral development imposed by the genetic diversity of the virus.

Ultimately, it is likely that a combination of multiple antivirals will be required to contain the emergence of drug-resistant HCV variants in the absence of concomitant interferon therapy. We found that the combination of anti-NS3 intrabodies with IFN-α had an additive effect in reducing HCV RNA replication (Fig. 5). The exact mechanism of this additive effect remains to be elucidated, as is whether such an effect can be expected with chemical inhibitors of the NS3/4A protease. Importantly, scFvs 35 and 171 compete with each other for binding to NS3, and thus probably engage similar or identical epitopes on NS3 and have a similar mechanism of inhibition (Gal-Tanamy et al., 2005). They showed no increased inhibitory effect when expressed simultaneously (Fig. 5).

In vitro data presented here (Fig. 6A–C) and previously (Gal-Tanamy et al., 2005) show that anti-NS3 intrabodies inhibit the enzymatic activity of the NS3 protease both in vitro and in transfected cells. Moreover, the presence of intrabodies in NS3-expressing cells inhibits NS3 catalysis as indicated by monitoring the cleavage efficiency of an NS3 substrate (Fig. 6D). Thus, the disruption of polyprotein processing is presumed to be the major mechanism by which these intrabodies might inhibit RNA replication and virus production. However, in the context of the virus, NS3 is a multifunctional protein with protein processing protease, helicase, and nucleoside triphosphatase (NTPase) activities located in two functionally and structurally distinct domains (Zhang et al., 2005). Previous studies suggest that that the HCV NS3 protein interacts with NS4B and NS5B (Piccininni et al., 2002) and that the NS3 helicase, NS5A, and NS5B are part of a membrane-bound replicase complex that is responsible for viral RNA synthesis (Binder et al., 2007). The specific role of the NS3 helicase is not known; one model is that it could unwind RNA secondary structures and/or a double-stranded RNA intermediate providing a single-stranded template for RNA synthesis by NS5B. Studies have demonstrated that the NTPase and thus helicase activity of the NS3 is essential (Piccininni et al., 2002). Furthermore, Zhang et al. (2005) have shown that the NS3 protease domain functions not only as an enzyme, but also mediates a specific interaction with NS5B which enhances the NS3 helicase activity. Thus, it is likely that the various protein components of the replicase affect and/or regulate each other's properties through multiple protein–protein interactions. This suggests the possibility that, by binding to the protease domain, intrabodies may have an allosteric effect on the NS3 helicase. If so, anti-NS3 intrabodies could have dual inhibitory functions, not only inhibiting the protease, but also the replicase complex, and subsequently directly inhibiting RNA replication.

To test this hypothesis, we determined the inhibitory activity of anti-NS3 intrabodies in a cell-free HCV replicase assay. An NS5B inhibitor included as a control showed an expected dose-dependent inhibition of RNA synthesis (Fig. 6E). In contrast, an NS3 protease-inhibitor (SCH6) was devoid of any inhibitory effect, suggesting that protease activity is not required for RNA synthesis by fully formed replicase complexes. Minimal inhibition was observed with the most potent anti-NS3 intrabody, scFv 171 (Fig. 6E). Thus the major mechanism by which these intrabodies inhibit replica-

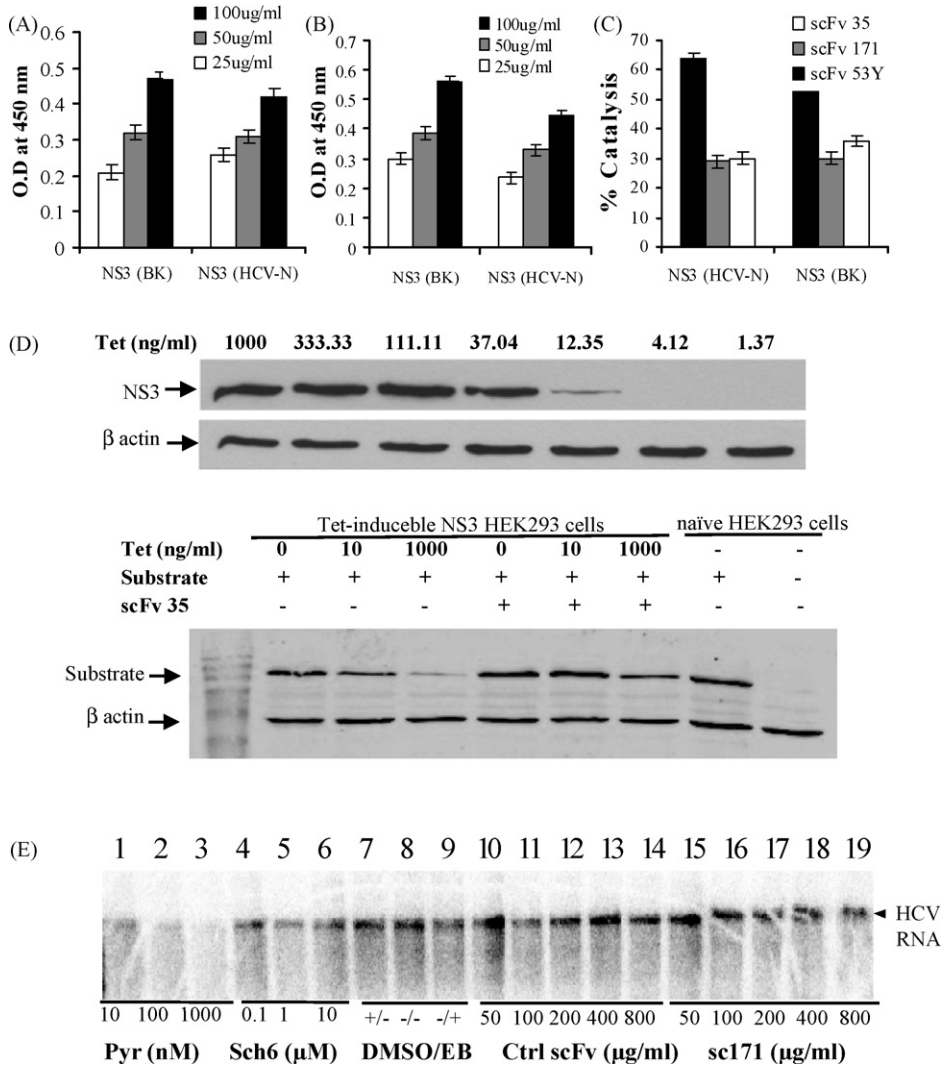

Fig. 6. Effect of intrabodies on NS3 catalysis and cell-free HCV RNA synthesis. (A and B) Analysis of NS3-inhibitory scFvs specific binding to MBP-scNS3 in an ELISA. A microtiter plate was coated with NS3 BK/HCV-N. Binding assays were performed with purified MBP-scFv 171 (in A) and 35 (in B). MBP-scFvs were detected with anti-myc and HRP-conjugated anti-mouse antibodies. Error bars represent the standard deviation of the data. (C) Inhibition of NS3 BK/HCV-N strains enzymatic activity in vitro by scFv inhibitors. Fluorescence in supernatant was used to calculate catalysis percentage. Error bars represent the standard deviation of the data. (D) Inhibition of NS3 catalysis by intrabodies. Upper panel: 30 ng of total protein from lysates of tetracycline-inducible NS3-expressing cells that were supplemented with 3-fold dilutions of tetracycline for 48 h were analyzed by immunoblotting with mouse anti-EGFP (for the detection of EGFP-NS3) and mouse anti-actin antibodies (loading control) followed by HRP-conjugated secondary antibodies and ECL development. Lower panel: immunoblot analysis of naïve HEK293 cells or tetracycline-inducible NS3 HEK293 cells transfected with MBP-EGFP-full 1b NS5AB-CBD substrate or co-transfected with the substrate and intrabody 35. Cells were induced with 0, 10 or 1000 ng/ml tetracycline. Full length substrate was detected with rabbit polyclonal anti-CBD and goat anti-rabbit antibodies. β actin was detected with mouse anti-β actin and goat anti-mouse antibodies. (E) Effect of intrabodies on the HCV in vitro RNA synthesis assay. Increasing concentrations of a pyrimidine inhibitor of NS5B polymerase activity (lanes 1–3), SCH6 (lanes 4–6), control, non-inhibitory intrabody 53Y (lanes 10–14), or intrabody 171 (lanes 15–19) were incubated with an optimal amount of a P16 heavy membrane preparation made from lysates of subgenomic replicon cells. For RNA synthesis, membranes were incubated under standard transcription reaction condition. Following the reaction, viral [^{32}P]CTP-labeled RNA was extracted, precipitated and separated on a 1% agarose gel containing Glyoxal. Bands were detected by exposure of the dried gel to X-ray films. Control reactions (lanes 7–9) were carried out either in the absence or presence of 1% DMSO, as required to solubilize many small-molecule compounds, or the elution buffer used in preparing the scFv proteins.

tion of the virus is likely to be suppression of polyprotein processing (by virtue of the ability of the intrabodies to inhibit protease activity), or possibly suppression of replicase assembly. It seems likely that the scFvs do not have access to their binding sites on the NS3 protein within the pre-assembled membrane-bound replicase complexes used in the assays shown in Fig. 6E. However, a better understanding of replicase complex structure and function will be needed to address these questions and fully understand how these intrabodies work.

There are many technical issues, particularly surrounding the means of delivery to the cell, that must be addressed before any clinical application of these intrabodies will be possible (Lobato and Rabbitts, 2004; Williams and Zhu, 2006). Our future study is focused on developing protein transduction technologies for the efficient delivery of these intrabodies into hepatocytes. Such challenges are generic to the field of genetic engineering today. Nonethelesss, the data presented here provide strong proof of principle supporting the further development of this novel class of therapeutic agents. These intrabodies may also serve as useful tools for developing a better understanding of the multiple roles of the NS3 protease in the HCV life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.08.001.

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