Antiviral Research 96 (2012) 245–255

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Fluorescent primuline derivatives inhibit hepatitis C virus NS3-catalyzed RNA unwinding, peptide hydrolysis and viral replicase formation

Jean Ndjomou [a], Rajesh Kolli [a], Sourav Mukherjee [a], William R. Shadrick [a], Alicia M. Hanson [a], Noreena L. Sweeney [a], Diana Bartczak [a], Kelin Li [b], Kevin J. Frankowski [b], Frank J. Schoenen [b], David N. Frick [a,*]

[a] Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, 3210 N. Cramer St., Milwaukee, WI 53211, United States
[b] University of Kansas Specialized Chemistry Center, University of Kansas, Lawrence, KS 66047, United States

ARTICLE INFO

Article history:
Received 4 May 2012
Revised 19 July 2012
Accepted 17 August 2012
Available online 25 August 2012

Keywords:
Motor protein
ATPase
RNA-binding proteins
Protease
Direct acting antiviral
Benzothiazole

ABSTRACT

The hepatitis C virus (HCV) multifunctional nonstructural protein 3 (NS3) is a protease that cleaves viral and host proteins and a helicase that separates DNA and RNA structures in reactions fueled by ATP hydrolysis. Li et al. (2012) recently synthesized a series of new NS3 helicase inhibitors from the benzothiazole dimer component of the fluorescent yellow dye primuline. This study further characterizes a subset of these primuline derivatives with respect to their specificity, mechanism of action, and effect on cells harboring HCV subgenomic replicons. All compounds inhibited DNA and RNA unwinding catalyzed by NS3 from different HCV genotypes, but only some inhibited the NS3 protease function, and few had any effect on HCV NS3 catalyzed ATP hydrolysis. A different subset contained potent inhibitors of RNA stimulated ATP hydrolysis catalyzed by the related NS3 protein from Dengue virus. In assays monitoring intrinsic protein fluorescence in the absence of nucleic acids, the compounds cooperatively bound NS3 with K_ds that reflect their potency in assays. The fluorescent properties of the primuline derivatives both *in vitro* and in cells are also described. The primuline derivative that was the most active against subgenomic replicons in cells caused a 14-fold drop in HCV RNA levels (IC_{50} = 5 ± 2 μM). In cells, the most effective primuline derivative did not inhibit the cellular activity of NS3 protease but disrupted HCV replicase structures.

1. Introduction

Hepatitis C virus (HCV) infects more than 170 million people worldwide, causing an epidemic of steatosis, cirrhosis, and hepatocellular carcinoma (Enserink, 2011; Murray and Rice, 2011). Host cell machinery translates the approximately 9000-nucleotide long HCV positive sense RNA genome into a single precursor polyprotein, which host and viral proteases cleave into 10 proteins. These mature HCV proteins include nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B, which are responsible for the replication and packaging of the HCV genome, and structural proteins (core, E1 and E2) that form the virus particle that coats the RNA genome (Lindenbach and Rice, 2005). All proteins encoded by HCV have been extensively studied as drug targets (Garber, 2011).

HCV nonstructural protein 3 (NS3) is a multifunctional protein that cleaves viral and host proteins and works as a helicase to separate or rearrange nucleic acid duplexes in a reaction fueled by ATP hydrolysis. Newly approved HCV drugs target the NS3 protease (Bacon et al., 2011; Zeuzem et al., 2011), but fewer compounds have been reported that inhibit the NS3 helicase function (Belon and Frick, 2009; Frick, 2007), and no helicase inhibitors have entered clinical trials for HCV. Replication of HCV in human cells requires the action of the NS3 helicase (Lam and Frick, 2006) and helicase actions must be coordinated with the protease (Beran and Pyle, 2008; Beran et al., 2007, 2009; Ding et al., 2011; Frick et al., 2004; Lindenbach et al., 2007), suggesting that compounds that inhibit both the NS3 helicase and NS3 protease might be useful as therapeutics. Specific NS3 helicase inhibitors and compounds targeting both NS3 functions would also be valuable reagents to study the precise role that NS3 helicase and related proteins play in viral replication.

Li et al. (2012) recently synthesized a series of potent HCV helicase inhibitors from the main component of the yellow dye primuline that inhibit the ability of HCV helicase to unwind DNA, but unlike other helicase inhibitors, they do not primarily exert their actions simply by binding the helicase DNA substrate. We have further characterized a subset of these compounds to reveal that they have three other important and useful properties. First, they inhibit the action of NS3 helicase derived from the HCV JFH1 strain and some are also potent inhibitors of Dengue virus NS3h. Second, the compounds potently inhibit RNA unwinding catalyzed by

* Corresponding author. Tel.: +1 414 229 6670; fax: +1 414 229 5530.
E-mail address: frickd@uwm.edu (D.N. Frick).

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.08.006

NS3. Third, analogs with certain *para* substituents in the terminal benzene moiety also inhibit the NS3 protease function. The more potent compounds directly interact with NS3 to quench its intrinsic protein florescence with K_ds that mimic their potency in enzyme assays. This report also details the optical properties of this series of benzothiazoles and shows how a representative can be used in cells as a fluorescent molecular probe that disrupts HCV replication complexes.

2. Experimental methods

2.1. Chemicals and reagents

All oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA). Primuline derivatives were synthesized and purified as described before (Li et al., 2012). Telaprevir was from Celia Schiffer (UMASS Medical School). Three different NS3 proteins were used in this study. Two were truncated C-terminally His-tagged NS3 proteins lacking the N-terminal protease, called NS3h, the third was a full length NS3 with the portion of NS4A needed for protease activation fused to its N-terminus, called scNS4A-NS3 (Howe et al., 1999), and the fourth was a 23 kDa scNS4A-NS3 protease fragment lacking the helicase domains, called scNS4A-NS3p (Protein One, Rockville, MD). NS3h was expressed from two different HCV strains. NS3h_1b(con1) was from the con1 strain of genotype 1b [Genbank accession AB114136], and NS3h_2a(JFH1) was from the JFH1 strain of HCV genotype 2a [Genbank accession AJ238799]. The genotype 1b(con1) strain is the basis for the HCV replicons used here (Lohmann et al., 1999) and genotype 2a(JFH1) is a unique strain capable of replicating in cell culture (Wakita et al., 2005). His-tagged recombinant NS3h_1b(con1), NS3h_2a(JFH1), and scNS4A-NS3 (also from genotype 1b(con1)) were expressed, and purified as previously described (Frick et al., 2010; Lam et al., 2003). The scNS4A-NS3p (genotype 1b) was from Protein One (catalog #P501). A plasmid expressing NS3h from dengue virus strain 2 (NS3h_DV2) [Genbank accession 2BMF] was obtained from Julien Lescar (Singapore) (Xu et al., 2005), and used to express and purify NS3h_DV2 as described before (Belon et al., 2010).

2.2. Helicase assays

The ability of compounds to inhibit helicase action was monitored using molecular beacons as described previously (Belon and Frick, 2008; Hanson et al., 2012). Assays contained 25 mM MOPS, pH 6.5, 1.25 mM $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 5 μg/ml BSA, 0.01% (v/v) Tween20, 0.05 mM DTT, 5 nM substrate, 5–30 nM NS3h, and 1 mM ATP. The partially-duplex DNA substrates used in helicase assays consisted of a 45-mer bottom strand 5′-GCT CCC CGT TCA TCG ATT GGG GAG CTT TTT TTT TTT TTT TTT-3′ and a 25-mer HCV top strand 5- /5Cy5/GCT CCC CAA TCG ATG AAC GGG GAG C/3IAbRQSp/-3. The 3-stranded RNA substrate used was made of two RNA strands, a 60-nucleotide long bottom strand 5′-rGrGrA rGrCrU rGrGrU rGrGrC rGrUrA rGrGrC rArArG rArGrU rGrCrC rUrUrG rArCrG rArUrA rCrArG rCrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU rUrUrU-3′, a 24 nucleotide long top strand 5′-rArGrU rGrCrG rCrUrG rUrArU rCrGrU rCrArA rGrGrC rArCrU-Cy5, and a third DNA top strand with the sequence /5IAbRQ/CCT ACG CCA CCA GCT CCG TAG G-3′.

To monitor helicase reaction kinetics and to calculate IC_{50} values, assays were performed in 60 μl in white half-area 96-well plates (Corning Lifesciences, catalog #3693) and measured in a Fluostar Omega multimodal plate reader (BMG Labtech Inc.) using 640 nm excitation wavelength and 680 emission wavelength filters. Reactions were performed by first incubating all components except for ATP for 2 min in 54 μl, then injecting 6 μl of ATP such that the final concentration of all components was as noted above. Initial reaction velocities were calculated by fitting a first order decay equation to data obtained after ATP addition and calculating an initial velocity from the resulting amplitude and rate constant. The concentration at which a compound caused a 50% reduction in reaction velocity (IC_{50}) was calculated using GraphPad Prism (v. 5).

2.3. ATP hydrolysis (ATPase) assays

A modified malachite green-based assay was used to measure ATP hydrolysis (Lanzetta et al., 1979). Reactions were performed in 30 μl and contained 25 mM MOPS pH 6.5, 1.25 mM $MgCl_2$, and 1 mM ATP, 33 μg/ml BSA, 0.07% (v/v) Tween 20, 0.3 mM DTT. The colorimetric reagent was prepared fresh by mixing three volumes 0.045% (w/v) malachite green, one volume 4.2% ammonium molybdate in 4 N HCl, and 0.05 volumes of 20% Tween 20. For IC_{50} value determination, reactions were performed in 10% DMSO with 2-fold dilution series of compound ranging from 200 to 1.6 μM in the absence of RNA with 100 nM of NS3h_1b(con1). Reactions were initiated by adding ATP, incubated for 15 min at 23 °C, and terminated by adding 200 μl of the malachite green reagent, followed by 30 μl of 35% sodium citrate. The color was allowed to develop for 30 min and absorbance at 630 nm was read. Percent inhibition was calculated by normalizing the data to assays without enzyme (100% inhibition) and reactions with DMSO only (0% inhibition).

RNA stimulated ATP hydrolysis was monitored under slightly different conditions to compare the effect of compounds on HCV NS3h to Dengue virus NS3h, which was notably less active than HCV helicase at pH 6.5. To compare the effect of compounds on HCV NS3h_1b (con1) to NS3h_DV2, reactions were performed with 2 nM of either enzyme in 25 mM Tris pH 7.5, 1.25 mM $MgCl_2$, 1.0 mM ATP, 10% DMSO, 5 μg/ml BSA, 0.01% (v/v) Tween 20, 0.05 mM DTT, and 10 μM poly(U) RNA at 37 °C.

2.4. NS3 protease assay

All protease assays were carried out using the 5-carboxyfluorescein-labeled substrate from the AnaSpec Enzolyte 520 Protease Assay Kit (AnaSpec, San Jose, CA). Each assay contained 5 nM scNS4A-NS3 or 50 nM scNS4A-NS3p, 30 mM DTT, 5% DMSO and 1X Anaspec HCV protease assay buffer. Assays were carried out in a total volume of 20 μl in black 384-well plates with fluorescence at 520 nm measured using a BMG FLUOstar Omega fluorescence spectrophotometer. Reactions were performed with eight concentrations of a 2-fold dilution series of each compound (in duplicate) starting at 100 μM. Compound concentration needed to reduce reaction velocity by 50% (IC_{50}) was calculated with GraphPad Prism (v. 5).

2.5. Compound effects on NS3 intrinsic protein fluorescence

Aliquots of each compound (1 μl of a 1 mM solution in DMSO) were added sequentially to 50, 100, or 200 nM of NS3h_2a(JFH1), or 100 nM scNS4A-NS3 dissolved in 2 ml of 25 mM MOPS pH 7, 1.25 mM $MgCl_2$ and, 0.01% (v/v) Tween 20, 0.3 mM DTT. The titrations were performed in a stirred temperature controlled 1 cm cuvette at 23 °C in a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). Intrinsic protein fluorescence was recorded by exciting the sample at 280 nm and reading emission at 340 nm. Excitation and emission slit widths were set to 5 and 10 nm, respectively.

All raw fluorescence data were corrected for sample dilution and inner filter effects using Eq. (1):

$$F_c = F_{obs}(V_0 + V_i)/V_0 * 10\hat{}(A_{ex} + A_{em}/2) \quad (1)$$

where F_c is corrected fluorescence, F_{obs} is observed fluorescence, V_0 is initial sample volume, V_i is total volume of titrant added, A_{ex} is the absorbance of the solution at the excitation wavelength (280 nm), and A_{em} is the absorbance of the solution at the emission wavelength (340 nm). Absorbance was calculated at each point in the titrations from extinction coefficients for each compound in titration buffer, which were calculated at 280 and 340 nm using four different compound concentrations. The resulting correct fluorescence values were fit to a Hill equation to calculate the macroscopic dissociation constant (K_d) describing the amount of compound needed to decrease NS3 fluorescence by 50%, using Eq. (2).

$$F_c = F_0 - (\Delta F_{max} * (F_c)\hat{}n/(K_d\hat{}n + (C)\hat{}n)) \quad (2)$$

In Eq. (2), F_c is corrected fluorescence, F_0 is fluorescence in the absence of compound, ΔF_{max} is the maximum change in fluorescence, n is the Hill coefficient, and C is compound concentration.

2.6. HCV Renilla luciferase reporter gene assay

Huh7.5/HCV Con1sg *R*luc replicon stable cells were established as described before (Li et al., 2012), and seeded at a density of 10,000 cells per well in 96-well plates and incubated for 4–5 h to allow the cells to attach to the plate. The compounds dissolved in DMSO were added at the indicated concentrations to cells (DMSO solvent final concentration was 0.5%) and the cells were incubated for 72 h at 37 °C under 5% CO_2 atmosphere. The effects of compounds on HCV replication were then assessed by measuring the *Renilla* luciferase activity in compound-treated versus DMSO-treated cells. At the end of the incubation period, the medium was aspirated and the cells were washed with 1x PBS. The *Renilla* luciferase reporter gene assay was performed using the *Renilla* luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the cells were lysed by addition of 50 μl of *Renilla* luciferase lysis buffer (Promega) followed by two cycles of freeze/thaw. The luciferase activity content of the lysate was measured with a FLUOstar Omega microplate reader instrument (BMG Labtech, Germany) after injecting 50 μl of luciferase substrate and reading for 5 s.

2.7. Cell viability assay

To assess compound toxicity towards Huh7.5/HCV Con1sg *R*luc replicon stable cells were plated and treated as above and cell viability was assessed using the CellTiter-Glo luminescent cell viability kit (Promega) following the manufacturer's instructions. Briefly, at the end of a 72 h, the medium was removed, cells were washed with PBS, and then an equal volume of growth medium and CellTiter-Glo reagent were added. After incubating for 30 min at 23 °C, luciferase activity was measured for 1 s using a FLUOstar Omega reader (BMG Labtech).

2.8. Cellular HCV RNA RT-qPCR assay

At the end of the treatment, cells were washed two times with PBS, harvested, and collected by centrifugation (1000*g*) for 5 min at 4 °C. Total RNA was then extracted using the TRIzol kit (Invitrogen) and suspended in 30 μl of nuclease-free water. RNA concentration was determined from A_{260}. Reverse transcriptase quantitative PCR (RT-qPCR) was performed using TaqMan probes, 1 μg total RNA and the qScript™ One-step Fast qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD). Reverse transcription was done at 50 °C for 20 min followed by one cycle at 95 °C for 5 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. HCV primers targeted the HCV 5′UTR (HCV forward: 5′-AGCCATGGCGTTAGTATGAGTGT-3′, HCV reverse: 5′-TTCCGCAGACCACTATGG-3′, HCV probe: 5′-/56-FAM/CCTCCAGGACCCCCCCTCCC/36-TAMSp/-3′), primers for 18S ribosomal (rRNA) were from the 18S rRNA control kit RT-CKFT-18S (Eurogentec, San Diego, CA). The amount of HCV RNA in each sample was determined by first calculating the ΔC_T of each sample, which was obtained by subtracting the threshold cycle (C_T) obtained with the rRNA primers obtained from that of the C_T with the HCV primers. $\Delta\Delta C_T$ values were obtained by subtracting ΔC_T values obtained in the presence of compound from ΔC_T obtained with cells treated with DMSO only. The relative changes in HCV RNA levels were then calculated by assuming that each C_T difference reflects a 2-fold difference in RNA level (*i.e.* $Expression = 2^{-\Delta\Delta C_T}$).

2.9. Cell lysate preparation and immunoblot analysis

To analyze effects on HCV proteins expression by western blotting, Huh7.5/HCV Con1sg *R*luc replicon stable cells were washed with PBS, harvested in PBS, and lysed with RIPA buffer for 20 min on ice and cleared lysates were obtained by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentrations were determined with the Bio-Rad protein assay kit (Bio-Rad) and 15 μg total proteins was resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membrane. The blots were probed with primary anti-NS5A mAb (Meridian life Sciences, Saco, ME) and a peroxidase conjugated secondary antibody (Cell Signaling, Danvers, MA) and were developed with enhanced chemiluminescence (ECL) detection reagents (Pierce, Rockford, IL). The blots were stripped and probed again with primary anti-tubulin antibody (Sigma) to confirm equal loading of protein and a peroxidase conjugated anti-mouse secondary antibody (Cell Signaling) and developed with ECL reagents (Pierce).

2.10. Indirect immunofluorescence staining

Huh-7.5/HCV Con1sg *R*luc cells were plated on cover slips in a 24 well plate. The next day, compounds were added at a 10 μM concentration and after 48 h from the first addition, the medium was supplemented with 10 μM compound. Interferon alpha-2b was used as positive control at 100 U/well and added at the same time points as compounds. After 72 h, indirect immunofluorescence staining was performed as described previously (Lindenbach et al., 2005). Cells were fixed in 1% PFA for 40 min, washed thrice with 5 ml PBS, permeabilized with 0.05% saponin (Sigma) and blocked with 3% BSA in PBS for 1 h at 37 °C. Subsequently, cells were washed with 2 ml PBS three times and incubated with mouse monoclonal antibody 9E10 against NS5A (from Charles Rice, Rockefeller University) (Jones et al. 2010) diluted in blocking buffer for 1 h at room temperature. Cells were washed three times with 2 ml PBS and once with 3% BSA. The cells were then incubated with Alexa 546 conjugated-goat F(ab′)2 anti-mouse immunoglobulin G (IgG) (Invitrogen) for 1 h at room temperature. Cells were washed twice with PBS and then mounted on glass slides using ProLong Gold anti-fade reagent (Invitrogen). Appropriate controls were performed to rule out non-specific binding of primary and secondary antibodies.

2.11. Cell-based protease assay

An IPS-1 (also known as MAVS) based reporter assay was used for cell-based protease assay. IPS-1 based reporter plasmid TRIP-RFP-NLS-IPS (a kind gift from Charles M. Rice, Rockefeller University) encodes the SV40 nuclear localization signal (NLS) and IPS fused to RFP (Jones et al., 2010). Pseudoparticles were generated by co-transfection of a mixture of 5 μg of the TRIP-RFP-NLS-IPS provirus plasmid, and the helper plasmids 5 μg of the HIV-1 Gag/Pol, 12 μg of the HIV-Rev, 1.5 μg of the vesicular stomatitis virus

envelope protein G (VSV-G) in 293T packaging cells by using a Calcium phosphate transfection kit (Sigma Aldrich) as described previously (Naldini et al., 1996; Zufferey et al., 1998). RFP-NLS-IPS pseudoparticles were transduced into Huh7.5/HCV Con1sg *Rluc* cells in a 24 well plate containing cover slips and compounds were added to the cells after 6 h at a 10 μM concentration and after 72 h from the first addition, cells were fixed in 1% PFA, washed twice with PBS, mounted on glass slides and microscopic analyses were done.

2.12. Microscopy

Wide-field microscopy was performed with a Nikon Ti-E inverted fluorescence microscope using a 20× and 40×/NA 1.4 objective. Compounds were imaged using DAPI filter 340/40 band-pass excitation and a 435/50 band pass emission filter. For Alexa 546 and RFP-NLS-IPS, a 528/25 nm band pass excitation filter and 590/60 nm band-pass emission filter were used. Image acquisition was performed with a Q imaging Rolera camera and the NIS elements basic research imaging software (Nikon). Images were processed uniformly by using NIH ImageJ 1.45 software.

3. Results

The fluorescent yellow dye primuline is a heterogeneous mixture of benzothiazole oligomers terminating with *p*-aminobenzene groups (Horobin et al., 2002). These benzothiazole oligomers inhibit the ability of the HCV helicase to separate duplex DNA, and the potency with which they inhibit the HCV helicase correlates with the benzothiazole chain length (Li et al., 2012). Because only small amounts of the most potent helicase inhibitor in primuline (compound **1**, Table 1) are present in commercial primuline batches, Li et al. used the notably less active dimeric benzothiazole (compound **2**, Table 1) to synthesize compound **1** analogs with a range of anti-helicase activities, a subset of which was selected for further characterization in this study (Table 1, compounds **3–13**). The goal was to examine the specificity of mechanism of action of each compound *in vitro* and in cells.

3.1. Specificity of primuline derivatives with regard to HCV genotype and helicase substrate composition

One of the challenges in developing compounds that target HCV proteins arises from the fact that compounds effective against certain HCV genotypes do not act on others. Compounds that specifically target less conserved regions of a HCV protein are often not potent inhibitors of the same protein derived from other genotypes, and they might be susceptible to rapid evolution of related viruses. Li et al. tested the primuline derivatives on HCV helicase isolated from the genotype 1b(con1) strain. Here, the subset of primuline analogs was tested on the helicase isolated from the genotype 2a(JFH1) strain. The two recombinant proteins differ at 83 of their 480 amino acids. The JHF1 strain is also of interest because it serves as the backbone for HCV strains capable of replicating in cell culture (Wakita et al., 2005). The same molecular beacon-based helicase assay (MBHA) (Belon and Frick, 2008) that was used by Li et al. (2012) was used to measure the ability of each compound to inhibit DNA unwinding catalyzed by NS3h isolated from genotype 2a(JFH1) (NS3h_2a(JFH1)). All compounds tested showed similar activity on NS3h_2a(JFH1) as they did with NS3h_1b(con1) (Table 1). None showed any specificity for one genotype or another. Selected compounds were also tested for the ability to inhibit helicase reactions catalyzed by full length NS3 and a single chain NS4A-NS3 protein (scNS4A-NS3) described below and used for protease assays. Again, no noteworthy differences in potency were observed in the presence of an intact protease domain or NS4A peptide (data not shown).

To act as antivirals in cells, it is also important for an HCV helicase inhibitor to inhibit the activity of the NS3h on RNA. The Li et al. study used DNA as a surrogate because the enzyme is more active on DNA than RNA despite the fact that there is no known DNA stage in the viral lifecycle. As noted by Li et al., it is possible that the compounds exert their action, in part by interacting with DNA. To test if the compounds inhibited RNA unwinding by NS3, a three-stranded substrate was used in which the helicase must separate a duplex RNA to enhance fluorescence (Fig. 1A). The least potent primuline derivative (Fig. 1B), the most potent derivatives (Fig. 1C and D) and the other compounds tested (Table 1) inhibited this RNA-based assay with IC_{50} values similar to those seen with the DNA substrate (Table 1). As with protein specificity, no differences were uncovered regarding substrate specificity for any of the compounds tested. In other words, none of the compounds preferred to act either on DNA or RNA substrates.

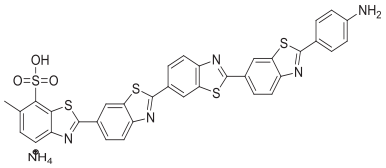
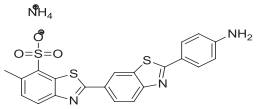
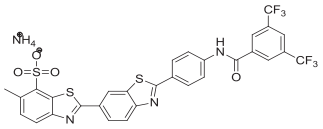
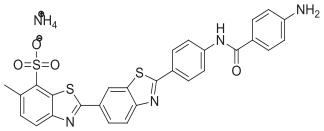
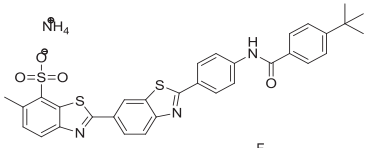
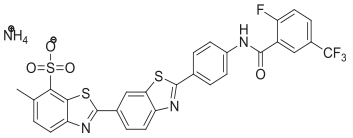
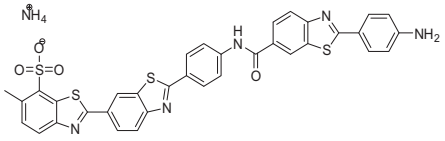
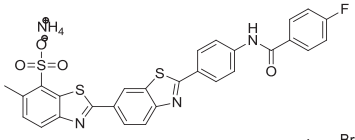
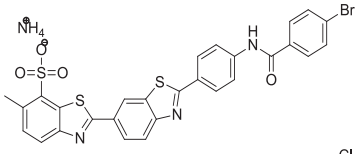
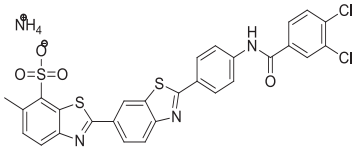
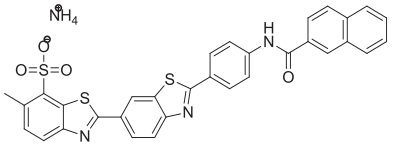
3.2. Effects of primuline derivatives on HCV NS3-catalyzed peptide cleavage and ATP hydrolysis

In stark contrast to the above results, major differences in compound specificity were observed with regard to the ability of the various primuline derivatives to inhibit both the NS3 protease and helicase function (Fig. 1E–G, Table 1). Interestingly, the benzothiazole tetramer (compound **1**), the most potent helicase inhibitor found in primuline, inhibited both the NS3 protease and helicase with a similar IC_{50} value, but the dimeric benzothiazole primuline component (compound **2**) did not inhibit the protease more than 50% at 100 μM, the highest concentration tested (Table 1). The discovery that compound **1** could simultaneously inhibit both NS3 functions was surprising because we know of no other compounds that inhibit both NS3 activities. Experiments with HCV protease inhibitors in clinical development (*e.g.* telaprevir) showed that such protease inhibitors do not inhibit the NS3 helicase action on DNA or RNA substrates (data not shown). Similarly, other helicase inhibitors, such as the symmetrical benzimidazoles do not affect NS3 protease function (Belon et al., 2010).

Like compound **1**, some primuline derivatives retained an ability to inhibit NS3 protease, but others did not (Table 1). Compounds **3** (Fig. 1E), **7**, **9** and **13** (Fig. 1G) inhibited protease with about the same potency as helicase, but compounds **8**, **11**, and **12** (Fig. 1F) were over 40 times less active against the protease than the helicase. The compounds inhibited the protease if they were added before or after the protease substrate, and inhibition was not simply due to the fact that the compounds were quenching the fluorescence of the protease reaction product. Compounds targeting the protease also inhibited peptide cleavage even in the absence of the NS3 helicase domain, as evidenced by the fact that similar IC_{50} values were obtained in assays containing a truncated scNS4A-NS3 protein that lacks most of the helicase domain (scNS4A-NS3p, Table 1). Compound **12** was the derivative that most specifically targeted the helicase function and was a 50-times less potent protease inhibitor than compound **1** (Fig. 1F, Table 1).

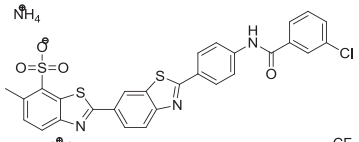
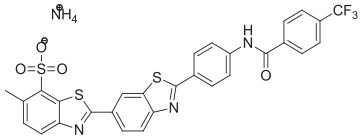
A simple explanation for the ability of some of the primuline derivatives to inhibit helicase and protease with similar potency would be that the compounds acted by simply aggregating or denaturing the enzyme. To test for irreversible inhibition, NS3h was incubated with 100 μM of each compound, diluted and then assayed for its ability to unwind DNA under standard conditions. Protein pre-incubated with the primuline derivatives retained an activity comparable to protein pre-incubated with DMSO-alone. In a second test for a simple aggregation mechanism, each compound was tested for its ability to inhibit NS3-catalyzed ATP hydrolysis. ATP hydrolysis fuels the unwinding reaction and NS3

Table 1
Activities and properties of compounds purified from primuline and their derivatives.

Cmpd	Structure	Absorbance λ_{max} (nm)	Fluor. Em_{max} (nm)	DNA Helicase NS3h_1b[a] *IC_{50} (μM) ± SD*	RNA Helicase[c] NS3h_2a *IC_{50} (μM) ± SD*	Protease scNS4A-NS3[d] *IC_{50} (μM) ± SD*	ATPase[f] No RNA NS3h_1b *IC_{50} (μM) ± SD*	ATPase w/ RNA[g] NS3h_1b *IC_{50} (μM) ± SD*
		ϵ (M^{-1} cm^{-1})	RFU @ 10 μM	NS3h_2a[b] *IC_{50} (μM) ± SD*		scNS4A-NS3p[e] *IC_{50} (μM) ± SD*		NS3h_DV2 *IC_{50} (μM) ± SD*
1		382 32,000	467 3	2 ± 0.1 1 ± 0.3	3 ± 1.3	2 ± 1 4.9	43 ± 10	N.D. 0.94 ± 0.7
2		366 38,550	553 15	45 ± 14 58 ± 51	32 ± 14	>100 >100	>200	>200 32 ± 17
3		355 45,650	497 121	22 ± 4.2 29 ± 5.2	14 ± 13	23 ± 15 7.6	141 ± 28	>200 11 ± 2
4		360 36,600	429 4	10 ± 2.4 5 ± 2.5	7 ± 3	39 ± 17 >100	194 ± 30	140 ± 3 12 ± 4
5		356 37,150	502 164	8 ± 1.0 5 ± 0.7	9 ± 9	56 ± 23 93	150 ± 21	150 ± 40 8 ± 1
6		355 41,750	497 159	6 ± 1.9 7 ± 0.9	10 ± 3.0	51 ± 25 >100	61 ± 13	19 ± 2 7 ± 0.1
7		361 42,000	532 2	6 ± 2.1 3 ± 1.2	6 ± 5.4	7 ± 1 20	>200	150 ± 2 0.5 ± 0.4
8		356 24,950	496 111	5 ± 0.6 3 ± 0.8	4 ± 2	89 ± 47 >100	67 ± 30	73 ± 13 6 ± 1
9		356 51,600	497 209	5 ± 3.9 7 ± 3.3	4 ± 2.0	6 ± 1 6.5	50 ± 4	21 ± 1 3 ± 0.8
10		356 44,000	501 203	4 ± 1.0 4 ± 0.5	4 ± 2.0	13 ± 4 6.4	44 ± 12	72 ± 13 4 ± 1
11		359 63,450	496 236	3 ± 0.7 2 ± 0.6	3 ± 2.0	>100 >100	30 ± 6	52 ± 6 3 ± 1

(*continued on next page*)

Table 1 (*continued*)

Cmpd	Structure	Absorbance λ_{max} (nm)	Fluor. Em_{max} (nm)	DNA Helicase NS3h_1b[a] IC_{50} (μM) ± SD	RNA Helicase[c] NS3h_2a IC_{50} (μM) ± SD	Protease scNS4A-NS3[d] IC_{50} (μM) ± SD	ATPase[f] No RNA NS3h_1b IC_{50} (μM) ± SD	ATPase w/ RNA[g] NS3h_1b IC_{50} (μM) ± SD
		ϵ (M^{-1} cm^{-1})	RFU @ 10 μM	NS3h_2a[b] IC_{50} (μM) ± SD		scNS4A-NS3p[e] IC_{50} (μM) ± SD		NS3h_DV2 IC_{50} (μM) ± SD
12		356 59,500	502 215	3 ± 0.8 4 ± 0.5	6 ± 3.1	>100 24	74 ± 7	51 ± 3 4 ± 0.6
13		356 42,750	498 158	2 ± 0.4 2 ± 1.0	3 ± 1	5 ± 1 7	24 ± 5	16 ± 5 2 ± 0.5

N.D., not determined.

[a] From Li et al. (2012).

[b] Average (±SD) IC_{50} value from three sets of DNA based molecular beacon based helicase assays performed with a 8 point 2-fold dilution series of each compound starting at 100 μM.

[c] Average (±SD) IC_{50} value from three sets of RNA helicase assays performed with a 8 point 2-fold dilution series starting at 100 μM.

[d] Average (±SD) IC_{50} value from two sets of protease assays performed with a 8 point 2-fold dilution series starting at 100 μM.

[e] IC_{50} value from one sets of protease assays performed with a 8 point 2-fold dilution series starting at 100 μM.

[f] Average (±SD) IC_{50} value from three sets of ATPase assays performed with a 8 point 2-fold dilution series starting at 200 μM.

[g] Average (±SD) IC_{50} value from three sets of ATPase assays performed in the presence indicated proteins (nM) and of 10 μM poly(U) RNA with a 8 point 2-fold dilution series starting at 200 μM.

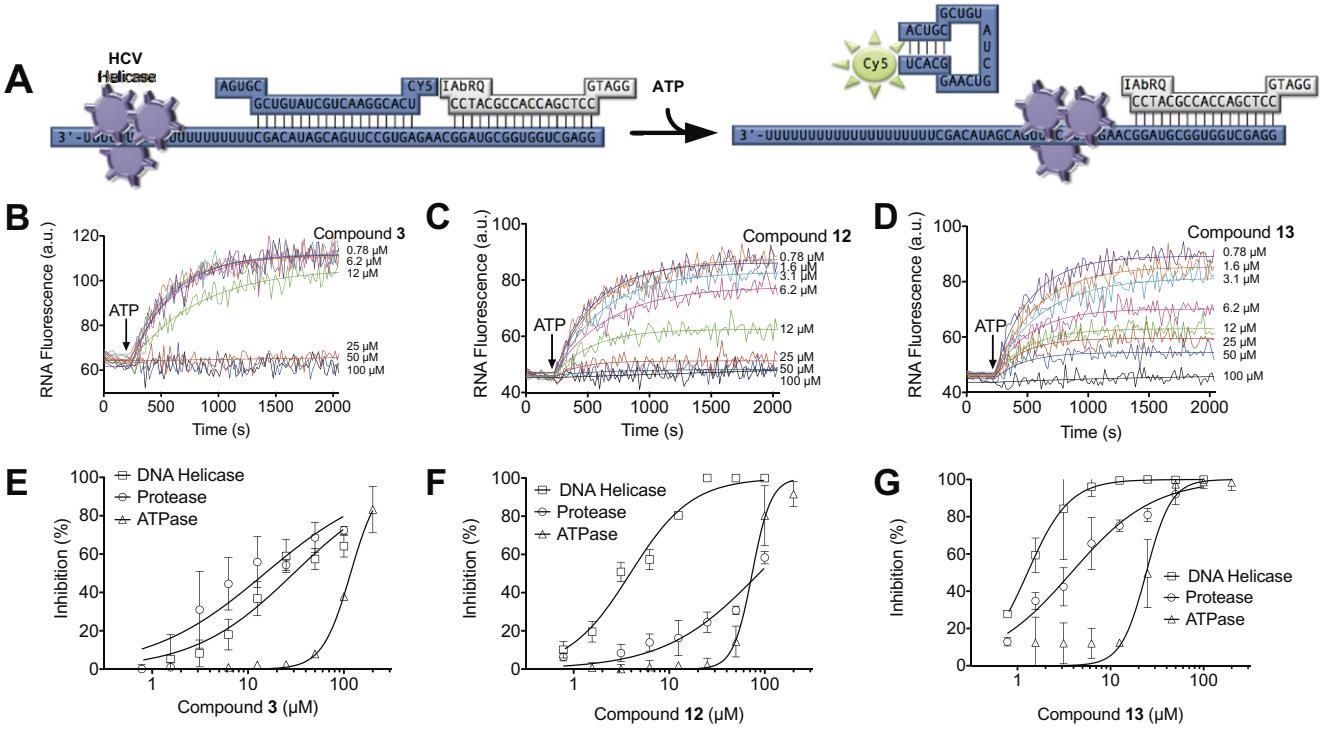


Fig. 1. Ability of primuline derivatives to inhibit NS3-catalyzed RNA unwinding, DNA unwinding, ATP hydrolysis and peptide cleavage. (A) An RNA-based NS3 helicase assay where NS3 helicase must separate an RNA duplex (blue) to enhance Cy5 fluorescence. Unwinding only occurs upon addition of ATP, which fuels helicase action. (B–D) Effects of various concentrations of primuline derivatives on the kinetics of NS3 catalyzed RNA unwinding. Data obtained after ATP addition were fitted to a first order rate equation (solid curves). (E–G) Comparison of the ability of various primuline derivatives (for structures see Table 1) to inhibit NS3 catalyzed DNA unwinding (squares), peptide cleavage (circles), and ATP hydrolysis (triangles). Points are averages of three independent assays and error bars show standard deviations. Data were fitted to a normalized dose response equation with IC_{50} values listed in Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrolyzes ATP both in the presence and absence of DNA or RNA; nucleic acid stimulates NS3 catalyzed ATP hydrolysis about 50-fold. If compounds aggregate the enzyme, they should also inhibit the ability of NS3 to hydrolyze ATP with the same potency as they inhibit its other activities. To test this hypothesis, ATPase assays were performed in the presence of various concentrations

of each primuline derivative, under conditions similar to those used to monitor effects on DNA and RNA unwinding. Far more of each compound was needed to inhibit NS3 catalyzed ATP hydrolysis than was needed to inhibit the helicase activity, or, in some cases, NS3 protease activity (Fig. 1, Table 1).

3.3. Some primuline derivatives are potent inhibitors of the NS3h encoded by Dengue virus

To further profile compound specificity, we next asked whether the compounds would inhibit homologous NS3h expressed by the Dengue virus (NS3h_DV2). The ability of each compound to inhibit RNA stimulated ATP hydrolysis catalyzed by NS3h_DV2 was examined under conditions where the Dengue enzyme is more active (pH 7.5) as has been previously described (Belon et al., 2010). The same assays were also repeated with HCV NS3h_1b(con1) for comparison (Table 1). Similar IC_{50} values were obtained with HCV NS3h as were seen in the absence of RNA (Table 1). However, most of the primuline derivatives were relatively potent inhibitors of RNA stimulated ATP hydrolysis catalyzed by the Dengue enzyme under the same conditions. The SAR with NS3h_DV2 roughly mirrored that seen with the HCV NS3h DNA and RNA based unwinding assays, with the notable exception that compound **7** was the most potent Dengue NS3h inhibitor (1.1 ± 0.1 μM).

3.4. Interactions of primuline derivatives with HCV NS3h

To examine the direct interaction of the compounds with NS3, we examined the effect of a select few on NS3 intrinsic protein fluorescence. This technique has been widely used to monitor the interaction of NS3 with its nucleic acid ligands (Lam et al., 2004; Levin and Patel, 2002; Preugschat et al., 1996) and other small molecules (Belon et al., 2010). Although such experiments were technically challenging due to the fact that the compounds absorb light at the wavelengths needed to monitor intrinsic protein fluorescence, careful correction for these "inner filter effects" (Eq. (1), methods) revealed clear interaction of compound **3** and the most potent inhibitors (compounds **11–13**) with apparent K_ds that mirrored their potencies in other helicase assays (Fig. 2A and B). Of note is the fact that, unlike compounds that simply compete for the nucleic acid binding site (*e.g.* $(BIP)_2B$ (Belon et al., 2010), the primuline derivatives bound NS3h cooperatively as evident from their sigmoid binding isotherms and Hill coefficients greater than one (Eq. (2)). When compounds were tested against scNS4A-NS3, binding again was detected and apparent affinity for the full-length complex was about five times higher than it was for NS3h (Fig. 2C).

3.5. Primuline derivatives as fluorescent molecular probes

Primuline is a fluorescent dye that is used to determine yeast cell viability (Graham and Caiger, 1969), suggesting that it might also be useful to stain NS3 helicase (or related proteins) in cells. The fluorescence of the primuline components is inversely proportional to their number of benzothiazole units (Li et al., 2012), and the most potent helicase inhibitor in primuline, compound **1** (Fig. 3A), is less fluorescent than compound **2**, which has 10–20 times lower affinity towards NS3 (Fig. 3B). Several of the primuline derivatives profiled are notably more fluorescent than compounds **1** and **2** (Table 1, Fig. 3). The derivative that Li et al. found was most active against the HCV subgenomic replicon (compound **3**) and the most potent helicase inhibitors (compounds **11**, **12**, and **13)** are highly fluorescent. When directly compared with **1**, the semi-synthetic primuline derivatives have a sharper absorbance peak centered near 360 nm, and they emit 3–15 times more light when excited at their absorbance maximum (Fig. 3A–E). The compounds stain live cells harboring HCV replicons, as can be seen using fluorescence microscopy (Fig. 3F–J). Interestingly, the compounds with different functional groups and specificities showed different subcellular localization in Huh7.5 cells/HCV Con1sg *Rluc* cells. The parent compound **2** showed diffused staining of both the nucleus and cytoplasm (Fig. 3G), but the helicase specific inhibitors like **11** and **12** were localized in the cytoplasm and not in the nucleus (Fig. 3H and I). The most potent replicon inhibitor, compound **3**, showed cytoplasmic staining with dot-like structures in the replicon cells (Fig 3F). Some compounds, like **13**, stained both the cytoplasm and nucleus, but they also stained glass coverslips (Fig. 3J).

3.6. Effect of compound 3 on cells harboring a HCV Rluc subgenomic replicon

To further characterize the antiviral activity of this class of compounds, the same HCV *Renilla* luciferase (*R*luc) subgenomic replicon cells used in the study by Li et al. (2012) (Fig. 4A) were exposed to different concentrations of compound **3** and the effect of **3** on HCV replication was assessed on HCV *Rluc* reporter gene activity. Compound **3** was chosen over the more active derivatives because it is more soluble in cell culture media than the others. The solubility of the other derivatives (*e.g.* **11**, **12**, and **13**) in the absence of non-ionic detergents used in *in vitro* assays limited the ability to administer more than 10 μM of each to cells. As a consequence, more than 90% of HCV replicon *R*luc activity was lost only in the presence of elevated concentrations (>10 μM) of compound **3**. Replicon luciferase activity was reduced in cells treated with compound **3** in a concentration-dependent manner with 5 ± 2 μM needed to reduce replicon-encoded luciferase by 50% (Fig. 4B). The other compounds in Table 1 were not as effective. Cells were exposed in parallel to the same doses of **3**, for the same incubation period, to test the effect of **3** on cell viability. Even at the highest concentration tested (50 μM), **3** was not toxic (Fig. 4B). The direct effect of compound **3** on HCV RNA was also examined with qRT-PCR, and **3** exposure reduced HCV RNA levels in replicon cells in a dose-dependent manner (Fig. 4C). To compare the effect of **3** to

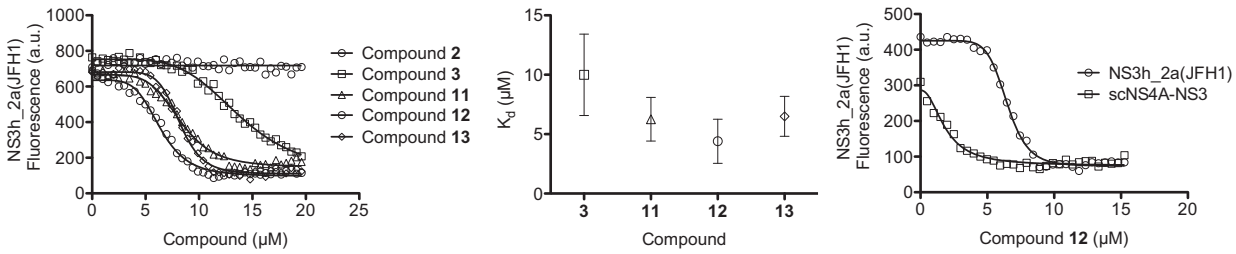


Fig. 2. Interaction of primuline derivatives with NS3h and scNS4A-NS3. (A) Effect of various compounds on NS3h intrinsic protein fluorescence. Corrected fluorescence values for titrations of 200 nM NS3h_2a(JFH1) (F_c, Eq. (1)). Data were fitted to Eq. (2). (B) Average K_d values from three titrations with each compound performed at 50, 100 and 200 nM NS3h_2a(JFH1). Error bars show standard deviations. (C) Corrected fluorescence values for titrations of either 100 nM NS3h_2a(JFH1) or scNS4-NS3. Data were fitted to Eq. (2).

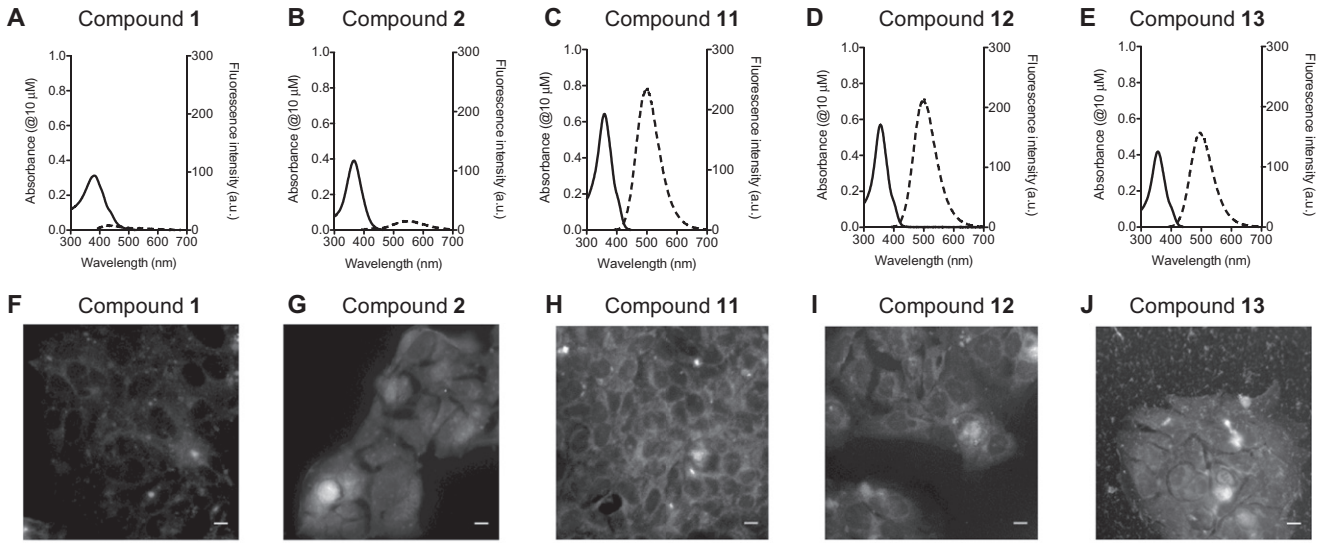


Fig. 3. Use of fluorescence microscopy to visualize helicase inhibitors in cells. (A) Absorbance (solid lines, left *y*-axis) spectra, and fluorescence emission spectra when excited at peak absorbance ±5 nm (see Table 1 for peak absorbance, dotted lines, right *y*-axis) of 10 μM aliquot of compound **1**, the most active helicase inhibitor isolated from primuline (Li et al., 2012). (B) Optical properties of compound **2**, which was used to synthesize compounds **3**–**13** (Table 1) (Li et al., 2012). (C–E) Optical properties of the most potent helicase inhibitors derived from primuline. (F–J) Fluorescence of cells exposed to the most potent derivatives (excitation 340 ± 40 nm, emission 435 ± 50). Scale bar – 10 μm.

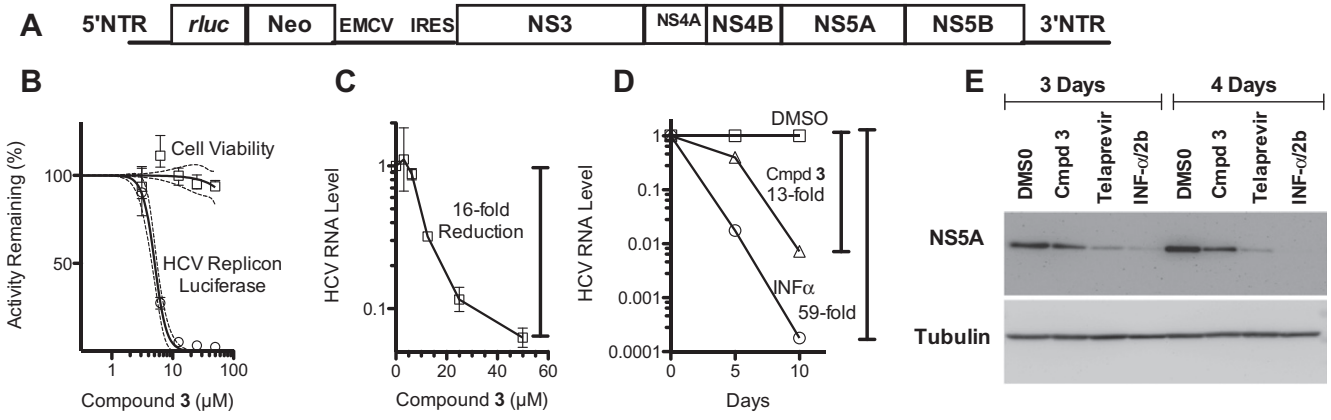


Fig. 4. Effect of compound **3** on Huh7.5 hepatoma cells harboring a stably transfected subgenomic *rLuc* HCV replicon. (A) Schematic genome map of the HCV replicon. (B) Percent *Renilla* luciferase remaining (circles) after 72 h exposure of replicon containing cells to **3** when grown in media containing 0.5% DMSO. Cell viability was measured with the Cell Titer-Glo luminescent cell viability kit (Promega) and is expressed compared to DMSO controls. (C) Amount of HCV RNA remaining in cells exposed to the indicated amounts of **3**. RNA was measured using 1 μg of total cellular RNA with specific Taqman probes and quantitative reverse transcriptase PCR (qRT-PCR). (D) Time-dependent clearance of HCV RNA in cells exposed to 10 μM of **3** (triangles) or 100 units of interferon (circles). RNA levels are expressed relative to RNA levels observed in cells grown in the presence of media and 0.5% DMSO. All assays were performed in triplicate, and error bars mark standard deviations. (E) Western blot of cell extracts treated with **3** or standard HCV drugs for indicated times.

that of interferon-α/2b (IFN-α/2b), cells were exposed to either for 10 days. In repeated experiments, **3** decreased HCV replicon RNA content about 13-fold, or about one-quarter that seen with interferon-α/2b after 10 days of treatment (Fig. 4D). To examine if reduced HCV RNA levels corresponded to lower amounts of HCV proteins, cellular NS5A amounts were examined after 4-day exposure to either compound **3**, IFN-α/2b, or telaprevir. Compound **3** reduced NS5A levels in a time dependent manner, but did so to a lesser extent than either IFN-α/2b or telaprevir (Fig. 4E).

*3.7. Effect of compound **3** on the formation of HCV replication complexes in cells stably transfected with subgenomic HCV replicons*

In replicon containing Huh7.5 cells, HCV replication occurs in a membranous web associated with the rough endoplasmic reticulum (Gosert et al., 2003). Having found that compound **3** inhibited HCV replication, we next investigated how compound **3** treatments might affect HCV replicase formation and cellular distribution in HCV *R*luc subgenomic replicon cells. Replicon cells treated with compound **3**, primuline, DMSO only (negative control), or IFN-α/2b (positive control), were stained with antibodies for NS5A. Compound **3** significantly affected HCV replication complexes. Cells treated with **3** had a lower number of replication complexes (Fig. 5D), and replicase number was comparable to that of IFN-α/2b (Fig. 5B). A clear cytoplasmic ER-like staining of HCV NS5A was present in the cells treated with compound **3** or IFN-α/2b. In contrast, primuline (Fig. 5C) did not influence the number of HCV replication complexes when compared to the DMSO control (Fig. 5A).

Because **3** is fluorescent (Fig. 5E) and can be directly observed in cells (Fig. 5F), the cellular location of **3** was compared with that of an antibody complex staining NS5A. Compound **3** stained the cyto-

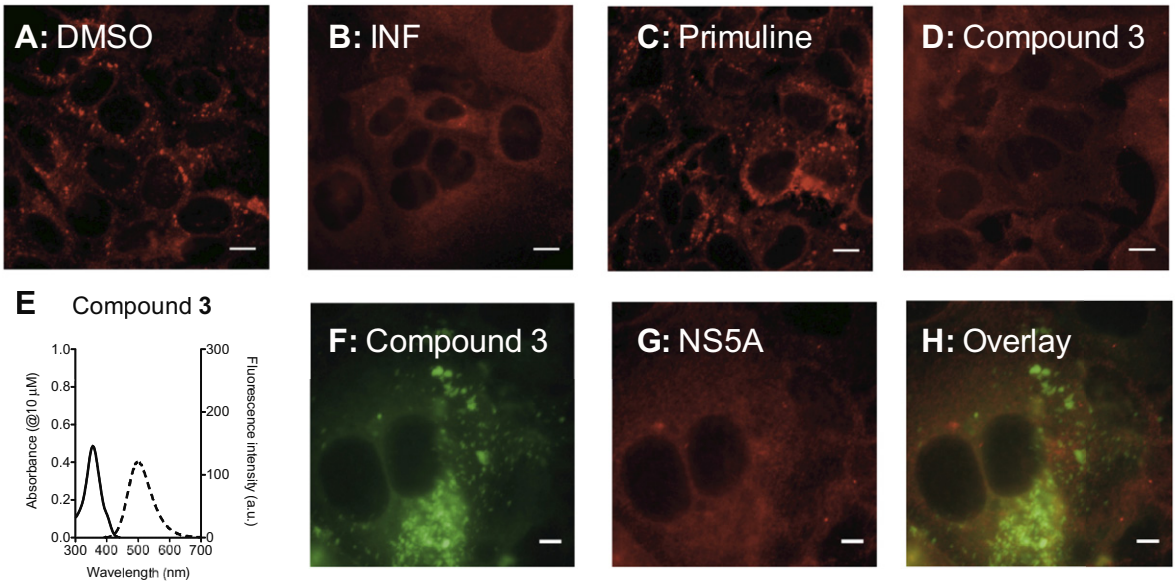


Fig. 5. Effect of compound **3** on the cellular location of HCV replication complexes observed in replicon-containing Huh7.5/Con1sg-*R*luc cells. Cells were fixed, permeabilized, and stained with 9E10 α-NS5A antibody (obtained from Charles Rice, Rockefeller University) and Alexa 546 secondary antibody 72 h after treatment with (A) 0.5% DMSO, (B) 100 units of interferon, (C) 10 μM primuline, or (D) 10 μM of compound **3**. Scale bar – 10 μm. (E) Compound **3** fluorescence. Note: scale is the same as the graphs for the more potent inhibitors shown in Fig. 2. To examine compound **3** location in cells, Huh7.5/Con1sg-*R*luc cells were treated with compound **3** for 72 h, permeabilized and stained with 9E10 α-NS5A antibody and Alexa 546 secondary antibody. (F) Localization of compound **3** fluorescence observed when the cells are excited at 350 nm. (G) NS5A localization observed when the same cells were excited to observe the NS5A replication complexes. (H) Overlay of the images with compound **3** colored green and NS5A complexes colored red. Scale bar – 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

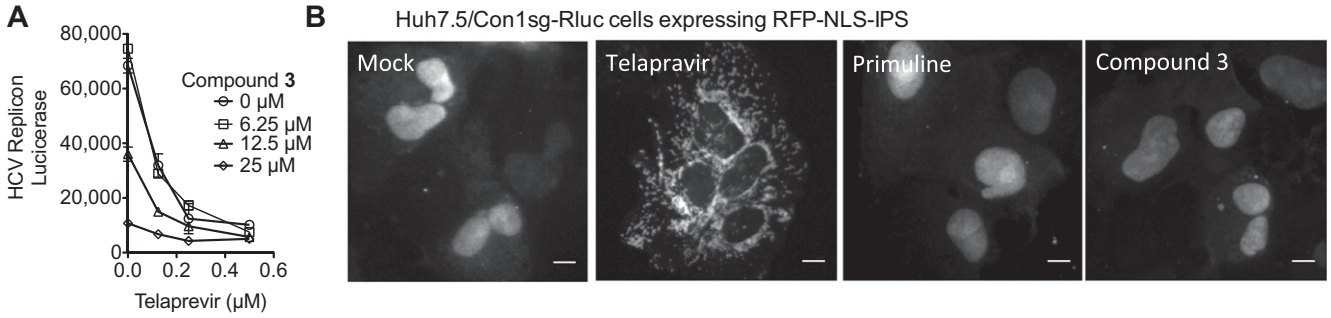


Fig. 6. Effects of helicase and protease inhibitors on cells harboring HCV replicons. (A) HCV replicon content in cells following treatment with various concentrations of telaprevir and **3** for 72 h. (B) Effect of 10 μM of various compounds (when administered in DMSO for 72 h) on the location of a recombinant NS3 protease substrate: red fluorescent-nuclear localization signal-interferon (IFN)-β promoter stimulator protein 1 (RFP-NLS-IPS) (Jones et al., 2010). Note that when NS3 is present, the reporter is in the nucleus, but in the presence of protease inhibitors, the reporter remains tethered to mitochondria. Scale bars – 10 μm.

plasm in a diffused dotted pattern and was not present in the nucleus (Fig. 4F–H). NS5A and **3** did not appear to co-localize, which would be expected if replication complexes had been disrupted (Fig. 4G and H).

3.8. Effect of primuline derivatives on the cellular activity of HCV protease

To examine how compound **3** behaves in the presence of HCV drugs that target NS3, we added both compound **3** and telaprevir to cells harboring the *Rluc* subgenomic HCV replicon. Telaprevir administration led to a similar concentration dependent reduction in replicon expression in the presence and absence of compound **3** (Fig. 6A). No synergy was observed between **3** and telaprevir.

A cell-based NS3 protease assay was also used to test the hypothesis that primuline derivatives exert their cellular effects *via* the NS3 protease. The assay chosen was developed by (Jones et al. (2010), and uses a fusion protein made from a NS3 protease cellular target called IPS-1 (also known as MAVS) fused to a red fluorescent protein (RFP) and a nuclear localization signal (NLS). Lentivirus expressing the RFP-NLS-IPS-1 was used to transduce the above *Rluc* subgenomic HCV replicon cell line, and the effect of compounds on the location of RFP-NLS was examined using fluorescence microscopy. RFP appeared in the nucleus of cells only in the presence of an active NS3 protease. Telaprevir prevented this translocation, but primuline did not. Compound **3** also did not inhibit nuclear translocation (Fig. 6B).

4. Discussion

The HCV multifunctional NS3 protein is both a protease and a helicase. NS3 protease inhibitors are an important new class of HCV drugs, but less progress has been made in developing specific compounds targeting the NS3 helicase function. Li et al. recently

reported a new class of NS3 helicase inhibitors synthesized from the main component of the fluorescent yellow dye primuline (Li et al., 2012). In the Li et al. study, the main primuline component (compound **2**) was substituted with aryl acid chlorides to produce the derivatives studied here (Table 1). The present study reports the unexpected discoveries that some of these new primuline derivatives retain fluorescence properties similar to those of the parent dye, that some compounds can inhibit both the helicase and protease functions of NS3, and that some are also potent inhibitors of Dengue virus NS3. The solubility of the more potent compounds in the absence of detergents used in enzyme assays presently limits their utility as cellular molecular probes, but a less potent, more soluble analog inhibits HCV RNA synthesis, protein synthesis, and replicase formation in a concentration-dependent and time-dependent fashion.

Unlike other HCV helicase inhibitors, the primuline derivatives are uniquely fluorescent, a characteristic that could be exploited both *in vitro* and *in vivo*. Unfortunately none of the most potent or specific benzothiazoles had a concentration-dependent effect on the HCV subgenomic replicon system used here, most likely due to their limited solubility and/or their inability to cross cell membranes (Li et al., 2012). As was found by Li et al., most of the primuline derivatives tested have some modest activity against the HCV replicon, but repeated experiments have revealed effects vary depending on the batch of cells used and the passage number of stably transfected replicon cells. Only compound **3** showed a batch-independent and concentration-dependent inhibition of replicon luciferase activity, with 5 ± 2 μM needed to reduce replicon-encoded luciferase by 50%. Even at the highest concentration administered to cells (50 μM), compound **3** was not toxic. In the same system, telaprevir inhibited the replicon with an IC_{50} of 0.62 ± 0.05 μM and reduced cell viability with a CC_{50} value of 28 ± 5 μM. In replicon containing Huh7.5 cells, HCV replication occurs in a membranous web associated with the rough endoplasmic reticulum (Gosert et al., 2003). Clear differences in such complexes were observed when mock-treated (DMSO only) cells were compared with cells treated with compound **3**, or interferon-α/2b. Like interferon, compound **3** treatment reduced the number of replication complexes. To further examine the anti-HCV activity of primuline derivatives in cells containing HCV replicons, we examined the effect of compound **3** and compound **12** exposures on HCV RNA levels. Assays with compound **12** (not shown) were confounded by its poor solubility in cell culture media. In contrast, compound **3** reduced HCV RNA levels, protein levels, and the amount of HCV replication foci seen in replicon cells in a concentration-dependent and time dependent manner.

There are two possible explanations for the ability of compounds to inhibit both NS3 helicase and protease. The first explanation is that certain compounds interact with the interface formed between the protease and helicase domains of NS3. The second explanation is that the compounds that inhibit the protease are simply more likely to interact with any protein. Although we have directly observed interactions with NS3 (Fig. 2), the later explanation seems somewhat more likely because (1) compounds inhibit the NS3 protease even when the helicase portion of NS3 is not present (Table 1), (2) some of the protease inhibitors also inhibit the unrelated single-stranded DNA binding (SSB) protein from *Escherichia coli* while compounds that target helicase only do not affect SSB (Mukherjee et al., in press), and (3) many potent protease inhibitors in this series also interact with nucleic acid polymers (Li et al., 2012).

The lack of clear specificity for compounds targeting both helicase and protease also confounds the interpretation of any antiviral effect seen with this series of compounds. Besides targeting NS3, compound **3** might inhibit cellular proteins like the DEAD-box helicases (*e.g.* DDX3), which have been shown to be involved in HCV replication (Angus et al., 2010; Oshiumi et al., 2010). The observation that Huh7.5 cells lacking HCV replicons show staining that was similar to that seen in HCV replicon cells, and the negative results of the cellular protease assays with compound **3** (Fig. 5) lead us to suspect that compound **3** might not exert its effect primarily by inhibiting NS3 in cells. Selection of HCV replicons resistant to compound **3**, which is an ongoing project in our lab, might provide better clues into the exact target of compound **3**.

Even though it appears that the compounds targeting protease and helicase lack specificity, the structure activity relationships reported here should provide a framework to design specific inhibitors to target the NS3 helicase from either HCV or Dengue virus. Since compounds directly interact with both NS3h and scNS4-NS3 (Fig. 2), it should also be possible to pinpoint the binding site using X-ray crystallography or another high-resolution biophysical technique. Such structures could be used to further optimize this scaffold. The recent structure of full-length NS3 bound to a reversible acylsulfonamide inhibitor (Schiering et al., 2011) or various Dengue NS3-ligand complexes (Luo et al., 2008) might also be helpful in this regard. Regardless, clues to specificity in this series are already clear from the structure activity relationships described in Table 1. The less specific inhibitors were obtained when CF_3 (compound **13**), Br (compound **9**), NH_2 (compound **7**), or Cl (compound **10**) were present in the *para* position of the benzamide. The most potent of these, was the 4-CF_3 analog (compound **13**), even though compound **7** was the one that most closely resembled compound **1**. Potent, specific helicase inhibitors were obtained only when F was present in the *para* position (compound **8**), or if no *para* substituent was present (Table 1). The most specific compounds for NS3 helicase were the 3-Cl analog (compound **12)** and one in which the substituted terminal benzene was replaced with naphthalene (compound **11**). A comparison of compounds **10** and **12**, where the addition of a single Cl causes a 10-fold more potent activity against the NS3 protease, clearly demonstrates the importance of a *para* substituent for protease inhibition.

Even though the exact target of compound **3** in cells is still uncertain, the results here suggest that experiments with the more potent and specific helicase inhibitors (*e.g.* compounds **11** and **12**) might be possible if they can be administered with a compatible excipient. Primuline derivatives might also exert more of an effect on an authentic HCV cell based replication system based on the JFH1 strain (*e.g.* HCVcc (Lindenbach et al., 2005)). We suspect that these compounds might be more potent inhibitors in the HCVcc system based on the recent demonstration that NS3 and the HCV structural protein core physically interact with one another (Mousseau et al., 2011). The core protein is not present in the subgenomic replicon, and it acts as an RNA chaperone to help pack the HCV genome in its envelope (Ivanyi-Nagy et al., 2006). One could envision how a chaperone and helicase might work together to package viral RNA, and it is possible that our compounds inhibit this process. It is also possible that the primuline derivatives might enter the cells *via* endocytosis along with the virus so higher compound concentrations would be available in the HCVcc system. If a system is found to deliver the primuline derivatives to cells, the more specific compounds could serve as powerful tools to yield insights into when and where the helicase acts in an infected cell, or to test the hypothesis that helicase activity on RNA or DNA is needed for HCV replication. Methods to administer the more potent derivatives and procedures to synthesize more soluble analogs that retain potency are currently under investigation in our laboratories. Ongoing experiments are also directed at investigating direct interaction of compound **3** and NS3 using fluorescent NS3 fusion proteins and monoclonal NS3 antibodies. If successful some of these compounds could then be used to directly monitor virus morphogenesis and egress in real time using live cell imaging.

Finally, it should be noted that some compounds in this series (*e.g.* compound **7**) are uniquely effective against the Dengue virus helicase, suggesting compounds in this series might act against other viruses or even as broad acting antivirals. Also noteworthy is the fact that the related dye, thioflavine S has been used as a tool to monitor neurofibrillary tangles and senile plaques in Alzheimer's disease (Guntern et al., 1992), suggesting some compounds described here might also be useful in neuroscience, or other areas not related to virology.

Acknowledgments

This work was supported by National Institutes of Health Grants U54 HG005031, R03 MH085690-01 and RO1 AI088001 and a Research Growth Initiative Award from the UWM Research Foundation. We also thank John J. Hernandez, Mahsa Ranji and Kevin Staniszewski (UW-Milwaukee, USA) for technical assistance. Celia Schiffer, Cihan Aydin (UMASS Medical School, USA), Dagmar Wirth (Helmholtz Centre for Infection Research, Germany) and Charles M. Rice (The Rockefeller University, USA) all provided valuable reagents.

References

Angus, A.G., Dalrymple, D., Boulant, S., McGivern, D.R., Clayton, R.F., Scott, M.J., Adair, R., Graham, S., Owsianka, A.M., Targett-Adams, P., Li, K., Wakita, T., McLauchlan, J., Lemon, S.M., Patel, A.H., 2010. Requirement of cellular DDX3 for hepatitis C virus replication is unrelated to its interaction with the viral core protein. J. Gen. Virol. 91, 122–132.

Bacon, B.R., Gordon, S.C., Lawitz, E., Marcellin, P., Vierling, J.M., Zeuzem, S., Poordad, F., Goodman, Z.D., Sings, H.L., Boparai, N., Burroughs, M., Brass, C.A., Albrecht, J.K., Esteban, R., 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1207–1217.

Belon, C.A., Frick, D.N., 2009. Helicase inhibitors as specifically targeted antiviral therapy for hepatitis C. Future Virol. 4, 277–293.

Belon, C.A., Frick, D.N., 2008. Monitoring helicase activity with molecular beacons. BioTechniques 45, 433–440, 442.

Belon, C.A., High, Y.D., Lin, T.I., Pauwels, F., Frick, D.N., 2010. Mechanism and specificity of a symmetrical benzimidazolephenylcarboxamide helicase inhibitor. Biochemistry 49, 1822–1832.

Beran, R.K., Lindenbach, B.D., Pyle, A.M., 2009. The NS4A protein of hepatitis C virus promotes RNA-coupled ATP hydrolysis by the NS3 helicase. J. Virol. 83, 3268–3275.

Beran, R.K., Pyle, A.M., 2008. Hepatitis C viral NS3-4A protease activity is enhanced by the NS3 helicase. J. Biol. Chem. 283, 29929–29937.

Beran, R.K., Serebrov, V., Pyle, A.M., 2007. The serine protease domain of hepatitis C viral NS3 activates RNA helicase activity by promoting the binding of RNA substrate. J. Biol. Chem. 282, 34913–34920.

Ding, S.C., Kohlway, A.S., Pyle, A.M., 2011. Unmasking the active helicase conformation of nonstructural protein 3 from hepatitis C virus. J. Virol. 85, 4343–4353.

Enserink, M., 2011. Infectious diseases. First specific drugs raise hopes for hepatitis C. Science 332, 159–160.

Frick, D.N., 2007. The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. Curr. Issues Mol. Biol. 9, 1–20.

Frick, D.N., Ginzburg, O., Lam, A.M., 2010. A method to simultaneously monitor hepatitis C virus NS3 helicase and protease activities. Methods Mol. Biol. 587, 223–233.

Frick, D.N., Rypma, R.S., Lam, A.M., Gu, B., 2004. The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. J. Biol. Chem. 279, 1269–1280.

Garber, K., 2011. Hepatitis C: move over interferon. Nat. Biotechnol. 29, 963–966.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H.E., Bienz, K., Moradpour, D., 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. 77, 5487–5492.

Graham, R.K., Caiger, P., 1969. Fluorescence staining for the determination of cell viability. Appl. Microbiol. 17, 489–490.

Guntern, R., Bouras, C., Hof, P.R., Vallet, P.G., 1992. An improved thioflavine S method for staining neurofibrillary tangles and senile plaques in Alzheimer's disease. Experientia 48, 8–10.

Hanson, A.M., Hernandez, J.J., Shadrick, W.R., Frick, D.N., 2012. Identification and analysis of inhibitors targeting the hepatitis C virus NS3 helicase. Methods Enzymol. 511, 463–483.

Horobin, R.W., Kiernan, J.A., Conn, H.J. (Eds.), 2002. Conn's Biological Stains: a Handbook of Dyes, Stains and Fluorochromes for Use in Biology and Medicine. BIOS, Oxford, pp. 357–358.

Howe, A.Y., Chase, R., Taremi, S.S., Risano, C., Beyer, B., Malcolm, B., Lau, J.Y., 1999. A novel recombinant single-chain hepatitis C virus NS3-NS4A protein with improved helicase activity. Protein Sci. 8, 1332–1341.

Ivanyi-Nagy, R., Kanevsky, I., Gabus, C., Lavergne, J.P., Ficheux, D., Penin, F., Fosse, P., Darlix, J.L., 2006. Analysis of hepatitis C virus RNA dimerization and core-RNA interactions. Nucleic Acids Res. 34, 2618–2633.

Jones, C.T., Catanese, M.T., Law, L.M., Khetani, S.R., Syder, A.J., Ploss, A., Oh, T.S., Schoggins, J.W., MacDonald, M.R., Bhatia, S.N., Rice, C.M., 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. Nat. Biotechnol. 28, 167–171.

Lam, A.M., Frick, D.N., 2006. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. J. Virol. 80, 404–411.

Lam, A.M., Keeney, D., Eckert, P.Q., Frick, D.N., 2003. Hepatitis C virus NS3 ATPases/helicases from different genotypes exhibit variations in enzymatic properties. J. Virol. 77, 3950–3961.

Lam, A.M., Rypma, R.S., Frick, D.N., 2004. Enhanced nucleic acid binding to ATP-bound hepatitis C virus NS3 helicase at low pH activates RNA unwinding. Nucleic Acids Res. 32, 4060–4070.

Lanzetta, P.A., Alvarez, L.J., Reinach, P.S., Candia, O.A., 1979. An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 100, 95–97.

Levin, M.K., Patel, S.S., 2002. Helicase from hepatitis C virus, energetics of DNA binding. J. Biol. Chem. 277, 29377–29385.

Li, K., Frankowski, K.J., Belon, C.A., Neuenswander, B., Ndjomou, J., Hanson, A.M., Shanahan, M.A., Schoenen, F.J., Blagg, B.S., Aube, J., Frick, D.N., 2012. Optimization of potent hepatitis C virus NS3 helicase inhibitors isolated from the yellow dyes thioflavine S and primuline. J. Med. Chem. 55, 3319–3330.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.

Lindenbach, B.D., Pragai, B.M., Montserret, R., Beran, R.K., Pyle, A.M., Penin, F., Rice, C.M., 2007. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J. Virol. 81, 8905–8918.

Lindenbach, B.D., Rice, C.M., 2005. Unravelling hepatitis C virus replication from genome to function. Nature 436, 933–938.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Luo, D., Xu, T., Watson, R.P., Scherer-Becker, D., Sampath, A., Jahnke, W., Yeong, S.S., Wang, C.H., Lim, S.P., Strongin, A., Vasudevan, S.G., Lescar, J., 2008. Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. EMBO J. 27, 3209–3219.

Mousseau, G., Kota, S., Takahashi, V., Frick, D.N., Strosberg, A.D., 2011. Dimerization-driven interaction of hepatitis c virus core protein with NS3 helicase. J. Gen. Virol. 92, 101–111.

Mukherjee, S., Hanson, A.M., Shadrick, W.R., Ndjomou, J., Sweeney, N.L., Hernandez, J.J., Bartczak, D., Li, K., Frankowski, K.J., Heck, J.A., Arnold, L.A., Schoenen, F.J., Frick, D.N., in press. Identification and analysis of hepatitis C virus NS3 helicase inhibitors using nucleic acid binding assays. Nucleic Acids Res, http://dx.doi.org/10.1093/nar/gks623.

Murray, C.L., Rice, C.M., 2011. Turning hepatitis C into a real virus. Annu. Rev. Microbiol. 65, 307–327.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263–267.

Oshiumi, H., Ikeda, M., Matsumoto, M., Watanabe, A., Takeuchi, O., Akira, S., Kato, N., Shimotohno, K., Seya, T., 2010. Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1-mediated IFN-beta induction. PLoS One 5, e14258.

Preugschat, F., Averett, D.R., Clarke, B.E., Porter, D.J., 1996. A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. J. Biol. Chem. 271, 24449–24457.

Schiering, N., D'Arcy, A., Villard, F., Simic, O., Kamke, M., Monnet, G., Hassiepen, U., Svergun, D.I., Pulfer, R., Eder, J., Raman, P., Bodendorf, U., 2011. A macrocyclic HCV NS3/4A protease inhibitor interacts with protease and helicase residues in the complex with its full-length target. Proc. Natl. Acad. Sci. U.S.A. 108, 21052–21056.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Xu, T., Sampath, A., Chao, A., Wen, D., Nanao, M., Chene, P., Vasudevan, S.G., Lescar, J., 2005. Structure of the dengue virus helicase/nucleoside triphosphatase catalytic domain at a resolution of 2.4 Å. J. Virol. 79, 10278–10288.

Zeuzem, S., Andreone, P., Pol, S., Lawitz, E., Diago, M., Roberts, S., Focaccia, R., Younossi, Z., Foster, G.R., Horban, A., Ferenci, P., Nevens, F., Mullhaupt, B., Pockros, P., Terg, R., Shouval, D., van Hoek, B., Weiland, O., Van Heeswijk, R., De Meyer, S., Luo, D., Boogaerts, G., Polo, R., Picchio, G., Beumont, M., 2011. Telaprevir for retreatment of HCV infection. N. Engl. J. Med. 364, 2417–2428.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., Trono, D., 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. 72, 9873–9880.