Antiviral Research 91 (2011) 102–111

Contents lists available at ScienceDirect

Antiviral Research

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

Development of hepatitis C virus chimeric replicons for identifying broad spectrum NS3 protease inhibitors

Joseph Binder *, Selwyna Tetangco [1], Megan Weinshank, Karen Maegley, Laura Lingardo, Wade Diehl, Robert Love, Amy K. Patick [2], George J. Smith III

Pfizer Worldwide Research and Development, 10777 Science Center Dr., San Diego, CA 92121, USA

ARTICLE INFO

Article history:
Received 22 June 2010
Revised 5 May 2011
Accepted 12 May 2011
Available online 19 May 2011

Keywords:
HCV
NS3 protease
Chimeric replicon

ABSTRACT

Several potent inhibitors of hepatitis C virus (HCV) NS3/4A protease have been identified that show great clinical potential against genotype 1. Due to the tremendous genetic diversity that exists among HCV isolates, development of broad spectrum inhibitors is challenging. With a limited number of lab strains available for preclinical testing, new tools are required for assessing protease inhibitor activity. We developed a chimeric replicon system for evaluating NS3 protease inhibitor activity against naturally occurring isolates. NS3/4A genes were cloned from the plasma of HCV-infected individuals and inserted into lab strain replicons, replacing the native sequences. The chimeric reporter replicons were transfected into Huh 7.5 cells, their replication monitored by luciferase assays, and their susceptibilities to inhibitors determined. Viable chimeras expressing heterologous genotypes 1, 2, 3, and 4 protease domains were identified that exhibited varying susceptibilities to inhibitors. Protease inhibitor spectrums observed against the chimeric replicon panel strongly correlated with published enzymatic and clinical results. This cell-based chimeric replicon system can be used to characterize the activities of protease inhibitors against diverse natural isolates and may improve the ability to predict dose and clinical efficacy.

1. Introduction

Hepatitis C virus (HCV) is a major global health concern, infecting nearly 3% of the world's population. While HCV infections can spontaneously resolve, most infections become chronic. Over time, persistent infection may lead to inflammation, fibrosis and cirrhosis of the liver, hepatic failure, hepatocellular carcinoma, and, ultimately, death. The current standard of care, coadministration of pegylated interferon and ribavirin, is only partially effective. Although sustained virologic response rates are high in patients infected with genotypes 2 or 3, combination therapy is effective in only 50% of patients with genotype 1 (Di Bisceglie and Hoofnagle, 2002; Manns et al., 2001). In addition, interferon and ribavirin can cause significant side effects, including flu-like symptoms, depression, and hemolytic anemia. Given the limited efficacy and high side effect burden associated with the current standard of care, novel broad-spectrum HCV-specific therapies are urgently needed.

HCV, a member of the Flaviviridae family, possesses a single-strand positive-sense RNA genome encoding a ~3100 amino acid polyprotein that is co- and post-translationally processed by host and viral proteases into four structural and six nonstructural proteins. The multifunctional NS3 protein plays a critical role in the viral life cycle. The amino-terminal chymotrypsin-like serine protease and carboxy-terminal helicase domains generally function independently and both are required for viral replication. The ~180 amino acid protease domain acts in association with NS4A to release mature nonstructural proteins from the polypeptide precursor, allowing the proteins to perform their critical replication functions (Lindenbach and Rice, 2001). NS3 protease has also been shown to help HCV evade host innate immunity (Li et al., 2005a,b). Given its essential roles in viral polyprotein processing and immune evasion, NS3 protease is a prime target for antiviral chemotherapy. The promising therapeutic potential of protease inhibitors has been confirmed in several clinical trials, with BILN-2061/ciluprevir (Hinrichsen et al., 2004; Lamarre et al., 2003), VX-950/telaprevir (Reesink et al., 2006), ITMN-191/danoprevir (Forestier et al., 2008), and SCH-503034/boceprevir (Sarrazin et al., 2007) demonstrating efficacy in genotype 1-infected patients.

HCV exhibits a high degree of natural genetic variation, with six major genotypes and over 50 subtypes identified thus far. Genotypes can differ by up to 30% at the nucleotide and amino acid levels while subtypes within each genotype can differ by as much as 20–25% (Robertson et al., 1998; Simmonds, 2004). In addition, HCV exists within each infected individual as a quasispecies population

* Corresponding author. Tel.: +1 858 526 4978; fax: +1 858 435 1839.
E-mail address: joe.binder@pfizer.com (J. Binder).
[1] Present address: Life Technologies, 5781 Van Allen Way, Carlsbad, CA 92008, USA.
[2] Present address: Adamas Pharmaceuticals, Inc., 1900 Powell St., Suite 1050, Emeryville, CA 94608, USA.

0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2011.05.007

of related viruses with distinct genetic sequences (Martell et al., 1992; Pawlotsky, 2003). The high genetic variation poses a significant challenge in developing broad spectrum antivirals. Structure-based drug design efforts have identified NS3 protease inhibitors active against genotype 1, but with limited spectrum of activity (Benhamou et al., 2009; Foster et al., 2010; Reiser et al., 2005; Tong et al., 2006). Unfortunately, few tools have been developed for effectively characterizing spectrum of activity prior to clinical trials. Unlike many viruses, HCV replicates poorly in tissue culture and as a result, direct antiviral assays with clinical isolates cannot be readily performed. Despite recent progress in HCV infectious cell culture systems (Kato et al., 2007), subgenomic replicons remain the primary tools for characterizing antiviral activity (reviewed in Blight and Norgard (2006)). However, only a few select replicons are readily available, with H77, con1, and JFH-1 serving as prototype strains for genotypes 1a, 1b, and 2a, respectively (Blight et al., 2003; Kato et al., 2003; Lohmann et al., 1999). In this study, we describe our efforts to develop a cell-based replicon system for evaluating the activities of HCV protease inhibitors against natural isolates from the four most common genotypes, with the ultimate goals of using this system to drive clinical drug candidate selection and better predict clinical efficacy.

2. Materials and methods

2.1. Cloning of clinical isolate NS3/4A genes

Serum samples from 28 HCV-infected patients were obtained from Cliniqa (Fallbrook, CA) and Teragenix Corporations (Fort Lauderdale, FL). The clinical isolate panel included 10 genotype 1a, five genotype 1b, six genotype 2b, two genotype 3a, and five genotype 4 samples. Viral RNA was extracted from the samples using a QIAamp Viral RNA Mini kit (Qiagen Inc., Valencia, CA) and reverse transcribed into cDNA with random hexamer primers and Superscript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA) according to the Manufacturer's instructions. Following RNAseH digestion, NS3/4A genes were PCR amplified with Platinum Taq High Fidelity (Invitrogen) and genotype-specific primers (Table 1) designed to anneal to conserved regions near the 3′ end of NS2 and central region of NS4B. Although PCR conditions were optimized for each primer set, standard initial PCR conditions included a 2-min denaturation step at 94 °C, 37 amplification cycles (94 °C for 30 s, 52–60 °C for 30 s, 68 °C for 2 min, 45 s), and a seven minute final extension step at 72 °C. Amplicons were separated by agarose gel electrophoresis and fragments of the expected size (2.3–2.9 kb) were extracted using a QIAquick Gel Extraction kit (Qiagen Inc.). Amplicons were ligated into pCR2.1-TOPO (Invitrogen) and the resulting plasmids were electroporated into either DH10B Electromax or One Shot Top10 Electrocomp *Escherichia coli* (Invitrogen). Bacteria were plated onto Luria–Bertani agar plates containing ampicillin (50–100 μg/ml) and X-galactose. Clones with NS3/4A sequences were identified by standard blue/white and colony PCR screening followed by sequencing with T3/T7 oligos.

2.2. Sequence analysis

Plasmids containing NS3/4A inserts were sequenced using a bidirectional primer walking method and sequences were analyzed with Lasergene software (DNAStar, Inc., Madison, WI). On average, eight clones (range 4–12) were sequenced for each clinical isolate (Table 2), with each base covered by at least two independent sequencing runs as needed to resolve ambiguities. Clone sequences were aligned with the MegAlign program and a consensus sequence for each isolate was determined. For each isolate, one clone was selected as the representative sequence. In most cases, the representative was identical to the isolate consensus at the amino acid level. For the six isolates without a perfect match, the clone with the greatest amino acid identity (at least 99.7%) to the consensus was selected as the representative.

2.3. Construction of chimeric replicons

The genotype 1b con1 parent replicon carries the humanized *Renilla* luciferase reporter gene linked to the neomycin resistance marker via a Foot-and-Mouth Disease Virus (FMDV) 2A autoprotease cassette (Hao et al., 2007a,b). The reporter-selectable replicon carries three adaptive mutations to increase replication capacity (E1202G in the NS3 protease domain, T1280I in the NS3 helicase domain, and S2197P in NS5A). Standard molecular biology techniques were used in constructing the chimeric replicons (Ausubel et al., 1999). While cloning procedures varied for each isolate, the general strategy involved amplifying clinical isolate representative clones by PCR, either the entire 2058-base NS3/4A region (NS3 b1 through NS4B b3) or the NS3 protease domain only (NS3 b1 through b555). The amplicons were inserted into the replicon backbone, precisely replacing the corresponding native con1 sequence, by tripartite overlapping PCR ligation and/or standard restriction fragment exchange techniques. Both monocistronic and dicistronic versions of the replicon were utilized. Representative clonal sequences from three clinical isolates (16, 17, and 21) were cloned into the dicistronic replicon-encoding plasmid, pBB7M4hRLuc, at positions 2806 through 3360 (protease domain) or 4863 (NS3/4A). Representative sequences from all other clinical isolates were cloned into the monocistronic replicon-encoding plasmid, pRB10HRSM, at bases 2673 through 3227 (protease

Table 1
Sequences of primers used to PCR amplify clinical isolate NS3/4A genes.

Genotype	NS2-specific forward primers	NS4B-specific reverse primers
1a	5′ ATGGAGACCAAGCTCATCACGTGG 3′ 5′ ACCGCCGCGTGCGGTGACATCATC 3′	5′ CCTYCTGCTTGAACTGCTCRGCGAGC 3′ 5′ GCTGGTGACRGCAGCTGTAAAAGCCATC 3′
1b	5′ GACATGGAGACCAAGATCATCACCTGG 3′ 5′ GACATGGAGACYAARRTYATCACNTGGGG 3′ 5′ ATGGAGACYAAGVTYATCACVTGGGG 3′ 5′ GAGCCYGTYGTCTTCTCYGAYATGGAGAC 3′	5′ GCATTCCYTGYTCGATGTAVGGRAGGTG 3′ 5′ CCGCTGATGAARTTCCACATRTGCTTYGCCC 3′ 5′ CCGCTGATGAARTTCCACATGTGCTTYGC 3′ 5′ GCTATCAGCCGGTTCATCCACTGCAC 3′
2b	5′ GTTCTGCTCGGCCCTGCCGAT 3′ 5′ TGGACCGGCACTTACATC 3′ 5′ TGGAGAAGAAGGTCATYGTGTGG 3′	5′ CCCCCACTAGGCCACTGACA 3′ 5′ GATGTTCAAGAGGATGGTGGTGCTG 3′
3a	5′ GCTGATGACTATCGGGAGATGG 3′	5′ GCTCGCTGCAGCAATCCAAG 3′
4	5′ AGATCTTGCTCGGACCAGCCGATACAG 3′	5′ GAATGCCGCTGATGAAATTCCACATG 3′

Degenerate bases: R = A/G, Y = C/T, V = A/C/G, N = A/C/G/T.

Table 2
Clinical isolates characterized by clonal sequence analysis.

Clinical isolate	Genotype	No. clones sequenced	GenBank accession No.[a]	Quasispecies diversity[b]	% Divergence from con1 NS3/4A[c]	
					nt	aa
1	1a	7	HM568417	0.26	19.6	8.0
2	1a	11	HM568418	0.19	20.1	7.0
3	1a	7	HM568419	0.06	19.6	7.6
4	1a	7	HM568420	0.61	19.8	8.3
5	1a	7	HM568421	0.57	19.7	7.4
6	1a	7	HM568422	0.79	19.7	7.9
7	1a	7	HM568423	0.14	20.1	6.9
8	1a	7	HM568424	0.23	19.8	7.7
9	1a	8	HM568425	0.36	19.7	8.0
10	1a	7	HM568426	0.21	20.0	8.0
11	1b	9	HM568427	0.34	7.3	2.3
12	1b	7	HM568428	0.4	6.4	2.8
13	1b	8	HM568429	0.09	8.8	2.6
14	1b	5	HM568430	1.3	8.3	3.5
15	1b	7	HM568431	0.64	7.0	2.8
16	2b	11	HM568432	0.27	29.6	18.5
17	2b	11	HM568433	0.46	29.9	19.0
18	2b	10	HM568434	0.82	29.9	19.4
19	2b	12	HM568435	0.85	29.7	19.1
20	2b	10	HM568436	0.78	30.1	19.4
21	2b	10	HM568437	0.47	30.5	19.0
22	3a	4	HM568438	0.50	30.3	18.2
23	3a	7	HM568439	0.15	30.3	18.2
24	4	5	HM568440	0.57	25.4	11.1
25	4	5	HM568441	0.48	25.4	11.2
26	4	7	HM568442	0.74	26.4	11.8
27	4	6	HM568443	0.87	25.3	12.0
28	4	7	HM568444	0.56	25.3	10.7

[a] Genbank accession number of representative clones used in chimeric replicon construction.
[b] Average percentage amino acid differences between clones of a particular isolate.
[c] Percentage nucleotide (nt) and amino acid (aa) differences within NS3/4A between representative clone and genotype 1b con1 replicon strain.

domain) or 4730 (NS3/4A). All plasmids were confirmed by sequencing.

The genotype 2a JFH-1 subgenomic bicistronic replicon has been previously described (Date et al., 2004). To create a reporter-selectable 2a replicon, the luciferase-2A-neo^R cassette was amplified from pBB7M4hRLuc by PCR. The amplicon was inserted into pSRG-JFH1/H2476L at positions 10684 through 1196 by bipartite overlapping PCR ligation and *EcoRI*/*PmeI* restriction fragment exchange to create p2aRep. The reporter-selectable replicon carries one adaptive mutation (H2476L) to increase replication capacity. A representative genotype 2b protease domain sequence (encoding NS3 amino acids 1–181) from clinical isolate 17 was cloned into p2aRep at positions 2778 through 3320.

2.4. Transient replication assay

Replicon plasmids were linearized with either *ScaI* (genotype 1b backbone) or *XbaI* (genotype 2a), ethanol precipitated, washed with 70% ethanol, and resuspended in nuclease-free water. RNA transcripts were prepared by *in vitro* transcription according to the Manufacturer's instructions (MEGAscript T7 kit, Ambion, Austin, TX). Purified RNA transcripts (15 μg) were transfected into 6×10^6 Huh 7.5 cells (Apath, LLC, St. Louis, MO) by electroporation at 220 volts/950 microfarad capacitance in a Gene Pulser II System (Bio-Rad, Hercules, CA). Electroporated cells were plated in 96-well, flat, clear-bottom black tissue culture plates at a density of 4.5×10^4 cells/well in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4.5 g/L D-glucose, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 0.1 mM non-essential amino acids). After 3 days of incubation at 37 °C under 5% CO_2, flash *Renilla* luciferase activity (in relative light units, RLU) was determined according to the Manufacturer's protocol (Promega Corporation, Madison, WI) on a Wallac MicroBeta TriLux luminometer (Perkin Elmer, Waltham, MA). In standard fitness experiments, the number of replicate wells for a given replicon varied from a minimum of four to a maximum of 36.

For antiviral dose response assays, transfected cells were plated as above and incubated for approximately two hours at 37 °C under 5% CO_2 while compounds were prepared. Inhibitors were dissolved in 100% dimethyl sulfoxide (DMSO), serially diluted in half-log increments, and added to the transfected cells in duplicate at nontoxic concentrations. *Renilla* luciferase activity was measured after three days of incubation and percent inhibition of luciferase activity (compared to "no drug" controls) was calculated at each concentration. The amount of compound required to inhibit luciferase activity by 50% (50% effective concentration, EC_{50}) was calculated using XLfit software model 205.

2.5. NS3/4A expression and purification

Representative NS3/4A sequences from each genotype were selected for protein expression and purification. Representatives included lab strains H77 (genotype 1a), con1 (1b) and JFH1 (2a), as well as clinical isolates 18 (2b), 23 (3a), and 25 (4). In order to reduce autoproteolysis during purification, two substitutions were introduced – T401S and C428S. Sequences were cloned into pAcSG2 (BD Biosciences, San Jose, CA), a baculovirus expression vector encoding an N-terminal hexahistidine tag. Protein was expressed according to the Manufacturer's instructions. Briefly, Sf9 cells were cotransfected with the NS3/4A plasmids and Baculogold viral DNA (BD Biosciences) to generate high titer recombinant viral stocks. Sf9 cells were infected (2×10^6 cells/ml with 1/500 viral stock dilution) in ESF-921 media (Expression Systems, Woodland, CA) and after a 72 h incubation, cells were lysed in buffer A (50 mM hepes pH 7.5, 500 mM NaCl, 10% glycerol, 20 mM

imidazole, 14 mM 2-Me) with 1% triton X-100 and 1% IGEPAL. Following a 45 min centrifugation at 186,000*g* in a Ti45 rotor (Beckman Coulter, Fullerton, CA), supernatants were loaded onto 5 ml Ni–NTA columns. Columns were washed with 20 column volumes of buffer A plus 0.2% triton X-100 before step elution with buffer A plus 250 mM imidazole and 0.2% triton X-100. Peak fractions were pooled, the protein concentrations determined by Bradford assays, and the purities characterized by polyacrylamide gel electrophoresis. Purified NS3/4A was frozen in liquid nitrogen prior to biochemical assays.

2.6. HCV Protease assay

Inhibition of HCV protease activity was monitored using a continuous fluorescence resonance energy transfer (FRET) assay. Compounds at various concentrations were added to assay buffer (50 mM MOPS pH 7.5, 50 mM NaCl, 20% glycerol, 0.1% Triton-X 100, 1 mM tris (2-carboxyethyl) phosphine) containing 3 μM depsipeptide FRET substrate S1 (AnaSpec, San Jose, CA) (Taliani et al., 1996) in white, non-binding 96-well plates (Corning, Lowell, MA). Reactions were initiated by the addition of full-length NS3/4A enzyme at a final concentration of 3 nM. The increase in fluorescence intensity following peptide cleavage was monitored using a Safire fluorescence plate reader (Tecan, Zurich, Switzerland) with excitation and emission wavelengths of 340 nm and 490 nm, respectively. Inhibition constants (K_i) were calculated by plotting rate as a function of inhibitor concentration and fitting the data to an equation derived for competitive inhibition. Due to inner filter effects present at high substrate concentrations, accurate K_m values could not be determined using the FRET assay. Instead, substrate titrations were performed using an HPLC assay under identical conditions to the FRET assay except that for some genotypes, the enzyme concentration was reduced to keep substrate turnover under 10% at the lowest substrate concentrations. In addition, reactions were stopped after 5 min by the addition of trifluoroacetic acid, 0.5% final concentration, prior to injection onto a C18 reverse phase HPLC column. Both products were separated from each other and the substrate using a 15 min linear gradient from 5% to 50% acetonitrile in 0.1% TFA/water. Product concentrations were determined using a standard curve derived from the EDANS labeled hydrolysis product (AnaSpec). K_m values were estimated by plotting the rate of product formation (pmol/minute) as a function of substrate concentration and fitting the data to the Michaelis–Menton equation.

2.7. Statistical analysis

The antiviral and biochemical activities of each compound were determined in at least three independent experiments. EC_{50} and K_i values were log transformed and analyzed using *t*-tests (implemented in PROC TTESTS of SAS v8.2 software, SAS Institute Inc., Cary, NC) to compare the activities against the various genotypes versus the genotype 1b reference strain. The group mean and the confidence interval were estimated and anti-log transformed back to the original scale to obtain the geometric mean of each group and the confidence interval of the geometric mean. Consequently, the *p*-values associated with testing the difference between the group means of log-transferred data became the *p*-values of testing the fold changes between geometric means.

3. Results

3.1. Clinical isolate NS3 sequences are genetically diverse

NS3/4A genes were amplified from the plasma of HCV-infected individuals by RT-PCR with genotype-specific primers designed against conserved regions within NS2 and NS4B (Table 1). Consensus sequences, derived from an average of eight clones per isolate (Table 2), were compared to each other as well as to prototype lab strains (H77, con1, and JFH-1) by phylogenetic analysis (Fig. 1). Considerable genetic diversity was observed among the isolates. Of the 685 amino acids comprising NS3/4A, 66.6% were completely conserved across all isolate consensus sequences, including critical amino acids of the active site catalytic triad (H57, D81, S139), P1 pocket (L135, F154, A157), zinc binding motif (C97, C99, C145, H149), and protease cleavage sites (NS2/3 and NS3/4A). Within a given genotype, isolates varied on average by 4.3% at the amino acid level, while isolates in different genotypes differed by an average of 17.1%. Quasispecies diversity, the mean percentage amino acid differences between clones of a particular isolate, was 0.5%. Genetic diversity was greater at the nucleotide level compared to the protein level, with the average divergence between any two clinical isolates at 23.1% (nucleotide) and 12.7% (protein).

While the core of the NS3 protease substrate/inhibitor binding pocket was completely conserved across all genotypes, genotype-specific differences were observed at eight locations around the periphery of the pocket (Fig. 2 and Table 3). Genotypes 1 and 4 binding pockets were highly conserved, with only two minor differences between them (positions 122 and 132). In contrast, the binding pockets of genotypes 2 and 3 proteases exhibited greater levels of divergence from genotype 1. Key differences were observed at positions 78, 79, 80 and 122 for genotype 2, as well as amino acids 123 and 168 for genotype 3.

3.2. Clinical isolate NS3/4A sequences severely impair replicon replication

To generate chimeric replicons, we first selected one clone for each isolate to serve as the representative sequence for that isolate. In most cases, the representative was identical to the isolate consensus at the amino acid level. The representative sequences were then cloned into either mono- or dicistronic genotype 1b con 1 strain replicons (Fig. 3) as precise replacements for the native NS3/4A genes. The replicons, each carrying the humanized *Renilla* luciferase gene to serve as a marker for viral replication, were transfected into highly permissive Huh 7.5 human hepatoma cells and luciferase activities were measured after three days. In this transient replication system, the parent replicon produces $\sim 10^5$ relative light units (RLU) per well with a signal: noise ratio of >1000. As shown in Fig. 4, replacement of the native NS3/4A with genotype 1b clinical isolate genes reduced replication capacity, or relative fitness, by 92.0% to 99.9%. Although severely impaired, the five intrasubtypic 1b/1b chimeras were replication competent. In contrast, most intersubtypic 1a/1b and all intergenotypic 2b/1b or 3a/1b chimeras failed to produce signals above background levels (genotype 4/1b chimeras were not characterized). Since the transient replication assay may not be sensitive enough to detect a low basal level of replication, we transfected the intergenotypic replicons into Huh-7.5 cells and selected for neomycin-resistant colonies with G418. If replication competent, the replicons would be expected to accumulate mutations during growth that increase fitness and allow colony formation in the presence of G418. Despite repeated attempts, no stable cell lines carrying intergenotypic replicons were ever observed, suggesting that replication levels were not sufficient to permit adaptive mutations.

3.3. NS3 protease domain chimeras replicate efficiently

The poor replication of the NS3/4A chimeras may have resulted from inefficient functioning of the heterologous polypeptides (NS3 protease, NS3 helicase, and/or NS4A) or the loss of two con1 adaptive mutations in NS3 (E1202G and T1280I). We hypothesized that

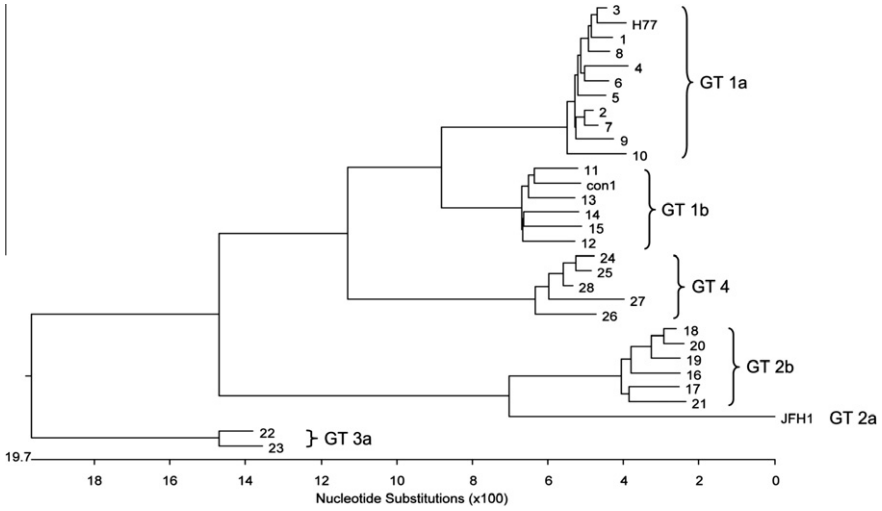

Fig. 1. Predicted phylogenetic relationships among naturally occurring HCV isolates and reference strains. Consensus NS3/4A protein sequences from clinical isolates and replicon strains were aligned with DNAStar's MegAlign program using the ClustalW method. The phylogenetic tree was constructed according to the neighbor-joining method, with distances calculated using the Kimura formula. The total horizontal branch length between isolates represents the genetic distance between those isolates in nucleotide substitutions per 100 bases. H77, con1, and JFH1 are the genotypes 1a, 1b, and 2a lab reference strains.

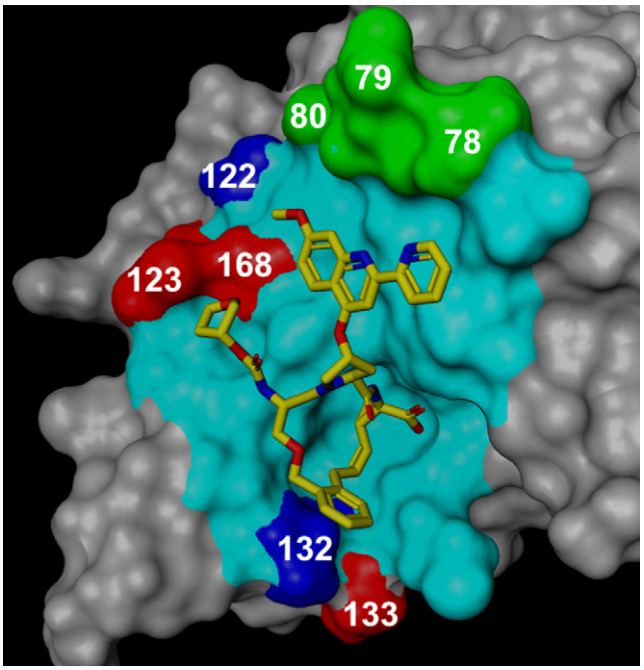

Fig. 2. Co-crystal structure of the genotype 1b (BK strain) NS3 protease with NS4A cofactor and noncovalently bound active site inhibitor PF-969597 at 2.8Å resolution. PF-969597 is identical to PF-968989 (see Fig. 5) except the P3 capping group contains a tetrahydrofuran ring instead of cyclopropane. Conserved active site residues within 6 Å of the inhibitor are colored light blue. Genotypes 2a and 2b differ from genotype 1 at sites colored green (78, 79, 80) and dark blue (122, 132) while genotype 3 differs from 1b at amino acids colored red (123, 133, 168) and dark blue (132). Genotype 4 differs from 1 only at dark blue residues (122, 132).

replication capacity could be significantly enhanced by exchanging only the NS3 protease domain while maintaining the native con1 helicase, including T1280I, and NS4A. Therefore, we created a second set of chimeric replicons in which only the con1 protease domain sequence (encoding NS3 amino acids 1–185) was replaced with the corresponding sequences from clinical isolates. When transfected into Huh-7.5 cells, intragenotypic protease domain chimeras replicated very efficiently to levels approaching that of the parent replicon (Fig. 4a). Only one of fifteen intragenotypic protease chimeras (isolate 4) failed to replicate efficiently. Luciferase activities produced by the protease domain chimeras were 49-fold (range 11–115) and 262-fold (range 2–530) higher than the corresponding 1b/1b and 1a/1b NS3/4A chimeras, respectively. Relative fitness levels of the intergenotypic protease chimeras were variable. While genotype 4/1b chimeras replicated as efficiently as the intragenotypic chimeras, none of the six genotype 2b/1b protease chimeras produced signals above background (Fig. 4b). The 2b protease domains were incompatible with the genotype 1b replicon backbone in both mono- (isolates 18, 19, and 20) and dicistronic (isolates 16, 17, and 21) contexts. The two 3a/1b chimeras were viable, although their relative fitness levels were reduced by 99% compared to the parent replicon.

The genetic divergence between isolate 2b and con1 protease domains averaged 27.0% at the amino acid level. We hypothesized that the 2b protease domains would be more compatible in a genotype 2a replicon backbone (14.1% mean divergence). To test this hypothesis, we inserted the protease domain sequence from a single representative 2b isolate (isolate 17) into a genotype 2a JFH-1 reporter replicon and monitored replication capacity. Although relative fitness was reduced 93% compared to the 2a parent replicon, the 2b/2a chimera generated a strong luciferase signal equivalent to the unmodified genotype 1b con1 replicon (Fig. 4b).

Table 3
Genotypic variability in protease inhibitor binding site.

Amino acid	Genotype					
	1a	1b	2a	2b	3a	4
55	V/I[a]	V	V	V	V	V
78	V	V	A	A	V	V
79	D	D	E	E	D	D
80	K/Q[b]	Q	G	G	Q	Q
122	S	S	K	R	S	T
123	R	R	R	R	T	R/K[d]
132	I	V	I	L	L	I
133	S	S	S	S	S/A[c]	S
168	D	D	D	D	Q	D

[a] I in 1 of 10 clones.
[b] Q in 3 of 10 clones.
[c] A in 1 of 2 clones.
[d] K in 1 of 5 clones.

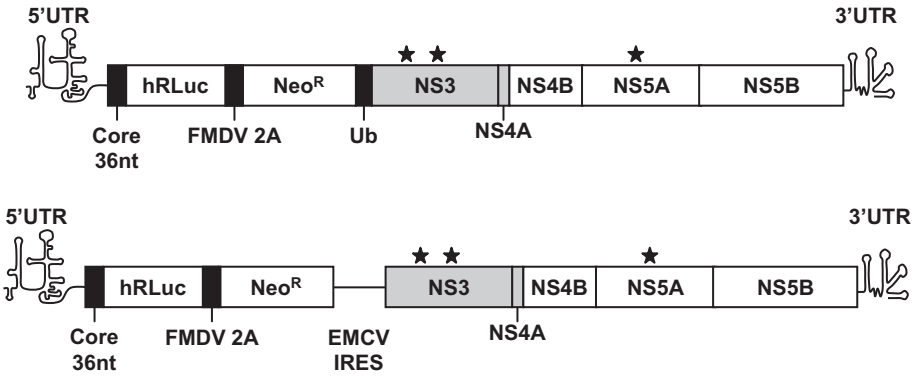

Fig. 3. Genetic organization of reporter selectable subgenomic replicons. Parental genotype 1b con1 replicons are depicted in monocistronic (top) and dicistronic (bottom) formats. Shaded areas indicate native NS3/4A sequences replaced by corresponding clinical isolate genes. Stars identify sites of adaptive mutations (E1202G, T1280I, and S2197P). The genotype 2a JFH-1 replicon mirrors the dicistronic format with a single adaptive mutation (H2476L). UTR, untranslated regions; hRLuc, humanized *Renilla* luciferase gene; Neo^R, neomycin resistance gene; FMDV 2A, Foot-and-Mouth Disease Virus 2A "autoprotease" cassette; Ub, ubiquitin hydrolase cleavage site; EMCV IRES, encephalomyocarditis virus internal ribosome entry site.

3.4. Clinical isolate chimeras exhibit varying susceptibilities to protease inhibitors

As demonstrated in both biochemical and clinical studies, the activity of a protease inhibitor can vary significantly depending on HCV genotype. To determine if the chimeric replicon phenotypic susceptibility assay can assess antiviral spectrum of activity, we characterized the activities of six protease inhibitors (Fig. 5) against the panel of protease domain chimeric replicons. Two protease inhibitors with proven clinical activity, BILN-2061 (ciluprevir) and VX-950 (telaprevir), as well as an NS5B polymerase inhibitor were included as controls. Four preclinical protease inhibitors, tripeptidic compounds varying in their P2 head groups, P3 capping groups, and in the presence or absence of a P1–P3 macrocyclic linkage, were also screened. The antiviral activities of the compounds against the chimeras are summarized in Table 4.

While the polymerase inhibitor was equipotent against all chimeras with a genotype 1b polymerase and backbone, the protease inhibitors exhibited one of two inhibition profiles. VX-950 generally demonstrated broad spectrum activity against the clinical isolates. The compound was at least as active against all 14 replication competent genotype 1 chimeras and the genotype 2a replicon compared to the reference con1 replicon. However, VX-950 was slightly less active against genotypes 2b, 3a, and 4 isolates, exhibiting EC_{50} values 2.5- to 15-fold higher than con1. In contrast, BILN-2061 and the four preclinical compounds demonstrated a more narrow inhibition profile. Although compounds from this class potently inhibited all genotype 4 and most genotype 1 isolates, they did not effectively inhibit genotypes 2a, 2b, and 3a. These compounds were at least 42- to 1200-fold less active against genotypes 2 and 3 compared to con1.

To provide additional confidence that the chimeric replicon system was properly characterizing the spectrum profiles of protease inhibitors, biochemical inhibition assays were developed. One representative sequence for each subtype or genotype was cloned into a baculovirus expression system and the NS3/4A proteins were expressed and purified. The inhibitors were tested against the panel of proteases and inhibition constants were determined. As shown in Table 5, the biochemical results were consistent with the chimeric replicon data. VX-950 exhibited broad spectrum activity against all genotypes, with at most a 5-fold reduction in activity (genotype 3a) compared to the reference 1b protease. In contrast, BILN-2061 and the four preclinical compounds potently inhibited genotypes 1 and 4, but not genotypes 2 and 3. Those compounds were 44- to 960-fold less active against genotypes 2a, 2b, and 3a compared to the 1b strain.

4. Discussion

The high degree of natural genetic variation within HCV, manifested in six major genotypes, over 50 subtypes, millions of isolates, and a nearly unlimited number of quasispecies variants, poses a significant challenge for antiviral chemotherapy. Despite recent progress in HCV culture techniques, researchers still lack the tools to effectively predict how antivirals will perform against naturally occurring isolates. Biochemical enzyme inhibition assays are useful tools, but the assays are highly artificial and labor-intensive to develop. Lab-adapted replicons and infectious systems can also be used to characterize spectrum of activity, but these tools cover only a small fraction of total HCV diversity. Recently, several groups have successfully developed cell-based NS5B chimeric replicons for characterizing polymerase inhibitor spectrum of activity (Herlihy et al., 2008; Shi et al., 2009; Tripathi et al., 2007). In such systems, NS5B genes are cloned from the plasma of HCV-infected individuals and the clinical isolate genes are subsequently inserted into a lab-adapted replicon, replacing the native polymerase gene. The abilities of antivirals to inhibit chimera replication can then be assessed. In this study, we describe our efforts to develop a robust chimeric replicon system for characterizing the activity of NS3 protease inhibitors against genotype 1a, 1b, 2, 3, and 4 isolates.

Unlike the NS5B chimeras, replicons carrying heterologous full-length NS3/4A sequences replicated poorly, if at all. Intrasubtypic (1b/1b) chimeras replicated to limited extents while intragenotypic (1a/1b) and intergenotypic (2b/1b, 3a/1b) chimeras were generally not viable. Although the poor fitness of NS3/4A chimeras may partly be caused by the loss of two adaptive mutations (E1202G in NS3 protease and T1280I in NS3 helicase), the heterologous sequences do not fully compensate for the essential replication functions of the native sequence. While heterologous proteases could theoretically fail to process con1 polyproteins, *in vitro* translation studies suggested that the isolate proteases were functional (data not shown). Alternatively, divergent NS3 helicases may not be able to properly interact with con1 replication proteins, replicon RNA, or essential host factors within the replication complex. To overcome potential obstacles with a heterologous helicase or NS4A, we created a second set of chimeras in which only the protease domain was replaced with clinical isolate sequences. We hypothesized that the NS3 protease domain chimeras would have higher replication capacities, enabling characterization of isolate protease inhibitor susceptibility. Indeed, the protease domain chimeras replicated very efficiently, generally over 100-fold better than the corresponding full-length NS3/4A chimeras. Nearly all intragenotypic

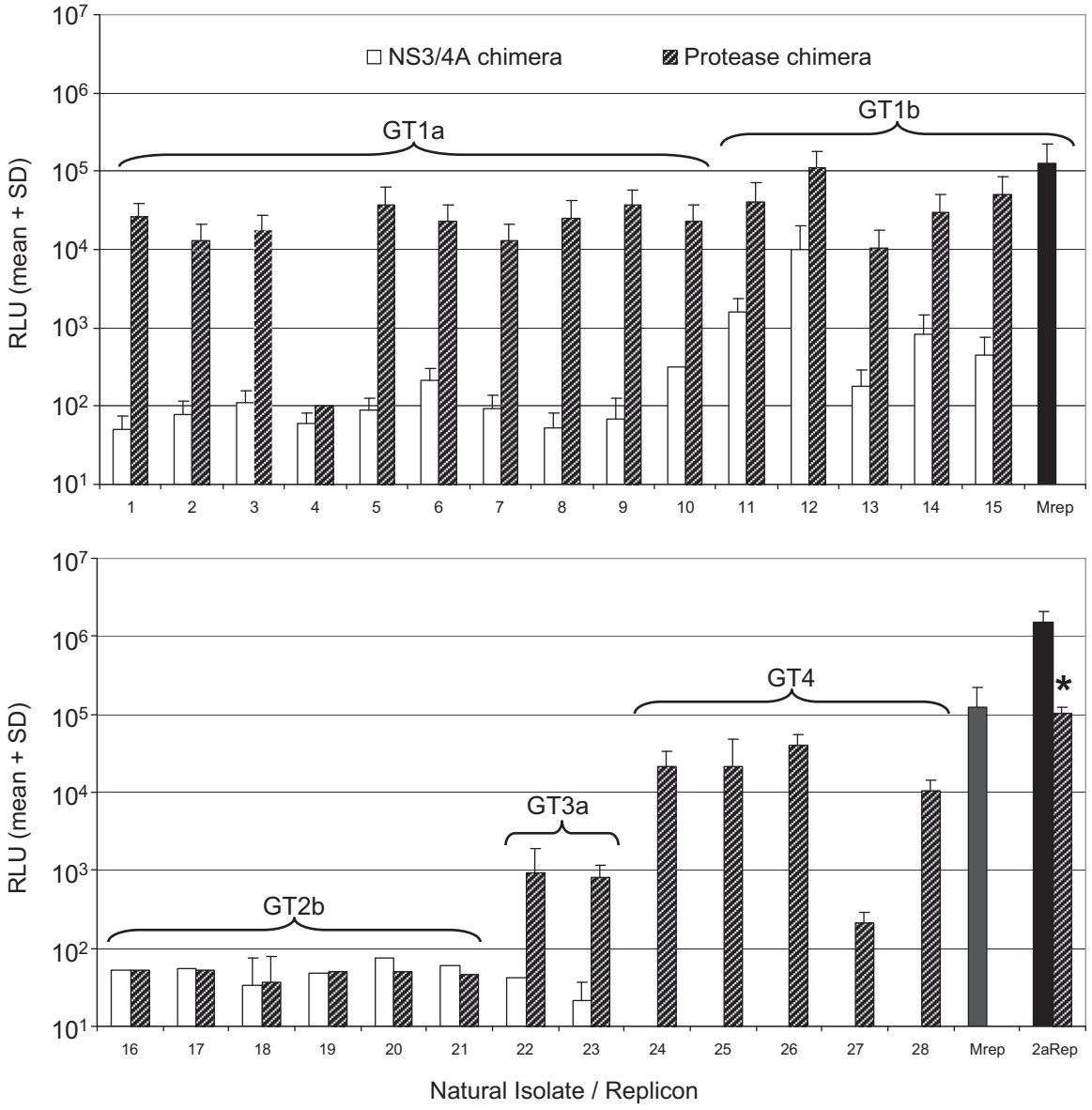

Fig. 4. Chimeric replicon fitness as measured by luciferase activity. Replicon transcripts were transfected into Huh7.5 cells and luciferase activity was measured after 3 days. Plotted values represent the means + standard deviations (SD) of at least three independent experiments. Background levels for nonreplicating controls (polymerase knockout and nonsense mutants) were typically 100 RLU or less (not shown). The clinical isolates from which the heterologous sequences were derived are indicated on the *X*-axes, with genotype 1 isolates shown in Upper and genotypes 2, 3, and 4 isolates in Lower. Chimeras with heterologous NS3/4A genes are represented by clear boxes while those with only heterologous protease domains are represented by hatched boxes. Parental genotype 1b con1 (Mrep) and 2a JFH-1 (2aRep) replicons are represented by black boxes. The 2aRep protease domain chimera (∗) carries the protease domain sequence from genotype 2b isolate 17. RLU, relative light units per well.

(1b/1b, 1a/1b, 2b/2a) chimeras, as well as certain intergenotypic chimeras (4/1b) replicated to levels approaching that of the parent replicon. Despite differing from the native sequence by up to 17% at the amino acid level, the clinical isolate protease domains provided sufficient proteolytic activity to support replication. However, as the divergence between isolate and lab strain protease domains increased, replication was significantly impaired (3a/1b chimeras with ∼23% divergence) or completely abolished (2b/1b chimeras with ∼27% divergence). Not surprisingly, clinical isolate 2b protease domains could support robust replication in a genotype 2a replicon context in which the divergence was only 13%. While additional studies are required to define why the highly divergent protease domains do not support replication, these results suggest that robust NS3 chimeras may be optimally created by inserting isolate genes into the most closely related parent replicon. By following this methodology, researchers may be able to create viable protease domain chimeras for most isolates, including those not explored in this study (genotypes 2a, 5, and 6).

We were able to produce viable protease domain chimeras from the majority of clinical isolates using a "consensus" sequence approach, believing this strategy would eliminate defective quasispecies variants, maximize replication signals, and provide a uniform source of constructs for future phenotypic susceptibility studies. Given the high signals observed, a "population" strategy is also likely to succeed. Such a strategy would entail amplifying isolate protease genes, cloning the amplicon population directly into the replicon without selecting or sequencing individual clones, and creating "population" transcripts for transient transfection. Since the population strategy is unbiased, less labor-intensive, and inclusive of rare, potentially resistant quasispecies variants that may exist within a patient, the population strategy may be better suited for a clinical phenotypic susceptibility assay than the consensus approach.

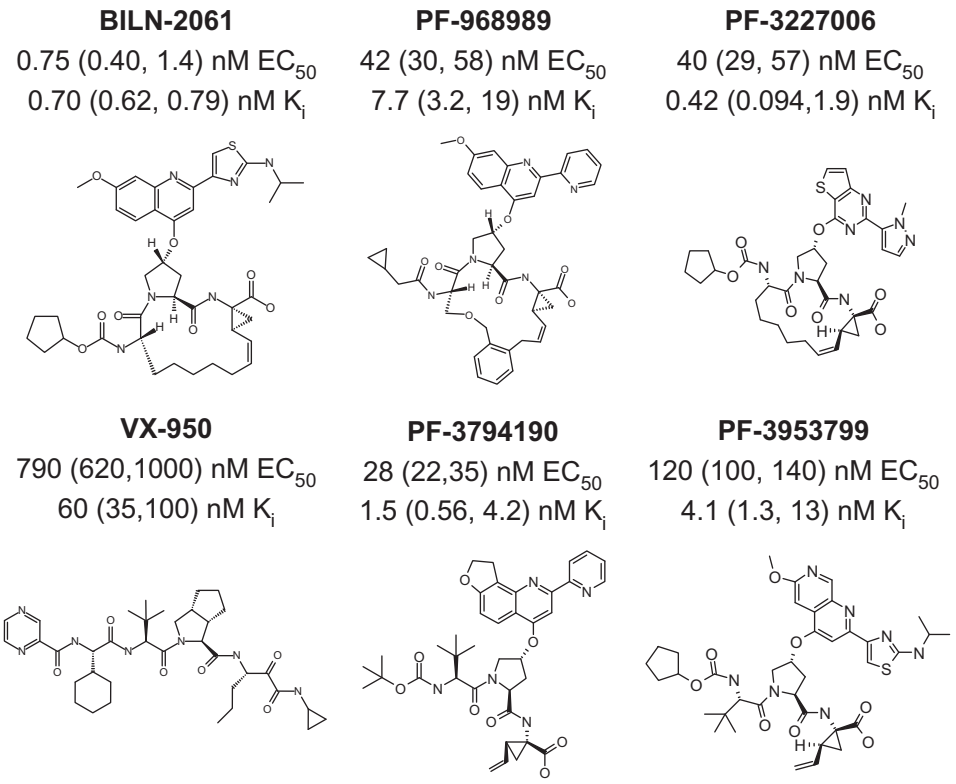

Fig. 5. Chemical structures of the protease inhibitors profiled against the chimeric replicon panel. Activities against the genotype 1b con1 reference strain replicon (50% effective concentration, EC_{50}) and protease (inhibitory constant, K_i) are indicated as geometric means with 95% confidence intervals.

Table 4
Antiviral activities of compounds against NS3 protease domain chimeric replicon panel.

Replicon (isolate No.) [b]	pro GT[c]	Chimera EC_{50}/1b parent replicon EC_{50}[a]						
		Pol 1[d]	VX-950	BILN-2061	PF-3794190	PF-968989	PF-3953799	PF-3227006
1	1a	2.1	1.0	5.0*	6.4*	2.9*	6.3*	7.5*
2	1a	1.8	0.63*	0.98	0.81	0.22*	0.89	1.0
3	1a	1.6	0.76	1.3	0.83	0.38	1.4	1.8*
5	1a	1.6	0.63	1.8	2.8*	0.99	2.7*	3.5*
6	1a	1.7	1.0	4.3*	4.9*	2.0	4.7*	5.3*
7	1a	1.4	0.41*	0.65	0.64	0.25	1.1	0.91
8	1a	1.9	0.71	3.7*	3.8*	1.3	3.8*	4.3*
9	1a	1.9	0.55*	1.9	2.7*	1.5	2.7*	3.2*
10	1a	1.6	0.33*	0.57	1.7	1.1	2.0*	1.5
11	1b	1.3	0.76	1.6	1.3	1.0	1.5*	2.3
12	1b	1.2	1.3*	3.1*	2.2*	2.0	1.6*	2.7*
13	1b	1.2	0.72*	1.1	0.76	0.58	0.96	1.2
14	1b	1.1	0.82	2.2*	1.2	1.4	1.5*	2.8*
15	1b	1.0	0.92	0.81	1.2	0.91	0.85	1.8
JFH-1[e]	2a	190*	1.0	220*	110*	240*	65*	150*
17[e]	2b	35*	3.7*	1200*	850*	>770*	130*	>790*
22	3a	1.0	15*	420*	110*	240*	>270*	650*
23	3a	0.88	2.5*	150*	42*	240*	>270*	400*
24	4	1.5	5.2*	<1	0.51	0.27*	0.56*	1.4
25	4	1.5	3.6*	<1	0.37	0.19*	0.41*	0.99
26	4	1.9	7.3*	<1	0.70	0.64	0.76*	1.3
27	4	1.7	8.6*	<1	1.4	0.14*	0.13*	0.55
28	4	1.6	2.9*	<1	0.31	0.12*	0.28*	0.42*

[a] Fold change in susceptibility (ratio of geometric mean 50% effective doses) for chimeric replicons compared to con1 reference strain replicon. EC_{50} values were determined in a minimum of three independent experiments.
[b] Identity of natural isolate or strain from which the protease domain was derived.
[c] Genotype of protease domain.
[d] NS5B polymerase inhibitor.
[e] Genotype 2a JFH-1 strain replicon backbone.
* Statistically significant difference from con1 replicon (*t*-test, $p < 0.05$).

Table 5
Biochemical activity of compounds against NS3 proteases of various genotypes.

Compound	Mean fold change in activity compared to genotype 1b protease[a]				
	1a	2a	2b	3a	4
VX-950	0.34*	0.64	0.48*	5.2*	1.0
BILN-2061	0.55*	72*	120*	110*	0.35*
PF-3794190	0.58	130*	200*	170*	0.51
PF-968989	1.1	44*	200*	100*	0.47
PF-3953799	1.8	110*	130*	960*	0.81
PF-3227006	1.3	210*	620*	670*	0.38

[a] Ratio of geometric mean inhibitory constants (K_i) for indicated genotype compared to 1b con1 reference protease. K_i values were determined in a minimum of three independent experiments.
* Statistically significant difference from 1b protease (*t*-test, $p < 0.05$).

We developed the protease chimera system as a tool to drive discovery efforts towards broad spectrum inhibitors, as well as to predict activity against natural isolates prior to entering clinical trials. We characterized the system with known protease inhibitors, including two that have reached clinical trials – VX-950 (telaprevir) and BILN-2061 (ciluprevir). In agreement with enzymatic inhibition data reported here and elsewhere (Lin et al., 2007; Reesink et al., 2006; Tong et al., 2006), VX-950 demonstrated broad spectrum activity against the chimeric replicon panel. The compound was widely active against genotype 1 isolates, inhibiting all genotype 1 chimeras at least as effectively as the parent 1b replicon with minimal variability between isolates (⩽4-fold range in EC_{50}). While VX-950 effectively inhibited other genotypes as well, the inhibitor did exhibit statistically significant reductions in activity (2.5- to 15-fold) against genotype 2b, 3a, and 4 isolates. These results suggest that VX-950 may be less efficacious in patients with those genotypes compared to genotype 1. Interestingly, the results of two recent Phase 2a clinical trials suggest this might be the case (Benhamou et al., 2009; Foster et al., 2010). In contrast, BILN-2061 and the four preclinical inhibitors exhibited narrow spectrum profiles. Like VX-950, they were highly active against all genotype 1 chimeras, showing ⩽7.5-fold reductions in activity compared to the parent replicon. The compounds also potently inhibited genotype 4, but they were dramatically less active against genotypes 2 and 3, with EC_{50} values typically over 100-fold greater than the parent. Consistent with these results, BILN-2061 has been reported to have a narrow spectrum of activity in both enzymatic assays (Thibeault et al., 2004; Tong et al., 2006) as well as clinical trials (Hinrichsen et al., 2004; Lamarre et al., 2003; Reiser et al., 2005). The compound proved highly efficacious in patients infected with genotype 1, but not genotypes 2 or 3.

Despite the consistencies between chimeric replicon data and early clinical results, the predictive value of the chimeric replicon system remains to be determined. The protease domain chimeras are artificial constructs consisting of both clinical isolate and lab-adapted sequences. Since NS3 proteolytic activity is likely influenced by elements outside the protease domain, including NS3 helicase, NS4A, and potentially other nonstructural proteins, a natural isolate may conceivably exhibit a different protease inhibitor susceptibility profile than the corresponding isolate/lab strain hybrid. Unfortunately, the natural isolates in this study were not obtained from patients in protease inhibitor trials, and therefore no direct correlation can be drawn between chimera susceptibility and clinical outcome. Ultimately, this type of study will be needed to determine the predictive value of the chimeric replicon system.

The chimeric replicon data illustrate a potency versus spectrum paradox for HCV protease inhibitors. While the inhibitor binding site core is highly conserved, several genotype-specific differences are observed around the periphery. Inhibitors designed to interact with the core and peripheral amino acids can have a high affinity for a specific protease, but they will likely be limited in their spectrum. Inhibitors designed primarily against the conserved core can have a broader spectrum, but lower affinity for any specific protease. For example, BILN-2061 and the four other noncovalent peptidomimetics described here have been optimized for high activity against genotype 1. Large aromatic P2 head groups interact favorably, either directly or indirectly, with genotype 1-specific peripheral amino acids such as valine-78, aspartate-79, glutamine-80, serine-122, and aspartate-168 (via arginine-155). While the head groups contribute to the high activity for genotype 1 (mean EC_{50} = 1.9 nM for BILN-2061 against the genotype 1 chimeric replicons), they also contribute to the reduced affinity for proteases that differ at these key sites (Thibeault et al., 2004; Tong et al., 2006). In contrast, VX-950 has a smaller bicycloproline in the P2 position that interacts less extensively with peripheral amino acids like 78, 79, 80 and 122. Consequently, VX-950 has lower activity against genotype 1 proteases (mean EC_{50} = 610 nM against the genotype 1 chimeric replicons), but the compound's activity is less affected by genotype-specific variations in this region, resulting in a better spectrum of activity.

In the last few years, the promising therapeutic potential of NS3 protease inhibitors has become apparent. As more protease inhibitors move into clinical trials and beyond, the need for a simple, robust, cell-based phenotypic assay has increased. This novel protease chimera shuttle vector system can be utilized to guide discovery efforts in the direction of broad spectrum antivirals. By providing a more realistic picture of the activity of a compound against naturally occurring variants, this system should improve the abilities of researchers to plan dosing regimens needed to achieve viral suppression, thereby increasing the likelihood of clinical success.

Acknowledgements

We thank Dr. Charles M. Rice (Apath, LLC, St. Louis, MO) for providing the genotype 1b con1 subgenomic replicon and Huh-7.5 cell line, Dr. Weidong Hao and Dr. Rohit Duggal for providing the reporter selectable con1 replicons, and Dr. Takaji Wakita (Tokyo Metropolitan Institute for Neuroscience, Tokyo) for providing the genotype 2a JFH-1 subgenomic replicon. We also thank Chi-Hse Teng for assistance with statistical analysis.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., 1999. Current Protocols in Molecular Biology. John Wiley and Sons, Inc., Hoboken, New Jersey.
Benhamou, Y., Moussalli, J., Ratziu, V., Lebray, P., Gysen, V., de Backer, K., Ghys, A., Van Heeswijk, R., Vangeneugden, T., Picchio, G., Beumont-Mauviel, M., 2009. Results of a proof of concept study (C210) of telaprevir monotherapy and in combination with peginterferon alfa-2a and ribavirin in treatment-naive genotype 4 HCV patients. J. Hepatol. 50 (Suppl. 1), 6.
Blight, K.J., McKeating, J.A., Marcotrigiano, J., Rice, C.M., 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77, 3181–3190.
Blight, K.J., Norgard, E.A., 2006. HCV replicon systems. In: Tan, S.L. (Ed.), Hepatitis C viruses: genomes and molecular biology. Horizon Bioscience, Norfolk, UK, pp. 311–351.

Date, T., Kato, T., Miyamoto, M., Zhao, Z., Yasui, K., Mizokami, M., Wakita, T., 2004. Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. J. Biol. Chem. 279, 22371–22376.

Di Bisceglie, A.M., Hoofnagle, J.H., 2002. Optimal therapy of hepatitis C. Hepatology 36, S121–S127.

Forestier, N., Larrey, D.G., Guyader, D., Marcellin, P., Rouzier, R., Patat, A.A., Bradford, W.Z., Porter, S., Zeuzem, S., 2008. Treatment of chronic hepatitis C virus (HCV) genotype 1 patients with the NS3/4A protease inhibitor ITMN-191 leads to rapid reductions in plasma HCV RNA: results of a phase 1b multiple ascending dose (MAD) study. Hepatology 48, 1132A.

Foster, G.R., Hezode, C., Bronowicki, J.P., Carosi, G., Weiland, O., Verlinden, L., van Heeswijk, R., Van Baelen, B., Picchio, G., Beumont-Mauviel, M., 2010. Activity of telaprevir alone or in combination with peginterferon alfa-2a and ribavirin in treatment-naive genotype 2 and 3 hepatitis-C patients: final results of study C209. 45th Annual Meeting of the European Association for the Study of the Liver, Vienna, Austria.

Hao, W., Herlihy, K.J., Zhang, N.J., Fuhrman, S.A., Doan, C., Patick, A.K., Duggal, R., 2007a. Development of a novel dicistronic reporter-selectable hepatitis C virus replicon suitable for high-throughput inhibitor screening. Antimicrob. Agents Chemother. 51, 95–102.

Hao, W., Weady, P., Maldonado, F., Patick, A., Duggal, R., 2007b. Discovery of two novel classes of inhibitors of hepatitis C virus (HCV) replication utilizing a dicistronic reporter HCV replicon high throughput assay. Antiviral Res. 74, A58.

Herlihy, K.J., Graham, J.P., Kumpf, R., Patick, A.K., Duggal, R., Shi, S.T., 2008. Development of intergenotypic chimeric replicons to determine the broad-spectrum antiviral activities of hepatitis C virus polymerase inhibitors. Antimicrob. Agents Chemother. 52, 3523–3531.

Hinrichsen, H., Benhamou, Y., Wedemeyer, H., Reiser, M., Sentjens, R.E., Calleja, J.L., Forns, X., Erhardt, A., Cronlein, J., Chaves, R.L., Yong, C.L., Nehmiz, G., Steinmann, G.G., 2004. Short-term antiviral efficacy of BILN 2061, a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. Gastroenterology 127, 1347–1355.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125, 1808–1817.

Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R.K., Murthy, K., Wakita, T., Liang, T.J., 2007. Production of infectious hepatitis C virus of various genotypes in cell cultures. J. Virol. 81, 4405–4411.

Lamarre, D., Anderson, P.C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D.R., Cartier, M., Cordingley, M.G., Faucher, A.M., Goudreau, N., Kawai, S.H., Kukolj, G., Lagace, L., LaPlante, S.R., Narjes, H., Poupart, M.A., Rancourt, J., Sentjens, R.E., St George, R., Simoneau, B., Steinmann, G., Thibeault, D., Tsantrizos, Y.S., Weldon, S.M., Yong, C.L., Llinas-Brunet, M., 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature 426, 186–189.

Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale Jr., M., Lemon, S.M., 2005a. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc. Natl. Acad. Sci. USA 102, 2992–2997.

Li, X.D., Sun, L., Seth, R.B., Pineda, G., Chen, Z.J., 2005b. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc. Natl. Acad. Sci. USA 102, 17717–17722.

Lin, C., Hanzelka, B.L., Muh, U., Kovari, L., Bartels, D.J., Tigges, A.M., Miller, J., Rao, B.G., Kwong, A.D., 2007. Telaprevir (VX-950) is a potent inhibitor of HCV-NS3 proteases derived from genotype non-1 HCV-infected patients, 42nd Annual Meeting of the European Association for the Study of the Liver (EASL). Barcelona, Spain.

Lindenbach, B., Rice, C., 2001. Flaviviridae: the viruses and their replication. Fields Virology, pp. 991–1041.

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.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M., Albrecht, J.K., 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358, 958–965.

Martell, M., Esteban, J.I., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J., Gomez, J., 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J. Virol. 66, 3225–3229.

Pawlotsky, J.M., 2003. Hepatitis C virus genetic variability: pathogenic and clinical implications. Clin. Liver Dis. 7, 45–66.

Reesink, H.W., Zeuzem, S., Weegink, C.J., Forestier, N., van Vliet, A., de Rooij, J., McNair, L., Purdy, S., Kauffman, R., Alam, J., Jansen, P.L., 2006. Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. Gastroenterology 131, 997–1002.

Reiser, M., Hinrichsen, H., Benhamou, Y., Reesink, H.W., Wedemeyer, H., Avendano, C., Riba, N., Yong, C.L., Nehmiz, G., Steinmann, G.G., 2005. Antiviral efficacy of NS3-serine protease inhibitor BILN-2061 in patients with chronic genotype 2 and 3 hepatitis C. Hepatology 41, 832–835.

Robertson, B., Myers, G., Howard, C., Brettin, T., Bukh, J., Gaschen, B., Gojobori, T., Maertens, G., Mizokami, M., Nainan, O., Netesov, S., Nishioka, K., Shini, T., Simmonds, P., Smith, D., Stuyver, L., Weiner, A., 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch. Virol. 143, 2493–2503.

Sarrazin, C., Rouzier, R., Wagner, F., Forestier, N., Larrey, D., Gupta, S.K., Hussain, M., Shah, A., Cutler, D., Zhang, J., Zeuzem, S., 2007. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. Gastroenterology 132, 1270–1278.

Shi, S.T., Herlihy, K.J., Graham, J.P., Nonomiya, J., Rahavendran, S.V., Skor, H., Irvine, R., Binford, S., Tatlock, J., Li, H., Gonzalez, J., Linton, A., Patick, A.K., Lewis, C., 2009. Preclinical characterization of PF-00868554, a potent nonnucleoside inhibitor of the hepatitis C virus RNA-dependent RNA polymerase. Antimicrob. Agents Chemother. 53, 2544–2552.

Simmonds, P., 2004. Genetic diversity and evolution of hepatitis C virus – 15 years on. J. Gen. Virol. 85, 3173–3188.

Taliani, M., Bianchi, E., Narjes, F., Fossatelli, M., Urbani, A., Steinkuhler, C., De Francesco, R., Pessi, A., 1996. A continuous assay of hepatitis C virus protease based on resonance energy transfer depsipeptide substrates. Anal. Biochem. 240, 60–67.

Thibeault, D., Bousquet, C., Gingras, R., Lagace, L., Maurice, R., White, P.W., Lamarre, D., 2004. Sensitivity of NS3 serine proteases from hepatitis C virus genotypes 2 and 3 to the inhibitor BILN 2061. J. Virol. 78, 7352–7359.

Tong, X., Guo, Z., Wright-Minogue, J., Xia, E., Prongay, A., Madison, V., Qiu, P., Venkatraman, S., Velazquez, F., Njoroge, F.G., Malcolm, B.A., 2006. Impact of naturally occurring variants of HCV protease on the binding of different classes of protease inhibitors. Biochemistry 45, 1353–1361.

Tripathi, R.L., Krishnan, P., He, Y., Middleton, T., Pilot-Matias, T., Chen, C.M., Lau, D.T., Lemon, S.M., Mo, H., Kati, W., Molla, A., 2007. Replication efficiency of chimeric replicon containing NS5A-5B genes derived from HCV-infected patient sera. Antiviral Res. 73, 40–49.