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Development of a replicon-based phenotypic assay for assessing the drug susceptibilities of HCV NS3 protease genes from clinical isolates

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ABSTRACT

Hepatitis C virus (HCV) protease inhibitors targeting HCV NS3 can efficiently suppress HCV replication. However, the selection of resistance has been observed both in vitro and in vivo. Here, we describe a new method for efficient analysis of the drug susceptibility of the NS3 protease genes from patient isolates. Luciferase-reporter 1b replicon shuttle vectors that allow cloning of either the HCV full-length NS3/4A gene or the NS3 protease domain gene only were created. Initially, chimeric replicons carrying patient-derived full-length NS3/4A failed to replicate in cell culture. However, the poor replication efficiency of the NS3/4A shuttle vector was enhanced by approximately 100-fold when the NS3 helicase domains of clinical isolates were substituted for that of the 1b Con1 lab strain. Chimeric replicons carrying only the patient-derived NS3 protease domains replicated at levels sufficient for phenotypic analysis in 20/20 clinical isolates. EC_{50} values for the NS3 inhibitor BILN-2061 ranged from 0.2 to 1.1 nM for 20 genotype 1 patient isolates. Significantly reduced susceptibility to BILN-2061 was observed with mutant/wild type mixtures of 5%/95% for the D168V or 50%/50% for A156T resistance mutations in the NS3. These shuttle vectors can be used to evaluate candidate drugs and assist in the development of new drugs targeting the NS3 protease.

1. Introduction

Hepatitis C virus (HCV) infection is estimated to affect 170 million individuals worldwide (Wasley and Alter, 2000) and is a leading cause of chronic liver diseases, including chronic hepatitis, liver fibrosis, cirrhosis and hepatocellular carcinoma (Hoofnagle, 2002). The limited efficacy and dose limiting side effects of pegylated interferon-alpha in combination with ribavirin (Fried, 2002; Hoofnagle et al., 2003; Kenny-Walsh, 2001) have stimulated the development of more efficacious and tolerable agents that specifically target viral genes, such as HCV NS3 protease and NS5B polymerase. Several small molecule inhibitors that specifically block NS3/4A protease-dependent HCV polyproprotein processing have been identified and shown in clinical trials to produce a rapid and profound decrease in viremia in genotype 1 infected patients. These include the non-covalent inhibitor BILN-2061 (Hinrichsen et al., 2004; Lamarre et al., 2003) and the covalent inhibitors, SCH-503034 (Sarrazin et al., 2007b) and VX-950 (Reesink et al., 2006).

However, mutants resistant to NS3 protease inhibitors have been shown to emerge rapidly both in vitro and in vivo. To date, NS3 mutations have been identified that confer resistance to BILN-2061 (R155Q, A156V/T and D168V/A) (Lin et al., 2004; Lu et al., 2004), VX-950 (V36M/A, T54A, R155K/T, and A156V/T) (Lin et al., 2004, 2005), SCH-503034 (T54A, A156S/T and V170A) (Tong et al., 2006) and ITMN-191 (D168A/V/E and A156S/V) (Seiwert et al., 2006) in vitro. Recent clinical studies have confirmed the selection of resistance mutations against VX-950 at positions 36, 54, 155 and 156 and against SCH-503034 at position 54 (Sarrazin et al., 2007a,b). Among these positions, amino acid changes at position 156 conferred high-level resistance to all HCV protease inhibitors in development. Multiple amino acid changes at position 168 confer high-levels of resistance to BILN-2061 and ITMN-191, but remained sensitive to VX-950.

Drug-resistant mutants are selected from minor pre-existing species that are generated due to the error-prone nature of HCV RNA polymerase as well as the high replication rate of HCV. Under drug pressure, drug-resistant mutants have a fitness advantage over the wild type virus and emerge as the dominant viral species. The frequency of drug-resistant mutants in the viral population, whether at low-level at baseline or enriched from drug treatment, varies from patient to patient and plays a critical role in determining the susceptibility and the virologic response of patients to antiviral therapies. It is therefore important to assess the activity spectrum of drug candidates against a diverse panel of HCV variants and

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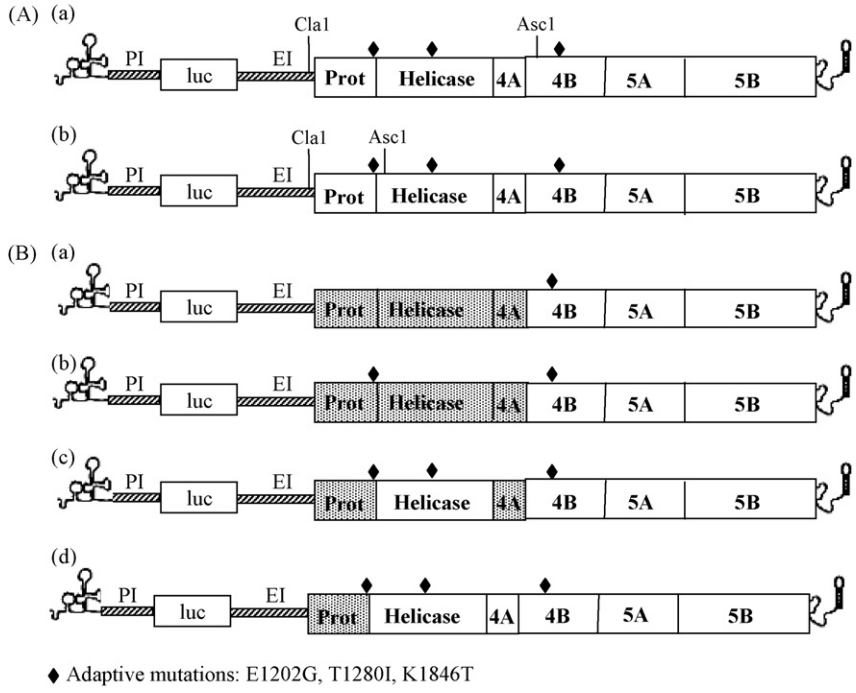

Fig. 1. Organization of the 1b parental replicon construct used for generating the shuttle vector. (A) Schematic drawing of full-length NS3/4A shuttle vector (a) or protease domain gene shuttle vector (b) with cloning sites and adaptive mutations indicated (black diamond). (B) Schematic drawing of shuttle vector containing patient-derived genes (dotted area). (a) Full-length NS3/4A; (b) full-length NS3/4A with E1202G; (c) protease domain with E1202G and NS4A; (d) protease domain alone with E1202G.

to monitor the rise of the drug-resistant mutants during the drug treatment that often lead to viral breakthrough and consequent loss of clinical benefits.

The HCV replicon system has been a useful tool to study the potency and mechanism of HCV inhibitors in a cell-based format. However, current subgenomic or full-length genomic replicons are established with laboratory-optimized strains that limit the assessment of antiviral agents to a few reference HCV sequences (e.g. genotype 1b Con1 or genotype 1a-H77). The limited number of replication competent reference sequences therefore limits the assessment of how genetic variation within viral population of an HCV-infected patient may affect response to an antiviral therapy. Several reports have demonstrated the successful use of shuttle vector approach to address this question for the NS5B gene encoding the RdRp in which the NS5B gene was isolated from the sera of HCV-infected patients and shuttled to a replicon vector deficient in RdRp activity to restore the RNA replication (Ludmerer et al., 2005; Middleton et al., 2007; Tripathi et al., 2007). When the replicons containing patient-derived NS5B from a panel of clinical isolates were tested against a polymerase inhibitor, a wide range of potency was observed for the inhibitor indicating this approach is capable of evaluating the phenotype of a mixed quasispecies pool. In this report, we explored the development of full-length NS3/4A and NS3 protease domain shuttle vectors for efficient analysis of drug susceptibilities of patient HCV isolates.

2. Material and methods

2.1. Plasmids

The genotype 1b Con1 subgenomic replicon construct used to create the shuttle vector has been described by Friebe et al. (2001). The components of the plasmid are depicted in Fig. 1. A poliovirus IRES element was added at the 5′-end after the HCV 5′UTR to increase firefly luciferase translation and RNA replication. Translation of HCV replicon from NS3 to NS5B is driven by EMCV IRES. Three adaptive mutations, two in NS3 (E1202G + T1280I) and one in NS4B (K1846T) were introduced for efficient replication (Lohmann et al., 2003).

Two unique restriction sites Cla1 and Asc1 were created for cloning the NS3/4A gene cassette. A Cla1 site was introduced at the linker region between EMCV IRES and the 5′-end of NS3 gene. An Asc1 site was created in NS4B gene, 43 amino acids downstream of the start of NS4B. This results in an amino acid substitution at position 1756 from threonine to alanine. All of the restriction sites were generated by site directed mutagenesis using QuikChange mutagenesis kit (Stratagene, La Jolla, CA). To prevent contamination of recombinant shuttle vector with the parental NS3/4A gene, a fragment of 1.5 kb length between two Sph1 sites within the NS3 gene was deleted to create a replication defective parental vector.

For shuttling the NS3 protease domain, the same Cla1 created for NS3/4A cloning was used for 5′-end cloning. A unique Asc1 site was introduced seven amino acids downstream of the end of protease domain, resulting in a two amino acids change at positions 1214 and 1215 from serine to glycine and serine to alanine, respectively. To generate the replication defective parental vector, another Asc1 was introduced at position 1071. The fragment between the two Asc1 sites (1071–1214) was then excised to create a deletion within the protease domain.

2.2. Amplification of NS3/4A gene from patient sera

The Qiagen RNA extraction kit (Qiagen Inc., Valencia, CA) was used to isolate the HCV RNA from 140 μL of plasma as described in the user's manual except the RNA is eluted in 50 μL at the final step. To synthesize cDNA, we use 0.125 μM of genotype specific primers (1a: 1a4a3′5735 or 1b: 1b4a3′5650, Table 1) and 10 μl of extracted RNA in a 20 μl reaction using MonsterScript reverse transcriptase as recommended by manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Reverse transcription was run on MJ Research PTC-100 thermal cycler using the following program: 1a: 50 °C or 1b: 54 °C for 10 min, 60 °C for 40 min, 90 °C for 2 min.

Table 1
Sequence of primers used for cDNA synthesis and gene amplification.

Primer	Sequence
1a4a3′5735	5′-TTGGCTAGTGGTTAGTGGGCTGG-3′
1b4a3′5650	5′-GTGGACAAGCCTGCTAAGTACTGTATCCCGC-3′
1a3-5′3181	5′-ATCAAGTTAGGGGCGCTTACTGGCAC-3′
1b3-5′3150	5′-GTCGCTGGGGGTCATTATGTCCAAATGG-3′
1b PCR NS3–4A F2	5′-ATTAGTCAATCGATACCATGGCGCCYATCACGGCCTACTC CCAACAGACGCG-3′
1b PCR NS3–4A R2	5′-CCCAGAAGGYCTCRAGGGCGCGCCACTTGGAYTCCACCA CGGGAGC-3′
1b PCR Prot R2 (E1202G)	5′-ATATGCTCAGGCGCGCCGTTGTCYGTGAAGACCGGRGAC CGCATRGTRGTTCCCAT-3′
1a PCR NS3–4A F2	5′-ATTAGTCAATCGATACCATGGCGCCCATCACGGCGTACGC CCAGCAGAC-3′
1a PCR NS3–4A R2	5′-TTCGCCCARAAGRCCTCGAGGGCGCGCCAGTTGGTCTGG ACAGCAGG-3′
1a PCR Prot R2 (E1202G)	5′-ATATGCTCAGGCGCGCCGTTGTCCGTGAACACCGGGGAC CTCATGGTTGTCCCTAGG-3′
1b PCR E1202G F	5′-CCCGTCGAGTCTATGGGAACCACTATGCGGTCCCCGGTC TTCAC-3′
1b PCR E1202G R	5′-GTGAAGACCGGGGACCGCATAGTGGTTCCCATAGACTCG ACGGG-3′
1b PCR helicase F	5′-CGGCTGACCTGGAGGTCGTCACGAGCACCTGGGTGCTGG TAGGCGG-3′
1b PCR helicase R	5′-CCGCCTACCAGCACCCAGGTGCTCGTGACGACCTCCAGG TCAGCCG-3′
1a PCR E1202G F	5′-CCTGTGGAGAACCTAGGGACAACCATGAGATCCCCGGTG TTCAC-3′
1a PCR E1202G R	5′-GTGAACACCGGGGATCTCATGGTTGTCCCTAGGTTCTCCA CAGG-3′
1a PCR helicase F	5′-GTGAACACCGGGGATCTCATGGTTGTCCCTAGGTTCTCCA CAGG-3′
1a PCR helicase R	5′-CCGCCAACGAGCACCCAGGTGCTCGTGACGACCTCCAGG TCGGCCG-3′

PCR was performed using 10 μl of cDNA with genotype-specific primers to amplify the full-length NS3/4A gene using the following primers in a 50 μl reaction; 1a:1a3-5′3181 and 1a4a-3′5735 or 1b: 1b3-5′3150 and 1b4a-3′5650 using platinum *Taq* DNA polymerase (Invitrogen) as recommended by manufacturer. PCR temperature cycles were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 1a: 60 °C or 1b: 65 °C for 30 s, 72 °C for 3 min, 72 °C for 7 min.

The first PCR products were used as template in the following nested PCR reactions to generate gene cassettes with cloning sites incorporated at both ends. Nested PCR was performed with High Fidelity PCR master kit (Roche Applied Science, Indianapolis, IN) as directed by the manufacturer. The NS3/4A gene cassette was generated using primer PCR NS3/4A F2 and PCR NS3/4A R2. To introduce the adaptive mutation E1202G into the NS3/4A gene cassette, mega-primer PCR was applied to first generate two separate PCR products with either primer pair PCR NS3/4A F2/PCR E1202G R or PCR E1202G F/PCR NS3/4A R2. The two PCR products were subsequently combined along with the outside primers PCR NS3/4A F2 and PCR NS3/4A R2 to generate the final NS3/4A cassette with E1202G in it. The chimeric NS3/4A gene cassette with helicase from 1b Con1 was generated by overlapping PCR strategy. Three rounds of separate PCR were performed to generate three fragments: 5′-end of NS3 to the end of protease domain with primer PCR NS3/4A F2/PCR E1202G R, helicase from Con1 using 1b Pi-luc plasmid as template with primer PCR E1202G F/PCR helicase R, and end of helicase to 3′-end of NS4A with primer PCR helicase F/PCR NS3/4A R2. The three fragments were then combined to generate the final NS3/4A gene cassette using the outside primers PCR NS3/4A F2/PCR NS3/4A R2. The protease domain cassette was generated using primer pair PCR NS3/4A F2 and PCR Prot R2 (E1202G) with the adaptive mutation E1202G incorporated in the reverse primer.

2.3. Transfer of gene cassette to shuttle vector and RNA synthesis

The nested PCR products of the NS3/4A gene or protease domain were treated with Cla1 and Asc1 (New England Biolabs) at 37 °C for 3 h and then cleaned up by MinElute Reaction cleanup kit (Qiagen). The shuttle vector DNA was similarly digested and the fragment of interest was isolated by gel electrophoresis and removed from the gel matrix with QIAEX II gel extraction kit (Qiagen). The vector was subsequently treated with shrimp alkaline phosphatase (Roche). Ligations were performed at 16 °C overnight. The ligation products were then precipitated with Pellet Paint (Novagen, Gibbstown, NJ) to remove the buffer and ligase and subsequently resuspended in H_2O. Transformation of the ligation reaction was done by electroporation into ElectroTen-Blue cells (Strategene) according to supplier's recommendations. Ten percent of the transformation mixture was plated on antibiotic selection plates to determine the transformation efficiency and the remaining transformants were expanded in liquid culture to propagate the quasispecies pool. The plasmid DNA was extracted and linearized by digestion with Sca1 at 37 °C overnight. RNA was synthesized using a T7 Megascript RNA synthesis kit (Invitrogen-Ambion) following manufacturer's instruction.

2.4. Transient replication assay and luciferase reading

Huh-7 cells used in the transient transfection assay was derived from a cured replicon cell clone (Friebe et al., 2005), designated Huh-7/Lunet. Lunet cells were grown in Dulbecco's modified Eagle medium (GIBCO, Carlsbad, CA) supplemented with 10% of fetal bovine serum (Hyclone, Logan, UT), 100 U/ml of penicillin, 100 μg/ml of streptomycin and non-essential amino acids. Cells were harvested by trypsinization and washed with cold PBS twice before electroporation. Cell density was adjusted to 1×10^7 cell/ml, and 0.4 ml of cells were transferred to a cold cuvette with a gap width of 0.4 cm (Bio-Rad, Laboratories, Hercules, CA) along with 5–10 μg of RNA. After one pulse at 960 μF and 270 V with a Gene Pulser II (Bio-Rad Laboratories), cells were transferred to 20 ml of complete medium. Cell suspension was seeded in a 96-well plate with a clear bottom (Costar) at 100 μl/well and allowed to attach overnight. An aliquot of 1 ml cell suspension was taken 4 h post-transfection and luciferase activity was measured to normalize the transfection efficiency. Cells were harvested 4 days after transfection to measure the luciferase activity. For EC_{50} determination, serially diluted compounds in DMSO were added to the plate the day after transfection (0.5% final DMSO concentration) and cells were harvested 3 days after adding the compounds. To measure the luciferase activity, medium was removed and plates were washed with PBS once followed by adding 50 μl of lysis buffer (Promega, Madison, WI). Lysis of the cells was completed in 20 min with rocking, then 50 μl of luciferase substrate (Promega) was added to the well. Luciferase was measured with a Victor Luminometer (PerkinElmer, Waltham, MA). For all experiments, EC_{50} values of test compounds were calculated by nonlinear regression using the GraphPad Prism program (GraphPad Software Inc., La Jolla, CA). Signal to noise window was determined as the ratio of luciferase activity from cells treated with 0.5% DMSO versus activity from cells treated with 500 nM of BILN-2061 in 0.5% DMSO.

3. Results

3.1. Design and construction of the shuttle vectors

A high level of replication of the parental replicon vector to be used for the shuttle vector is required for assessing the drug susceptibility of patient isolates as the chimeric replicons carrying the heterogeneous genes from clinical isolates will likely have reduced RNA replication. The plasmid used for the construction of the shuttle vector, designated as 1b Pi-luc, includes three adaptive mutations (E1202G, T1280I, and K1846T) and a poliovirus IRES element that has been shown to enhance both translation of the luciferase gene and RNA replication (Friebe et al., 2001). This replicon plasmid gives rise to a strong luciferase signal in a transient transfection assay with a signal to noise ratio of 500–2000 in a 96-well plate and is therefore suitable for the purposes of this study. Two unique restriction sites, a Cla1 for 5′ cloning and an Asc1 for 3′ cloning were created for the insertion of the NS3/4A genes (Fig. 1A-a). Addition of the Asc1 site at 43 amino acids downstream of the start of NS4B gene resulted in a T1756A substitution. The replicon containing the modified restriction sites replicated at 45% of the wild type 1b Pi-luc level (Fig. 2A). Nevertheless, a robust signal to noise window of ~900-fold was retained for the shuttle vector (data not shown). With such a high signal, a dose–response curve can be generated even if the replicons containing patient-derived genes only replicate at 1% of the lab stain efficiency. When the NS3/4A gene from the 1b Con1 strain was amplified and shuttled to the replication defective parental vector that has the 1.5 kb deletion in the NS3 gene, replication efficiency was restored to 80% of the original shuttle vector level suggesting that the cloning approach taken here enables restoration of the majority of the replication efficiency from a completely inactive parental replicon (Fig. 2A).

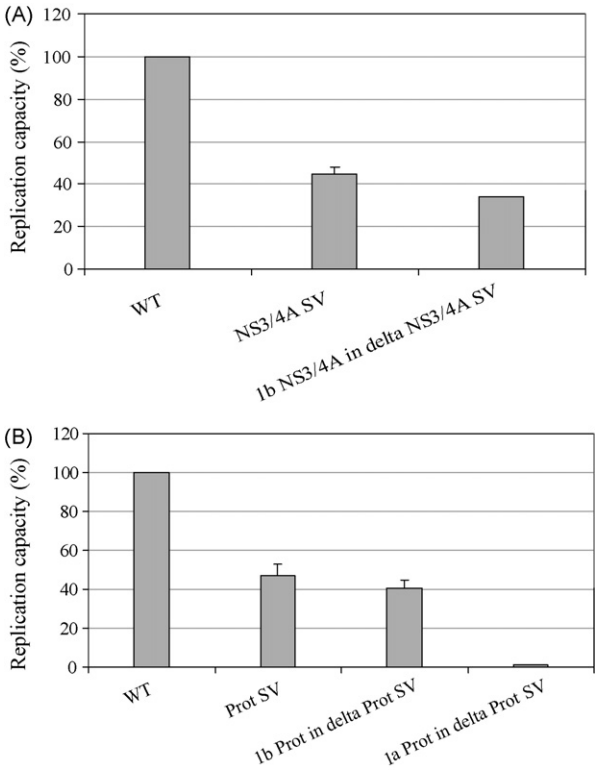

Fig. 2. Replication capacity of (A) full-length NS3/4A shuttle vector (SV) and (B) protease domain gene shuttle vector compared to wild type 1b Pi-luc and restoration of the replication capacity of the defective delta SV with 1b Con1 full-length NS3/4A or protease domain gene shuttled in. A signal to noise ratio of 500–2000-fold was achieved for the wild type 1b Pi-luc construct in a 96-well plate.

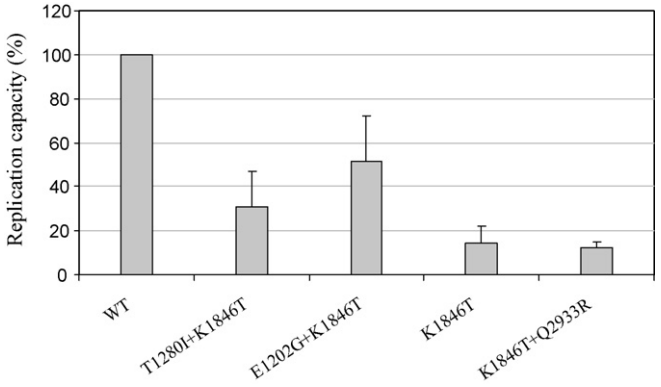

Fig. 3. Impact of the adaptive mutations on the replication capacity of 1b Pi-luc.

For the NS3 protease domain shuttle vector, an Asc1 site was inserted seven amino acids downstream of the junction between the protease and helicase domain to enable 3′-end cloning (Fig. 1A-b). The 5′-end cloning site uses the same Cla1 site generated for NS3/4A gene shuffling. Adding the Asc1 site into the helicase domain caused two amino acids change, S1214G and S1215A, respectively. These modifications adversely affected the replication efficiency by approximately 50% (Fig. 2B). To create a replication defective parental construct, another Asc1 was generated 45 amino acids downstream of the NS3 start codon to take out a 0.4 kb fragment between the two Asc1 sites. When the protease domain from the 1b Con was put back to the parental vector, up to 87% of the replication capacity of the wild type was rescued, consistent with the results observed for the NS3/4A shuttle vector. However, when the protease gene from genotype 1a-H77 was transferred to the shuttle vector, the replication level of this chimeric replicon was reduced to 1.4% of the wild type 1b Pi-luc (Fig. 2B).

3.2. Replication of the full-length NS3/4A gene cassette

The initial strategy for phenotyping the patient samples to HCV NS3 protease inhibitors was to clone the full-length NS3/4A gene from the sera of HCV-infected patients to the shuttle vector and determine the drug susceptibility since NS4A is a cofactor of NS3 and an indispensable part of the NS3 protease function (Bartenschlager et al., 1995; Failla et al., 1995; Tanji et al., 1995). One question arising from this strategy is how the loss of two adaptive mutations, E1202G and T1208I located in the NS3 gene, would impact the replication efficiency when the NS3/4A gene of the lab strain replicon is replaced by that of the clinical samples. It has been reported that the three adaptive mutations, E1202G, T1280I and K1846T present in the 1b Pi-luc can enhance the transient HCV replication by more than 200-fold compared to the replicon with no adaptive mutations while the K1846T mutation in NS4B alone can enhance the replication by 34-fold (Lohmann et al., 2003). To address this question, the two adaptive mutations E1202G and T1208I in the Pi-luc were reverted to the wild type residue glutamic acid and threonine, individually or in combination, and their replication efficiency were assessed (Fig. 3). Loss of a single adaptive mutation at position 1202 or 1280 led to a decrease of replication efficiency by ~70% and ~50%, respectively. This result suggested that a replicon with E1202G + K1846T is more adaptive than a replicon with T1280I + K1846T. Loss of both the E1202G and T1280I adaptive mutations decreased the replication by 86%. This observation is in agreement with the value reported by Lohmann et al. (2003) that replicon containing K1846T alone is 16% replication competent with respect to the one with K1846T in addition to E1202G and T1280I. Nevertheless, the signal to noise ratio for the replicon with the single K1846T adaptive mutation retained

Table 2
Replication efficiency and signal to noise (S/N) ratio of NS3–4A genes isolated from the sera of five genotype 1 patients.

Patient ID	Genotype	Replication efficiency	S/N ratio
1b Con1	1b	100	1747.5 ± 487
#1	1b	0.51 ± 0.3	8.1 ± 2.7
#2	1b	0.16 ± 0.07	2.7 ± 0.5
#3	1a	0.15 ± 0.02	2.5 ± 0.3
#4	1a	0.08 ± 0.004	1.3 ± 0.4
#5	1a	0.07 ± 0.02	1.4 ± 0.7

Table 3
Replication efficiency of patient-derived NS3/4A genes with adaptive mutation E1202G or the NS3/4A chimeric gene cassette with helicase domain from 1b Con1 strain.

Patient	Replication efficiency of NS3/4A (%)[a]		
	−E1202G	+E1202G	+E1202G and Con1 helicase
#1	0.2 ± 0.15	0.18 ± 0.14	19.0 ± 15.1
#2	0.13 ± 0.06	0.52 ± 0.47	13.2 ± 9.8

[a] % normalized to the replication efficiency of the wild type NS3/4A shuttle vector.

at least 50-fold which would be sufficient for drug susceptibility testing if shuttling the NS3/4A from patient isolates does not further reduce the replication capacity. With this in mind, the feasibility of shuttling the whole NS3/4A gene cassette was explored. The full-length NS3/4A gene was amplified from the sera of five HCV-infected patients among which two were genotype 1b and three were genotype 1a by two rounds of PCR. The first round used primers annealing to a relatively conserved region of the specific genotypes and the second round was a nested PCR to incorporate the restriction sites to the PCR products for insertion into the shuttle vector. The replicons containing the NS3/4A gene from either the 1b or 1a infected patients in the Con1 background (Fig. 1B-a) were subsequently transfected into Lunet cells, a replicon-cured Huh7 cell line, and luciferase was measured at 4 h and 4 days post-transfection. The day 4 reading was normalized to the 4 h reading for transfection efficiency. The replication efficiency was defined as the ratio of the day 4 luciferase value of the patient replicons compared to the lab strain, normalized to the 4 h luciferase values of each. As shown in Table 2, all five replicons with NS3/4A isolated from the genotype 1 patients replicated very poorly with an efficiency of less than 1% of the wild type lab strain. With these constructs, signal to noise ratio was too low to test for the inhibitory effects of HCV inhibitors.

3.3. Rescue of replication efficiency of the shuttle vector containing NS3/4A derived from HCV-infected patients

Alternative strategies were sought to boost the replication of the shuttle vector containing full-length patient-derived NS3/4A gene since its signal from the transient assay was too low for phenotypic analysis. One solution was to identify adaptive mutations in the NS4B to 5B region that are capable of restoring the replication efficiency comparable to wild type 1b. Although several highly adaptive mutations have been mapped to the NS4B–5B region, few single substitutions have been shown to be more effective than the K1846T in NS4B and when combined together they have been found to be incompatible with each other and have an antagonistic effect on replication in general (Blight et al., 2003; Krieger et al., 2001; Lohmann et al., 2003). Lohmann et al. reported a Q2933R conserved mutation in NS5B that was observed in conjunction with adaptive mutations in NS4B in 2 of the 26 replicon clones they sequenced (Lohmann et al., 2003), suggesting it might be synergistic with the K1846T. Based on this observation, Q2933R was introduced into the shuttle vector and tested for RNA replication. Fig. 3 shows adding Q2933R on top of the K1846T failed to enhance the replication of the shuttle vector. In light of this result, we resorted to introducing the adaptive mutation E1202G to the patient-derived NS3/4A gene by a mega-primer PCR strategy as we have demonstrated that replicons with E1202G + K1846T replicate more efficiently than replicon with T1280I + K1846T (to approximately 50% of the wild type Pi-luc level, Fig. 3). The final construct is illustrated in Fig. 1B-b. This idea was tested on two previously tested genotype 1b patients and the results are shown in Table 3. The results show that having the E1202G adaptive mutation in the NS3/4A gene of the clinical isolates had very little or no positive impact on their replication efficiency compared to not having this adaptive mutation. These results indicated that some critical interactions between the protease, helicase or the 4A peptide and the rest of the nonstructural proteins or untranslated regions (UTR) were disrupted in the chimera resulting in poor progeny RNA synthesis. In this regard, HCV helicase has been reported to bind specifically to the 3′UTR (Banerjee and Dasgupta, 2001), to interact with NS5B and modulate the polymerase activity (Piccininni et al., 2002; Zhang et al., 2005), and more recently, has been demonstrated to be an essential component of the initiation complex in recognizing and regulating positive-strand RNA synthesis (Binder et al., 2007). It was possible that the helicase domain from patient isolates was incompatible with the NS5B and 3′UTR from the 1b Con1 strain in this chimera. Based upon this assumption, we proposed to retain the Con1 helicase domain in the cassette to restore the replication. An overlapping PCR strategy was employed to first amplify the NS3 protease and NS4A gene from the clinical samples individually. These two PCR fragments were then combined with the PCR product of helicase from Con1 and outside primers containing the restriction sites in the second round PCR to obtain full-length NS3/4A gene fragment. The E1202G adaptive mutation was incorporated into the primer used for NS3 protease amplification which is located just five amino acids upstream of 3′-end of protease domain. The final construct is illustrated in Fig. 1B-c. Replicon constructs were made again from genotype 1b patients #1 and #2 and the replication results are shown in Table 3. Our results showed that retaining the 1b Con1 helicase domain in the patient replicons improved the replication efficiency by as much as 100-fold in both cases and enabled an acceptable signal to noise ratio to test the inhibitory effect of anti-HCV compounds. These data suggest that helicase is highly specific in recognizing and interacting with other components of the RNA synthesis complex from the same viral strain and disturbance of that recognition specificity seems to be detrimental to the RNA replication.

3.4. Replication of NS3 protease catalytic domain cassette

Because phenotyping full-length NS3/4A of clinical samples has been demonstrated to be difficult without retaining the helicase corresponding to the replicon, we pursued a more straightforward strategy of shuttling in only the NS3 protease domain from the clinical isolates. Since the mutations conferring resistance to the protease inhibitors were observed in NS3 protease domain, none in NS4A, this strategy would allow us to study the activity of most protease inhibitors targeting the active site of the NS3 protease enzyme from HCV-infected patients. The NS3 protease domain was amplified from sera of 20 HCV-infected patients including 10 genotype 1a and 10 genotype 1b using a primer that contains the E1202G mutation. The final PCR products were then ligated to the protease domain shuttle vector to generate a replicon construct illustrated in Fig. 1B-d. Replication and susceptibility of HCV quasispecies pools of protease molecules to BILN-2061 were determined with the transient transfection assay (Fig. 4). The replication capacity of 1b patient samples is on average superior to that of the 1a patient samples likely due to a higher homology and compatibility with the backbone. A few of the 1a samples replicated at an activity just

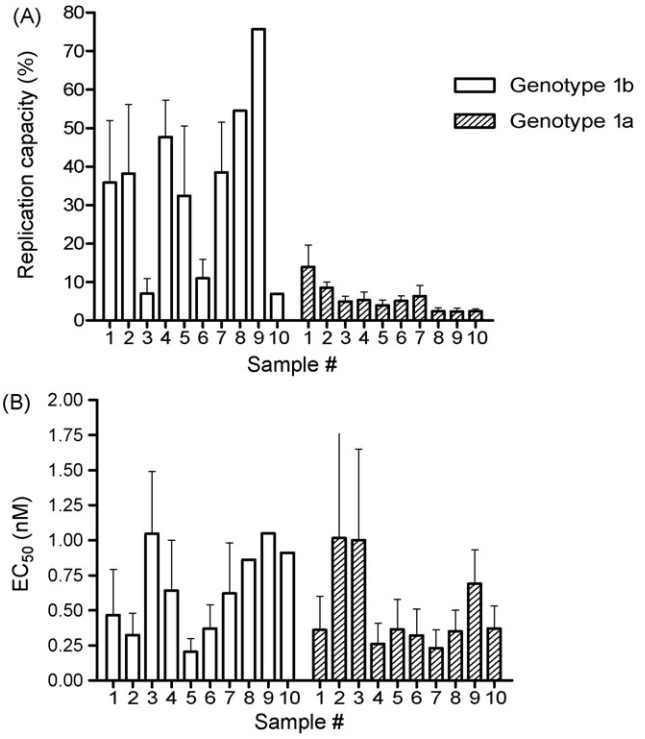

Fig. 4. Replication efficiency of replicons containing patient-derived NS3 protease genes normalized to wild type protease shuttle vector (A) and their susceptibility to BILN-2061 (B).

above the minimum signal to noise ratio of 10 in order to test drug susceptibility. However, the extent of inhibition was not correlated with the level of replication activity as demonstrated in Fig. 4. The sensitivities of the subtype 1b isolates to BILN-2061 were very similar to that of the 1a isolates with 1b EC_{50} ranging from 0.21 to 1.1 nM and the 1a EC_{50} ranging from 0.23 to 1.0 nM. Since NS4A from the isolates was not included in the protease phenotypic assay, replication capacity and sensitivity to BILN-2061 were compared between replicons with or without patient-derived NS4A to examine whether it would cause any difference in replication or the EC_{50} value. The comparison was made for the same two subtype 1b patients (patients #1 and #2 in Table 3 and Fig. 4). As shown in Table 3 and Fig. 4, shuttling only the protease domain from these two patients resulted in a slightly better replication efficiency than shuttling in both the protease domain and NS4A, but the EC_{50} of BILN-2061 was found to be similar for the two replicons (data not shown).

3.5. Sensitivity of the protease phenotypic assay for detecting resistance in mixed populations

Emergence of drug resistance mutations during the course of antiviral treatment will lead to a reduction in drug susceptibility for HCV isolates from an infected patient although the level of decrease will vary among different mutations. The measured change in drug susceptibility is affected by several factors including the prevalence of the drug-resistant virus in the mixed population of viral quasispecies, the replication efficiency of the mutant virus, and the level of resistance conferred by the specific mutations. When the change in drug susceptibility is evaluated in an in vitro phenotypic assay, there is a limitation on the assay sensitivity in differentiating the sensitive and resistant phenotype at which point the existence of a resistance mutation in the viral population can no longer be detected. Therefore, the sensitivity of the assay defines the lowest frequency of a resistance mutation in a viral pool that confers an

Table 4
EC_{50} values of BILN-2061 to mixtures of wild type with either D168V or A156T mutant.

Percent of mutant	EC_{50} (μM)	
	D168V	A156T
0	0.00077 ± 0.00038	0.00077 ± 0.00038
5	0.019 ± 0.009	0.0014 ± 0.0005
20	2.8 ± 0.6	0.0024 ± 0.0019
50	3.5 ± 0.6	0.032 ± 0.017
80	4.0 ± 1.2	1.7 ± 0.8
100	4.0 ± 0.8	2.1 ± 1.2

EC_{50} shift beyond the experimental variation of EC_{50} for wild type virus.

In order to characterize the sensitivity of mutant detection in mixtures of mutant and wild type populations in the protease phenotypic assay developed here, in vitro transcribed RNA of the shuttle vector containing wild type protease was mixed in varying proportions with vector containing protease with either the D168V or A156T, both NS3 mutations confer high levels of resistance to BILN-2061. The susceptibility of BILN-2061 was then tested against the mixture in a transient replication assay. As shown in Table 4, significant changes in EC_{50} values were observed in mutant/wild type mixtures of 5%/95% for the D168V or 50%/50% for A156T resistance mutations. Fig. 5 shows that a distinguishable bi-phasic titration curve can be readily observed for a mixture of 5% of either D168V or A156T and 95% wild type protease when compared to 100% wild type. The two distinct portions of the curve resulted from first inhibition of the wild type strain followed by inhibition of the mutant

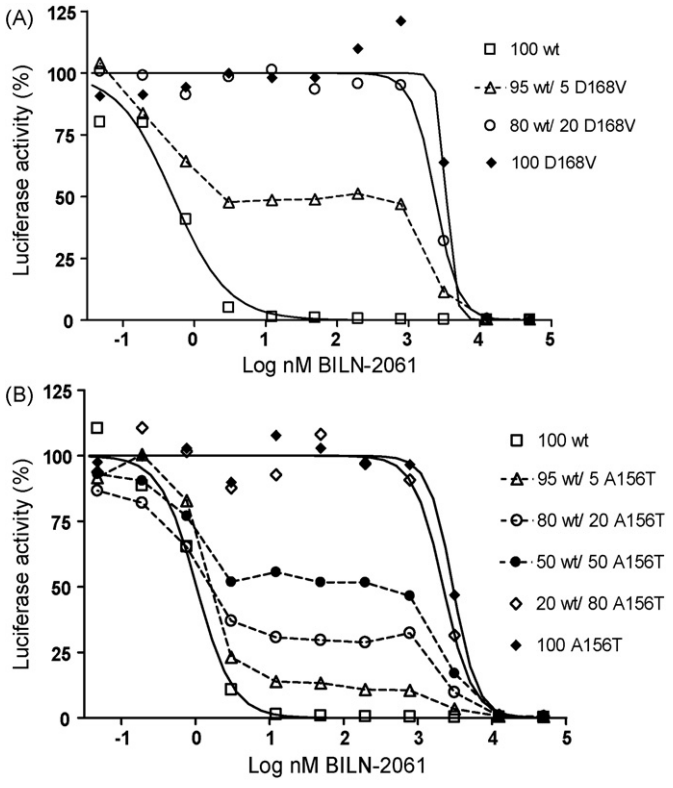

Fig. 5. Titration curve of BILN-2061 to mixtures of wild type (wt) protease and protease containing the D168V or A156T mutants. In vitro transcribed RNA of wt 1b protease shuttle vector was mixed with shuttle vector containing the D168V (A) or A156T mutation (B) at different ratios. Mixed RNA was then transiently transfected to cured Huh-7 cells (Lunet). Cells were incubated with BILN-2061 for 4 days before measuring the luciferase activity. Titration curves of the 50/50 and 20/80 mixture of wt/D168V were not shown in the figure as they were indistinguishable from 100 D168V curve.

at higher drug concentration. For the same percentage of resistant mutant in the mixture, the D168V mutation exhibited a much stronger shift in the dose–response curve than for the A156T mutation suggesting a higher sensitivity to BILN-2061. In the case of D168V, 20% mutant mixed with 80% wild type was enough to confer an EC_{50} indistinguishable from 100% mutant, whereas for A156T it takes 80% mutant mixed with 20% wild type to show the same phenomenon.

4. Discussion

Current drug discovery of novel anti-HCV therapy is extensively dependent on evaluating the antiviral activity of compounds in laboratory-optimized standard replicons. The limitation of a standard replicon assay is that the efficacy of the novel compounds can only be assessed against a few laboratory strains of HCV. It does not reflect the range of activity of a compound against a heterogeneous viral population that exists in an HCV-infected patient. This issue is particularly important for HCV given its high degree of genetic variability that leads to a great diversity of viral quasispecies circulating within and among HCV-infected individuals. The replicon-based shuttle vector approach described here offers an advantage over the standard replicon assay that enables investigation of the activity of compounds against a panel of HCV isolates of interest in a transient assay without having to establish a stable replicon over a long period of time. In this report, we focused on the NS3 gene of the HCV genome although this approach should be generalizable to any nonstructural gene represented in a replicon. Utility of this assay can be extended to phenotypic analysis of NS3 isolates from HCV-infected patients in clinical trials of a compound targeting the NS3 protease. In addition to pharmacokinetics, understanding the sensitivity of individual NS3 genes derived from clinical subjects to a protease inhibitor is critical in interpreting the treatment outcome. Sequence variation and the presence of pre-existing resistance mutations at baseline in subjects could alter the drug susceptibility and affect the responsiveness to the therapy. Emergence of resistance mutations during the treatment would confer reduced susceptibility to the drug. Ultimately, the level of decrease in sensitivity combined with the pharmacokinetics of the drug determines the different profiles of virologic response observed among subjects. Therefore, this replicon-based shuttle vector assay for the phenotypic analysis of clinical samples offers a powerful tool in evaluating the heterogeneity of HCV and treatment response in clinical settings.

The full-length NS3/4A gene derived from patient sera was initially attempted for the shuttle vector as it represents the most relevant construct to study the protease activity. It was expected that replication fitness would be drastically affected by replacing the NS3/4A gene of Con1 with patient-derived sequences as it has been determined that reverting the two adaptive mutations in the NS3 region to wild type sequence decreased the replication efficiency by approximately 80% (Fig. 3). However, this 20% wild type replication level is sufficient for testing the drug susceptibility if the chimeric NS3/4A replicons do not have further decreases in replication efficiency. Surprisingly, transfer of a heterologous NS3/4A gene resulted in non-functional replicons for all the five genotype 1 samples tested, two of which were subtype 1b and three were subtype 1a. Assuming the lack of adaptive mutations was the cause for the loss of replication, the adaptive mutation E1202G was introduced to two genotype 1b NS3/4A patient sequences. However, no improvement of replication efficiency was observed (Table 3) indicating there is a broader compatibility between the patient-derived NS3/4A gene and the other components from the laboratory strains in these chimeras.

Interestingly when the NS3 helicase domain was substituted with the one from the Con1 strain in the NS3/4A cassette, replication efficiency increased by approximately 100-fold (Table 3). The significant increase in replication generated a satisfactory signal to noise window to allow determination of susceptibility to any compound with a potential mechanism of action involving NS4A. Several studies have already demonstrated direct or indirect interaction of helicase with other NS proteins or the untranslated region plays a critical role in viral RNA replication. Our finding highlights that these interactions take place in a highly strain-specific and regulated manner at either the amino acid or nucleotide level. It seems that substitution of helicase even within the same subtype was not tolerated and completely debilitated the RNA replication. Our result is also in line with the finding of Binder et al. (2007) from their intergenotypic replicase chimeras study in which the construct containing NS3–4B of Con1 in a genotype 2a JFH backbone failed to replicate whereas replacing the helicase of Con1 with the helicase of JFH resulted in a fully competent replicon, also indicating that interaction of helicase with other components in the replicon is critical for the replication. Taken together, these results suggest that phenotyping the full-length NS3/4A would be challenging with helicase being the hurdle to achieve an acceptable replication window for analysis.

We then successfully constructed a shuttle vector for the protease domain of NS3 gene as an alternative to full-length NS3/4A. This reduces the technical complexity and is suitable for most protease inhibitors that target the catalytic active site of the NS3 protease. Inclusion of the matching NS4A peptide from the patient isolates to the protease domain does not seem to be critical for evaluating the activity of protease inhibitors as our results showed that the presence of matching NS4A in two 1b isolates did not increase the replication efficiency and had no impact on their susceptibility to BILN-2061. Moreover, no resistance mutations to active site protease inhibitors have been mapped to NS4A. All 20 samples we tested, including 10 genotype 1b and 10 genotype 1a, generated functional replicons. Although the genotype 1a samples on average showed a less robust replication than genotype 1b samples, the signal to background window obtained was sufficient to determine the drug susceptibility. In addition, we have managed to further improve the signal to background ratio in later experiments by switching from clear bottom to opaque bottom assay plates. Although these plates do not allow microscopic assessment of cell morphology, they significantly increase the signal by reflecting the emitted light to the detector more efficiently. We were able to achieve a minimum 50-fold signal to noise ratio on all the 1a samples tested using the opaque plates. Attempts were also made to generate a genotype 1a-specific shuttle vector to better support the protease activity of 1a isolates. When using the most common 1a laboratory strain H77 as the shuttle vector backbone in which a number of adaptive mutations were introduced (Q1067R, E1202G, K1691R and S2204I), the replication of this 1a-H77 replicon was much lower than the Con1 strain even using an optimal Huh-7 cell clone for transfection (~3% of the 1b Con1 replication capacity). As a matter of fact, the replication of the 1a-H77 was similar to or lower than the chimeric 1b Con1 replicons carrying the NS3 protease domain from 1a patient isolates. Therefore, the attempt to develop a 1a-H77 replicon-based NS3 shuttle vector was discontinued. Beyond genotype 1 samples, the protease domain from the 2a JFH strain was also tested in this shuttle vector and found to be completely incompetent in replication (data not shown), suggesting that the compatibility of NS3 protease with other components of the replicon is poor across different genotypes. Further investigation is necessary to determine whether this assay is robust enough to accommodate resistance-conferring substitutions with poor fitness or even natural variants existing in a larger sample size.

In this report, we assessed the sensitivity of this phenotypic assay for the D168V and A156T mutants with BI-2061. Results from this experiment clearly demonstrated that assay sensitivity differs

from one mutant to another and has to be determined individually for each mutant. A stronger fitness or higher fold of resistance conferred by a mutant usually correlates to a more sensitive limit of detection of that mutant in a mixed population. Both the D168V and A156T substitutions confer similar level of resistance (>2500-fold) to BILN-2061, but the in vitro replication efficiency of D168V is about 2–3 fold better than A156T and it therefore displayed a more readily observed titration curve shift for the same mutant percentage in a mixture with wild type. Based on this observation, the assay sensitivity for detection of mutant in a mixed population may be reduced for resistance mutations less fit or with lower levels of resistance. It was noted that the titration curves shown in Fig. 5 possess a bi-phasic characteristic in which the lower drug concentration portion of the curve results from inhibition of wild type and the higher drug concentration portion from the mutant, so determining the EC_{90} is more representative of the phenotype of the resistant species than the conventional EC_{50}. In the case of A156T, the EC_{50} of BILN-2061 is barely distinguishable between 100% wild type (Fig. 5, open square) and 20% A156T mixed with 80% wild type (open circle), however the EC_{90} of the latter is approximately three magnitudes of order higher. This suggests that reporting EC_{90} for the assay instead of EC_{50} would serve to increase the sensitivity of assay.

In conclusion, we have found that chimeric replicons carrying patient-derived full-length NS3/4A failed to support replication in cell culture. The poor replication of the NS3/4A chimeras appeared to be due to the incompatibility between patient-derived helicase and other components of laboratory strain in the 1b Con1 replicon shuttle vector. We then developed replicon-based shuttle vectors to allow rapid phenotypic analysis of the NS3 protease domain or NS3 protease domain plus NS4A from serum samples of a large number of HCV-infected patients which reflects the heterogeneity of the HCV quasispecies. We demonstrated that similar results were obtained with the NS3 protease domain versus NS3 protease plus NS4A shuttle vectors for protease inhibitor BILN-2061. The potency of BILN-2061 against 20 patient-derived NS3 protease genes (10 genotype 1a and 10 genotype 1b) using the NS3 protease shuttle vector was comparable to the 1b lab strain. Significantly reduced susceptibility to BILN-2061 was observed with mutant/wild type mixtures of 5%/95% for the D168V or 50%/50% for A156T resistance mutations. This cell-based methodology is useful for characterizing the intrinsic genetic diversity of NS3 of chronically infected patients and for monitoring the development of novel mutations during treatment.

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