Selection of Clinically Relevant Protease Inhibitor-Resistant Viruses Using the Genotype 2a Hepatitis C Virus Infection System^{▽†}

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Treatment of patients infected with hepatitis C virus (HCV) with direct acting antivirals can lead to the emergence of drug-resistant variants that may pose a long-term threat to viral eradication. HCV replicons have been used to select resistance mutations; however, genotype 2a JFH-1-based viruses provide the opportunity to perform resistance selection in a *bona fide* infection system. In this study, we used a tissue culture-adapted J6/JFH-1 virus to select resistance to the NS3 protease inhibitors BILN-2061 and VX-950. Lunet-CD81 cells were infected with J6/JFH-1 virus and maintained in the presence of inhibitors until high-titer viral supernatant was produced. Viral supernatants were passaged over naive cells at escalating drug concentrations, and the resulting viruses were then characterized. Three NS3 resistance mutations were identified in BILN-2061-resistant viruses: A156G, D168A, and D168V. Interestingly, D168A, D168V, and A156T/V, but not A156G, were selected in parallel using a genotype 2a replicon. For VX-950, the T54A and A156S NS3 resistance mutations were identified in the virus selections, whereas only A156T/V emerged in genotype 2a replicon selections. Of note, VX-950 resistance mutations selected using the 2a virus (T54A and A156S) were also observed during VX-950 clinical studies in genotype 2 patients. We also performed viral fitness evaluations and determined that the mutations selected in the viral system did not confer marked reductions in virus production kinetics or peak titers. Overall, the HCV infection system is an efficient tool for drug resistance selections and has advantages for the rapid identification and characterization of clinically relevant resistance mutations.

Chronic hepatitis C virus (HCV) infection represents a significant and immediate worldwide health burden (2, 40). Accordingly, tremendous resources have been directed toward discovering and developing novel therapies to treat HCV infection. Currently, a number of direct-acting antiviral agents (DAAs) are under clinical development (7, 35, 37). Various DAAs have been designed to target distinct HCV viral proteins (3, 4, 6), including NS3 protease, NS5A, and the NS5B RNA-dependent RNA polymerase. During short-term monotherapy studies in HCV-infected patients, many of these DAAs have elicited pronounced antiviral effects (e.g., ≥ 3 log HCV viral load reductions in as few as 3 days of treatment). However, even in these short-term studies the selection of viral resistance was apparent and thus poses a significant challenge to the long-term efficacy of these novel agents (6, 11, 32, 35).

The HCV replicon has been a useful *in vitro* tool for identifying and characterizing resistance mutations for multiple classes of DAAs (11, 32, 35). For example, the NS3 mutations R155K, A156T/V, and D168A/V were selected in replicons using the first clinically active NS3 protease inhibitor, BILN-2061 (a prototype noncovalent NS3 inhibitor) (16, 19). Unfortunately, the resistance profile of BILN-2061 in the clinic has not been reported, making it impossible to compare *in vitro*

and *in vivo* results (15, 28). However, structurally related protease inhibitors have selected mutations at R155, A156, and D168 in the clinic (13, 27, 30, 36). A partially overlapping *in vitro* resistance profile was identified for a structurally distinct protease inhibitor, VX-950 (telaprevir, a prototype covalent NS3 inhibitor) (16, 19, 20, 31, 44). R155 and A156 substitutions are cross-resistant to VX-950, whereas D168 mutants remain fully sensitive to VX-950. Mutations at positions R155 and A156 were selected with VX-950 in the replicon system and also in patients during clinical studies. However, additional mutations that were not identified *in vitro* (e.g., T54 and V36 mutations) were also commonly identified in patients (31).

Although the replicon system has proven partially predictive of clinical resistance, it has conceptual limitations. First, the replicon, by nature, is limited to evaluation of the RNA replication, translation, and protein processing steps of the HCV life cycle (18). Inherently virus entry, assembly, egress, and cell-to-cell spread cannot be studied in the replicon system. As a result, the replicon is only useful for HCV inhibitors targeting viral replication. Second, the replicon limits the evaluation of mutation fitness to the replication portion of the HCV life cycle. Since resistant viruses need to both replicate and spread through cultures to be viable, it is conceivable that limiting fitness evaluations to the replication portion of the life cycle could overestimate or, perhaps more likely, underestimate fitness impacts. Related is the fact that resistant mutants in the replicon system need only to replicate to levels sufficient to confer G418 resistance (and thus colony survival) to be detected (16, 38). In contrast, mutant viruses not only need to replicate intracellularly but also to be assembled, be secreted,

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FIG. 1. Drug resistance selection strategy with HCVcc. (A) Diagrams of the JFH-1 subgenomic replicon and adapted J6/JFH-1 adapted virus. The JFH-1 subgenomic replicon encodes a firefly luciferase (*luc*)-neomycin phosphotransferase (*neo*) fusion reporter. An asterisk (*) indicates the location of the adaptive mutations in the virus genome. A triangle (Δ) indicates an insertion of a FLAG epitope into the NS5A protein. (B) Strategy for HCVcc drug resistance selection. Naïve Lunet-CD81 cells were infected with the adapted J6/JFH-1 virus at an MOI of 1 and maintained in the presence of BILN-2061 or VX-950. The infected cells were passaged every 3 to 4 days until high-titer viral supernatant was produced or a viral cytopathic effect was observed. Viral supernatants were then passaged over naïve Lunet-CD81 cells at escalating drug concentrations for continuous selection.

and be capable of infecting naïve cells to establish new rounds of replication and become the dominant species. Therefore, the replicon system may enable the selection of mutants that are unfit in the context of the whole virus or may bias the frequency and/or variety of mutations compared to what might occur in the clinic (14, 16, 19, 31). Third, resistance selections using the replicon system typically require a significant period of time (e.g., 2 to 4 weeks of drug selection, at which point cell clones can be picked but requires another 2 to 4 weeks of expansion prior to genotypic and phenotypic analyses). Prolonged exposure of actively dividing replicon cells to antiviral drugs potentially enables the selection of host cell variants that become resistant to antivirals or to G418 (which is included in selection as a dominant-selectable marker) (1, 29). However, the occurrence of cell-based resistance in Huh-7 cells is unlikely to have clinical relevance and may also obscure or prevent the identification of viral resistant mutants.

The recently described JFH-1 cell culture infection model (HCVcc) provides a novel opportunity for drug resistance studies and should address the major issues associated with the replicon resistance selections discussed above (17, 39, 43). Recent studies have demonstrated that passaging JFH1-infected or -transfected cells does result in the selection of drug resistance. However, the relatively low titers of unadapted JFH-1 virus makes it difficult to efficiently select resistant virus by continuously passaging viral supernatant onto naïve cells at escalating drug concentrations (9, 10, 41). Our tissue culture adaptation has enabled JFH-1-based viruses to robustly propagate in cells, efficiently expand from cell-to-cell, and reach high supernatant titers ($>10^6$ 50% tissue culture infective doses [TCID₅₀]) (24); these properties make drug resistance

selections based on passaging viral supernatant (and not just infected cells) feasible and practical. We report here that *in vitro* selection using the HCV infection system is an efficient approach for identifying drug-resistant mutants. Using cell culture-adapted J6/JFH-1 virus and permissive Lunet-CD81 cells, infectious viral mutants with resistance to BILN-2061 or VX-950 were rapidly selected. Furthermore, we have demonstrated that resistant mutants selected in the infection system possess high replication capacity and high viral fitness. The VX-950 mutations identified in this genotype 2 infection system also closely mimic those recently reported in genotype 2 HCV patients treated with VX-950 (5).

MATERIALS AND METHODS

Cell culture. Huh-Lunet cells were obtained from ReBLikon GmbH (Mainz, Germany) (12). Lunet-CD81 cells were previously established in our laboratory by transducing Huh-Lunet cells with a lentivirus encoding the human CD81 gene (24). 2aLucNeo-25 cells were established as described below. All cell lines were maintained in Dulbecco modified Eagle medium (DMEM) with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U of penicillin/ml, 100 μ g of streptomycin/ml, 10 mM HEPES buffer, and 10 mM nonessential amino acids (Invitrogen). 2aLucNeo-25 cells were cultured in the presence of 0.5 mg of G418 (Invitrogen)/ml. All cell lines were maintained in humidified incubators at 37°C and 5% CO₂.

Construction of a luciferase-encoding genotype 2a subgenomic replicon cDNA. Plasmid pLucNeo2a encodes a bicistronic genotype 2a subgenomic replicon with the firefly luciferase reporter in the first cistron (Fig. 1A). The plasmid was derived from pJFH-1 (Toray, Japan), a plasmid containing the full-length genotype 2a (JFH-1 strain) genome, as follows: the HCV non-structural genes, along with the plasmid backbone in pJFH-1, were amplified and self-ligated to generate p2aSG by PCR using the primer set HCV2aCoreAfeIrev and HCVMLu2aNS3fw. HCV2aCoreAfeIrev has the sequence 5'-TCTAGA AGCGCT tggcgc acggtt ggtgtt tctttt GG-3' (with the HCV

sequence indicated in lowercase) and encodes an AfeI site (underlined). HCVmlu2aNS3fw has the sequence 5'-GAGCTT ACGCGT atgctt cccatc atctgt tatg-3' (with the HCV sequence in lowercase) and an MluI site (underlined). The AfeI and MluI sites were introduced at the twentieth residue of core protein and upstream of NS3, respectively, to allow the insertion of the luciferase reporter gene and the neomycin phosphotransferase (*neo*) gene. The fragment encoding the reporter, the *neo* gene, and EMCV IRES was amplified by PCR from the pFKi389lucubineoNS3-3' ET replicon (ReBLikon) using primers with AfeI and MluI sites at the ends and subsequently cloned into a pTA TOPO vector (Invitrogen). An XbaI site was knocked out of the luciferase/*neo*/IRES fragment by site-directed mutagenesis so that the final replicon construct would have only one XbaI site. The plasmid pLucNeo2a was then generated by ligation of the cloned AfeI-MluI fragment containing luciferase/*neo*/IRES into plasmid p2aSG after digestion with AfeI and MluI. The current replicon sequence was confirmed by DNA sequencing.

Generation of a genotype 2a subgenomic replicon stable cell line. Plasmid pLucNeo2a was transcribed into RNA as described below. A 3- μ g portion of RNA was transfected into Huh-Lunet cells by using the DMRIE C lipofection reagent (Invitrogen) in six-well culture dishes. At 2 days posttransfection, the cells were treated with trypsin and seeded in 100-mm culture dishes. Transfected cells were selected in the presence of 1 mg of G418/ml for 2 weeks. G418-resistant colonies were isolated and expanded, and HCV replication was quantified by using a commercial firefly luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions. Clones positive for HCV replication were further expanded. Clone 2aLucNeo-25 was selected for further studies based on its robust luciferase expression.

Antiviral compounds. BILN-2061, VX-950, SCH-503034, and 2'-C-methyl adenosine (2'-CMA) were purchased from Acme Biosciences (Belmont, CA).

Selection of protease inhibitor resistant J6/JFH-1 viruses. A tissue culture-adapted strain of J6/JFH-1 was previously established by our laboratory (Fig. 1A) (24). Naive Lunet-CD81 cells were infected with the adapted J6/JFH-1 virus at a multiplicity of infection (MOI) of 1.0 and maintained in the presence of BILN-2061 or VX-950. The infected cells were passaged every 3 to 4 days until a high-titer viral supernatant was produced. Viral supernatants were then passaged over naive Lunet-CD81 cells at escalating drug concentrations for continuous selection (Fig. 1B).

Selection of protease inhibitor resistant replicon cell lines. 2aLucNeo-25 cells were seeded in 100-mm dishes for protease inhibitor resistance selections at a density of 2.5×10^5 cells per dish. Cell culture medium containing 0.5 mg of G418/ml plus either 5 μ M BILN-2061 or VX-950 was added to the cells and replaced every 3 days. Approximately 3 weeks later, widespread cell death occurred, and individual colonies were visible on the culture dishes. Cell clones were isolated, expanded, and cryopreserved. Resistant replicon clones were subsequently subjected to phenotypic and genotypic analyses as described below.

Virus titer determination (TCID₅₀ assay). Lunet-CD81 cells were seeded at a concentration of 4×10^3 cells per well in 96-well plates in a total volume of 100 μ l of complete DMEM/well. The next day, 3-fold serial dilutions of virus-containing supernatant were prepared, and 100 μ l of each dilution was added to four wells per dilution. Three days later, cells were fixed and stained for the presence of NS5A by indirect immunofluorescence (as described below). Wells positive for NS5A protein were counted, and virus titers (TCID₅₀/ml) were calculated based on the method of Reed and Muench (24, 26).

Detection of NS5A protein by indirect immunofluorescence. Infected cells in 96-well plates were fixed in 4% paraformaldehyde (50 μ l/well) at room temperature for 20 min. Cells were then washed three times with phosphate-buffered saline (PBS) and blocked with 3% bovine serum albumin (BSA), 0.5% Triton X-100, and 10% FBS. Immunostaining of NS5A was performed with a mouse monoclonal antibody (9E10; Apath, Brooklyn, NY) at a dilution of 1:10,000 in PBS with 3% BSA for 1 h at room temperature. After three washes with PBS, bound primary antibodies were detected using an anti-mouse antibody conjugated to Alexa Fluor 555 at a dilution of 1:1,000 in PBS containing 3% BSA in the dark at room temperature for 1 h. At the same time, DNA was stained with Hoechst 33342 (Invitrogen) at a concentration of 2 μ g/ml. Finally, cells were washed three times with PBS and imaged by using a Zeiss microscope with fluorescence capabilities (Thornwood, NY), through Cy3 (for NS5A) and DAPI (4',6'-diamidino-2-phenylindole; for nucleus) channels.

Isolation, reverse transcription-PCR (RT-PCR) amplification, and sequencing of HCV genomes. For viral resistance selections, total RNA was isolated from cell culture supernatants by using a virus RNA QIAamp kit (Qiagen, Valencia, CA) as recommended by the manufacturer. For replicon resistance selections, total RNA was isolated from replicon cells by using an RNeasy minikit (Qiagen) as recommended by the manufacturer. For cDNA synthesis, 1 μ g of total RNA and 50 pmol of primers were used, along with a MonsterScript First-Strand

cDNA synthesis kit (Epicentre Biotechnologies, Madison, WI) as recommended by the manufacturer. Six overlapping PCR products that covered the entire viral coding region, or four products that covered the replicon viral nonstructural region, were amplified from the synthesized cDNA. Sequence analysis was performed by Elim Biopharmaceuticals (Hayward, CA) with a set of primers covering the HCV genome. Sequences for drug-selected and control/parental viruses or replicons were aligned to identify emerging mutations by using Vector NTI software (Invitrogen).

Replicon antiviral activity assays. Replicon antiviral assays were performed as described previously (22). Briefly, replicon cells were seeded into 96-well plates at a density of 5×10^3 cells per well in 100 μ l of complete DMEM, excluding G418. After overnight incubation, HCV inhibitors were added to the cell culture. Compounds were serially diluted in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) in 3-fold steps and added to cells at a 1:200 dilution, achieving a final DMSO concentration of 0.5% in a total volume of 200 μ l/well. The 50% effective concentrations (EC₅₀s) were determined after incubation of the compounds with replicon cells for 72 h. The luciferase activity was quantified by using a commercially available assay (Promega, Madison, WI) and a Victor Light instrument (Perkin-Elmer, Waltham, MA). The data were converted into percentages relative to untreated controls (defined as 100%), and EC₅₀s were calculated by nonlinear regression of two replicate data sets using XLfit 4 software (IDBS, Emeryville, CA). Resistance fold changes were calculated as the ratio of mutant to wild-type replicon EC₅₀.

Infectious virus antiviral activity assays. Lunet-CD81 cells were seeded into 96-well plates at a density of 4×10^3 cells per well in 100 μ l of complete DMEM. Cells were allowed to attach overnight, and then compounds were added in a volume of 50 μ l of medium. Compounds were diluted serially in 100% DMSO in a 3-fold step and then added at a 1:50 dilution to complete DMEM before being transferred to the cell culture wells. Immediately after compound addition, virus was added to cells in a volume of 50 μ l and at an MOI of 0.5. The final compound dilution was therefore 1:200 with a final DMSO concentration of 0.5%. The cell plates were incubated at 37°C for 3 days, after which the culture medium was removed, and the cells were assayed for viral replication levels. The proteolytic activity of virally encoded NS3 protease was measured as a marker for viral replication using a europium-labeled NS3 peptide substrate as described previously with slight modifications (42). Briefly, the medium was aspirated from virus-infected cells and replaced with 90 μ l of 1 $\times$ Promega luciferase lysis buffer supplemented with 150 mM NaCl per well in a 96-well plate. The plates were shaken for 10 min at room temperature, and 10 μ l of 1 μ M europium-labeled NS3 substrate in lysis buffer was then added to each well. NS3 europium-labeled substrate was purchased from Perkin-Elmer. Time-resolved fluorescence was measured by using a Victor 3 (Perkin-Elmer) at an excitation of 340 nm and an emission of 610 nm. Initial slopes were measured using the maximum slope algorithm of the instrument software (Workout 1.5; DAZDAQ, East Sussex, United Kingdom). EC₅₀s and resistance fold changes were determined as described above for replicon antiviral activity assays.

NS3 protease assay for biochemical resistance phenotyping. Lunet-CD81 cells were seeded in 96-well plates at a concentration of 4×10^3 cells per well and were infected with either the parental adapted J6/JFH-1 virus or the protease inhibitor-resistant viruses at an MOI of 0.5. The cells were incubated for 3 days, after which culture media were removed, and the cells were washed once with PBS. The infected cells were then lysed with 80 μ l of 1 $\times$ Promega luciferase lysis buffer supplemented with 150 mM NaCl at room temperature for 20 min. Antiviral drug dilutions were prepared in DMSO at 10 times the final desired concentrations. Portions (10 μ l) of diluted drugs were then added to each well of cell lysates, and the plates were incubated on a plate shaker for 10 min at room temperature. Then, 10 μ l of 1 μ M europium-labeled NS3 substrate was added to each well. Protease activity data were collected and analyzed to determine the biochemical IC₅₀s as described above for the NS3 protease EC₅₀ determination assay.

Construction of mutant replicons and viruses. Mutations identified from resistance selections were introduced into pLucNeo2a (a plasmid encoding the 2a subgenomic replicon as described above) or J6/JFH-All 7 (a plasmid encoding a cDNA for the adapted J6/JFH virus) using the QuikChange Lightning mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by DNA sequencing (Elim Biopharmaceuticals).

In vitro RNA transcription. *In vitro* transcripts were generated as previously described (22). Briefly, plasmids were linearized by XbaI and purified by using a MinElute column (Qiagen). RNA was transcribed from 1 μ g of purified template by using a T7 Megascript kit (Ambion, La Jolla, CA). Reactions were incubated at 37°C for 2 h and then digested with 3 U of DNase I (Ambion) for 15 min. RNA was purified by using an RNeasy kit (Qiagen) and then quantified by determining

FIG. 2. Representative selection chart for BILN-2061- and VX-950-resistant viruses. The selection of drug-resistant viruses started at a drug concentration of $1 \times EC_{50}$ and was performed as described in Fig. 1B. The drug concentrations were increased in a stepwise fashion until either cellular toxicity was observed or the viruses failed to reach a high titer after 12 days of infection. Each symbol (except the last one for each selection) represents the passage of viral supernatant onto naive cells. The last symbols of both selections indicate the viral supernatants for genotypic and phenotypic analyses.

the absorbance at 260 nm. RNA integrity was determined by agarose gel electrophoresis.

Transient transfection of replicon and viral RNAs. Transcribed viral or replicon RNAs (5 μ g) were mixed with a suspension of Huh-Lunet cells (10^7 cells/ml in a volume of 400 μ l). Cells were then electroporated at 260 V and 950 μ F in a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Electroporated cells were allowed to recover for 10 min at room temperature prior to addition of cell culture medium. For replicon RNAs, 10^4 cells were seeded onto 96-well plates per well for EC_{50} determination. For infectious viral RNAs, 2×10^6 cells were seeded in T75 flasks and passaged on day 4 at a ratio of 1:6. On day 7 posttransfection, viral supernatants were collected for virus titer determination. To measure the percentage of NS5A-positive cells, an aliquot of cells were subjected to NS5A immunostaining on days 2 and 7 posttransfection and analyzed with an ImageXpress Micro (Molecular Devices, Sunnyvale, CA) equipped with MetaXpress 2.0 software.

Viral infection kinetics assays. A total of 10^6 naive Lunet-CD81 cells in T75 flasks were infected with either the parental J6/JFH-1 virus or mutant viruses at an MOI of 0.05. Cells were passaged on day 4, and viral supernatants were collected on days 1, 2, 4, 6, and 7. Virus titers for each viral supernatant were determined by TCID₅₀ assays as described above.

RESULTS

Selection of viruses resistant to the protease inhibitors BILN-2061 and VX-950. To select HCVcc resistant to BILN-2061 and VX-950, we infected a Huh-7 derived cell line (Lunet-CD81) supporting high levels of infection with a tissue culture adapted strain of J6/JFH-1 virus (24) at an MOI of 1.0 (Fig. 1A). The infected cells were then maintained in the presence of BILN-2061 and VX-950 at a concentration of $1 \times EC_{50}$ until high-titer viral supernatants ($>10^4$ TCID₅₀/ml) were produced (Fig. 1B). Viral supernatants were then passaged over naive Lunet-CD81 cells, and the selections were continued at escalating drug concentrations. Passaging of viral supernatants was halted when either drug concentrations became toxic to the cells or the virus failed to reach a high titer after 12 days on the cells. Viral supernatants collected from the preceding drug concentrations were then amplified for phenotypic and genotypic analyses.

Five independent selections were performed with BILN-2061, and four selections were performed with VX-950. A representative selection plot for each inhibitor is shown in Fig. 2. For BILN-2061, the selections started at a concentration of

50 nM, its EC_{50} [consistent with the reported values (22)] against the tissue culture-adapted genotype 2a virus ($1 \times EC_{50}$). After approximately 40 days of selection and five incremental increases in drug concentration, the virus demonstrated a significantly reduced susceptibility to BILN-2061. The selected virus was able to proliferate efficiently in the presence of 2 μ M BILN-2061 ($40 \times EC_{50}$), although it failed to do so at higher drug concentrations ($80 \times EC_{50}$). Similarly, for VX-950, the starting drug concentration was 150 nM ($1 \times EC_{50}$), and at the conclusion of the selection process (36 days) the virus was able to expand robustly in the presence of 3 μ M VX-950 ($20 \times EC_{50}$).

To confirm the resistant phenotype of the selected viruses, naive Lunet-CD81 cells were infected with the parental virus, drug-selected viruses, or a control virus from DMSO mock selection (hereafter referred to as the "DMSO control virus") in the presence or absence of protease inhibitors. At 3 days postinfection, cells were immunostained with antibody against HCV NS5A protein. As shown in Fig. 3, without drug treatment, all viruses efficiently infected Lunet-CD81 cells as indicated by the expression of NS5A protein. In contrast, in the presence of 2 μ M BILN-2061, only the BILN-2061 resistant virus (depicted in Fig. 3 as a representative) was able to infect the cells successfully, while infection by both the parental and DMSO control viruses were completely abolished. These results thus provide direct evidence that viruses resistant to either BILN-2061 or VX-950 could be readily selected using the HCV infection system.

Phenotypic analyses of protease inhibitor-resistant viruses.

To measure the loss in inhibitor susceptibility of the drug selected viruses, EC_{50} assays were performed with a panel of HCV inhibitors. Both parental and DMSO control viruses were included as controls and to allow precise determinations of the fold changes in drug susceptibility. As summarized in Table 1, the BILN-2061-resistant viruses showed 33- to 60-fold decreases in BILN-2061 susceptibility, and VX-950-resistant viruses showed 10- to 15-fold decreases in VX-950 susceptibility compared to the parental virus. In contrast, the DMSO control virus remained sensitive to all of the HCV inhibitors. Interestingly, all VX-950-resistant viruses were cross-resistant to SCH-503034 but had only very minor or no cross-resistance to BILN-2061. Furthermore, there was no cross-resistance of BILN-2061-resistant viruses to VX-950, suggesting that the resistance mutations selected by the two inhibitors were different. All resistant viruses remained sensitive to the HCV nucleoside NS5B inhibitor 2'-C-methyl-adenosine (2-C-MeA), suggesting that the resistance phenotype was specific to the drugs used for selection.

To investigate whether resistant phenotypes were due to decreases in the biochemical susceptibility of the NS3-4A protease, enzymatic NS3-4A protease assays were performed with lysates of cells infected with parental or resistant viruses (Table 2). In this assay, target cells were infected with resistant or parental virus, and 3 days later the cells were lysed and the NS3-4A protease activity in the lysates was quantified by monitoring cleavage of an NS3 FRET peptide substrate. The results of this assay were consistent with the resistant phenotypes observed in cellular EC_{50} assays; specifically, the NS3-4A protease activities from the lysates prepared from resistant virus-infected cells demonstrated levels of resistance and cross-re-

FIG. 3. Reduced susceptibility of the BILN-2061 selected viruses. Lunet-CD81 cells were seeded at a density of 4×10^3 cells/well in a 96-well plate. After overnight attachment, cells were infected with parental virus, a control virus from DMSO mock selection (DMSO control virus), or a representative BILN-2061-resistant virus at an MOI of 0.5. Cells were fixed 3 days postinfection, and the extent of infection was visualized by NS5A specific immunofluorescence (red stain indicates presence of NS5A protein). Nuclear DNA was stained with DAPI (blue).

sistance that closely matched the EC_{50} results presented in Table 1. Overall, these biochemical data suggest that the resistant viruses emerged by acquiring mutations in viral NS3-4A protease that conferred phenotypic resistance.

Genotypic analyses of the protease inhibitor-resistant viruses. To identify genetic changes in the resistant viruses, viral RNA was extracted from the viral supernatants and was subjected to RT-PCR and population sequencing over the entire coding region of the viral genome. None of the nine resistant viruses had identical genomic sequences; multiple point mutations were apparent throughout the various HCV genomes, confirming the independence of each viral selection (data not shown). Since the changes in NS3-4A protease were responsible for the resistance phenotype (based on the biochemical analysis presented in Table 2), subsequent analyses focused on the coding regions of the HCV NS3 protease domain and

NS4A. All amino acid changes identified within these regions are listed at the bottom of Table 1.

For each resistant virus, at least one amino acid substitution in the NS3 protease domain was identified and occurred at a residue previously reported to confer protease inhibitor resistance (T54, A156, or D168). In the BILN-2061 selections, we observed mutations primarily at position D168 (to alanine or valine); however, mutation of residue A156 to glycine was observed in one resistant virus. Mutations at these locations were accompanied by additional NS3 mutations in some of the selected viruses. For VX-950, we observed the T54A or A156S mutations or the combination of both T54A and A156S. A V102A mutation was observed in one virus in conjunction with A156S; V102A was also observed in one BILN-2061-resistant virus with D168V. A mutation of A19S in NS4A was observed in seven of the nine resistant viruses, but it was not studied

TABLE 1. Phenotypic and genotypic analyses of resistant viruses^a

Inhibitor or protease	Avg EC ₅₀ (μM)		Avg EC ₅₀ fold resistance ± SD								
	WT	DMSO	BI-R1	BI-R2	BI-R3	BI-R4	BI-R5	VX-R1	VX-R2	VX-R3	VX-R4
Inhibitor											
BILN-2061	0.06	0.10	53 ± 1.5	37 ± 9.7	33 ± 9.4	49 ± 16	60 ± 28	0.8 ± 0.1	2.4 ± 0.1	3.6 ± 0.4	1.0 ± 0.4
VX-950	0.19	0.25	0.2 ± 0.0	1.1 ± 0.2	1.0 ± 0.3	0.7 ± 0.0	0.3 ± 0.0	15 ± 5.2	11 ± 2.7	14 ± 5.3	10 ± 5.3
SCH-503034	0.16	0.25	0.7 ± 0.4	0.6 ± 0.2	1.2 ± 0.4	0.7 ± 0.4	ND	7.3 ± 1.8	7.7 ± 3.9	6.4 ± 2.0	9.3 ± 2.0
2-C-MeA	3.3	3.8	1.7 ± 0.4	1.2 ± 0.6	1.2 ± 0.1	1.5 ± 0.5	0.7 ± 0.1	1.4 ± 0.1	1.5 ± 0.2	1.9 ± 0.2	1.3 ± 0.1
Mutation(s)											
Protease	WT	DMSO	BI-R1	BI-R2	BI-R3	BI-R4	BI-R5	VX-R1	VX-R2	VX-R3	VX-R4
NS3	NM	NM	D168A	D168V	D168V, E79D, V102A	D168V	A156G, T63S, K122R	T54A	T54A, A156S	A156S, V102A	T54A
NS4A	NM	A19S	A19S	A19S	A19S	A19S	NM	A19S	A19S	A19S	A19S

^a These EC_{50} and fold resistance values represent averages of two independent experiments. WT, wild type. The fold resistance is calculated as the ratio of the mutant virus EC_{50} to the wild-type virus EC_{50} . DMSO, BI-R, and VX-R indicate DMSO control selection, BILN-2061-resistant, and VX-950-resistant viruses, respectively. NM, no mutation; ND, not determined.

TABLE 2. Biochemical analyses of NS3 protease drug susceptibility in lysates infected with wild-type or resistant viruses^a

Inhibitor	WT IC ₅₀ (μM)	Fold resistance								
		BI-R1	BI-R2	BI-R3	BI-R4	BI-R5	VX-R1	VX-R2	VX-R3	VX-R4
BILN-2061	0.29	111	151	61	70	135	0.9	1.8	3.9	0.9
VX-950	0.05	1.0	0.9	0.9	0.7	1.3	10	10	27	15
SCH-503034	0.07	1.0	1.0	1.4	0.6	ND	7.0	6.0	13	13

^a The fold resistance is calculated as the ratio of the mutant virus IC₅₀ to the wild-type virus IC₅₀. Values of IC₅₀ and fold resistance represent averages of two independent experiments. BI-R and VX-R indicate BILN-2061-resistant and VX-950-resistant viruses, respectively. ND, not determined.

further since it also appeared in the DMSO control virus. Overall, the genotyping results agreed well with the phenotypic analyses presented above.

Phenotypic analyses of the selected protease inhibitor-resistant mutations. Protease inhibitor resistance mutations were previously identified at residues T54, A156, and D168 (16, 19, 31); however, they were characterized in genotype 1 replicons, and little is known about their role in resistance in genotype 2 HCV. Therefore, the mutations T54A, A156G, A156S, D168A, and D168V were introduced individually by site-directed mutagenesis into a JFH-1 Luc-neo subgenomic replicon that possesses the same nonstructural region as the parental HCVcc used to select the resistant viruses. Mutation V102A was also introduced because it was selected in two independent selections. The other amino acid changes were not tested, e.g., E79D, T63S, and K122R, because they appeared only once and were in combination with known resistance mutations.

The BILN-2061 mutation D168V was most frequently identified (3/5 selections) and conferred 28-fold resistance as an individual mutation (Table 3). The other BILN-2061 resistance mutations, D168A and A156G, each conferred ~20-fold resistance to BILN-2061. None of these BILN-2061 resistance mutations conferred cross-resistance to VX-950. The VX-950-selected mutations, T54A and A156S conferred low to medium levels of resistance (4- to 8-fold) to VX-950 (Table 3). T54A did not confer cross-resistance to BILN-2061; however, A156S conferred low-level cross-resistance to BILN-2061 (3-fold) in

the genotype 2a replicon. Overall, the phenotypic drug susceptibility results obtained with the site-directed mutants in the 2a replicon system were highly consistent with those observed for the originally selected viruses.

Selection of protease inhibitor-resistance mutations in JFH-1 replicon cells. Next, we sought to select BILN-2061 and VX-950 resistance in the genotype 2a replicon system for two reasons: (i) to compare mutations selected in the 2a virus system to those selected in the 2a replicon system and (ii) to compare mutations selected in the 2a replicon system to those reported in genotype 1 replicon systems. Stable JFH-1 subgenomic replicon cells were therefore treated with either BILN-2061 or VX-950 at a concentration of 5 μM for 3 weeks. This concentration was chosen to apply similar antiviral pressure (in multiples of EC₅₀) between the replicon and HCVcc selections. Eight colonies were isolated from each selection and subjected to phenotypic and genotypic analyses.

As shown in Table 4, all selected colonies had significantly reduced susceptibility to the protease inhibitors used to select them. The major resistance mutations for BILN-2061 were A156V (4/8), D168A (2/8), and D168V (2/8). A156T (5/8) and A156V (3/8) were the only resistance mutations identified for VX-950. There were no other mutations found in the NS3 protease domain from the selections with either drug. The mutations selected in genotype 2a replicon are thus comparable to those reported in genotype 1 replicons.

Resistance mutations selected with genotype 2a virus are highly fit. Mutations identified during the genotype 2a virus and 2a replicon selections did not overlap entirely; specifically, different mutations were observed at position A156, and T54 was only observed in the virus selections. We hypothesized that the resistant mutants emerging in the viral system must remain sufficiently fit to enable these viruses to spread efficiently and become the dominant species. However, less-fit mutations

TABLE 3. Phenotypic analyses of protease inhibitor resistance mutations in JFH-1 replicons and infectious virus by site-directed mutagenesis

Inhibitor(s)	Mutation	Fold resistance ^a to:				Replication relative to WT (%) ^b
		BILN-2061		VX-950		
		Replicon	Virus	Replicon	Virus	
BILN-2061	A156G	17	16	0.3	0.5	100
	D168A	22	27	0.4	0.7	99
	D168V	28	26	0.4	0.6	89
VX-950	T54A	0.5	0.4	4.2	4.7	70
	A156S	3.1	2.2	8.9	6.9	118
BILN-2061, VX-950	V102A	0.8	NA	1.0	NA	97

^a The fold resistance is calculated as the ratio of the mutant EC₅₀ to the wild-type EC₅₀ in either replicon or virus. Values represent the averages of two independent experiments.

^b The replication capacity is presented as the percentage of replication level of mutant to wild type (WT) in the replicon.

TABLE 4. Phenotypic and genotypic analyses of protease inhibitor resistant replicon clones

Inhibitor	Mutation	Frequency	Fold resistance ^a to:	
			BILN-2061	VX-950
BILN-2061	A156V	4/8	49	36
	D168A	2/8	72	0.7
	D168V	2/8	61	1.1
VX-950	A156V	3/8	96	45
	A156T	5/8	82	46

^a The fold resistance is calculated as the ratio of the mutant EC₅₀ to the wild-type EC₅₀ in the replicon. The values represent the averages of two independent experiments.

could potentially be selected in replicon systems since replicons do not spread between cells and resistant replicons only need to replicate to levels sufficient to confer G418 resistance for colonies to survive.

To investigate this hypothesis, all five protease inhibitor-resistant mutations selected using the virus system (T54A, A156S, A156G, D168V, and D168A) were examined for their replication capacity (RC) in the replicon and their fitness in viral infection assays. To determine replicon RC, we transfected the site-directed mutant replicons into Huh-Lunet cells and measured the luciferase levels at 3 days posttransfection. As shown in Table 3, all mutants had RC comparable to the wild type, except T54A which had a slightly lower replication capacity (70% of the wild type). In parallel, all five of these mutations were individually introduced into a molecular clone of the parental J6/JFH adapted virus genome. Parental and mutant viral RNAs were then transfected into Lunet-CD81 cells. Viral infection was monitored by immunostaining the transfected cells with an antibody against NS5A. At 2 days posttransfection, <20% of the cells were NS5A positive. By day 7, however, >90% of cells were NS5A positive for all transfected cultures, suggesting all mutant viruses were able to replicate and spread efficiently throughout the cultures (data not shown). The day 7 virus supernatants were harvested and titrated (Fig. 4A). All mutants produced virus titers greater than 5×10^4 TCID₅₀/ml and were thus comparable to the parental virus. These results indicate that all of the selected resistance mutations remain compatible with efficient viral infection *in vitro*.

To ensure that the mutations did not revert to wild-type during these assays, all mutant viruses were genotyped in the NS3 protease domain. The expected mutations were present in each viral supernatant, and no additional nucleotide changes were observed (data not shown). The resistance phenotypes of each virus were also confirmed in drug susceptibility assays (Table 3); these results indicated that resistance levels (fold EC₅₀ changes) for the site-directed mutant viruses were similar to those for the selected viruses (Table 1). More importantly, each of the mutant viruses was used to infect naive Lunet-CD81 cells at a low MOI (i.e., 0.05), and their infection kinetics were monitored and compared to that of the parental virus. As shown in Fig. 4B, similar to the parental virus, all of the mutant viruses expanded exponentially from day 1 to day 4 and reached virus titers of approximately 5×10^4 TCID₅₀/ml. After that, the virus production leveled off through day 7; during this period of time, all of the cultures were fully infected, as determined by immunostaining for NS5A (data not shown). In addition, all mutants had intracellular virus titer and specific infectivity comparable to the parental virus on day 7 (see Fig. S1a and b in the supplemental material). Taken together, the results of both transfection and infection studies indicate that the fitness of the resistant mutants is not markedly different from the wild type.

DISCUSSION

In this study, we present evidence that the HCV infection system is an efficient tool for identifying drug-resistant mutants. Using a tissue culture-adapted J6/JFH-1 virus and permissive Lunet-CD81 cells, infectious variants with significant

FIG. 4. Resistance mutations selected with genotype 2a virus are highly fit. (A) Efficient virus production by resistant variants after transfection. All five protease inhibitor-resistant mutations selected using the viral system (T54A, A156S, A156G, D168V, and D168A) were individually introduced into a molecular clone of the parental virus genome shown in Fig. 1A. Parental and mutant viral RNAs were then transfected into Lunet-CD81 cells. On day 7, virus supernatants were harvested and titrated. (B) Infection kinetics of resistant variants are similar to the parental virus. Each of the mutant viruses harvested from transfection experiment (shown in panel A) were used to infect naive Lunet-CD81 cells at a low MOI (i.e., 0.05). Infection kinetics were monitored and compared to that of the parental virus by harvesting and titrating the viral supernatants at days 1, 2, 4, 6, and 7 postinfection. The results represent the average of two independent experiments.

resistance to two HCV NS3 protease inhibitors (BILN-2061 or VX-950) were selected in approximately 40 days (Fig. 1 and Fig. 2). Phenotypic analyses, including NS5A immunostaining and EC₅₀ assays, confirmed that the selected viruses were resistant specifically to the protease inhibitors (Fig. 3 and Table 1). Genotypic analyses identified mutations in the NS3 protease domain that were subsequently confirmed to confer phenotypic resistance when transferred into JFH-1 subgenomic replicons and infectious viruses (Table 3). Furthermore, we also conducted multiple independent selections (five for BILN-2061 and four for VX-950) to investigate whether different patterns of resistance would emerge for the same drugs. Of the nine selections, none of the selected viruses had identical genomic sequences, and multiple distinct resistance mutations were identified to the protease inhibitors (Table 1). These results indicate that the *in vitro* infectious virus has a diverse quasispecies population, and the selection process supports the emergence of resistance along different mutational pathways.

The resistance mutations selected against BILN-2061 were D168V, D168A, and A156G. In contrast, T54A and A156S were identified in the four independent virus resistance selections against VX-950. To measure resistance levels, all five mutations were introduced individually into JFH-1 sub-

genomic replicons and infectious viruses (Table 3). Phenotypic analyses of these mutants confirmed that each of the five mutations conferred significant protease inhibitor resistance. Interestingly, drug susceptibility changes were very similar between the replicon and virus systems. Furthermore, the resistance levels of site-directed mutant viruses were also very similar to those of the selected viruses (Table 1). For instance, the EC_{50} s of BILN-2061 against the selected viruses carrying D168V were 2 to 3 μ M; similarly, the EC_{50} s against site-directed D168V mutant virus and replicon were $\sim$ 5 μ M. Overall, these results indicate that, at least for protease inhibitors, the infection system is a valid and efficient way to identify and assay drug-resistant mutations; furthermore, drug susceptibility changes agree well between replicon and virus systems.

Compared to the replicon, the infection system has a number of advantages for resistance selections. First, selection of resistant virus is time and resource efficient. In the present study, it took less than 40 days (and was as quick as 30 days at $20\times EC_{50}$) and only the maintenance of a few small flasks to identify multiple resistance mutations to the protease inhibitors (Fig. 2). The virus selection did not require tedious colony picking and labor-intensive cell line expansion as typically needed by replicon selections (38). Furthermore, it may be possible to select resistance mutations even faster through different selection schemes (e.g., starting at higher drug concentration or escalating drug concentrations more rapidly). Second, virus selections may facilitate the identification of resistance mutations to HCV inhibitors that are prone to selecting cell-based resistance (1, 29). This may include drugs that require multiple viral mutations, drugs that require metabolism (e.g., prodrugs), or drugs whose antiviral activity may involve host functions (e.g., cyclosporine or its analogues). Resistance selections for such inhibitors appear very challenging in the replicon system, due to the selection of host variants during prolonged passaging of the same cells under two selective pressures (antiviral plus G418). In contrast, during virus selections, supernatants are continuously passaged over naive cells, precluding the possibility that the host cells are adapting to the antiviral over time. Third, the virus selection approach makes it possible to identify resistance mutations for inhibitors that target steps other than replication (38). This will likely become increasingly important as antiviral strategies targeting HCV entry, assembly, NS2 or structural proteins emerge (21, 25).

To compare resistance selections between virus and replicon systems, we selected BILN-2061 and VX-950 resistance using genotype 2a replicons at antiviral pressures comparable to those used to select virus resistance (i.e., similar EC_{50} multiples). Although there was some overlap in mutation selection, there were also marked differences. The major resistance mutations for BILN-2061 were A156V (4/8), D168A (2/8), and D168V (2/8) in the replicon. D168A and D168V were also selected in the virus system; however, A156G but not A156V (the most frequent replicon resistance mutation) was selected in the virus system. Unfortunately, there are no clinical virology analyses available from BILN-2061 studies in genotype 2 patients to compare to our *in vitro* results (15, 28).

In the case of VX-950, viral resistance mutations were T54A and A156S. However, in genotype 2a replicons, A156T and A156V were identified as the common resistance mutations. A

recent resistance analysis from a study of VX-950 in genotype 2 patients indicated that T54A, R155K, and A156S emerged in patients with viral breakthrough (5). It is striking how similar the resistance mutations were between genotype 2 patients and our *in vitro* virus selection (identification of T54A and A156S). In contrast, the genotype 2a replicon mutations, A156T and A156V were not identified in genotype 2 patients. Although we did not observe R155K during our virus selections, this likely reflects the codon usage in JFH-1, since selection of R155K would require two nucleotide changes (data now shown); it is possible, and perhaps likely, that patients that selected this mutation during the VX-950 clinical study were genotype 2b, which would require only one nucleotide change. Overall, although the data are limited, it is remarkable how much more predictive the virus (versus the replicon) was for clinical resistance.

The selection of different resistance mutations in viruses and replicons led us to hypothesize that the two systems have different fitness constraints. Specifically, viruses must remain sufficiently fit to enable efficient spread throughout cultures to become the dominant species. However, this requirement is not imposed during replicon selection because there is no spread between cells (38). Less-fit mutants could potentially be selected in replicons as long as they replicate to levels sufficient to confer G418 resistance. To investigate this hypothesis, all five protease inhibitor resistance mutations selected in viruses (T54A, A156S, A156G, D168V, and D168A) were examined for their replication capacity using the replicon and for their fitness in viral infection assays. All mutants had replication capacity in the replicon system comparable to the wild type (70 to 118% of the wild-type replication levels) (Table 3). When viral RNAs were transfected into Lunet-CD81 cells, all mutant viruses were able to replicate and spread efficiently throughout the cultures (data not shown) and produced virus titers comparable to the parental virus ($>5 \times 10^4$ TCID₅₀/ml) (Fig. 4A). Furthermore, when naive Lunet-CD81 cells were infected with the mutant viruses at low MOI (i.e., 0.05), both virus secretion kinetics and peak titers were comparable to the parental virus. These results support our hypothesis that the selected resistance mutations are not markedly different from the wild type in terms of fitness. One caveat with these studies is that *in vitro* viral fitness constraints may differ from *in vivo* fitness constraints, especially when adapted viruses and target cells selected to be highly permissive for viral infection and replication are used.

Although the data in the present study highlight several advantages of viral resistance selections, a few notable limitations exist for this approach. All HCVcc strains that replicate to high titers *in vitro* are currently based on genotype 2a (8, 23, 33). Progress has been made in developing 1a/2a and 1b/2a chimeric viruses; however, these still use the nonstructural region of genotype 2a (23). Thus, resistance selection for the prime drug targets of NS3, NS5A, and NS5B would be limited to genotype 2a. Selections conducted on genotype 2a viruses may be problematic since many clinically relevant inhibitors lose significant potency against this genotype (22). However, this limitation may be overcome, at least in part, by the successful establishment of chimeric infectious viruses encoding nonstructural regions from other genotypes, e.g., NS3 protease and NS5A protein (9, 34). Furthermore, resistance mutations identified in genotype 2a HCVcc may not be representative of those that would emerge in genotype 1 patients; it is also

possible that key resistance mutations in genotype 1 may not be identified in genotype 2a due to genetic differences (e.g., codon usage). Nevertheless, our results are encouraging in that genotype 2a resistance for both BILN-2061 and VX-950 mapped to known loci from genotype 1 *in vitro* or clinical resistance studies. In fact, our selection of T54A with VX-950 marks the first time this mutation was frequently selected *in vitro* in any system and this mutation is frequently observed in genotype 1 patients.

In summary, while the HCV replicon remains useful for drug resistance studies, the HCVcc system is an efficient tool for drug resistance studies and has potential advantages over the replicon. Future studies with different classes of inhibitors will provide a more complete picture of advantages and potential disadvantages of the viral system for resistance studies. Given the intense drug discovery and development efforts ongoing for HCV, we anticipate that the virus infection system will play an important role in assessing drug resistance.

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