In Vitro Resistance Studies of Hepatitis C Virus Serine Protease Inhibitors, VX-950 and BILN 2061

STRUCTURAL ANALYSIS INDICATES DIFFERENT RESISTANCE MECHANISMS*

We have used a structure-based drug design approach to identify small molecule inhibitors of the hepatitis C virus (HCV) NS3-4A protease as potential candidates for new anti-HCV therapies. VX-950 is a potent NS3-4A protease inhibitor that was recently selected as a clinical development candidate for hepatitis C treatment. In this report, we describe in vitro resistance studies using a subgenomic replicon system to compare VX-950 with another HCV NS3-4A protease inhibitor, BILN 2061, for which the Phase I clinical trial results were reported recently. Distinct drug-resistant substitutions of a single amino acid were identified in the HCV NS3 serine protease domain for both inhibitors. The resistance conferred by these mutations was confirmed by characterization of the mutant enzymes and replicon cells that contain the single amino acid substitutions. The major BILN 2061-resistant mutations at Asp168 are fully susceptible to VX-950, and the dominant resistant mutation against VX-950 at Ala156 remains sensitive to BILN 2061. Modeling analysis suggests that there are different mechanisms of resistance to VX-950 and BILN 2061.

It is estimated that 170 million patients worldwide and about 1% of the population in developed countries are chronically infected with hepatitis C virus (HCV)1 (1). The majority of acute HCV infections become chronic, some of which progress toward liver cirrhosis or hepatocellular carcinoma (2, 3). The current standard of care is pegylated interferon plus ribavirin, which has a sustained viral response rate of 40–50% in genotype 1 HCV-infected patients, which accounts for the majority of the hepatitis C population in the United States and Japan, and of 80–90% in patients infected with genotype 2 or 3 HCV (4, 5) (for a review, see Ref. 6). Thus, more effective therapeutic drugs with fewer side effects and shorter treatment durations are needed for patients infected with HCV.

HCV is an enveloped, single-stranded RNA virus with a 9.6-kb positive-polarity genome, which encodes a polyprotein precursor of about 3,000 amino acids. The HCV polyprotein is proteolytically processed by cellular and HCV proteases into at least 10 distinct products, in the order of NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (for a review, see Ref. 7). NS3 serine protease and helicase as well as NS5B RNA-dependent RNA polymerase are believed to be components of a replication complex responsible for viral RNA replication and have been shown to be essential for the HCV replicon in chimpanzees (8). These HCV enzymes have been the major targets for the development of HCV-specific therapeutics during the past decade (for a review, see Ref. 9). However, successful discovery of a new HCV-specific drug candidate has been hampered by the lack of a robust, reproducible infectious virus cell culture system. The development of a HCV replicon system was achieved by Lohmann et al. (10) and subsequent optimization by several laboratories (11, 12) has enabled quantitative evaluation of the antiviral potency of HCV inhibitors.

The HCV NS3-4A protease is responsible for cleavage at four sites within the HCV polyprotein to generate the N termini of the NS4A, NS4B, NS5A, and NS5B proteins (13–17). It has been shown that the central region (amino acids 21–30) of the 54-residue NS4A protein is essential and sufficient for the enhancement of proteolytic activity of the NS3 serine protease (18–22). The central region of NS4A forms a tight heterodimer with the NS3 protein (21), for which the first x-ray crystal structure was solved in 1996 (23). BILN 2061 is the first HCV serine protease inhibitor (PI) in clinical trials for hepatitis C (24). In phase I trials, a 2–3-log reduction of HCV viral load was observed after a 2-day treatment, which provided the first proof-of-concept evidence that HCV NS3-4A protease inhibitors could be a new therapeutic option for hepatitis C patients (24). Recently, another HCV NS3-4A protease inhibitor, VX-950 (25), was selected as a clinical candidate for hepatitis C (26).

Resistance to specific antiviral drugs is a major factor limiting the efficacy of therapies against many retroviruses or RNA viruses, due to the error-prone nature of the viral reverse transcriptases or RNA-dependent RNA polymerases. As new HCV-specific inhibitors enter clinical trials, resistance could become a major problem in patients treated with drugs targeting HCV NS3-4A serine protease or NS5B RNA polymerase. In this report, we used the HCV subgenomic replicon system to identify resistance mutations against two HCV protease inhibitor clinical candidates, BILN 2061 and VX-950. The in vitro resistance mutations selected against either inhibitor resulted in a significant reduction in susceptibility to the inhibitor itself. However, the primary resistance mutations against BILN 2061 were fully susceptible to VX-950, and the major resistance mutation against VX-950 remained sensitive to BILN 2061.

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The abbreviations used are: HCV, hepatitis C virus; PI, protease inhibitor; DMEM, Dulbecco’s modified essential medium; FBS, fetal bovine serum; RT, reverse transcriptase; FRET, fluorescence resonance energy transfer; HIV, human immunodeficiency virus.
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EXPERIMENTAL PROCEDURES

Plasmid Construction—A DNA fragment encoding residues Ala1–Ser106 of N502 (GenBank accession number CAA46913) was obtained by PCR from the HCV Con1 replicon plasmid, P1 neo/neo/NS3/3'-tet (renamed as pBR322-HCV-Neo in this study) (10) and inserted into pBE111 for expression of the HCV proteins with a C-terminal hexahistidine tag in Escherichia coli. Resistance mutations against the HCV NS3-4A PI were introduced into this construct by PCR-based, site-directed mutagenesis. To generate the HCV replicon containing the PI-resistant mutations, a 1.2-kb HindIII/BstXI fragment derived from the HCV Con1 replicon was subcloned into a TA cloning vector, pCR2.1 (Invitrogen). The PI-resistant mutations in the NS3 serine protease domain were introduced into the pCR2.1 vector containing the HindIII/BstXI HCV fragment by PCR, and a 579-bp BglII/BstXI fragment containing the mutated residue was subcloned back into a second generation pCR1 replicon plasmid containing three adaptive mutations, pBR322-HCV-Neo-mADE (see below). All constructs were confirmed by sequencing.

Generation of HCV Replicon Cells—The Con1 subgenomic replicon plasmid, pBR322-HCV-Neo, was digested with ScaI (New England Biolabs). Full-length HCV subgenomic replicon RNA was generated from the linearized DNA template using a T7 Mega-script kit (Ambion) and treated with DNase to remove the template DNA. The run-off RNA transcripts were electroporated into Huh-7 cells, and stable HCV replicon cell lines were selected with 0.25 or 1 mg/ml G418 (Geneticin) in DMEM containing 10% FBS. HCV replicon-stable cells were maintained in DMEM, 10% FBS, and 0.25 mg/ml G418. During the course of generation of the HCV subgenomic replicon stable cell lines, several different patterns of adaptive mutations were identified. One of these patterns contains three substitutions in the HCV nonstructural proteins, which were introduced into the original pBR322-HCV-Neo plasmid by site-directed mutagenesis to generate the second generation subgenomic replicon plasmid, pBR322-HCV-Neo-mADE. When the T7 run-off RNA transcripts from the ScaI-linearized pBR322-HCV-Neo-mADE plasmid were electroporated into Huh7 cells, stable replicon cell colonies were formed at a much higher efficiency than the original Con1 replicon RNA. The resistance mutations identified in this study were introduced into the pBR322-HCV-Neo-mADE replicon plasmid by site-directed mutagenesis. Stable replicon cell lines were generated using the T7 transcripts derived from either wild type pBR322-HCV-Neo-mADE or the ones with the resistance mutations.

IC50 Determination of HCV PIs in the HCV Replicon Cell Assay—HCV Con1 subgenomic replicon cells were maintained in DMEM containing 10% FBS and 0.25 mg/ml G418. The day prior to the assay, 10,000 HCV replicon cells/well were plated in a 96-well plate in DMEM plus 10% FBS. The next day, the medium was removed, and a compound serially diluted in DMEM, 2% FBS, and 0.5% MeSO was added. The replicon cells were incubated with the compounds for 48 h. Total cellular RNA was extracted using RNeasy-96 (Qiagen), and the copy number of the replicon RNA was determined by a quantitative, real-time RT-PCR (Taqman) assay. The cytotoxicity of the compounds was measured using a mitochondrial enzyme-based cell viability assay, CellTiter 96 AQone Solution Cell Proliferation Assay (Promega). The IC50 and CC50 values of the compounds were calculated using four-paramater curve fitting (SoftMax Pro).

Selection of HCV PI-resistant Replicon Cells—The HCV Con1 subgenomic replicon stable cells were serially passaged in the presence of 0.25 mg/ml G418 and slowly increasing concentrations of VX-950 (series A) or BILN 2061 (series B). The concentrations of VX-950 ranged from 3.5 μM (or 10 × IC50) in the 48-h assay (see above), to 28 μM (80 × IC50). For BILN 2061, the starting concentration was 80 nM (80 × IC50), and the final concentration was 12 μM (12,500 × IC50). During this course, selection, replicon cells were split twice per week when a 70–90% confluence was reached. Fresh HCV PI was added every 3–4 days regardless of the cell culture status was split.

Identification of HCV PI Resistance Mutations—During the selection of HCV PI-resistant replicon cells, pellets were collected every time the replicon cells were split. Total cellular RNA was extracted using the RNeasy miniprep kit (Qiagen). A 1.7-kb-long cDNA fragment encompassing the HCV NS3 serine protease region was amplified with a pair of HCV-specific oligonucleotides (5’-CCTTCTAAGCTCTTCTTG-3’ and 5’-CTCATATGGTCTGGAGT-3’) using the Titan One-Step RT-PCR kit (Roche Applied Science). The amplified products were purified using the QIAquick PCR purification kit (Qiagen). To monitor the emergence of the HCV PI-related mutations in the HCV NS3 serine protease domain during the selection, the purified 1.7-kb RT-PCR products of PI-treated replicons from several different culture time points were subjected to sequence determination. To determine the frequency of PI-resistant mutations, the 1.7-kb RT-PCR products of HCV RNA of the VX-950 or BILN 2061-resistant replicon cells were ligated into the TA cloning vector pCR2.1 (Invitrogen). For each time point, multiple individual bacterial colonies were isolated, and the HCV NS3 protease coding region of the purified plasmid DNA was sequenced.

Expression and Purification of the HCV NS3 Serine Protease Domain—Each of the expression constructs for the HCV NS3 serine protease domain containing the wild type sequence or the resistance mutant was subcloned into the pCR2.1 (Invitrogen) and H18528 for expression in E. coli cells (Stratagene). Freshly transformed cells were grown at 37 °C in a BHI medium (Difco) supplemented with 100 μg/ml carbenicillin and 35 μg/ml chloramphenicol to an optical density of 0.75 to 600 nm. Induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside was performed for 4 h at 24 °C. Cell pastes were harvested by centrifugation and flash frozen at −80 °C prior to protein purification. All purification steps were performed at 4 °C. For each of the HCV NS3 protease proteins, 100 g of cell paste was lysed in 1.5 liters of buffer A (50 mM HEPES (pH 8.0), 300 mM NaCl, 0.1% n-octyl-β-D-glucopyranoside, 5 mM β-mercaptoethanol, 10% (v/v) glycerol) and stirred for 30 min. The lysates were homogenized using a microfluidizer (Microfluidics, Newton, MA) and followed by ultracentrifugation at 55,000 g for 48 h at 4 °C. Imidazole was added to the supernatants to a final concentration of 5 ml and then washed with 20 column volumes of buffer A plus 5 ml imidazole. The HCV NS3 proteins were eluted in buffer A containing 300 mM imidazole. The eluates were concentrated and loaded onto a Hi-Load 16/60 Superdex 200 column, pre-equilibrated with buffer A. The appropriate fractions of the purified HCV proteins were pooled and stored at −80 °C.

Enzymatic Assays for the HCV NS3 Serine Protease Domain—Enzymatic activity was determined using a modification of the assay described by Taliani et al. (27). An internally quenched fluorogenicdepsipeptide (FRET substrate), Ac-DED(EDANS)EE(Abu)KAS[COO][COO]SK (DABCYL)-NH2, was purchased from AnaSpec Inc. (San Jose, CA). The assay was run in a continuous mode in a 96-well microtiter plate format. The buffer was composed of 50 mM HEPES (pH 7.8), 100 mM NaCl, 2% glycerol, 5 mM dithiothreitol, and 15 μM KRAA peptide (KGKSVVIVGRYLSDK). The KRAA peptide represents the central region of the NS4A cofactor from genotype 1a with lysine residues added for improved solubility (28). The reaction was initiated by the addition of the FRET substrate after a 10-min preincubation of the buffer components with a 2 mM concentration of the NS3 protease at room temperature. The reaction was monitored at 30 °C for 20 min in a Molecular Devices fluorometric plate reader. Excitation and emission wavelengths were 355 and 495 nm, respectively. For determination of substrate kinetic parameters, concentrations of the FRET peptide were varied from 0.5 to 7.0 μM. Intermolecular quenching was not observed in this range. The substrate kinetic parameters, Kcat and Vmax, were determined by fitting the data to the Michaelis-Menten equation. Inhibition constants (K1) were determined by titration of enzyme activity using the assay described above, except that compound dissolved in MeSO (no greater than 2% (v/v) MeSO; solvent only was used as control) was added to the buffer components and enzyme after the initial 10-min preincubation as described above. This mixture was incubated for an additional 15 min at room temperature prior to an incubation in the FRET substrate for 20 min at 30 °C. Seven or eight concentrations of compound were assayed, and the resulting data were fitted to the integrated form of Morrison’s equation for tight binding inhibition (29). All substrate and inhibitor data were fitted using Marquardt-Levenberg nonlinear regression with GraphPad Prism software.

Bilining—VX-950 and BILN 2061 were modeled into the active site of the NS3 serine protease domain using the crystal structure of a full-length HCV NS3 protein fused with a NS4A polypeptide, which was published by Yao et al. (30) (Protein Data Bank code 1CU1). The coordinates of the protease domain of the A segment in this structure showed that the C-terminal strand of the NS3 protein binds in the catalytic subsite of the protease. The termini of this strand is located near active site residues His27, Asp59, and Ser139 as well as the backbone amides of residues 137 and 139, which form the oxyanion hole. Additionally, the last six residues (residues

8 S. Chamber, personal communication.
9 C. Lin, unpublished results.
626–631) of the NS3 protein form an extended, antiparallel β strand along the edge of the E2 strand of the protease β barrel (31) and makes 12 backbone-to-backbone hydrogen bonds. A product-based inhibitor like BILN 2061 is expected to bind to the NS3 protease in a similar fashion. Therefore, we utilized the coordinates of this crystal structure to build our models of inhibitor-protease co-complexes. BILN 2061 molecule was built using QUANTA molecular modeling software (Accelrys Inc., San Diego, CA), and manually docked into the active site such that its carbonyl group overlaps with the C-termoinal carbonylate of the full-length NS3 protein. The inhibitor molecule was then rotated such that it makes all of the following backbone hydrogen bonds: P1 NH with Arg155 carbonyl, P3 carbonyl with Ala157 NH, and P3 NH with Ala157 carbonyl. This mode of binding placed the large P2 group of the BILN 2061 in direct clash with the Arg155 side chain. To avoid the clash, the Arg155 side chain was modeled in an extended conformation, which was observed in a crystal structure of NS3 protease complexed with a close analogue of BILN 2061 (32). The inhibitor was energy-minimized in two stages. In the first stage, only the inhibitor and the side-chain atoms of Arg155, Arg157, and Asp156 of the protease were allowed to move during energy minimization for 1000 steps. In the second stage, all of the side-chain atoms of the protease were allowed to move along with the inhibitor for 1000 additional steps. This modeled structure closely mimics the published structure of the BILN 2061 analog (32). A similar procedure was adopted for modeling VX-950 into the NS3 protease active site. VX-950 was modeled as a covalent adduct with si-face attachment of the Ser139 side chain to the keto carbonyl of the inhibitor. This binding mode was observed for analogous ketoamide inhibitors (33) and ketoacid inhibitors (34). The main chain of the inhibitor was overlaid with residues 626–631 of the C-terminal strand of the full-length NS3 protein such that it makes all of the following backbone hydrogen bonds: P1 NH with Arg155 carbonyl, P3 carbonyl with Ala157 NH, P3 NH with Ala157 carbonyl, and P4 cap carbonyl with NH of Cys159. In this binding mode, the P2 group of VX-950 was placed in the S2 pocket without any need to move the Arg155 side chain. The t-butyl and the cyclohexyl groups were placed in S3 and S4 pockets, respectively. To be consistent, we used the same two-stage energy minimization protocol used for the BILN 2061 model. The side chain of Asp156 is exposed to solvent. The valine side chain of the D168V mutant can adopt three canonical conformations with x1 = 60, -60, or 180°. All three orientations of the Val168 side chain were modeled. The interaction energy of the D168V mutant enzyme and the inhibitor was minimized by allowing the inhibitor and Val168 atoms to move while fixing positions of all of the other atoms of the protein molecule. In all cases, the Val168 side chain does not cause any steric clash with the inhibitor atoms. The serine mutation at Ala156 was modeled by the following procedure. The Ala156 side chain is in van der Waals contact with the P2 group of both of the inhibitors. The serine side chain of the A156S mutant was modeled at three canonical conformations of x1 = 60, -60, and 180°, and the energy was minimized by holding the conformation of the rest of the protein fixed. These models were used to examine the effects of this mutation on inhibitor binding. The x = 60° conformation was found to have the lowest energy as it forms a hydrogen bond with the neighboring Asp155 carbonyl, but it causes the maximal number of unfavorable contacts with both inhibitors. The 60 and 180° conformations are energetically equivalent, but the 60° conformation has fewer unfavorable contacts and was used in our analysis.

RESULTS

Development of Resistance to VX-950 in HCV Replicon Cells—VX-950 (Fig. 1) (25) was recently selected as a clinical candidate for hepatitis C treatment (26). VX-950 is a reversible, covalent inhibitor of the HCV NS3/4A serine protease. Although competitive with the peptide substrate in the active site, it exhibits apparent noncompetitive inhibition as a result of its tight binding properties and time-dependent inhibition mechanism.4 Incubation of the HCV Con1 subgenomic replicon cells with VX-950 resulted in a concentration-dependent decline of the HCV RNA level, as measured by the real time RT-PCR (Taqman) method (Fig. 2B). The IC50 value of VX-950 is 354 nM in the 48-h assay.

To identify VX-950 resistance mutations, the Con1 subgenomic replicon cells were serially passed in the presence of G418 and increasing concentrations of VX-950. Replicon cells were split, and fresh VX-950 was added to medium twice a week, as indicated by filled diamonds. The open rectangle indicates the time period in which the replicon cells had little or no overall growth accompanied by a concurrent massive cell death. Total cellular RNA of replicon cells at various time points (indicated by open arrows) during the resistance selection was extracted and the RT-PCR product covering the HCV NS3 serine protease was sequenced either directly or after being subcloned into the TA vector. B, dose-dependent inhibition of the wild type (filled triangle) or the series A (VX-950-resistant) (open circle) replicon cells at day 56 by VX-950 was shown. HCV RNA level was determined after 48-h incubation with VX-950.

^4 C. A. Gate and Y.-P. Luong, unpublished data.
Replicon cells in series A grew normally for the first 10 days in the presence of 3.5 μM VX-950. After 10 days, the series A cells grew significantly more slowly, and massive cell death was observed between days 10 and 17 (Fig. 2A). Normal growth did not resume until day 21. The IC50 of VX-950 against the series A replicon cells at day 56 was determined to be 8.1–12.0 μM, which is 23–34-fold higher than the IC50 (354 nM) against wild-type replicon cells (Fig. 2B).

Total cellular RNA from the series A cells at days 10, 21, and 56, was extracted and subjected to RT-PCR to amplify the coding region of the NS3 serine protease domain. The RT-PCR product was bulk-sequenced to identify the position(s) of potential mutations that could be responsible for the observed reduction in sensitivity to VX-950. No VX-950-related mutation was observed in the NS3 serine protease domain of the series A replicon cells at day 10 when compared with the wild type Con1 replicon cells cultured in the absence of VX-950. At days 21 and 56 in series A, substitutions at Ala156 in the protease domain were observed, suggesting that mutations at residue 156 might be critical for the reduced sensitivity to VX-950. No mutation was found at any of the four proteolytic sites in the HCV nonstructural protein region that are cleaved by the NS3/4A serine protease. To delineate the identity and frequency of the substitutions, a 1.7-kb RT-PCR product of the series A replicon cells at day 7 or 56 was subcloned into the TA vector, and multiple clones were sequenced for both samples. All clones derived from the day 7 samples contained the wild type Ala156. In the day 98 sample of the series A replicon cells, which had been cultured in the presence of 28 μM VX-950 for 63 days, 79% or 60 out of 76 clones had an alanine to serine (A156S) substitution.

Development of Resistance to BILN 2061 in HCV Replicon Cells—Another HCV NS3/4A protease inhibitor, BILN 2061 (Fig. 1) is the first PI to demonstrate efficacy in hepatitis C patients (24). HCV replicon cells resistant to BILN 2061 (series B) were selected in a similar manner as for VX-950. Again, wild-type Con1 subgenomic HCV replicon cells were serially passed in the presence of 0.25 mg/ml G418 and slowly increasing concentrations of BILN 2061 (Fig. 3A). Series B replicon cells grew normally for the first 7 days in the presence of 80 nM BILN 2061 or 80-fold above the IC50. However, the proliferation of series B cells slowed down significantly after day 7, and massive cell death was observed between days 7 and 17. As before, normal growth did not resume until day 21. BILN 2061 had an IC50 value of 1.0–1.8 μM against the series B cells at day 59, which is 1,000–1,800-fold higher than the IC50 (1 nM) against wild-type replicon cells (Fig. 3B).

No BILN 2061-related mutation was observed in the NS3 serine protease domain at day 7. By day 24, a variety of substitutions were observed at amino acid 168 of the NS3 protein, suggesting that substitutions at residue 168 may account for the resistance against BILN 2061. No mutation at the four sites in the HCV nonstructural protein region that are cleaved by the NS3/4A serine protease was observed. To determine the frequency of various substitutions at the NS3 residue 168, the HCV serine protease of the series B replicon at day 98, which was cultured in the presence of 3.2 μM BILN 2061, was sequenced. 60 of 94 clones or 64% had an Asp168 → Val (D168V) substitution, and 23 clones or 24% had an Asp168 → Ala (D168A) mutation.

The Dominant VX-950-resistant Mutant, A156S, Remains Susceptible to BILN 2061—To confirm whether the observed mutations at either Ala156 or Asp168 are sufficient to confer resistance against VX-950 or BILN 2061, respectively, site-directed mutagenesis was used to introduce each individual mutation at residue 156 or 168 into the wild type NS3 protease domain.

The kinetic parameters for the FRET substrate for the wild type NS3 protease domains from genotype 1a and 1b were identical (Table I) under our assay conditions. Although the NS4A peptide co-factor was from HCV genotype 1a, no discernible difference in the kinetic parameters was observed. This is consistent with molecular modeling, which suggests that the conservative variations in the central region of NS4A between genotypes 1a and 1b do not affect the interaction between the NS4A core peptide and the NS3 protease domain. K values of VX-950 and BILN 2061 were determined using genotypes 1a and 1b wild type protease, and there were no statistically significant differences between the two wild type proteases (Table II).

The kinetic parameters of the FRET substrate for the A156S mutant protease were virtually the same as those of the wild type protease (Table I). However, the K value of VX-950 was 2.9 μM against the A156S mutant protease, which is 29-fold higher than that against the wild type protease (0.1 μM) (Table II). BILN 2061 had a K value of 112 nM against the A156S mutant, which was 6-fold higher than that against the wild type protease, 19 nM (Table II).

The HCV RNA level in the replicon cells containing the A156S substitution was similar to that of wild type replicon
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TABLE I

| HCV protease | Km | kcat | kcat/Km |
|--------------|----|------|---------|
|              | μM | s⁻¹ | s⁻¹/μM |
| Wild type (1a) | 1.3 | 1.2 | 8.97 × 10⁵ |
| Wild type (1b) | 1.1 | 1.0 | 9.47 × 10⁵ |
| A156S        | 0.9 | 0.6 | 6.64 × 10⁵ |
| D168V        | 1.2 | 0.6 | 4.98 × 10⁵ |
| D168A        | 2.0 | 0.3 | 1.50 × 10⁵ |

TABLE II

| Mutant | KI | BILN 2061 | VX-950 |
|--------|----|-----------|--------|
|        | μM |           |        |
| Wild type (1a) | 0.006 | 0.047 |        |
| Wild type (1b) | 0.019 | 0.100 |        |
| A156S | 0.112 | 2.9 |        |
| D168V | >1.2 | 0.043 |        |
| D168A | >1.2 | 0.150 |        |

The K values of VX-950 and BILN 2061 were determined against the five purified HCV NS3 serine protease domains, including the wild type proteases from genotype 1a or 1b as well as three mutants, A156S, D168V, and D168A, of the genotype 1b, using the KK-NS4A peptide and the FRET substrate. The average of two independent assays is shown.

The Major BILN 2061-resistant Mutants, D168V and D168A, Remain Fully Susceptible to VX-950—The substrate kinetic parameters were not affected by the D168V mutation and showed only minor changes (less than 10-fold) for the D168A mutant as indicated by the comparison of the kcat and kcat/Km values of the wild type and the two mutant NS3 serine proteases (Table I). Similarly, no significant effect of either substitution at Asp168 was observed on the Ki value of VX-950 (Table II). However, the substitution of valine or alanine for aspartic acid at position 168 resulted in a mutant NS3 protease that was not inhibited by up to 1.2 μM BILN 2061 (Table II). These data indicate that either mutant protease is at least 63-fold less susceptible to BILN 2061 as compared with the wild type protease. The actual magnitude of resistance cannot be determined, since BILN 2061 was not fully soluble at concentrations greater than 1.2 μM in the assay buffer, as measured by the absorbance at 650 nm. The D168V or D168A mutation was also introduced into the wild type HCV replicon by site-directed mutagenesis, and a stable replicon cell line carrying either substitution was generated. BILN 2061 had an IC50 of 5.09 μM against the D168V replicon cells, which is more than 1,300 times higher than that against wild type replicon cells (4 μM) (Table III). The IC50 of BILN 2061 was 1.86 μM against the D168A mutant replicon. There was little change in IC50 values of VX-950 against the D168V, the D168A, and the wild type replicon cells (data not shown), which is consistent with the similar enzymatic catalytic efficiency of the A156S mutant and the wild type NS3 serine proteases. The IC50 value of VX-950 against the A156S replicon cells was 65 μM, which is 12 times higher than that against the wild type replicon cells (0.40 μM) (Table III). The difference between the IC50 values of BILN 2061 against the A156S (7 μM) and the wild type replicon (4 μM) cells was not significant (Table III).

In this study, we identified in vitro resistance mutations in HCV replicons against two clinical candidates for the hepatitis C treatment, VX-950 and BILN 2061, both peptidomimetic inhibitors of the HCV NS3-4A protease. The dominant resistance mutation observed against VX-950 was a substitution of Ala156 (green), Asp168 (orange), and Arg123 (orange). The Arg155 side chain of the BILN 2061-protease model (R155-BI) is shown in cyan, and that of the VX-950-protease model (R155-VX) is shown in orange. These side chains are highlighted with dot surfaces. The catalytic triad, Ser135-His28, and Asp102, are shown in gray. This figure was created by PyMOL Molecular Graphics Systems; DeLano Scientific LLC (San Carlos, CA).

The Major BILN 2061-resistant Mutants, D168V and D168A, Remain Fully Susceptible to VX-950—The substrate kinetic parameters were not affected by the D168V mutation and showed only minor changes (less than 10-fold) for the D168A mutant as indicated by the comparison of the kcat and kcat/Km values of the wild type and the two mutant NS3 serine proteases (Table I). Similarly, no significant effect of either substitution at Asp168 was observed on the Ki value of VX-950 (Table II). However, the substitution of valine or alanine for aspartic acid at position 168 resulted in a mutant NS3 protease that was not inhibited by up to 1.2 μM BILN 2061 (Table II). These data indicate that either mutant protease is at least 63-fold less susceptible to BILN 2061 as compared with the wild type protease. The actual magnitude of resistance cannot be determined, since BILN 2061 was not fully soluble at concentrations greater than 1.2 μM in the assay buffer, as measured by the absorbance at 650 nm. The D168V or D168A mutation was also introduced into the wild type HCV replicon by site-directed mutagenesis, and a stable replicon cell line carrying either substitution was generated. BILN 2061 had an IC50 of 5.09 μM against the D168V replicon cells, which is more than 1,300 times higher than that against wild type replicon cells (4 μM) (Table III). The IC50 of BILN 2061 was 1.86 μM against the D168A mutant replicon. There was little change in IC50 values of VX-950 against the D168V, the D168A, and the wild type replicon cells (Table III).
the co-complex model of BILN 2061. The Ala^{156} side chain is in van der Waals contact with the P2 group of these inhibitors. In our A156S mutant model, the terminal oxygen of Ser^{166} is too close to the P4 cyclohexyl group of VX-950, and it is also close to the terminal cyclopentyl cap of BILN 2061. Since the cyclopentyl cap of BILN 2061 is at the flexible end of the inhibitor, it can be moved away from this unfavorable contact without losing much of the binding. A similar movement of the P4 cyclohexyl group of VX-950 causes destabilization of the interactions between the inhibitor and S4 and S5 substates of the protease. Therefore, a larger loss in binding is expected for VX-950 than for BILN 2061 with the A156S mutant protease.

Asp^{168} is located in the P2 strand of the NS3 protease structure and is involved in salt bridge interactions with the side chains of Arg^{123} and Arg^{155} (Fig. 4). It is also part of the S4 binding pocket. The aliphatic part of this side chain is in van der Waals contact with the terminal cyclopentyl group of BILN 2061, which is not expected to be affected by the D168V mutation, since a valine side chain at this position does not cause any steric clash with the inhibitor. However, this D168V substitution results in the loss of salt bridge interaction with the Arg^{155} side chain on the neighboring E2 strand, which in turn makes multiple contacts with the large P2 group of BILN 2061 in our model. The conformation of the Arg^{155} (Fig. 4, color-coded in cyan) in the model of the BILN 2061-wild type NS3 protease complex is no longer energetically favored in the D168V mutant for two reasons. First, it cannot remain close to the backbone of the E2 strand in the absence of the salt bridge interaction between Arg^{155} and Asp^{168}. Second, an uncompensated and solvent-exposed positive charge of Arg^{155} side chain will seek a larger solvation shell, as observed in the crystal structures of the apoprotease and the two protease-inhibitor complexes that are available in the Protein Data Bank (codes 1DY8 and 1DY9) (34). These conformations of Arg^{155} are in direct clash with the P2 quinoline group of BILN 2061 and destabilize its binding. Therefore, substitution of Asp^{168} with any amino acid, other than glutamate, will disrupt the salt bridge interactions with Arg^{155} and result in reduction of BILN 2061 binding. On the other hand, the conformation of Arg^{155} in the two published crystal structures of the NS3 protease-inhibitor complex is similar to that in our VX-950-protease complex model (color-coded in orange in Fig. 4). In addition, this conformation of Arg^{155} confers stabilization of VX-950 binding as it allows the maximal number of van der Waals contacts between the Arg^{155} side chain and the inhibitor. Therefore, VX-950 is not expected to be affected by the substitutions at Asp^{168} as compared with BILN 2061.

A Blast search of the GenBank™ data base was conducted using the amino acid sequences of the HCV NS3 protease domain from the Con1 replicon. A total of 437 HCV isolates from all six major genotypes were identified, and Ala^{156} is absolutely conserved in all of the isolates. The lack of polymorphism at amino acid 156 of the NS3 serine protease suggests that substitution at this position might be unfavorable for viral replication. It remains to be examined if the substitution at Ala^{156} has a deleterious effect on the virus life cycle. Three naturally occurring variants were observed at amino acid 168 of the HCV NS3 serine protease. The vast majority (over 96%) of the 437 HCV isolates reported in GenBank™ have aspartic acid at position 168. Glutamate was found at the residue 168 in 10 isolates of genotypes 1b or 5. Since glutamate 168 is expected to be able to maintain the salt bridge with Arg^{155} and Arg^{123}, these genotype 1b or 5 isolates with Glu^{168} would remain susceptible to both HCV protease inhibitors, BILN 2061 and VX-950. However, six isolates of genotype 3 reported in GenBank™ have glutamate at position 168. For these geno-

type 3 HCV strains, no salt bridge between Gln^{168} and Arg^{155} is expected to form, and the Arg^{155} side chain would cause interference with binding of the large P2 group of BILN 2061 as was suggested by our modeling analysis of the D168V mutant. The Gln^{168} NS3 serine protease is expected to have a reduced susceptibility against BILN 2061 but not against VX-950. However, these six isolates of genotype 3 with a Gln^{168} have a threonine instead of arginine at position 123. It is not obvious whether such a double mutant will have the same differential effect on the binding of the protease inhibitors, VX-950 and BILN 2061.

One of the major factors limiting the efficacy of virus-specific therapies against many retroviruses and RNA viruses is the development of resistance to antiviral drugs. Resistance to inhibitors of HIV reverse transcriptase or protease is caused by specific mutations in the viral enzymes (for a review, see Ref. 36). Due to the error-prone nature of the HIV reverse transcriptase, resistance mutations were selected under the pressure of HIV inhibitors in patients who were on monotherapy. It is estimated that all possible single mutations can be randomly generated within 1 day in an HIV-infected patient. Although elimination or cure of HIV infection in patients remains an elusive task, multidrug combination or “cocktail” therapies are much more effective than monotherapy to reduce HIV viral load and to suppress the emergence of resistance mutations. Drug-resistant strains of hepatitis B virus containing specific mutations in the viral polymerase are the primary cause of treatment failure of lamivudine or 3TC, the first approved hepatitis B virus-specific drug. It was reported that the frequency of resistance mutations against 3TC increased from 24% in the first year to 67% in the fourth year in the hepatitis B patients treated with lamivudine (37).

From these lessons, it is clear that as new HCV-specific inhibitors enter clinical trials, resistance could become a major problem in patients treated with drugs targeting the HCV enzymes. The replication rate of HCV in patients was reported to be in the range of 10^{10} to 10^{12} viral particles per day, higher than the viral replication rate in HIV-infected patients (38). In this study, we demonstrate that the major in vitro resistant mutations against BILN 2061 remain fully susceptible to VX-950, and the dominant VX-950 resistance mutation is still sensitive to BILN 2061. In vitro resistance mutations against the HCV polymerase inhibitors have also been identified in the replicon system (39, 40). These studies suggest that future hepatitis C therapy involving small molecule inhibitors of HCV enzymes might require multidrug combination, as in the case of the current HIV treatments. Clearly, combinations of small molecule, HCV-specific inhibitors with either interferon α or other HCV-specific inhibitors will represent an important strategy to suppress the emerging of resistance and increase the efficacy of HCV therapy. Our current findings should prove to be useful in optimization of future protease inhibitor-based therapies against hepatitis C.

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In Vitro Resistance Studies of Hepatitis C Virus Serine Protease Inhibitors, VX-950 and BILN 2061: STRUCTURAL ANALYSIS INDICATES DIFFERENT RESISTANCE MECHANISMS

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