JBC Papers in Press. Published on September 12, 2005 as Manuscript M506462200

IN VITRO STUDIES OF CROSS-RESISTANCE MUTATIONS AGAINST TWO HEPATITIS C VIRUS SERINE PROTEASE INHIBITORS, VX-950 AND BILN 2061

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Running Title: *In vitro* HCV NS3•4A protease inhibitor resistance mutations

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VX-950 is a potent, small-molecule, peptidomimetic inhibitor of the hepatitis C virus (HCV) NS3•4A serine protease, which has recently demonstrated antiviral activity in a phase I trial in patients chronically infected with genotype 1 HCV. In a previous study, we described *in vitro* resistance mutations against either VX-950 or another HCV NS3•4A protease inhibitor, BILN 2061, which has also demonstrated antiviral activity in phase I clinical trials in hepatitis C patients. Single amino acid substitutions were identified in the HCV NS3 serine protease domain that conferred drug resistance, distinct for either inhibitor. The dominant resistance mutation against VX-950, A156S, remains sensitive to BILN 2061. The major BILN 2061-resistant mutations, D168V and D168A, are fully susceptible to VX-950. Modeling analysis suggested that there are different mechanisms of resistance for these mutations, induced by VX-950 or BILN 2061, respectively. In the current study, we identified mutations that are cross-resistant to both HCV protease inhibitors. The cross-resistance conferred by substitutions of Ala[156] with either Val or Thr was confirmed by characterization of the purified enzymes and reconstituted replicon cells that contain the single amino acid substitution, A156V or A156T. Both cross-resistance mutations, A156V and A156T, displayed significantly diminished fitness (or replication capacity) in a transient replicon cell system.

Chronic hepatitis C has become one of the most common liver diseases and is estimated to affect 170 million patients worldwide and about 1% of the population in developed countries (1). In many patients, hepatitis C virus (HCV) infection leads to liver cirrhosis or hepatocellular carcinoma (2,3). The current standard of care, a 48-week treatment with pegylated interferon (IFN)-α in combination with ribavirin, has a sustained viral response rate of 40 to 50% in the difficult-to-treat genotype 1 HCV-infected patients (4,5) [for a review, see Ref. (6,7)], which accounts for the majority of the hepatitis C patient population in the developed countries. A more effective treatment with fewer side effects and shorter treatment durations is urgently needed for HCV-infected patients.

HCV is an enveloped virus containing a single-stranded, positive-polarity RNA that encodes a polyprotein precursor of about 3,000 amino acids. The HCV polyprotein is proteolytically processed by cellular and viral proteases into at least 10 distinct products, in the order of NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH [for a review, see Ref. (8)]. The structural proteins are processed by host signal peptidases, whereas the nonstructural (NS) proteins are processed by two virally encoded proteases, the NS2•3 and NS3•4A proteases. The NS2•3 protease is responsible for the cleavage between NS2 and NS3 proteins, whereas the NS3•4A serine protease is responsible for the release of the remaining four nonstructural proteins, NS4A, NS4B, NS5A, and NS5B (9-13).

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The essentiality of the NS3•4A serine protease for viral replication has been demonstrated by the nonproductive infection following liver inoculation of chimpanzees with a genomic HCV RNA containing a mutation in the NS3 protease active site (14). 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 (15-19). The central region of NS4A forms a tight heterodimer with the NS3 protein (18), for which the first X-ray crystal structure was solved in 1996 (20). The NS3•4A serine protease has been one of the major targets for the development of HCV-specific therapeutics during the past decade [for a review, see Ref. (21)]. VX-950, a small-molecule, potent, and selective inhibitor of HCV NS3•4A serine protease, was discovered using structure-based drug design techniques (22). Clinical proof-of-concept for HCV protease inhibitors (PIs) has been demonstrated by Boehringer Ingelheim and Vertex Pharmaceuticals, Inc. using BILN 2061 (23) and VX-950 (24), respectively. Both compounds reduced HCV viral load in patients by ~ 2–3 $\log_{10}$ in the first 3 days of dosing. In some patients treated with VX-950, the HCV viral load dropped by more than 4 $\log_{10}$ to below the limit of detection (<10 IU/mL) during 14 days of dosing (24).

Due to the error-prone nature of the viral reverse transcriptase of retroviruses or RNA-dependent RNA polymerase of RNA viruses, drug resistance frequently emerges in patients treated with antiviral drugs and therefore limits the efficacy of these therapies. For these new HCV NS3•4A serine protease inhibitors, resistance could become a major issue in treated patients. In our previous report, we used the HCV subgenomic replicon system to identify resistance mutations against two HCV protease inhibitor clinical candidates, BILN 2061 and VX-950 (25). The *in vitro* resistance mutations selected against either inhibitor resulted in a significant reduction in susceptibility to the same inhibitor. 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. In this study, we identified mutations that are cross-resistant to both protease inhibitors. Analysis of structural models of these mutants indicates that steric hindrance is the primary reason for resistance of these mutations against both HCV PIs. HCV replicon containing either cross-resistance mutation, A156T or A156V, displayed significantly reduced fitness (or replication capacity) and remained as sensitive to IFN-α or ribavirin as the wild-type replicon in cell assay.

EXPERIMENTAL PROCEDURES

Plasmid Construction. An *Escherichia coli* expression plasmid containing a DNA fragment encoding residues Met^{1}–Ser^{181} of the HCV NS3 protease (GenBank™ CAB46913) of the HCV Con1 replicon, I_{377}neo/NS3–3'/wt (26) (re-named as pBR322-HCV-Neo in this study), followed by a C-terminal hexa-histidine tag has been described (25). Cross-resistance mutations against both HCV NS3•4A PIs were introduced into this construct by PCR-based site-directed mutagenesis. The PI-resistant mutations in the NS3 serine protease domain were also introduced into a second generation Con1 replicon plasmid containing three adaptive mutations, pBR322-HCV-Neo-mADE, as described before (25). For transient transfection, the neomycin phosphotransferase gene in these pBR322-HCV-Neo-mADE plasmids was replaced in-frame with a firefly luciferase gene to generate the corresponding pBR322-HCV-Luc-mADE plasmids. All constructs were confirmed by sequencing.

Generation of Stable HCV Replicon Cells HCV Con1 subgenomic replicon stable cells (26) or its variants with resistance mutations were generated and maintained in Dulbecco's modified minimal essential medium (DMEM; JRH Biosciences, Lenexa, Kansas) containing 10% fetal bovine serum (FBS; JRH Biosciences) and 0.25 mg/ml G418 (Geneticin; Invitrogen, Carlsbad, California). The cross-resistance mutations identified in this study were introduced into the pBR322-HCV-Neo-mADE replicon plasmid by site-directed mutagenesis as described before (25). Stable replicon cell lines were generated using the T7 transcripts derived from either wild type pBR322-HCV-Neo-mADE or the ones with the cross-resistance mutations.

IC_{50} Determination of Antiviral Agents in the HCV Replicon Cell Assay. IC_{50} values of antiviral agents were determined in a 48 h assay with the HCV Con1 subgenomic replicon cells as described before (25). Briefly, 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 antiviral agents serially diluted in DMEM, 2% FBS and 0.5% DMSO was added. The replicon cells were incubated with the antiviral agents for 48 h. Total cellular RNA was extracted using RNeasy-96 (Qiagen; Valencia, California), and the copy number of the HCV 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 AQ_{ueous} One Solution Cell Proliferation Assay (Promega; Madison, Wisconsin). The IC_{50} and CC_{50} of the antiviral agents were calculated using four-parameter curve fitting (SoftMax Pro; Molecular Devices, Sunnyvale, California).

Selection of HCV PI Cross-Resistant Replicon Cells. Several schemes were employed to generate HCV replicon cell lines that were cross-resistant to both HCV serine protease inhibitors, VX-950 and BILN 2061. First, a replicon cell line, which was resistant to VX-950 [series A in Ref. (25)], was serially passaged in the presence of 0.25 mg/ml G418 and 14 µM VX-950 plus slowly increasing concentrations of BILN 2061 from 40 nM to 6.4 µM (designated as series C). Second, another replicon cell line, which was resistant to BILN 2061 [series B in Ref. (25)], was serially passaged in the presence of 0.25 mg/ml G418 plus slowly increasing concentrations of both VX-950 (from 7 µM to 14 µM) and BILN 2061 (from 160 nM to 6.4 µM) (designated as series D). Last, the naïve HCV Con1 sub-genomic replicon stable cells were serially passaged in the presence of 0.25 mg/ml G418 and slowly increasing concentrations of both VX-950 (from 3.5 µM to 14 µM) and BILN 2061 (from 80 nM to 1.6 µM) (designated as series E). During the course of selection, replicon cells were split twice per week when a 70-90% confluence was reached. Fresh medium and HCV PIs were added every 3 or 4 days regardless of whether the cell culture was split.

Identification of HCV PI Cross-Resistance Mutations. During the selection of HCV PI-resistant replicon cells, cell pellets were collected whenever the cell culture was 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 and purified as described before (25). The purified RT-PCR products were subjected to bulk sequence determination. To determine the frequency of PI-resistant mutations, the 1.7-kb RT-PCR products of HCV RNA of the PI cross-resistant replicon cells were ligated into the TA cloning vector pCR2.1 (Invitrogen), and multiple individual bacterial colonies were isolated for each time point and the HCV NS3 protease coding region of the purified plasmid DNA was sequenced.

Expression and Purification of the HCV NS3 Serine Protease Domain. HCV NS3 serine protease domain containing the wild type sequence or the cross-resistance mutations (A156V or A156T) were expressed in BL21/DE3 pLysS *E. coli* cells (Stratagene; La Jolla, California) as described before (25). Briefly, freshly transformed cells were grown at 37°C in a BHI medium (Difco Laboratories; Kansas City, Missouri) supplemented with 100 µg/ml carbenicillin and 35 µg/ml chloramphenicol to an optical density of 0.75 at 600 nM followed by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 24°C. All purification steps were performed at 4°C as described before (25). Cell paste was lysed in 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 homogenized using a microfluidizer (Microfluidics; Newton, MA), followed by ultracentrifugation at 54,000 x g for 45 min. A final concentration of 5 mM imidazole along with 2 ml of pre-equilibrated Ni-nitrilotriacetic acid resin (Clontech; Palo Alto, California) was added to the supernatants and the mixtures were rocked for 3 h and washed with 20 column volumes of buffer A plus 5 mM 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 an assay described by Taliani et al., (27) with modification. An internally quenched fluorogenic depsipeptide (FRET substrate), Ac-DED(EDANS)EEαAbuψ[COO]ASK (DABCYL)-NH_2, was purchased from AnaSpec Incorporated (San Jose, California). The assay was carried out in a continuous mode in a 96-well microtiter plate format with 2 nM HCV NS3 protease domain protein, 25 µM KK4A peptide (KKGSVVIVGRIVLSGK) (28), and the FRET substrate in 50 mM HEPES (pH 7.8), 100 mM NaCl, 20% glycerol, 5 mM DTT. The reaction was initiated by the addition of the FRET substrate after a 10-min pre-incubation of the buffer components with NS3 protease and KK4A peptide at room temperature and run at 30°C for 20 min using a Molecular Devices fmax fluorometric plate reader as described before (25). For determination of substrate kinetic parameters, concentrations of the FRET peptide were varied from 0.5 to 7.0 µM. The substrate kinetic parameters, K_m and V_{max}, were determined by fitting the data to the Michaelis-Menten equation. Inhibition constants (Ki) were determined by titration of enzyme activity using the assay described above, except that compound dissolved in DMSO was added to the mixture, with the final concentration of DMSO at 2%. 2% DMSO was used as controls. This reaction was run for 20 min at 30°C as described before (25). Seven to 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.

Fitness of HCV NS34A PI-resistant Mutants in Replicon Cells. T7 RNA run-off transcripts were generated from the ScaI-linearized pBR322-HCV-Luc-mADE plasmids with or without the PI-resistance mutations, and transfected into PI-cured Con1 subgenomic replicon cells by electroporation as described before (26). Transfected cells were plated in DMEM plus 10% FBS in 96-well plates, and incubated for up to 4 days. The cells were lysed with passive lysis buffer and the luciferase activity was determined using a luciferase assay kit (Promega). The normalized replication efficiency of either the wild-type or PI-resistant mutant replicon was the ratio of the luciferase signal at 4 days post-electroporation (replication efficiency) divided by the luciferase signal at 4 h post-electroporation (transfection efficiency). The fitness of a PI-resistant mutation was the percentage of the normalized replication efficiency of the mutant replicon to that of the wild-type replicon.

Computational Modeling. Modeling of VX-950 and BILN 2061 into the active site of the NS3 serine protease domain using the crystal structure of a full-length HCV NS3 protein (30) (Protein Data Bank code: 1CU1) was previously described (25). The Ala^{156} side chain on the E2 β-strand of the HCV NS3•4A protease separates the S4 and S2 pockets of the enzyme active site and is in van der Waals contact with the P2 group of the two inhibitors (Fig. 5). The Val or Thr substitution of Ala^{156} extends the side chain with two additional (methyl or hydroxyl) groups into the compact space between the wild type enzyme and the inhibitors. The Val^{156} or Thr^{156} side chain was modeled at all the three possible canonical conformations of $\chi_1 = 60^\circ$, -60° and 180° following the procedure outlined previously for the modeling of A156S mutation (25). The resulting models of the mutants were energy-minimized by allowing all the protein atoms to move during minimization. The inhibitors, VX-950 and BILN 2061, were docked into these mutant enzyme active sites to model the effect of the mutations on inhibitor binding.

RESULTS

HCV NS3•4A Serine Protease Inhibitors, VX-950 and BILN 2061

VX-950 (Fig. 1) has recently demonstrated antiviral activity in chronic hepatitis C patients in a phase I trial (24). 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 (C. A. Gate and Y.-P. Luong, unpublished data). 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, with an average IC_{50} value of

354 nM in the 48-h assay (22,25). Another HCV NS3•4A protease inhibitor, BILN 2061 (Fig. 1) is the first PI to demonstrate proof-of-concept in hepatitis C patients (23). Its average IC_{50} value in the 48-h replicon cell assay is about low single digit nM.

Development of PI Cross-Resistant HCV Replicons from VX-950-Resistant Cells

To identify resistance mutations that are cross-resistant to both VX-950 and BILN 2061, several schemes of selection were employed. First, a VX-950-resistant replicon cell line [series A in Ref. (25)] was initially developed by serial passage of HCV subgenomic replicon cells in the presence of 0.25 mg/ml G418 and increasing concentrations of VX-950. After the series A replicon cells became resistant to 14 μM VX-950, the cells were then serially passaged in the presence of slowly increasing concentrations of BILN 2061, in addition to 0.25 mg/ml G418 and 14 μM VX-950 (designated as series C) (Fig. 2A). For BILN 2061, the starting concentration was 40 nM and the final concentration was 6.4 μM. Every 3 or 4 days, replicon cells were split and the medium was replenished, and fresh VX-950 and BILN 2061 were added. Since HCV PIs inhibit NS3•4A serine protease activity and consequently block replication of HCV RNA, the steady state levels of HCV proteins and neomycin phosphotransferase protein gradually decline over time and eventually become undetectable in the presence of high concentrations of HCV PI (data not shown). Cells with low or no neomycin phosphotransferase protein proliferate at a gradually decreasing rate and eventually die in the presence of G418. Replicon cells with the dominant VX-950-resistant mutation, A156S, are expected to die in the presence of increasing concentrations of BILN 2061 since it has been shown to be susceptible to inhibition by BILN 2061 (25). Only HCV RNA with mutations that are cross-resistant to both VX-950 and BILN 2061 can replicate in the presence of high concentrations of both HCV PIs and support the growth of the replicon cells harboring them. During the development of series A (VX-950-resistant) or series B (BILN 2061-resistant) replicon cell lines, the cell growth became stalled for a period of 7–10 days, concurrent with massive cell death. However, replicon cells in series C grew normally for the entire selection process, which lasted for 2 months. The IC_{50} of BILN 2061 against the series C replicon cells at day 52 was determined to be 3.9 μM in the 48-h assay, which is 390-fold higher than the IC_{50} against the series A (VX-950-resistant) replicon cells (10 nM) (Fig. 2C). The series C replicon cells at day 52 remained resistant to VX-950, with an IC_{50} value larger than 30 μM (Fig. 2B). Therefore, the series C replicon cells at day 52 are cross-resistant to both VX-950 and BILN 2061.

Total cellular RNA of the replicon cells in series C at day 32, which had been cultured in the presence of 14 μM VX-950 and 0.32 μM of BILN 2061, was extracted and subjected to RT-PCR to amplify the coding region of the HCV 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 both HCV PIs. Substitutions at Ala^{156} in the protease domain were observed, suggesting that mutations at residue 156 might be critical for the reduced sensitivity to both PIs. No amino acid substitution was observed in the NS4A coding sequences or 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 C replicon cells at day 32 was sub-cloned into the TA vector and 10 individual colonies were subjected to sequencing. 6 clones had an Ala^{156} to Thr (A156T) substitution, and 3 other clones had a substitution of Ala^{156} with Val (A156V). The 10th clone retains the A156S mutation.

Development of PI Cross-Resistant HCV Replicons from BILN 2061-Resistant Cells

The second selection scheme was to grow BILN 2061-resistant replicon cells in the presence of both BILN 2061 and VX-950. In this case, a BILN 2061-resistant replicon line [series B in Ref. (25)] was initially developed by serial passage of the HCV subgenomic replicon cells in the presence of 0.25 mg/ml G418 and increasing concentrations of BILN 2061. Then the BILN 2061-resistant series B replicon cells was subsequently serially passaged in the presence of 0.25 mg/ml G418, and slowly increasing concentrations of VX-950 and BILN 2061 (designated as series D) (Fig. 3A). For BILN 2061, the concentrations ranged from 160 nM to 6.4 μM. Only two concentrations of VX-950 were

used: 7 μM and 14 μM. Replicon cells containing the major BILN 2061-resistant mutations, D168V or D168A, are expected to die in the presence of high concentrations of VX-950 since they have been shown to be susceptible to inhibition by VX-950 (25). Again, only HCV RNA with mutations that are cross-resistant to both VX-950 and BILN 2061 can replicate in the presence of high concentrations of both HCV PIs and support the growth of the replicon cells harboring them. However, the replicon cells in series D grew normally for most of the selection process, which lasted for 2 months. Since 30 μM VX-950 did not result in more than a 50% reduction of HCV RNA in the series D replicon cells at day 52, the actual IC_{50} values of VX-950 could not be determined, but will be more than 100-fold higher than the IC_{50} (0.26 μM) against the series B (BILN 2061-resistant) replicon cells (Fig. 3B). The IC_{50} values of BILN 2061 against the series D replicon cells at day 52 were determined to be 2.3 μM, which indicates the series D replicon cells at day 52 remain resistant to BILN 2061 (Fig. 3C). Therefore, the series D replicon cells are cross-resistant to both VX-950 and BILN 2061 at day 52.

Total cellular RNA from the cells in series D at day 32, which had also been cultured in the presence of 14 μM VX-950 and 0.32 μM of BILN 2061, was extracted and subjected to RT-PCR to amplify the coding region of the HCV 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 both HCV PIs. Again, substitutions at Ala^{156} in the protease domain were observed, confirming that mutations at residue 156 might be critical for the reduced sensitivity to both PIs. No amino acid substitution was observed in the NS4A coding sequences or 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 from the series D replicon cells at day 32 was sub-cloned into the TA vector and 14 individual colonies were subjected to sequencing. 12 clones had the A156V substitution, and the 13th clone had the A156T mutation. The 14th clone has two mutations, A156S and D168V.

Development of PI Cross-Resistant HCV Replicons from Naïve Replicon Cells

In our previous studies of resistance mutations against a single HCV PI, either VX-950 or BILN 2061, cell growth became stalled for several days, during which massive cell death was observed (25), which signaled the emergence of resistance mutant replicon cells and concurrent death of non-resistance replicons. However, no such cell death or slow-down in cell growth was observed during selection of the cross-resistant replicon series C or D as described above. It is possible that the cross-resistance mutations, A156T and A156V, may have already existed in VX-950-resistant (series A) or BILN 2061-resistant (series B) replicon cells as a minor population. If so, these two selection schemes could provide bias toward the A156T or A156V mutation over other potential cross-resistance mutations. Thus, a third selection scheme was performed using the naïve HCV replicon cells that have not been exposed to either inhibitor.

The Con1 subgenomic replicon cells derived from pBR322-HCV-Neo-mADE (25) were serially passaged in the presence of 0.25 mg/ml G418 and slowly increasing concentrations of both VX-950 and BILN 2061 (designated as series E) (Fig. 4). The starting concentration of VX-950 was 3.5 μM and the highest concentration was 14 μM. For BILN 2061, the starting concentration was 80 nM and the final concentration was 1.6 μM. Replicon cells were split or the medium was replenished every 3 or 4 days, and fresh VX-950 and BILN 2061 were added. Replicon cells in series E grew normally for the first 10 days in the presence of 3.5 μM VX-950 and 160 nM BILN 2061. After 10 days, the series E cells grew significantly slower and massive cell death was observed between days 10 and 21 (Fig. 4). Normal growth did not resume until day 21. Total cellular RNA from the series E cells at days 10, 21, and 48, was extracted and subjected to RT-PCR to amplify the coding region of the HCV NS3 serine protease domain. No HCV PI-related mutation was observed in the NS3 serine protease domain of the series E replicon cells at day 10 when compared to the wild type Con1 replicon cells cultured in the absence of both HCV PIs. To delineate the identity and frequency of the substitutions, a 1.7-kb RT-PCR product from the series E replicon cells at day 21 or 48 was sub-cloned into the TA

vector and multiple clones were sequenced for both samples. In the day 21 sample of the series E replicon cells, which had been cultured in the presence of 3.5 μM VX-950 and 0.32 μM of BILN 2061 for 14 days, 65% or 30 out of 46 clones had the A156T substitution, while another substitution, A156V, was found in 35% or 16 out of 46 clones. For the day 48 sample of the series E, which had been cultured in the presence of 14 μM VX-950 and 1.6 μM of BILN 2061 for 14 days, 80% or 35 out of 44 clones had the A156T substitution, while the A156V substitution was found in 20% or 9 out of 44 clones. In either case, no other mutation in the NS3 serine protease domain was found in more than 10% of the TA plasmid clones, indicating that A156T and A156V are the only two mutations that confer cross-resistance to both VX-950 and BILN 2061.

Either A156V or A156T Mutation is Sufficient to Confer Cross-Resistance to both VX-950 and BILN 2061

To confirm whether the observed mutations at Ala^{156} are sufficient to confer cross-resistance against both VX-950 and BILN 2061, site-directed mutagenesis was used to replace Ala^{156} with either Val or Thr in the wild type NS3 protease domain. The NS3 serine protease domain containing either mutation was expressed in *Escherichia coli* and purified for enzymatic characterization. These mutations were also introduced into high-efficiency subgenomic replicon plasmids for characterization in HCV replicon system.

The catalytic efficiency (k_{cat}/K_m) of A156T or A156V mutant protease against the FRET substrate was about 7- or 4-fold lower than that of the wild type protease, respectively (Table I). The K_i value of VX-950 was 9.9 μM or 33 μM against the A156T or A156V mutant protease, respectively, which is 99- or 330-fold higher than that against the wild type protease (0.1 μM), respectively (Table II). Both mutant proteases were apparently unaffected 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 to the wild type protease. The actual magnitude of resistance cannot be determined since solubility of BILN 2061 was limited at concentrations greater than 1.2 μM in the assay buffer, as measured by the absorbance at 650 nm (data not shown).

The fitness or replication capacity of the PI cross-resistant mutations was determined in a transient transfection system using the luciferase activity as the surrogate readout. Since the luciferase mRNA is part of the HCV replicon RNA, the amount of luciferase protein or its activity, as a direct consequence of its mRNA translation, can be used as an indirect readout of the HCV replicon RNA levels in the transiently transfected cell. The normalized replication capacity, or fitness, of the HCV replicon containing the A156T or A156V mutation was about 5% or 3%, respectively, of that of the wild-type replicon in the luciferase transient transfection assay. These results are consistent with the lower enzymatic catalytic efficiency of the two mutants as compared to that of the wild type HCV NS3 serine protease.

The HCV RNA level in the stable replicon cells containing the A156T or A156V substitution was also lower than that of stable wild type replicon cells (data not shown), which is not unexpected given the reduced replication capacity or fitness of the mutant replicons and lower enzymatic catalytic efficiency of the mutant proteases. No significant reduction of HCV replicon RNA by up to 30 μM VX-950 was observed in either mutant replicon cell line, indicating at least 75-fold decrease in sensitivity conferred by either mutation (Table III). The IC_{50} value of BILN 2061 against the A156T replicon cells was 1.09 μM, which is 272-times higher than that against the wild type replicon cells (4 nM). For the A156V mutant replicons, BILN 2061 has an IC_{50} value of 10 μM, indicating a more than 2,500-fold decrease in sensitivity conferred by the A156V mutation (Table III).

Since the current standard of care is a combination of pegylated IFN-α and ribavirin, it is interesting to know whether these PI cross-resistant HCV replicon cells remain sensitive to either IFN-α or ribavirin. As shown in Table III, the IC_{50} values of either IFN-α or ribavirin remained virtually the same for HCV replicon cells containing A156T or A156V as for the wild-type replicon cells. These results suggest that combination with IFN-α, or even ribavirin, could be a potential therapeutic strategy to suppress the emergence of resistance mutants against HCV PIs.

DISCUSSION

In our previous work, we have shown that the A156S mutation is resistant to VX-950, but not to BILN 2061. Substitution of Asp^{168} with Val or Ala causes resistance to BILN 2061, but remains susceptible to VX-950 (25). Since there is no apparent overlap between the *in vitro* dominant resistance mutation profiles of VX-950 and BILN 2061, it is likely that a combination of VX-950 and BILN 2061 would suppress the appearance of their dominant resistance mutations. Indeed, none of these three single residue substitution was observed when the HCV replicon cells were treated with both HCV protease inhibitors, as we reported in the current study. Instead, we found that substitution of Ala^{156} with either Val or Thr in HCV serine protease domain confers cross-resistance against both the inhibitors. The Ala^{156} is located on the E2 β-strand of the HCV NS3•4A protease, which is involved in backbone-to-backbone hydrogen bond interactions with the inhibitor, and its side chain divides the S2 and S4 subsites of the substrate-binding site of the protease. The Ala^{156} side chain is in van der Waals contact with the P2 group of the two inhibitors (Fig. 5). The A156S substitution puts the Ser side chain too close to the P4 group of the two inhibitors. Because the P4 group of BILN 2061 is a terminal group, it could avoid the repulsive interaction by moving out without losing any other interactions between BILN 2061 and HCV serine protease. On the other hand, any movement of the P4 group of VX-950 would cause loss of the hydrogen bond with cap carbonyl as well as hydrophobic interactions of the P4 group of the inhibitor (25).

Of the three possible conformations of the Ser side chain at position 156, the conformation with $\chi_1 = -60^\circ$ (see Fig. 6) has the least number of unfavorable contacts with VX-950 and BILN 2061. The other two conformers (with $\chi_1 = 180^\circ$ and 60°) (see Fig. 6) have unfavorable contacts with both the inhibitors either at the P2 side chain or P3 carbonyl group. In the A156T or A156V mutation, the additional hydroxyl or methyl group, respectively, at the C_β atom of the residue 156 side chain is forced to occupy one of these two positions with $\chi_1 = 180^\circ$ or 60°, which makes unfavorable interactions with the inhibitors. The three possible conformations of Thr^{156} residue are shown schematically in Fig. 6. In all cases, the additional methyl and hydroxyl groups of Thr^{156} have repulsive interaction with the inhibitor and/or enzyme backbone atoms. By energy minimization, we found that the $180^\circ/-60^\circ$ conformation (Fig. 6) has the least repulsive interaction and the main cause of the repulsion is the close clash of the terminal hydroxyl or methyl group of the Thr^{156} or Val^{156} side chain, respectively, against the P3 carbonyl group of the inhibitors. Therefore, A156T and A156V mutations are resistant to both inhibitors.

A Blast search of the GenBank™ database 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 seen if the substitution at Ala^{156} has a deleterious effect on the virus life cycle *in vivo*. However, several lines of evidence suggest that HCV protease with either cross-resistance mutation, A156V or A156T, may have lower fitness and be more compromised in its ability to support viral replication than the wild type protease or other PI-resistant protease mutants containing the A156S or D168V/A mutations. First, the catalytic efficiency of the A156V and A156T mutant proteases is 4- and 7-fold lower, respectively, than that of the wild type protease. Second, A156S was found to be the dominant resistance mutation against VX-950, even though A156T and A156V are at least as resistant to VX-950 as A156S. The same applies to BILN 2061 resistance mutations: D168V and D168A were found to be dominant mutations even though A156T and A156V are as resistant to BILN 2061 as the substitutions at Asp^{168}. Finally, both cross-resistance mutations were shown to have a significantly reduced replication capacity or fitness in transiently transfected replicon cells.

Several *in vitro* studies suggest that HCV NS3•4A serine protease may block IFN signal transduction pathway (31-33) and therefore interfere with host innate immune response, which could be one of the reasons that HCV escapes immune clearance and maintains chronic infection.

One may expect the HCV PI-resistant mutants, with a significantly diminished fitness and a less efficient NS3•4A protease, may be less capable to interfere with IFN pathway. If so, the HCV replicon cells containing these PI-resistant mutations may become more sensitive to inhibition by IFN-α. However, no change in IC_{50} values was observed for A156T or A156V replicon cells as compared to the wild-type replicon in our study, which is similar to what has been previously reported for other HCV PI-resistant mutations (34,35).

One of the major factors limiting the efficacy of virus-specific therapies against many retroviruses and RNA viruses is the development of resistance to anti-viral 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 emerged quickly in patients who were on monotherapy of HIV-specific inhibitors. It is estimated that all possible single mutations can be randomly generated within one day in an HIV-infected patient. Even though elimination or cure of HIV infection in patients remains an elusive goal, multi-drug combinations or cocktail therapies have been shown to be more effective than mono-therapy 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, the first hepatitis B virus-specific drug. It was reported that the frequency of resistance mutations against lamivudine increased from 24% in the first year to 67% in the fourth year in the hepatitis B patients treated with lamivudine (37).

From these examples, 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, especially in monotherapy. 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 replication rate of HIV in patients (38). *In vitro* resistance mutations against the HCV protease or polymerase inhibitors have also been identified in the replicon system (25,34,35,39,40). These studies suggest that future hepatitis C therapy involving small molecule inhibitors of HCV enzymes might require multi-drug combination, as in the case of the current HIV treatments. Since there is no overlap between the *in vitro* dominant resistance mutation profiles of VX-950 and BILN 2061 (25), it is possible that a combination of VX-950 and BILN 2061 would suppress the appearance of the dominant resistance mutations against each inhibitor. Indeed, the dominant resistance mutations, A156S, D168V, or D168A, either did not emerge or disappeared when various types of HCV replicon cells were incubated with both VX-950 and BILN 2061. However, a Thr or Val substitution at a single amino acid (Ala^{156}) in the serine protease domain, which resulted in cross-resistance to both inhibitors, appeared in the replicon cell system under the selective pressure of both inhibitors.

It should be noted that all the HCV PI-resistant replicon cells were selected under increasing concentrations of inhibitors, which would be ideal for the development of resistance. When a relatively high concentration of HCV PI was used at the beginning of selection, a 4–5 $\log_{10}$ reduction in HCV RNA levels was observed and no replicon cells were recovered after two weeks of treatment (41). It remains to be seen whether treatment of hepatitis C patients with a single HCV PI or a combination of different PIs will be able to suppress the virus so that no resistant virus will appear. Finally, since all these PI-resistance mutant replicons remained sensitive to IFN-α, combinations of HCV protease inhibitors with IFN-α, or inhibitors targeting other host factors that support viral replication, or inhibitors against a different HCV protein or nucleic acid target could raise the barrier to the emergence of resistance against protease inhibitor(s) and therefore increase the efficacy of anti-HCV therapy.

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ACKNOWLEDGMENTS

We are grateful to Hsun-Mei Hsiao, Judith Lippke, Kevin Cottrell, John Maxwell, Yee-Lan Wang, Ming Di, and John Alford for their technical assistance. We also thank Steve Lyons, John Thomson, and Michael Briggs for the critical reading of the manuscript.

FIGURE LEGENDS

Fig. 1. Chemical structures of the HCV NS3•4A protease inhibitors, VX-950 and BILN 2061.

Fig. 2. Development of cross-resistant replicons from VX-950-resistant replicon cells. (**A**) VX-950-resistant replicon cells were serially passaged in the presence of 0.25 mg/ml G418, 14 μM VX-950 (filled

rectangle) and increasing concentrations of BILN 2061 (filled triangle). Replicon cells were split, and fresh VX-950 and BILN 2061 were added to medium twice a week, as indicated by filled rectangles and triangles, respectively. Total cellular RNA of replicon cells at day 32 (open arrow) during the resistance selection was extracted and the RT-PCR product covering the HCV NS3 serine protease domain was sequenced either directly or after being sub-cloned into the TA vector. (**B**) Titration of VX-950 against the series A (VX-950-resistant) (filled rectangle) or the series C (cross-resistant) (open rectangle) replicon cells at day 52 is shown. HCV RNA level was determined after a 48-h incubation with VX-950. (**C**) Titration of BILN 2061 against the series A (VX-950-resistant) (filled triangle) or the series C (cross-resistant) (open triangle) replicon cells at day 52 is shown. HCV RNA level was determined after a 48-h incubation with BILN 2061.

Fig. 3. Development of cross-resistant replicons from BILN 2061-resistant replicon cells. (**A**) BILN 2061-resistant replicon cells were serially passaged in the presence of 0.25 mg/ml G418, 7 or 14 μM VX-950 (filled rectangle) and increasing concentrations of BILN 2061 (filled triangle). Replicon cells were split, and fresh VX-950 and BILN 2061 were added to medium twice a week, as indicated by filled rectangles and triangles, respectively. Total cellular RNA of replicon cells at day 32 (open arrow) during the resistance selection was extracted and the RT-PCR product covering the HCV NS3 serine protease was sequenced either directly or after being sub-cloned into the TA vector. (**B**) Titration of VX-950 against the series B (BILN 2061-resistant) (filled rectangle) or the series D (cross-resistant) (open rectangle) replicon cells at day 52 is shown. HCV RNA level was determined after a 48-h incubation with VX-950. (**C**) Titration of BILN 2061 against the series B (BILN 2061-resistant) (filled triangle) or the series D (cross-resistant) (open triangle) replicon cells at day 52 is shown. HCV RNA level was determined after a 48-h incubation with BILN 2061.

Fig. 4. Development of cross-resistant replicons from naive replicon cells. Naïve HCV subgenomic replicon cells were serially passaged in the presence of 0.25 mg/ml G418, and increasing concentrations of VX-950 (filled rectangle) and BILN 2061 (filled triangle). Replicon cells were split, and fresh VX-950 and BILN 2061 were added to medium twice a week, as indicated by filled rectangles and filled triangles, respectively. Total cellular RNA from replicon cells was extracted at various time points, indicated by open arrows, during the resistance selection and the RT-PCR product covering the HCV NS3 serine protease domain was sequenced either directly or after being sub-cloned into the TA vector.

Fig. 5. The computational models of protease-inhibitor complexes. The protein is shown as a cartoon based on its secondary structure in light blue. The inhibitors are shown as ball-and-stick models (VX-950 in purple and BILN 2061 in yellow) with nitrogens in blue, oxygens in red, and sulfur in cyan. Side chains of key residues are shown as sticks with different colors: Ala^{156} (green) and Asp^{168} (orange). The side chain of Ala^{156} is highlighted with green dot surfaces. The catalytic triad, Ser^{139}, His^{57}, and Asp^{81} are shown in blue. (The figure was created by PyMOL Molecular Graphics Systems, DeLano Scientific LLC, San Carlos, California, U.S.A. Copyright © 1998-2003).

Fig. 6. Schematic drawing of the Thr^{156} side chain conformations in relationship to the inhibitor binding. The thick lines represent the side chain of Thr^{156} of the mutant enzyme and the thin lines represent the backbone of E2 strand of the protease. The backbone carbonyl of the amino acid 165 and the backbone amide of residue 167 of the protease form hydrogen bonds with P1 amide and P3 carbonyl of the inhibitor, respectively. The three diagrams represent Newman projections of the three staggered conformations of χ_1 torsion angle. The two values shown at the top of diagram presented for the χ_1 torsion angle of Thr^{156} side chain are measured as $N\text{-}C_\alpha\text{-}C_\beta\text{-}O_\gamma$ and $N\text{-}C_\alpha\text{-}C_\beta\text{-}C_\gamma$, respectively. The same three conformations were also considered for Val^{156} side chain. The last conformation, $180^\circ/-60^\circ$, has the lowest energy for either mutation, but remains repulsive to both the inhibitors.

Table I

Enzymatic properties of the wild type or the mutant HCV NS3 serine protease domains

HCV Protease	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
Wild type	1.1	1.0	9.47×10^5
A156T	1.3	0.2	1.36×10^5
A156V	2.6	0.6	2.34×10^5

Three HCV NS3 serine protease domain proteins of the Con1 strain, including the wild type proteases and two mutants, A156T and A156V, were expressed and purified. The k_{cat} and K_m values of these NS3 proteases were determined using the KK-NS4A cofactor peptide and the FRET substrate, and the average of two independent assays is shown.

Table II

Confirmation of resistance in enzymatic assay

Mutant	K_i (μM)	
	BILN 2061	VX-950
Wild type	0.019	0.100
A156T (n=2)	> 1.2	9.9
A156V (n=1)	> 1.2	33

The K_i values of VX-950 and BILN 2061 were determined against the three purified HCV NS3 serine protease domains, including the wild type protease, as well as two mutants, A156T and A156V, using the KK-NS4A cofactor peptide and the FRET substrate. The solubility of BILN 2061 in the reaction buffer was limited at concentrations above 1.2 μM. No inhibition was observed for either A156T or A156V mutant NS3 protease in the presence of 1.2 μM BILN 2061.

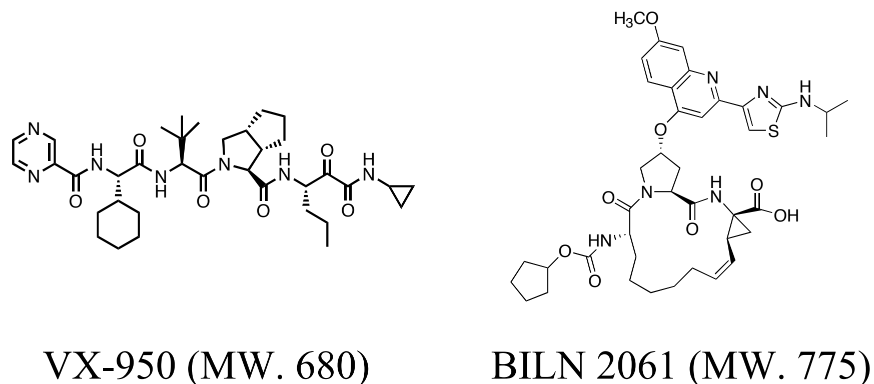
Table III

Confirmation of resistance in HCV replicons

Mutant	Replicon IC_{50}			
	BILN 2061 (μM)	VX-950 (μM)	Ribavirin (μM)	IFN-α (U/ml)
Wild type	0.004	0.402	40.5	1.01
A156T	1.09	> 30	49.7	1.03
A156V	10.0	> 30	75.1	1.20

Three HCV sub-genomic replicon stable cell lines, including the wild type, and two mutants, A156T and A156V, were generated using the T7 RNA runoff transcripts from the corresponding high efficiency Con1 replicon plasmids. The IC_{50} values of antiviral agents were determined against the three HCV replicon cell lines in the standard 48-h assay. The average IC_{50} of two independent assays is shown for VX-950 or BILN 2061, and IC_{50} in a single assay for ribavirin or IFN-α.

Fig. 1



VX-950 (MW. 680) BILN 2061 (MW. 775)

Fig. 2

A

[BILN 2061] (μM)
[VX-950] (μM)
Days of Treatment
0 10 21 32 42 52 63

B

Replicon RNA (% of untreated)
[VX-950] (μM)

C

Replicon RNA (% of untreated)
[BILN 2061] (nM)

Fig. 3

A

100
10
1
0.1
0.01
[BILN 2061] (μM)
30
20
10
0
[VX-950] (μM)
0 10 21 32 42 52 63
Days of Treatment

B

125%
100%
75%
50%
25%
0%
Replicon RNA (% of untreated)
0.01 0.1 1 10 100
[VX-950] (μM)

C

125%
100%
75%
50%
25%
0%
Replicon RNA (% of untreated)
0.01 0.1 1 10 100
[BILN 2061] (μM)

Fig. 4

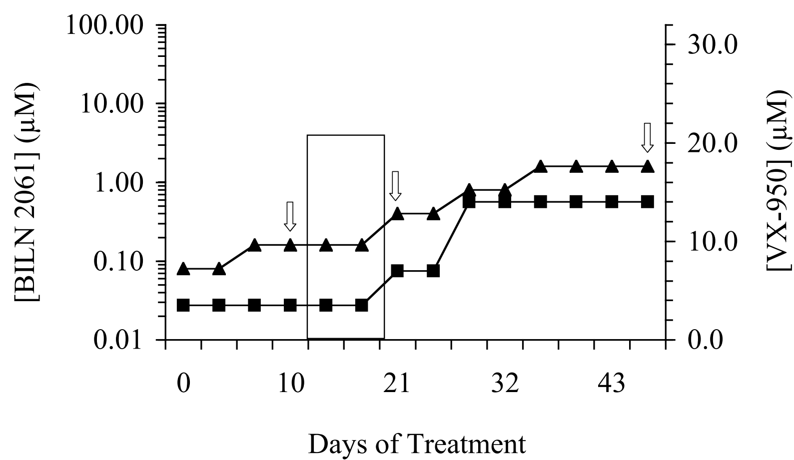


Fig. 5

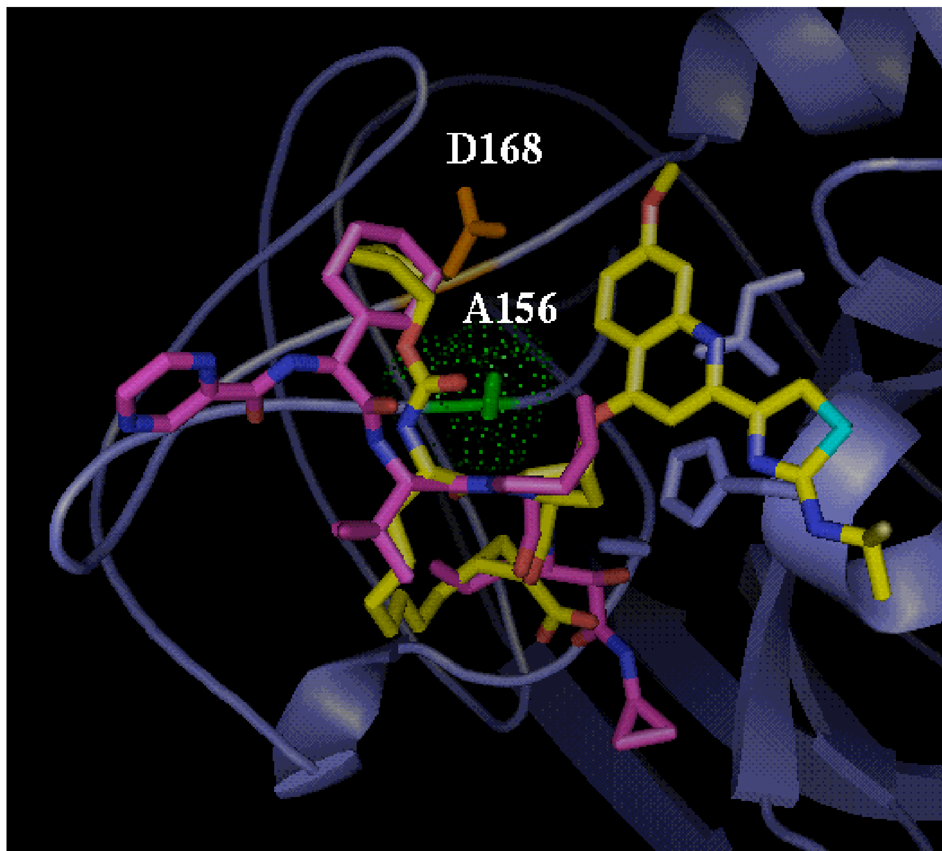


Fig. 6