In Vitro Studies of Cross-resistance Mutations against Two Hepatitis C Virus Serine Protease Inhibitors, VX-950 and BILN 2061

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VX-950 is a potent, small molecule, peptidomimetic inhibitor of the hepatitis C virus (HCV) NS3/4A serine protease and has recently been shown to possess 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 (Lin, C., Lin, K., Luong, Y.-P., Rao, B. G., Wei, Y.-Y., Brennan, D. L., Fulghum, J. R., Hsiao, H.-M., Ma, S., Maxwell, J. P., Cottrell, K. M., Perni, R. B., Gates, C. A., and Kwong, A. D. (2004) J. Biol. Chem. 279, 17508–17514). Single amino acid substitutions that conferred drug resistance (distinct for either inhibitor) were identified in the HCV NS3 serine protease domain. The dominant VX-950-resistant mutant (A156S) remains sensitive to BILN 2061. The major BILN 2061-resistant mutants (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. In this study, we identified mutants that are cross-resistant to both HCV protease inhibitors. The cross-resistance conferred by substitution of Ala156 with either Val or Thr was confirmed by characterization of the purified enzymes and reconstituted replicon cells containing 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 ~1% of the population in developed countries (1). In many patients, hepatitis C virus (HCV)2 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 40–50% in the difficult-to-treat genotype 1 HCV-infected patients (Refs. 4 and 5; for a review, see Refs. 6 and 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 ~3000 amino acids. The HCV polyprotein is proteolytically processed by cellular and viral proteases into at least 10 distinct products in the order of NH2-C-terminal of the NS2/NS3, the NS2/NS3-NS4A protease; the NS3/NS4A 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-950,3 respectively. Both compounds reduced HCV viral load in patients by ~2–3 log10 in the first 3 days of dosing. In some patients treated with VX-950, the HCV viral load dropped by >4 log10 to below the limit of detection (<10 IU/ml) during 14 days of dosing.3 Because of the error-prone nature of the viral reverse transcriptase of retroviruses or the 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 study, we used the HCV subgenomic replicon system to identify resistance mutations against two HCV PI clinical candidates, BILN 2061 and VX-950 (25). The in vitro resistance mutations selective against either inhibitor result in a significant reduction in susceptibility to the same inhibitor. However, the primary BILN 2061-resistant mutants are fully susceptible to VX-950, and the major VX-950-resistant mutant remains sensitive to BILN 2061. In this study, we identified mutants that are cross-resistant to both PIs. Analysis of structural models of these mutants indicated

2 The abbreviations used are: HCV, hepatitis C virus; IFN, interferon; NS, nonstructural; PIs, protease inhibitors; RT, reverse transcription; FRET, fluorescence resonance energy transfer; HIV, human immunodeficiency virus; EDANS, 5-[2′-aminoethyl]amino]-naphthalenesulfonic acid; DABCYL, 4-[4′-(dimethylamino)phenyl]azo]benzoic acid.

3 Reesink, H. W., Zeuzem, S., Weezeink, C. J., Forestier, N., van Vliet, A., van de Wetering de Rooij, J., McNair, L., Purdy, S., Chu, H.-M., and Jansen, P. L. M., 36th Annual Digestive Disease Week, Chicago, IL, May 15–19, 2005.
that steric hindrance is the primary reason for the resistance of these mutants against both HCV PIs. HCV replicon cells 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 assays.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction—*An Escherichia coli expression plasmid containing a DNA fragment encoding Met1–Ser181 of the HCV NS3 protease (GenBank™ accession number CAB46913) of the HCV Con1 replicon (designated series C). Second, another replicon cell line that was resistant to a DNA fragment encoding Met1–Ser181 of the HCV NS3 protease inhibitors were introduced into this construct by PCR-based site-directed mutagenesis. PI resistance mutations in the NS5 serine protease domain were also introduced into a second generation Con1 replicon plasmid containing three adaptive mutations (pBR322-HCV-Neo-mADE) as described previously (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—*Stable HCV Con1 subgenomic replicon cells (26) or variants with resistance mutations were generated and maintained in Dulbecco’s modified Eagle’s medium (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (JRH Biosciences) and 0.25 mg/ml G418 (Geneticin; Invitrogen). The cross-resistance mutations identified in this study were introduced into the pBR322-HCV-Neo-mADE replicon plasmid by site-directed mutagenesis as described previously (25). Stable replicon cell lines were generated using the T7 transcripts derived from pBR322-HCV-Neo-mADE with or without the cross-resistance mutations.

*IC_{50} Determination of Antiviral Agents in the HCV Replicon Cell Assay—*The IC_{50} values of the antiviral agents were determined in a 48-h assay with the HCV Con1 subgenomic replicon cells as described previously (25). Briefly, HCV replicon cells (10,000/well) were plated in a 96-well plate in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 0.5% Me2SO was added. The replicon cells were incubated with the antiviral agents for 48 h. Total cellular RNA was extracted using the RNeasy miniprep kit (Qiagen Inc.). A 1.7-kb-long cDNA fragment encompassing the HCV NS3 serine protease domain was amplified with a pair of HCV-specific oligonucleotides and purified as described previously (25). The purified RT-PCR products were subjected to bulk sequence determination. To determine the frequency of PI resistance mutations, the 1.7-kb RT-PCR products of HCV RNA from the PI-cross-resistant replicon cells were ligated into the TA cloning vector pCR2.1 (Invitrogen); 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—*The HCV NS3 serine protease domain containing the wild-type sequence or the cross-resistance mutation A156V or A156T was expressed in *E. coli* BL21(DE3)/pLysS cells (Strategene, La Jolla, California) as described previously (25). Briefly, freshly transformed cells were grown at 37 °C in brain heart infusion medium (Difco) supplemented with 100 μg/ml carbenicillin and 35 μg/ml chloramphenicol to an absorbance of 0.75 at 600 nm, followed by induction with 1 mM isopropyl-1-thio-β-d-galactopyranosidase for 4 h at 24 °C. All purification steps were performed at 4 °C as described previously (25). The cell paste was lysed in buffer A (50 mM HEPES (pH 8.0), 300 mM NaCl, 0.1% n-octyl β-d-glucopyranosidase, 5 mM β-mercaptoethanol, and 10% (v/v) glycerol) and homogenized using a Microfluidizer processor (Microfluidics, Newton, MA), followed by ultracentrifugation at 54,000 × g for 45 min. A final concentration of 5 mM imidazole and 2 ml of pre-equilibrated nickel-nitrilotriacetic acid resin (Clontech) were 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 HiLoad 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.

*Enzyme Assays for the HCV NS3 Serine Protease Domain—*Enzyme activity was determined using the assay described by Taliani et al. (27) with modifications. An internally quenched fluorogenic depsipeptide (fluorescence resonance energy transfer (FRET) substrate), Ac-Asp-Glu-Asp-(EDANS)-Glu-Glu-D-galactopyranosidase (Clontech) were 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 HiLoad 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.

**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 PIs VX-950 and BILN 2061. First, a replicon cell line that 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 series C). Second, another replicon cell line that 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 to 14 μM) and BILN 2061 (from 160 nM to 6.4 μM) (designated series D). Last, the stable naïve HCV Con1 subgenomic replicon cells were serially passaged in the presence of 0.25 mg/ml G418 and slowly increasing concentrations of both VX-950 (from 3.5 to 14 μM) and BILN 2061 (from 80 nM to 1.6 μM) (designated series E). During the course of selection, replicon cells were split twice per week when 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-cross-resistant replicon cells, cell pellets were collected whenever the cell culture was split. Total cellular RNA was extracted using the RNeasy miniprep kit (Qiagen Inc.). A 1.7-kb-long cDNA fragment encompassing the HCV NS3 serine protease domain was amplified with a pair of HCV-specific oligonucleotides and purified as described previously (25). The purified RT-PCR products were subjected to bulk sequence determination. To determine the frequency of PI resistance mutations, the 1.7-kb RT-PCR products of HCV RNA from the PI-cross-resistant replicon cells were ligated into the TA cloning vector pCR2.1 (Invitrogen); multiple individual bacterial colonies were isolated for each time point; and the HCV NS3 protease coding region of the purified plasmid DNA was sequenced.
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.1 Incubation of the HCV Con1 subgenomic replicon cells with VX-950 resulted in a concentration-dependent decline in the HCV RNA level as measured by real-time RT-PCR (TaqMan), with an average IC_{50} 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 in the low single digit nanomolar range.

**Development of PI-cross-resistant HCV Replicons from VX-950-resistant Cells**—To identify mutants that are cross-resistant to both VX-950 and BILN 2061, several selection schemes 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 series C) (Fig. 2A). For BILN 2061, the starting and final concentrations were 40 nM and 6.4 μM, respectively. Every 3 or 4 days, replicon cells were split; the medium was replenished; and fresh VX-950 and BILN 2061 were added. Because 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 declined over time and eventually became undetectable in the presence of high concentrations of HCV PI (data not shown). Cells with low or no neomycin phosphotransferase protein proliferated at a gradually decreasing rate and eventually died in the presence of G418. Replicon cells with the dominant VX-950 resistance mutation (A156S) were expected to die in the presence of increasing concentrations of BILN 2061 because they have been shown to be susceptible to inhibition by BILN 2061 (25). Only HCV RNA with mutations that were cross-resistant to both VX-950 and BILN 2061 could 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 for 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} for 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 IC_{50} > 30 μM (Fig. 2B). Therefore, the series C replicon cells at day 52 were cross-resistant to both VX-950 and BILN 2061.

Total cellular RNA was extracted from the series C replicon cells (which had been cultured in the presence of 14 μM VX-950 and 0.32 μM BILN 2061) at day 32 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

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**RESULTS**

HCV NS3-4A Serine Protease Inhibitors VX-950 and BILN 2061—VX-950 (Fig. 1) has recently been shown to possess antiviral activity in chronic hepatitis C patients in a phase I trial.3 VX-950 is a reversible

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1 C. A. Gates and Y.-P. Luong, unpublished data.
Development of PI-cross-resistant HCV Replicons from Naïve Replicon Cells—In our previous study of resistance mutations against a single HCV PI (VX-950 or BILN 2061), cell growth became stalled for several days, during which time a massive cell death was observed (25), signaling the emergence of resistant mutant replicon cells and concurrent death of nonresistant replicon cells. However, no such cell death or slow down in cell growth was observed during selection of the cross-resistant series C and D replicon cells 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 had not been exposed to either inhibitor.

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 RNA at day 32 was subcloned into the TA vector, and 10 individual colonies were subjected to sequencing. Six clones had the A156T substitution; three clones had the A156V substitution; and the last clone retained the A156S mutation.
In Vitro HCV NS3-4A Protease Inhibitor-resistant Mutants

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 series E) (Fig. 4). The starting and final concentrations of VX-950 were 3.5 and 14 \( \mu M \), respectively. For BILN 2061, the starting and final concentrations were 80 nM and 1.6 \( \mu M \), respectively. Every 3-4 days, replicon cells were split if they were confluent, the medium was replenished, 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 \( \mu M \) 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 was extracted from the series E cells at days 10, 21, and 48 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 compared with 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 RNA at day 21 or 48 was subcloned 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 \( \mu M \) VX-950 and 0.32 \( \mu M \) BILN 2061 for 14 days), 65% or 30 of 46 clones had the A156T substitution, whereas 20% or 9 of 44 clones had the A156V substitution. In the day 48 sample of the series E (which had been cultured in the presence of 14 \( \mu M \) VX-950 and 1.6 \( \mu M \) BILN 2061 for 14 days), 80% or 35 of 44 clones had the A156T substitution, whereas 20% or 9 of 44 clones had the A156V substitution. In either case, no other mutation in the NS3 serine protease domain was found in >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 Mutation A156V or A156T Is Sufficient to Confer Cross-resistance to both VX-950 and BILN 2061—To confirm that the observed mutations at Ala\(^{156}\) are sufficient to confer cross-resistance to 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 E. coli and purified for enzyme characterization. These mutations were also introduced into high efficiency subgenomic replicon plasmids for characterization in the HCV replicon system.

The catalytic efficiency (\( k_{cat}/K_m \)) of the A156T or A156V mutant protease for the FRET substrate was ~7- or 4-fold lower than that of the wild-type protease, respectively (TABLE ONE). The \( K_m \) values of VX-950 were 9.9 and 33 \( \mu M \) for the A156T and A156V mutant proteases, respectively, which are 99- or 330-fold higher than that for the wild-type protease (0.1 \( \mu M \)), respectively (TABLE TWO). Both mutant...
The sensitivity conferred by either mutation (TABLE THREE). The IC50 of either mutant replicon cell line, indicating at least a 75-fold decrease in 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 Ala156 side chain is in van der Waals contact with the P2 group of the two inhibitors. The three possible conformations of Thr156 are shown schematically in Fig. 6. In all cases, the atom of the residue 156 side chain is forced to the A156T or A156V mutant, the additional hydroxyl or methyl group, respectively, at the C-atom of the residue 156 side chain is forced to appear of their dominant resistance mutations. Indeed, not one of these single residue substitutions was observed when the HCV replicon cells were treated with both HCV PIs, as we reported in this study. Instead, we found that substitution of Ala156 with Val or Thr in the HCV serine protease domain conferred cross-resistance to both inhibitors. Ala156 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 Ala156 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 the HCV serine protease. On the other hand, any movement of the P4 group of VX-950 would cause loss of the hydrogen bond with the 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 of χ1 = −60° (Fig. 6) has the least number of unfavorable contacts with VX-950 and BILN 2061. The other two conformations (χ1 = 180° and 60°) (Fig. 6) have unfavorable contacts with both inhibitors either at the P2 side chain or at the P3 carbonyl group. In the A156T or A156V mutant, 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 χ1 = 180° or 60°, which has unfavorable interactions with the inhibitors. The three possible conformations of Thr156 are shown schematically in Fig. 6. In all cases, the additional methyl and hydroxyl groups of Thr156 have a repulsive interaction with the inhibitor and/or enzyme backbone atoms. By energy minimization, we found that the 180°/−60° conformation (Fig. 6) has the least repulsive interaction and that the main cause of the repulsion is resistant HCV replicon cells remain sensitive to either IFN-α or ribavirin. As shown in TABLE THIRD, the IC50 of either IFN-α or ribavirin remained virtually the same for HCV replicon cells containing A156T or A156V compared with 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 HCV PI-resistant mutants.

### DISCUSSION

We have previously shown that the A156S mutant is resistant to VX-950, but not to BILN 2061 (25). Substitution of Asp168 with Val or Ala causes resistance to BILN 2061, but susceptibility to VX-950 remains. Because 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, not one of these three single residue substitutions was observed when the HCV replicon cells were treated with both HCV PIs, as we reported in this study. Instead, we found that substitution of Ala156 with either Val or Thr in the HCV serine protease domain conferred cross-resistance to both inhibitors. Ala156 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 Ala156 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 the HCV serine protease. On the other hand, any movement of the P4 group of VX-950 would cause loss of the hydrogen bond with the cap carbonyl as well as hydrophobic interactions of the P4 group of the inhibitor (25).

The fitness or replication capacity of the PI cross-resistance mutations was determined in a transient transfection system using the luciferase activity as the surrogate readout. Because 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 ~5 or 3%, respectively, of that of the wild-type replicon in the luciferase transient transfection assay. These results are consistent with the lower catalytic efficiency of the two mutants compared with 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 in the stable wild-type replicon cells (data not shown), which was not unexpected given the reduced replication capacity or fitness of the mutant replicons and the lower catalytic efficiency of the mutant proteases. No significant reduction in HCV replicon RNA by up to 30 μM VX-950 was observed in either mutant replicon cell line, indicating at least a 75-fold decrease in sensitivity conferred by either mutation (TABLE THIRD). The IC50 of BILN 2061 for the A156T replicon cells was 1.09 μM, which is 272-fold higher than that for the wild-type replicon cells (4 nM). The IC50 of BILN 2061 for the A156V mutant replicons was 10 μM, indicating a >2500-fold decrease in sensitivity conferred by the A156V mutation (TABLE THIRD).
In Vitro HCV NS3-4A Protease Inhibitor-resistant Mutants

![Diagram of protease-inhibitor complexes](image-url)

The inhibitors VX-950 (purple) and BILN 2061 (yellow) are shown as stick models, with nitrogens in blue, oxygens in red, and sulfur in cyan. The Ala156 (green) and Asp168 (orange) side chains are shown as sticks. The side chain of Ala156 is highlighted with green dots. The catalytic triad Ser139, His57, and Asp81 is shown in orange. The catalytic triad side chains are shown as thin lines. The E2 strand of the protease is shown as thick lines. The inhibitors VX-950 and BILN 2061 have the lowest energy for either mutation, but remain repulsive to both inhibitors.

Several in vitro studies suggest that the HCV NS3-4A serine protease may block the IFN signal transduction pathway (31–33) and therefore interfere with host innate immune responses, which could be one of the reasons that HCV escapes immune clearance and maintains chronic infection. One may expect that the HCV PI-resistant mutants, with a significantly diminished fitness and a less efficient NS3-4A protease, may be less able to interfere with the IFN pathway. If so, the HCV replicon cells containing these PI resistance mutations may become more sensitive to inhibition by IFN-α. However, no change in IC50 values was observed for A156T or A156V replicon cells compared with wild-type replicon cells in our study, which is similar to what has been previously reported for other HCV PI resistance 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 antiviral drugs. Resistance to inhibitors of human immunodeficiency virus (HIV) reverse transcriptase or protease is caused by specific mutations in the viral enzymes (for a review, see Ref. 36). Because of the error-prone nature of the HIV reverse transcriptase, resistance mutations emerge quickly in patients who are on monotherapy with HIV-specific inhibitors. It is estimated that all possible single mutations can be randomly generated within 1 day in an HIV-infected patient. Even though elimination or cure of HIV infection in patients remains an elusive goal, multidrug combination therapies have been shown to be more effective than monotherapy in reducing HIV viral load and suppressing the emergence of resistance mutants. 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 has been reported that the frequency of resistance mutations against lamivudine increases from 24% in the first year to 67% in the fourth year in 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 1010 to 1012 viral particles/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 resistance to HCV therapy involving small molecule inhibitors of HCV enzymes might require multidrug combination, as in the case of the current HIV treatments. Because 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, and D168A either did not emerge or disappeared when various types of HCV replicon cells were incubated with both VX-950 and BILN 2061. However, Thr or Val substitution at a single amino acid (Ala156) 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 of 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 2 weeks of treatment (24). 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, because all of these PI-resistant mutant replicons remained sensitive to IFN-α, combinations of HCV PIs with IFN-α, 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 PI(s) and therefore increase the efficacy of anti-HCV therapy.

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