Susceptibility of Treatment-Naive Hepatitis C Virus (HCV) Clinical Isolates to HCV Protease Inhibitors

Andrew Bae, Siu-Chi Sun, Xiaoping Qi, Xiaowu Chen, Karin Ku, Angela Worth, Kelly A. Wong, Jeanette Harris, Michael D. Miller, and Hongmei Mo*

Department of Clinical Virology, Gilead Sciences, 333 Lakeside Drive, Foster City, California 94404

Received 7 June 2010/Returned for modification 26 July 2010/Accepted 1 September 2010

In order to assess the natural variation in susceptibility to hepatitis C virus (HCV) NS3 protease inhibitors (PIs) among untreated HCV patient samples, the susceptibilities of 39 baseline clinical isolates were determined using a transient-replication assay on a panel of HCV PIs, including two α-ketoamides (VX-950 and SCH-503034) and three macrocyclic inhibitors (MK-7009, ITMN-191, and TMC-435350). Some natural variation in susceptibility to all HCV PIs tested was observed among the baseline clinical isolates. The susceptibility to VX-950 correlated strongly with the susceptibility to SCH-503034. A moderate correlation was observed between the susceptibilities to ITMN-191 and MK-7009. In contrast, the phenotypic correlations between the α-ketoamides and macrocyclic inhibitors were significantly lower. This difference is partly attributable to reduced susceptibility of the HCV variants containing the NS3 polymorphism Q80K (existing in 47% of genotype 1a isolates) to the macrocyclic compounds but no change in the sensitivity of the same variants to the α-ketoamides tested. Our results suggest that the natural variation in baseline susceptibility may contribute to different degrees of antiviral response among patients in vivo, particularly at lower doses.

Hepatitis C virus (HCV) is characterized by a high degree of genetic diversity because of its rapid replication rate and turnover, combined with the poor fidelity of the HCV RNA-dependent RNA polymerase (RdRp) (3, 5, 32). The nucleotide sequences among the six different genotypes (GTs) differ at 30 to 35% of nucleotide sites (25, 26). Each of the six major GTs of HCV contains a series of closely related subtypes whose nucleotide sequences typically differ from each other by 20 to 25%. Furthermore, 5 to 8% sequence divergence is present between individual strains (variants) of HCV within a given subtype. A comprehensive analysis of HCV NS3 sequences from a larger number of GT-1 isolates found that amino acid polymorphisms were detected all along the protease sequence, including residues associated either with resistance to HCV protease inhibitors (PIs) (V36, I170, and D168) or with compensatory mutations (I72, T72, Q86, and I153) (1, 2, 30). However, many questions remain, including whether natural variation in the NS3 protease sequence impacts the susceptibility of HCV to PIs currently in development and whether there are any relationships among the chemotypes of the PIs and their baseline susceptibilities.

Hepatitis C virus NS3/4A serine PIs have demonstrated potent antiviral activity in subjects infected with HCV GT-1 by specifically blocking NS3/4A protease-dependent HCV polyprotein processing. Among these PIs, VX-950 (telaprevir) and SCH-503034 (boceprevir), the two most clinically advanced NS3/4A serine PIs, are both α-ketoamide compounds that covalently bind to the active-site serine of the protease (6, 9, 10, 14, 16, 22). These drugs also have similar resistance profiles. Mutations V36A/M, T54A, R155K, and A156S in the NS3 protease gene, conferring a low level of resistance to VX-950, were identified in HCV GT-1-infected patients treated with VX-950 monotherapy or in combination with alpha interferon (IFN-α) and ribavirin (11, 21). In addition, the single mutant A156T or A156V or the double mutant at positions 36/155 or 36/156, all conferring high-level resistance, were observed in some VX-950-treated patients. Similarly, samples from SCH-503034-treated patients revealed the emergence of the low- to moderate-level resistance mutations T54A, V170A, and A156S and, less commonly, the high-level resistance mutant A156T (27, 28).

More recently, other HCV PIs, including ITMN-191, TMC-435350, and MK-7009, have progressed to the early stages of clinical evaluation (12, 15, 19, 20, 23, 29). These compounds are structurally related macrocyclic inhibitors that are chemically distinct from VX-950 and SCH-503034. The mutations that display a reduced susceptibility to ITMN-191 include D168A/E/V, A156S/V, F43S, Q41R, and S138T in the NS3 protease; S489L in the NS3 helicase; and V23A in NS4A (24). S498L in the helicase domain buttresses the P2 pocket, and the substitution V23A is at a site in the NS4A peptide cofactor that borders the P1* pocket (24). The F43S, Q41R, R155K, A156T, and D168Y mutations in NS3 also displayed low- to high-level reduced sensitivity to MK-7009 (12). Since these PIs had some overlapping and some nonoverlapping resistance profiles, it would be of interest to compare their potencies against the natural HCV variants derived from treatment-naïve patient isolates.

Standard replicon assays limit the assessment of the inhibitory activities of anti-HCV compounds to a few laboratory-optimized strains. These assays may not reflect the range of activities of a compound against the heterogeneous viral population that exists in HCV-infected patients. Previously, we
have reported the development of a replicon-based shuttle vector to allow rapid phenotypic analysis of the NS3 protease domain from samples of a large number of HCV-infected patients (18). We demonstrated a high success rate and reproducibility of the novel replicon-based phenotypic assay. However, the assay sensitivity for detection of mutants in a mixed population may be reduced for resistance mutations that are less fit or with lower levels of resistance or samples containing only a small proportion of a mutant in a population (18). For example, reduced susceptibility to BILN-2061 was observed for mutant/wild-type mixtures of 5%/95% for the D168V mutation but was observed for only 50%/50% mixtures for the A156T resistance mutation. Using this phenotypic-analysis assay, the susceptibilities of a panel of HCV treatment-naïve clinical isolates to VX-950 (telaprevir), SCH-503034 (boceprevir), ITMN-191, MK-7009, and TMC-435350 were purchased from Acme Bioscience, Ltd. (Belmont, CA) (Fig. 1). All compounds were dissolved in dimethyl sulfoxide (DMSO) prior to use. GS-9132 was synthesized by Achillion Pharmaceuticals (New Haven, CT). Compounds were dissolved in DMSO at a concentration of 10 mM.

**MATERIALS AND METHODS**

**Compounds.** The PIs VX-950, SCH-503034, MK-7009, ITMN-191, and TMC-435350 were purchased from Acme Bioscience, Ltd. (Belmont, CA) (Fig. 1). All compounds were dissolved in dimethyl sulfoxide (DMSO) prior to use. GS-9132 was synthesized by Achillion Pharmaceuticals (New Haven, CT).

**Plasmids.** The GT 1b-Con-1 subgenomic replicon construct used to create the shuttle vector has been described by Friebe et al. (8). A poliovirus internal ribosome entry site (IREs) element was added to the 5′ end after the HCV 5′ untranslated region (UTR) to increase firefly luciferase translation and RNA ribosome entry site (IRES) element was added at the 5′ end of the shuttle vector to allow rapid phenotypic analysis of the NS3 protease domain. To generate the replication-defective parental vector, another Ascl site was introduced at position 1071. The fragment between the two Ascl sites (1071 to 1214) was then excised to create a deletion within the protease domain.

**Amplification of the protease gene from patient sera.** A Qiagen RNA extraction kit was used to isolate the HCV RNA, as described in the user’s manual. To synthesize cDNA, we used GT-specific primers (1a, 1a4a3’5′735; 1b, 1b4a3’5′650) (18) (see Table S1 in the supplemental material) and 10 μl of extracted RNA in a 20-μl reaction mixture using MonstSer Script reverse transcriptase as recommended by the manufacturer. Reverse transcription was run on an MJ Research PTC-100 thermal cycler (Bio-Rad) using the following program: 50°C (1a) or 54°C (1b) for 10 min, 60°C for 40 min, and 90°C for 2 min. PCR was performed with 10 μl of cDNA and GT-specific primers to amplify the full-length NS3/4A gene, using the following primers in a 50-μl reaction mixture with platinum Taq DNA polymerase (Invitrogen) as recommended by the manufacturer: 1a, 1a3’5′3181 and 1a4a3’5′735, or 1b, 1b3’5′3150 and 1b4a3’5′650. PCR temperature cycles were as follows: 94°C for 2 min (1a) and 35 cycles of 94°C for 30 s, 60°C (1a) or 65°C (1b) for 30 s, 72°C for 3 min, and one cycle of 72°C for 7 min.

The first PCR products were used as templates in the following nested PCRs to generate gene cassettes with cloning sites incorporated at both ends. All the nested PCRs were performed with High Fidelity PCR master mix (Roche Applied Science) as directed by the manufacturer. The protease domain cassette was generated using the primer pair PCR NS3/4A F2 and PCR Prot R2 (E1202G), with the adaptive mutation E1202G incorporated in the reverse primer. The sequences of the above-mentioned primers are described in Table S1 in the supplemental material and reference 18.

**Transfer of the gene cassette to a shuttle vector and RNA synthesis.** The nested-PCR products of the NS3/4A gene were treated with Clal and Ascl (New England Biolabs) at 37°C for 3 h and then cleaned up with a MinElute Reaction cleanup kit (Qiagen). The shuttle vector DNA was similarly digested, and the fragment of interest was isolated by gel electrophoresis and removed from the gel matrix with a Qiaex II gel extraction kit (Qiagen). The shuttle vector DNA was ligated and linearized by digestion with Scal at 37°C overnight. The ligation products were then precipitated with pellet paint (Novagen), washed, and subsequently resuspended in H2O. Transformation of the ligation reaction was done by electroporation into ElectroTen-Blue cells (Agilent) according to the supplier’s recommendations. Ten percent of the transformation mixture was plated on antibiotic selection plates to determine the transformation efficiency, and the remaining transformationants were expanded in liquid culture to propagate the quasispecies pool. The plasmid DNA was extracted and linearized by digestion with Scal at 37°C overnight. RNA was synthesized using a T7 Megascript RNA synthesis kit (Ambion) following the manufacturer’s instructions.

**Transient-replication assay and luciferase reading.** HuH-7 cells used in the transient-transfection assay were derived from a cured replicon cell clone (7) designated HuH-7/Lunet. The Lunet cells were grown in Dulbecco’s modified...
Eagle medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Cells were harvested by trypsinization and washed with cold phosphate-buffered saline (PBS) twice before electroporation. The cell density was adjusted to 1 × 10⁷ cells/ml, and 0.4 ml of cells was transferred to a cold cuvette with a gap width of 10 μl and allowed to attach overnight. A 1-ml aliquot of the cell suspension was added to 20 ml of complete medium. The cell suspension was seeded in a 96-well plate at 100 cells/ml, and 0.4 ml of cells was transferred to a cold cuvette with a gap width of 10 μl and allowed to attach overnight. A 1-ml aliquot of the cell suspension was measured for luciferase activity 4 h posttransfection to normalize for transfection efficiency. For 50% effective concentration (EC₅₀) determination, serially diluted compounds were added to the plate the day after transfection (0.5% final DMSO concentration). After 3 days of incubation, the cells were lysed by addition of 50 μl of lysis buffer (Promega). Fifty microliters of luciferase substrate (Promega) was then added to each well, and the luciferase signal was read with a Victor luminometer (Perkin-Elmer). For all experiments, the EC₅₀ of test compounds were calculated by nonlinear regression using the GraphPad Prism program. The signal-to-noise window was determined as the ratio of luciferase activity from cells treated with 0.5% DMSO to the activity from cells treated with 500 nM GS-9190 in 0.5% DMSO.

**RESULTS**

Activities of HCV PIs against genetically diverse GT-1 baseline clinical isolates. In order to determine the susceptibilities of diverse GT-1 clinical isolates, the NS3 protease domain was amplified from 39 treatment-naïve patient sera, including 25 GT-1a and 14 GT-1b isolates. The final PCR products were ligated into the protease domain shuttle vector, which also expresses firefly luciferase. The replication efficiencies and the susceptibilities of the quasispecies pool of proteases to PIs were determined in transient-transfection assays (Fig. 2). All 39 chimeric replicons carrying patient-derived NS3 protease genes replicated at levels sufficient for phenotypic analysis (firefly luciferase signal/noise ratios were at least 10 and up to 2,000), and their replication efficiencies ranged from 0.1 to 50% of that of the 1b-Con replicon (data not shown). The replication capacity of GT-1b samples are on average higher than those of 1a samples, possibly due to higher homology and compatibility with the backbone. A few of the GT-1a samples replicated at a level just high enough to give a minimum signal/noise ratio of 10 to test susceptibility to drugs. The extent of drug inhibition was not correlated with the level of replication, since similar EC₅₀ values were obtained for the control compound GS-9132 against all baseline clinical isolates, although their replication capacities varied from 0.1 to 50% (Fig. 2).

With the laboratory HCV 1b replicon, excellent reproducibility in EC₅₀ values was observed with all five PIs and GS-9132 (Table 1) tested in different experiments. The EC₅₀ from independent experiments differed by less than 2-fold with respect to the mean EC₅₀ for all 5 PIs (with a maximum of 1.8-fold). No significant difference in the degree of variation in EC₅₀ was seen between different PIs. When the chimeric replicons carrying NS3 proteases from 20 different baseline isolates were tested against GS-9132, an NS4A antagonist, there was little difference between two groups (a group of isolates containing Q80K versus another group lacking Q80K) that is greater than what can be attributed to random sampling variation. The clinical “isolates” used here refer to a mixture, for example, what was actually isolated from the patient sample, not a mutant generated by site-directed mutagenesis. The null hypothesis is that the two samples were not drawn from populations with different medians. The rank sum test is a nonparametric procedure, which does not require assuming normality or equal variance.

**Molecular modeling.** The crystal structures of TMC-435350 and SCH-503034 bound to HCV protease were obtained from the Protein Data Bank (PDB codes 3KEE and 2OCS, respectively) (4, 17). The analysis was performed with the program Pymol (Schrödinger Inc., New York, NY).

**TABLE 1.** Susceptibilities of HCV NS3 proteases from baseline clinical isolates to HCV inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean 1b-Con-1 EC₅₀ ± SD (nM)</th>
<th>EC₅₀ of baseline clinical isolates (nM; n = 39 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median (mean) 5th percentile 95th percentile 95th percentile/5th percentile</td>
</tr>
<tr>
<td>VX-950</td>
<td>376.7 ± 154.5 (1.5)</td>
<td>298 (166) 59 643 11</td>
</tr>
<tr>
<td>SCH-503034</td>
<td>275.8 ± 3.3 (1.4)</td>
<td>256 (150) 52 744 14</td>
</tr>
<tr>
<td>ITMN-191</td>
<td>0.6 ± 0.7 (1.7)</td>
<td>0.5 (0.4) 0.1 1.2 12</td>
</tr>
<tr>
<td>MK-7009</td>
<td>0.7 ± 0.4 (1.8)</td>
<td>0.5 (0.4) 0.2 1.0 6</td>
</tr>
<tr>
<td>TMC-435350</td>
<td>3.3 ± 0.7 (1.1)</td>
<td>7.4 (2.7) 0.9 29 32</td>
</tr>
<tr>
<td>GS-9132</td>
<td>28.2 ± 22 (2.3)</td>
<td>67 (62) 42 114 3</td>
</tr>
</tbody>
</table>

*The mean ± SD from 3 to 5 independent experiments; the values in parentheses indicate the maximum fold change of an individual experiment relative to the mean EC₅₀.

* n = 39 for the 5 protease inhibitors; n = 20 for GS-9132.
variation in EC_{50} among the genetically diverse GT-1 baseline isolates (Fig. 2 and Table 1), with the 95th percentile EC_{50} only 3-fold higher than the 5th percentile EC_{50}.

In contrast, some variation in susceptibilities to all five HCV PIs tested was observed among the baseline clinical isolates (Fig. 2 and Table 1). For example, the increases in 95th percentile EC_{50}s compared to the 5th percentile were 6-, 11-, 12-, 14-, and 32-fold for MK-7009, VX-950, ITMN-191, SCH-503034, and TMC-435350, respectively. Despite the variation, the mean EC_{50}s of the 39 baseline clinical isolates were similar to that of the laboratory strain 1b-Con-1 for all five PIs (Table 1). In addition, it appeared there were two groups of samples for TMC-435350, and one group had higher EC_{50}s than the other group (Fig. 2). Careful analysis found that these two clusters of isolates had different amino acids at position 80 of the HCV protease. For example, the higher-end EC_{50} cluster consisted of 11 genotype 1a isolates containing the Q80K substitution. The EC_{50}s of the remaining 14 genotype 1a isolates and the 14 genotype 1b isolates (all lacking Q80K) overlapped and formed the lower-end EC_{50} cluster (Fig. 2).

**NS3 protease sequence analysis of the baseline clinical isolates.** In order to define the genetic basis for the drug susceptibilities of the baseline clinical isolates, the NS3 protease genes from all 39 isolates were population sequenced. The amino acid sequence of the NS3 protease gene from each sample was aligned against its respective subtype reference, i.e., H77 (GenBank accession number AF009606) (1a) and Con1 (GenBank accession number AJ238799) (1b) (Fig. 3). The isolates were arranged in ascending order based on the EC_{50}s against TMC-435350 shown in Fig. 3. As described above, among the 25 GT-1a isolates, 11 isolates with higher EC_{50}s against TMC-435350 contained a Q80K substitution in the NS3 protease gene. Further analysis identified the presence of Q80K in 121 out of 268 (47%) genotype 1a isolates within the combined HCV Los Alamos and Gilead Science databases (data not shown). However, the prevalence of Q80K in genotype 1b was low (0.3%; 1/372). In addition to Q80K, a T54S substitution was identified in 2 out of 39 baseline samples, one GT-1a and another GT-1b. Mutations at position 54 have been identified in HCV-infected subjects treated with VX-950 (21). The T54S-containing GT-1a isolate also had the Q80K substitution. In contrast to Q80K, only 1.4% of genotype 1 (9/640; 1a and 1b) isolates from the above-mentioned database contained T54S. No known PI resistance mutations at positions 155, 156, and 168 were seen in any of the 39 samples.

**Correlation of the susceptibilities of the baseline clinical isolates between drug pairs.** All PIs studied here target the active site of HCV protease; however, whether there is a subtle difference in the binding mode among different PIs has not been well characterized. In order to address this question, the susceptibilities of the baseline isolates to VX-950, SCH-503034, ITMN-191, MK-7009, and TMC-435350 were compared between each drug pair (Fig. 4). The correlation was highest between VX-950 and SCH-503034 (R^2 = 0.92) susceptibilities and intermediate between ITMN-191 and MK-7009 (R^2 = 0.68) susceptibilities. In contrast, the correlations between the susceptibility to TMC-435350 and either ITMN-191 (R^2 = 0.23) or MK-7009 (R^2 = 0.17) were low. Furthermore, the correlations between susceptibility to VX-950 and either ITMN-191, MK-7009, or TMC-435350 were also very low (R^2 < 0.12). Similar low correlations were observed between the susceptibilities to SCH-503034 and either ITMN-191, MK-7009, or TMC-435350 (Fig. 4). Interestingly, a number of samples were less sensitive to TMC-435350 but more sensitive to SCH-503044 and VX-950, and vice versa (Fig. 4d and g). A more detailed analysis found that these samples all contain the Q80K polymorphism (Fig. 2 and 5). Similar results or slightly improved correlations were obtained when the EC_{50} plots against two PIs were further separated either by subtype (1a versus 1b) or by isolates containing versus lacking Q80K (data not shown). However, the sample sizes in the genotype 1b (total, 14) and Q80K (11 isolates) groups were relatively small. It would be interesting to assess the correlations of different subtype or Q80K presence in a larger sample size.

**Effects of chimeric replicons carrying Q80K- and T54S-containing NS3 protease genes from patient samples on susceptibility to PIs.** To investigate the role of Q80K in drug susceptibility, the 25 GT-1a isolates were divided into two groups: one containing Q80K (11 isolates) and the other lacking Q80K (14 isolates with Q80Q). As shown in Fig. 5 and Table 2, the Q80K-containing isolates displayed significantly reduced susceptibility to TMC-435350, with a mean EC_{50} 7.5-fold higher than the average EC_{50} of the Q80Q isolates. In addition, the baseline isolates containing Q80K were also on average less sensitive to MK-7009 and ITMN-191 but were equally or slightly more sensitive to VX-950 and SCH-503034 than the isolates lacking this mutation (P values ranged from >0.015 to <0.001) (Fig. 5 and Table 2). In addition, one isolate (B10; GT-1b) containing a T54S NS3 variant displayed significantly reduced susceptibility to VX-950 and SCH-503034 (6- to 9-fold) but maintained wild-type susceptibility to the other three macrocyclic PIs (Table 3). Interestingly, one of the GT-1a isolates (A25) containing both Q80K and T54S showed wild-type susceptibility to VX-950 and SCH-503034 but 11-fold resistance to TMC-435350 (B10) (Table 3). A final isolate with Q80L showed near-wild-type susceptibility to all drugs tested, indicating that this polymorphism has no phenotypic consequence.

**Characterization of mutations conferring resistance to PIs.** To confirm the effect of the Q80K and T54S NS3 mutations on susceptibility to PIs, the single mutants Q80K and T54S and the double mutant Q80K/T54S were engineered into the wild-type 1a-H77 replicons and characterized in a transient-repli- case assay (Table 4). The 1a-H77 replicon rather than 1b-Con-1 was used as a backbone because Q80K was identified in ~47% of genotype 1a isolates but not in GT 1b isolates. The T54S mutant exhibited 4.7- and 5.6-fold reduced susceptibility to SCH-503035 and VX-950, respectively, but was not cross-resistant to ITMN-191, MK-7009, or TMC-435350 (Table 4). In contrast, the Q80K mutant displayed 6.3- to 10.9-fold-reduced susceptibility to ITMN-191, MK-7009, or TMC-435350 yet retained wild-type susceptibility to SCH-503034 and VX-950 (Table 4). Results similar to those for the Q80K mutant were obtained with the double mutant Q80K/T54S.

**Molecular modeling of protease mutations with HCV protease inhibitors.** To understand the potential impacts of the Q80K and T54S mutations on protease inhibitor susceptibilities, we analyzed the TMC-534350/HCV protease (4) and SCH503034/HCV protease (17) co-crystal structures (Fig. 6).
FIG. 3. HCV NS3 protease amino acid sequence (1 to 181) from baseline clinical isolates aligned with EC50s of TMC-435350 in ascending order. (a) GT-1a clinical isolates. (b) GT-1b clinical isolates.
and performed molecular modeling. As shown in Fig. 1, all of the PIs share a peptidomimetic backbone, which binds similarly in the active site of NS3 protease. However, macrocyclic inhibitors all contain a large P2 moiety off the proline ring (the cyclopentyl ring for TMC-43350). The crystal structure (Fig. 6A) shows that the P2-substituted quinoline moiety of TMC-43350 induces a buried salt bridge formed by R155-D168, which is absent in apo structures (structure not shown). Therefore, mutations at either position 155 or 168 could severely disrupt this salt bridge and affect its interaction with TMC-43350, resulting in resistance. Furthermore, Q80 is in direct contact with R155. The Q80K mutation introduces a positively charged lysine next to the positively charged R155. The unfavorable repulsive charge interactions here likely weaken the interaction between the protease and the P2 moiety of the inhibitor, resulting in reduced susceptibility. Molecular modeling studies suggest that MK-7009 and ITMN-191 make similar interactions (data not shown).

In contrast, H9251-ketoamide inhibitors, like VX-950 and SCH-503034, do not possess a large P2 moiety that forces the formation of a buried R155-D168 salt bridge (Fig. 6B); both R155 and D168 side chains adopt solvent-exposed conformations.

FIG. 4. Correlation of susceptibilities between different HCV PIs. The x and y axes represent mean EC_{50}s from at least two independent experiments for the indicated drugs for the individual baseline clinical isolates. The lines represent the best unconstrained linear regression through the data points. The coefficient of determination (R^2) and the P value of each correlation plot are indicated.
These inhibitors make only a few contacts with R155 and D168 side chains, which is consistent with low-level resistance for R155K mutations. T54 lies in a buried position and does not directly interact with these inhibitors (Fig. 6C); it is relatively close to S139, a catalytic residue that forms a covalent bond directly with these inhibitors (Fig. 6C); it is relatively close to S139, a catalytic residue that forms a covalent bond directly with S139. The Q80K/T54S double mutant does not significantly affect macrocyclic inhibitors, since they do not rely on covalent interactions with S139. The Q80K/T54S double mutant does not significantly affect macrocyclic inhibitors, since they do not rely on covalent interactions with S139. The Q80K/T54S double mutant does impact macrocyclic inhibitors in a manner similar to that of the individual Q80K mutation. However, there is no clear structural evidence to explain why the Q80K/T54S double mutant is slightly less resistant to VX-950 and SCH-503034 than T54S alone.

**DISCUSSION**

Given the high degree of HCV genetic variability, which leads to a diversity of viral quasispecies circulating among infected individuals, it is particularly important to determine the range of activity of a compound against a heterogeneous viral population that exists in HCV-infected subjects. In this report, the susceptibilities of 39 HCV treatment-naïve clinical isolates to VX-950 (telaprevir), SCH-503034 (boceprevir), ITMN-191, MK-7009, and TMC-435350 were evaluated using a replicon-based NS3 protease phenotypic-analysis assay. Some natural variation in susceptibilities among these isolates was observed with all five HCV PIs tested (VX-950, SCH-503034, ITMN-191, MK-7009, and TMC-435350). The order of variation from least to greatest was MK-7009 < VX-950 < ITMN-191 < SCH-503034 < TMC-435350. The increases in EC_{50} in the 95th percentile compared to the 5th percentile EC_{50} ranged from 6- to 32-fold for the five PIs. This is in contrast to only a 3-fold difference between the 95th percentile and 5th percentile EC_{50} for GS-9132, an NS4A antagonist used as a control. The small variation in GS-9132 EC_{50} among the patient isolates showed a minimal effect of N-terminal NS3 sequence variation on the sensitivity to the NS4A antagonist GS-9132. Of note, the NS4A sequences were identical among the 39 HCV recombinant isolates created here (data not shown).

Our assay generated EC_{50} from independent experiments that differed by less than 2-fold with respect to the mean EC_{50} for the laboratory strain 1b-Con-1, showing that the assay itself also has a high degree of precision. Altogether, these findings indicate that the natural variation in susceptibility to the PIs observed among baseline clinical isolates is likely caused by the genetic diversity of the clinical isolates. This information has important implications for the interpretation of the clinical response and the dose selection for these inhibitors. In addition to the pharmacokinetics of the drug, the natural variation in susceptibility of baseline isolates may account for different responses in clinical studies. Indeed, variable response to some of the HCV PIs among different patients has been observed in clinical studies, especially in the lower-dose groups (20, 21, 22). Theoretically, the optimal clinical doses for these inhibitors should achieve C_{trough} (minimum concentration after dosing) values that are in excess of the EC_{50} obtained from a panel of patient isolates. Therefore, knowing the range of EC_{50} from a panel of patient isolates will be helpful in selecting doses for clinical trials. For example, knowledge of the low level of resistance to TMC-435350 conferred by the Q80K polymorphism helped determine the 200-mg once-a-day (QD) dose of TMC-435350 in a 5-day monotherapy trial (20). The mean plasma concentration of TMC-435350 at day 8 (72 h after the last dose of 200 mg QD) was still 3,360 ng/ml and thus 280 times above the wild-type replicon EC_{50} (plasma protein binding corrected), which would be ≥25 times the EC_{50} against Q80K variants. In addition, knowledge of the natural variation in baseline susceptibility for a given drug could provide a threshold for defining significant alterations in drug susceptibility that could serve as an indicator of potential drug resistance.

In order to identify the residues that may be potentially associated with reduced susceptibility to PIs, the amino acid sequence of the NS3 protease gene from each sample was aligned against its respective subtype reference with the EC_{50}.

**TABLE 2. Susceptibilities of the Q80Q versus Q80K genotype 1a baseline clinical isolates**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VX-950</td>
<td>SCH-503034</td>
</tr>
<tr>
<td>Mean EC_{50} (nM) of Q80Q isolates (n = 14)</td>
<td>176.2</td>
</tr>
<tr>
<td>Mean EC_{50} (nM) of Q80K isolates (n = 11)</td>
<td>72.0</td>
</tr>
<tr>
<td>Fold change in EC_{50}</td>
<td>0.5</td>
</tr>
<tr>
<td>P value</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*Fold change in EC_{50} = mean Q80K EC_{50}/mean Q80Q EC_{50}.*
for TMC-435350 in ascending order. Interestingly, a Q80K polymorphism was identified in isolates with higher EC50s against TMC-435350. Further analysis demonstrated that Q80K, seen in ≥47% of genotype 1a isolates, was associated with significantly reduced susceptibility to TMC-434350 and low-level resistance to MK-7009 and ITMN-191 but wild-type susceptibility to VX-950 and SCH-503034. Unlike VX-950 and SCH-503034, the other three PIs (MK-7009, ITMN-191, and TMC-435350) were fully active against the T54S-containing variant. The resistance profiles of the Q80K and T54S NS3 mutants were further confirmed by introducing these mutants into the 1a-H77 replicon. It would be interesting to further analyze the impacts of these two NS3 mutants on viral response in patients, particularly patients in lower-dose groups.

The HCV PIs could be divided into two chemically distinct classes: the ω-ketoamides (VX-950 and SCH-503034) and the macrocyclic inhibitors (MK-7009, ITMN-191, and TMC-435350). Consistent with the similarity of the chemical structures of the two ω-ketoamide inhibitors, the phenotypic susceptibilities of the 39 baseline clinical isolates were strongly correlated between VX-950 and SCH-503034. Similarly, good correlation was observed between the susceptibilities to ITMN-191 and MK-7009. The strong correlations of susceptibilities observed here suggest similarity in the binding modes between VX-950 and SCH-503034 or between ITMN-191 and MK-7009. However, the phenotypic correlations between VX-950 and SCH-503034 and the other PIs (ITMN-191, MK-7009, and TMC-435350) were low, consistent with their distinct chemical classes and different resistance profiles. The correlations among the susceptibilities to TMC-435350 with either MK-7009 or ITMN-191 were also low, which may be due in part to the fact that Q80K caused a slightly higher level of resistance to TMC-435350 than MK-7009 or ITMN-191. In fact, the baseline isolates were clearly separated into two groups based on their susceptibilities to TMC-435350: the variants with Q80K clustered together at the high end of EC50s versus the Q80Q variants at the low end of EC50s (Fig. 2 and 5). Similarly, the correlations between the susceptibilities to the ω-ketoamide inhibitors and macrocyclic inhibitors were low, confirming that these two classes of PIs may interact with the HCV protease differently, although they all bind to the same active site. The different binding modes of the two classes of PIs was further supported by (i) low-level resistance of Q80K to the macrocyclic inhibitors, particularly TMC-435350, but wild-type susceptibility to the ω-ketoamide inhibitors; (ii) the fact that the T54S/A and V36M/A mutations conferred low-level resistance to ω-ketoamides (21, 27), but not to the macrocyclic inhibitors, as demonstrated in the present and previous studies; and (iii) previous reports showing that mutations at D168 and R155 confer high-level resistance to the macrocyclic inhibitors (12, 23, 29) but remain fully susceptible (mutations at D168) or show only low-level resistance (mutations at R155) to ω-ketoamide inhibitors (21, 27). Taken together, the low susceptibility correlations and some nonoverlapping resistance of the ω-ketoamide and macrocyclic inhibitors suggest that there is a subtle difference in the interaction between these two classes of PIs with some of the residues in the active site of HCV protease, and a combination of these two classes of PIs may have clinical value.

Structural analysis of the cocrystal structures of NS3 protease complexed with either SCH-503034 (17) or TMC-533550 (4) indicated that the above findings are highly consistent with structural observations (Fig. 6). The P2-substituted quinoline moiety of TMC-435350 induces a buried salt bridge formed by R155-D168, which is absent in apo structures (structure not shown). Q80 is in direct contact with R155. Q80K may affect the interactions between the protease and inhibitor P2 moiety via R155. Molecular modeling suggests that MK-7009 and ITMN-191 make similar interactions (data not shown). Furthermore, the impact of the Q80K/T54S double mutant has been shown to be additive of the individual Q80K and T54S mutations and primarily driven by Q80K; this is consistent with the observation that the positions of Q80K and T54S are about 14 Å apart. On the other hand, ω-ketoamide inhibitors, like VX-950 and SCH-503034, make contacts only with the R155 and D168 side chains, resulting in minimal negative impact of the Q80K mutation. T54 could affect the conformation of the S139 position, a catalytic residue that forms a covalent bond with only ω-ketoamide inhibitors. Overall, the structural evidence is highly consistent with the observed resistance profiles for the two classes of inhibitors.

The present study determined the population sequence and range of activities of a panel of HCV PIs against the viral NS3 protease that exists in 39 HCV-infected subjects. It should be

### Table 3. Susceptibilities of baseline clinical isolates containing mutations at positions 80 and/or 54

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutations in NS3*</th>
<th>Fold change in EC50 compared to 1b-PI-lucb</th>
</tr>
</thead>
<tbody>
<tr>
<td>B13</td>
<td>R26K, V46I, Q80L</td>
<td>VX-950: 1.0  SCH-503034: 1.0  TMC-435350: 2.3  MK-7009: 1.0  ITMN-191: 1.0</td>
</tr>
<tr>
<td>B10</td>
<td>S7A, R26K, V48I, T54S</td>
<td>VX-950: 2.2  SCH-503034: 3.0  TMC-435350: 2.3  MK-7009: 1.0  ITMN-191: 1.0</td>
</tr>
<tr>
<td>A25</td>
<td>T40A, T54S, V55I, I64L, P67S, Q80K, Q88H, S90A, L153I</td>
<td>VX-950: 1.0  SCH-503034: 1.0  TMC-435350: 2.2  MK-7009: 1.0  ITMN-191: 1.0</td>
</tr>
</tbody>
</table>

* Mutations at position 54 or 80 are in boldface.

b Fold change in EC50 = chimeric mutant replicon EC50/wild-type replicon EC50. The values represent the means from 3 to 5 independent experiments; values in boldface represent >3-fold increases in the EC50.

### Table 4. Susceptibilities of mutants generated by site-directed mutagenesis

<table>
<thead>
<tr>
<th>NS3 mutant</th>
<th>Fold change in EC50a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VX-950</td>
</tr>
<tr>
<td>T54S</td>
<td>5.6</td>
</tr>
<tr>
<td>Q80K</td>
<td>1.0</td>
</tr>
<tr>
<td>Q80K/T54S</td>
<td>2.3</td>
</tr>
</tbody>
</table>

a Fold change in EC50 = chimeric mutant replicon EC50/wild-type replicon EC50. The values represent the means from 2 or 3 independent experiments; the values in boldface represent >3-fold increases in the EC50.
noted that this replicon-based assay allows us to study only the predominant quasispecies in a given sample, since some minor quasispecies may not replicate at a level sufficient for the EC_{50} determination. Clonal phenotypic analysis using this replicon-based assay would be challenging because (i) a large number of clones would be needed to fully represent the minor quasispecies (80 clones for a variant of 5% in the population), (ii) some clones would replicate too poorly to be tested in the EC_{50} assay, and (iii) the assay is very labor-intensive, with multiple steps. In addition to the limitations of the population-based phenotypic analysis, this study is also limited by HCV population sequence information from only 39 treatment-naïve subjects. Nevertheless, the available data provided insightful information about the susceptibilities of the predominant quasispecies from these 39 subjects at baseline.

In summary, the activities of five HCV PIs currently under clinical development against a panel of 39 baseline clinical isolates were evaluated using a replicon-based transient-replication assay. Some variation in drug susceptibility was observed for all five PIs, including two α-ketoamide inhibitors (VX-950 and SCH-503034) and three macrocyclic inhibitors (MK-7009, ITMN-191, and TMC-435350). These findings suggest that the existing natural variation in baseline susceptibilities may result in variable clinical responses and should be taken into account for the optimal dose selection of PIs. In addition, the lack of baseline susceptibility correlations and some nonoverlapping resistance between the α-ketoamide and macrocyclic inhibitors suggest that there is a subtle difference in the binding modes of these two classes of PIs with some of the residues in the active site of HCV protease and that a combination of these two classes of PIs, together with other HCV inhibitors, may have some value in the clinic. However, the selection of mutants that are cross-resistant to both classes of PIs may limit the benefit of multiple PIs in combination.

REFERENCES


ACKNOWLEDGMENTS

We gratefully acknowledge Christopher Sheng for purchasing the PIs and Milind Deshpande for providing GS-9132. We also gratefully acknowledge Hans Reiser, William Delaney, Weidong Zhong, and Bill Lee for their support and discussions. Lastly, we gratefully acknowledge Heidi Fisher for her editorial assistance.


