Phenotypic and Structural Analyses of Hepatitis C Virus NS3 Protease Arg\textsuperscript{155} Variants

SENSITIVITY TO TELAPREVIR (VX-950) AND INTERFERON $\alpha^\#$*

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Telaprevir (VX-950) is a highly selective, potent inhibitor of the hepatitis C virus (HCV) NS3-4A serine protease. It has demonstrated strong antiviral activity in patients chronically infected with genotype 1 HCV when dosed alone or in combination with peginterferon alfa-2a. Substitutions of Arg\textsuperscript{155} of the HCV NS3 protease domain have been previously detected in HCV isolates from some patients during telaprevir dosing. In this study, Arg\textsuperscript{155} was replaced with various residues in genotype 1a protease domain proteins and in genotype 1b HCV subgenomic replicons. Characterization of both the purified enzymes and reconstituted replicon cells demonstrated that substitutions of Arg\textsuperscript{155} with these residues conferred low level resistance to telaprevir (<25-fold). An x-ray structure of genotype 1a HCV protease domain with the R155K mutation, in a complex with an NS4A co-factor peptide, was determined at a resolution of 2.5 Å. The crystal structure of the R155K protease is essentially identical to that of the wild-type apoenzyme (Protein Data Bank code 1A1R) except for the side chain of mutated residue 155. Telaprevir was docked into the x-ray structure of the R155K protease, and modeling analysis suggests that the P2 group of telaprevir loses several hydrophobic contacts with the Lys\textsuperscript{155} side chain. It was demonstrated that replicon cells containing substitutions at NS3 protease residue 155 remain fully sensitive to interferon $\alpha$ or ribavirin. Finally, these variant replicons were shown to have reduced replication capacity compared with the wild-type HCV replicon in cells.

Hepatitis C virus (HCV)\textsuperscript{3} is an important human pathogen that causes chronic infection in a majority of patients after an initial, acute infection. It is estimated that about 170 million patients worldwide and $\sim$1% of the population in developed countries are chronically infected with HCV (1). Chronic hepatitis C can lead to severe liver diseases, including fibrosis, cirrhosis, or hepatocellular carcinoma (2, 3). The majority of the hepatitis C patient population in developed countries is infected with HCV genotype 1. A sustained viral response was achieved in only 40–50% of the difficult to treat genotype 1 HCV-infected patients after a 48-week treatment with peginterferon alfa plus ribavirin (4, 5) (for a review, see Refs. 6 and 7). This treatment has considerable adverse effects, including depression, fatigue, and flu-like symptoms that are associated with interferon $\alpha$ and hemolytic anemia caused by ribavirin. A more effective treatment with fewer side effects and shorter treatment duration is urgently needed for HCV-infected patients. HCV has a single-stranded, positive polarity RNA that encodes a polyprotein precursor of $\sim$3,000 amino acids. The polyprotein precursor is proteolytically cleaved into four structural proteins (C, E1, E2, and p7) followed by six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (for a review, see Ref. 8). NS3-4A serine protease, one of two HCV-encoded proteases, is responsible for the release of the N terminus of four nonstructural proteins (NS4A, NS4B, NS5A, and NS5B) (9–13) and is essential for HCV replication in chimpanzees (14). It is a noncovalent heterodimer that contains the NS3 serine protease catalytic domain and a cofactor peptide (the central region or residues 21–30 of the 54-residue NS4A protein) (15–19). The x-ray crystal structure of the HCV H strain NS3 serine protease domain, in a complex with an NS4A cofactor, was first determined in 1996 (20).

Proof of concept for HCV NS3-4A serine protease inhibitors (PIs) has been obtained in clinical trials with three different inhibitors, including BILN 2061 (21, 22), telaprevir (VX-950) (23), and SCH 503034 (24). Telaprevir, a potent, reversible, and highly selective HCV PI, was discovered using structure-based drug design techniques (25, 26). In a phase 1b trial, the average reduction in plasma viral load after a 2-day dosing in genotype 1 HCV-infected patients was $\sim$3.0 log\textsubscript{10} for a telaprevir dosage of 750 mg every 8 h (2,250 mg/day) (23). The average maximal reduction in plasma viral load during a 14-day dosing period was 4.65 log\textsubscript{10} for the group that received 750 mg of telaprevir every 8 h. For some patients dosed with telaprevir, the HCV plasma viral load dropped by $>$4 log\textsubscript{10} to below the limit of detection (<10 IU/ml) during the 14 days of dosing (23). However, a breakthrough in the plasma viral load was seen in other patients receiving telaprevir alone.

Due to the error-prone character of the viral reverse transcriptase of retroviruses or the RNA-dependent RNA polymerase of RNA viruses, drug-resistant variants may exist at a low

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The atomic coordinates and structure factors (code 2OIN) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org/) contains supplemental Schemes 1 and 2 and Fig. 1.

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3 The abbreviations used are: HCV, hepatitis C virus; PI, protease inhibitor; IFN, interferon; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; MES, 4-morpholineethanesulfonic acid.
frequency in untreated patients, as part of the viral quasispecies. In patients treated with potent direct antiviral drugs, which lead to a significant reduction of wild-type virus, drug-resistant virus may be selected. Persistence of drug-resistant virus could limit the efficacy of the direct antiviral therapies. In vitro PI-resistant variants have been identified for several HCV NS3-4A PIs using a genotype 1b HCV replicon cell system (27–32). These in vitro resistance mutations include A156S, A156T, and A156V against telaprevir (27, 30); R155Q, A156T, A156V, D168A, and D168V against BILN 2061 (27, 28, 30); T54A, A156S, A156T, and V170A against SCH 503034 (32); R109K and A156T against SCH6 (31); D168A/V/E/H/G/N, A156S/V, F43S, Q41R, S138T, and S498L of NS3 protein, and V23A of NS4A protein against ITMN-191 (33). It is unclear whether these mutations would confer resistance to these PIs in a different HCV genotype or subtype, such as genotype 1a or 2a, for which HCV replicon or infectious cell culture is available. It also remains to be determined whether any new PI-resistant substitution could be selected in vitro in a different HCV genotype or subtype. Although the A156T or, possibly, A156V variant confers cross-resistance against multiple PIs, the HCV replicon containing these two mutations displayed severely reduced replication capacity in replicon cells (28, 30–32, 34) and remained as sensitive to IFN-α or ribavirin as the wild-type replicon in cell assays (28, 30, 34).

A highly sensitive, clonal sequencing method was recently used to identify telaprevir-related variants in patients dosed with telaprevir alone (35). Substitutions of residue 36 (V36A/M), 54 (T54A), 155 (R155K/T), or 156 (A156S/T/V) were observed in some patients dosed with telaprevir alone. The selection of different groups of HCV protease variants seems to be associated with the pattern of antiviral response as well as the plasma exposure of telaprevir observed in these patients.

In the current report, we carried out extensive studies of several variants with substitutions at Arg155 of the HCV NS3 protease domain, including enzymatic characterization, replicon cell studies, x-ray crystallography, and computational modeling. Our data demonstrate that NS3 protease variants at Arg155 confer low level resistance to telaprevir (~25-fold) in both enzyme and replicon cell assays. The change in three-dimensional structure was subtle when the Arg155 was replaced with a Lys. Computational modeling analysis suggests that the impact on binding of telaprevir to the HCV NS3 protease is not expected to be dramatic, which is consistent with the low level loss of sensitivity to telaprevir observed in enzyme and replicon cell assays. In addition, these variants have decreased replication capacity in replicon cells, which is consistent with the reduced in vivo fitness shown previously in telaprevir-dosed hepatitis patients (35).

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Substitutions of Arg155 of the HCV NS3 protease domain were introduced into four sets of plasmids for replicon cell, enzymatic, or x-ray crystallography characterizations. For studies using replicon stable cells, substitutions at NS3 residue 155 were engineered by site-directed mutagenesis into a genotype 1b subgenomic HCV replicon plasmid, pBR322-HCV-Neo-mADE. As described previously, pBR322-HCV-Neo-mADE is a second generation, high efficiency replicon plasmid that contains three adaptive mutations (27) and was derived from a Con1 strain subgenomic replicon, I377neo/NS3-3/WT (GenBank accession number CAB46913) (36). The codon change at residue 155 of the genotype 1b HCV replicon was as follows: Arg (CGG) to Lys (AAG), Thr (ACG), Ser (AGT), Met (ATG), Ile (ATC), or Gly (GGG). The same set of substitutions at Arg155 was then subcloned into pBR322-HCV-Luc-mADE (30) for replication capacity assay in replicon cells using transient transfection. All constructs were confirmed by sequencing.

For expression of protein used in enzymatic studies, HCV cDNA was amplified using reverse transcription-PCR from the viral RNA isolated from a genotype 1a HCV-infected patient who was enrolled in a phase 1b clinical study in which patients were treated with telaprevir (VX-950) alone (35). A cDNA fragment encoding HCV NS3 residues Ala1–Ser181 from this patient (Fig. 1) (GenBank accession number AM489456) was then subcloned into a pBEV10 expression vector containing a C-terminal His6 tag. In each expression construct, NS3 protease residue Leu13 (codon CTC) was replaced with Lys (codon AAA) to improve the solubility of the protein. The HCV cDNA containing mutations at residue 155 of the NS3 protease domain was derived either by reverse transcription-PCR from serum samples of this patient (R155K) or constructed via site-directed mutagenesis (R155T, R155S, or R155I). The R155K and R155T variants were observed in this patient at either the end of the 14-day telaprevir dosing period or in the 7–10-day follow-up period after the last dose, whereas the R155S, R155I, R155M, and R155G variants were seen in other patients (35). The codon change for residue 155 was as follows: Arg (AGG) to Lys (AAG), Thr (ACG), Ser (AGC), or Ile (ATC).

To solve the x-ray crystal structure of R155K variant protease, a wild-type HCV-H strain (genotype 1a) cDNA fragment encoding NS3 residues Ala1–Ser181 was cloned into a different pBEV10 plasmid. The resulting expression construct encodes an NS3 protease flanked by a T7 tag at the N terminus and a His6 tag at the C terminus, similar to what has been previously described for a pET-based expression plasmid (20). The substitution of Arg155 (AGG) with Lys (AAG) was generated via site-directed mutagenesis.

**Generation of Stable HCV Replicon Cells**—Full-length HCV subgenomic replicon RNA was generated from ScaI-linearized DNA template using a MEGAscript T7 kit (Ambion, Austin, TX), treated with RNase-free DNase I in the kit to remove the DNA template, and purified by LiCl precipitation. RNA run-off transcripts were electroporated into naïve Huh-7 cells, and G418-resistant HCV replicon cells were selected with 0.5 mg/ml G418 (Genetix; Invitrogen) in Dulbecco’s modified minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 1% nonessential amino acids, and 100 units/ml penicillin plus 100 μg/ml streptomycin for 2–3 weeks. (All reagents were purchased from Invitrogen, except FBS, which was purchased from JRH Biosciences (Lenexa, KS)). The cells were split whenever they reached confluence. Replicon cells were then maintained in DMEM containing 10% FBS, 2 mM l-glutamine, 1% nonessential amino acids, and 0.25 mg/ml G418. Reverse tran-
HCV NS3 Protease Telaprevir (VX-950)-resistant Variants

Expression and Purification of the HCV NS3 Serine Protease Domain—HCV NS3 serine protease domain containing the wild-type (base-line) sequence of the patient (Fig. 1) or mutants (R155K, R155S, R155T, and R155I) were expressed from the corresponding pBEV10/HCV-3201/NS3181-His6 plasmids in BL21/DE3 Escherichia coli cells (Stratagene, La Jolla, CA) and purified as described before (27), with minor modifications. Briefly, freshly transformed cells were grown at 37 °C in a BHI medium (Difco) supplemented with 100 μg/ml carbenicillin to an optical density of 0.75 at 600 nm, followed by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 25 °C. All purification steps were performed at 4 °C as described before (27), with minor modifications. Briefly, 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, and 10% (v/v) glycerol) supplemented with 5 mM imidazole, using BugBuster Reagent (Novagen, Madison, WI), followed by centrifugation at 16,000 × g for 30 min. The clarified lysate was passed over pre-equilibrated TALON affinity resin (Clontech) and washed with 30 column volumes of buffer A plus 5 mM imidazole. The HCV NS3 proteins were eluted in buffer A containing 300 mM imidazole, concentrated, and loaded onto a Hi-Load 16/60 Superdex 200 column that was pre-equilibrated with buffer A. The appropriate fractions of purified HCV proteins were pooled and stored at −80 °C. The purity of these proteases was determined to be either over 90% (wild type and R155K) or around 80% (R155T and R155S) by SDS-PAGE with Coomassie Blue staining.

The HCV-H NS3 serine protease domain, which contains the R155K variation and is fused to a T7 tag at the N terminus and a His6 tag at the C terminus, was expressed from pBEV10/HCV-H/NS3181-His6 containing the R155K variation in BL21/DE3 E. coli cells as described above with the following modification. The concentration of isopropyl-1-thio-β-D-galactopyranoside for induction of protein expression was 0.2 mM instead of 1 mM. It was purified as described above with minor modifications. Cell paste was lysed in buffer using the microfluidizer, followed by ultracentrifugation at 54,000 × g for 45 min. A final concentration of 10 mM imidazole along with pre-equilibrated Ni2+-nitrilotriacetic acid resin (Sigma) was added to the supernatants, and the mixtures were rocked for 3 h and washed with buffer A plus 10 mM imidazole. The proteins were eluted in buffer A containing 300 mM imidazole, concentrated, and separated using a Hi-Load 16/60 Superdex 200 column. The pooled NS3 protease domain protein was bound with 2 eq of the NS4A peptide cofactor and then loaded onto a Hi-Load Sephacryl S100 column in buffer B (15 mM MES (pH 6.5), 500 mM NaCl, 20 mM β-mercaptoethanol). The pooled protein eluates were concentrated to 8.0 mg/ml for crystallization experiments.

Determination of $V_{max}$ and $K_m$ of HCV NS3 Serine Protease Domain Variants—Substrate kinetic parameters were determined with a 5A/5B peptide substrate (EDVY-Abu-CSMSY) (38). Protease was preincubated with 5 μM co-factor peptide KK4A (KKGSVVIVGRVIGSK) (39) in 50 mM HEPES (pH 7.8), 100 mM NaCl, 20% glycerol, 5 mM dithiothreitol at 25 °C for 10 min and then at 30 °C for 10 min. The reaction was initiated by the addition of the 5A/5B peptide substrate (Proteos, Kalamazoo, MI) and incubated for 20 min at 30 °C. Total assay volume was 100 μl. The reaction was quenched by the addition of 25 μl of 10% trifluoroacetic acid. Reaction products were separated on a reverse phase microbore high performance liquid chromatography column (Penomenex Jupiter 5 μ C18 300 μm column; 150 × 2.0 mm), which was heated to 40 °C. The flow rate was 0.2 ml/min, with 0.1% trifluoroacetic acid in H2O (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). A linear gradient was used as follows: 5−60% solvent B over 12 min and then 60−100% solvent B over 1 min, 3 min isocratic, followed by 100 to 5% B in 1 min and equilibration at 5% B for 10 min. The SMSY product peak, which typically had a retention time around 14 min, was analyzed using data collected at 210 nm. Substrate kinetic parameters, $K_m$ and $V_{max}$, were deter-
mained by fitting the data to the Michaelis-Menten equation with Prism software from GraphPad (San Diego, CA).

**Determination of Telaprevir (VX-950) Enzymatic IC_{50(1 h)} of the HCV NS3 Serine Protease Domain Variants—**Sensitivity of the NS3 protease domain variants to telaprevir was determined in 96-well microtiter plates (NBS 3990; Corning Glass) using an internally quenched fluorogenic depsipeptide, RET-S1 (DABCYL) (Anaspec Inc., San Jose, CA), as published previously (27). Briefly, the NS3 protease domain was preincubated with 5 μM KK4A peptide in 50 mM HEPEs (pH 7.8), 100 mM NaCl, 20% glycerol, 5 mM dithiothreitol at 25 °C for 10 min and at 30 °C for 10 min. Telaprevir, serially diluted in Me2SO, was added to the protease mixture and incubated for an additional 60 min at 30 °C. The reaction was started by the addition of 5 μM RET-S1 substrate and incubated at 30 °C for 10 min. Product release was monitored for 20 min (excitation at 360 nm and emission at 500 nm) in a Tecan SpectraFluorPlus plate reader (Tecan US, Durham, NC). Total assay volume was 100 μL. Protease concentration was chosen such that 10–20% of the substrate was turned over during the course of the assay. To calculate 50% inhibitory concentration (IC_{50}) values, data were fit to a simple IC_{50} equation using the Prism software.

**X-ray Structure of the R155K Variant Protease—**Purified R155K variant of the HCV-H strain protease domain, in a complex with an NS4A peptide co-factor (KKGSVIVGRIVLGSK-PAIIPKK) (20), at a concentration of 8.0 mg/ml was used for crystallization trials. The protein crystals were grown over a reservoir liquid of 0.1 M MES (pH 6.2), 1.4 M NaCl, 0.3 M KH₂PO₄, and 10 mM β-mercaptoethanol. Single crystals were obtained in the hanging droplets after equilibrating over 2 days. A single crystal with dimensions of 0.15 × 0.15 × 0.35 mm was transferred into cryoprotectant solution of mother liquid with 25% glycerol added shortly prior to being cooled to 100 K in a flush of nitrogen gas stream. The diffraction images were collected using a CCD4 image plate instrument mounted on ALS beam line 5.01. Data at 2.5 Å resolution was indexed and integrated using HKL2000 (HKL Inc., Charlottesville, VA) and CCP4 software. The crystals belong to space group R32 with unit cell dimensions of \(a = 225.31\, \text{Å}, b = 225.31\, \text{Å}, c = 75.66\, \text{Å}, \alpha = 90.00°, \beta = 90.00°, \gamma = 120.00°\). 5% of data are assigned for testing free R-factor in the later refinements. The crystals of the R155K variant studied here have an identical crystallographic lattice to that of the wild-type NS3-4A protease published previously (20). The published NS3-4A protease domain (Protein Data Bank code 1A1R) was used to perform the initial rigid body and positional refinement of the model. The difference in the side chains of residue 155 was confirmed to be a Lys instead of Arg in the electron density map. The protein molecule was visually inspected against the electron density map using QUANTA programs. Further inclusion of solvent molecules in the refinement and individual B-factor refinement at a resolution range of 20.0 to 2.5 Å reduced the R-factor and free R-factor to 22.0 and 24.7%, respectively. Residues included in the refined model range from amino acid 1 to 181 of the NS3 protease domain and from residue 21 to 39 of the NS4A cofactor for crystallographic independent molecule and two zinc metal ions.

**Computational Modeling—**Telaprevir was modeled into the active site of the R155K variant NS3 protease domain using the x-ray crystal structure of the R155K variant apoenzyme in a complex with an NS4A cofactor peptide, following the procedure described previously (27). The ketoamide group of telaprevir was modeled to form a covalent adduct with the Ser³¹³9 side chain with a si-face attachment. This binding mode was observed for analogous ketoamide inhibitors (40) and ketoacid inhibitors (41). The main chain of the inhibitor was overlaid with the analogous main chain of these ketoamide and ketoacid inhibitors such that the telaprevir main chain makes all of the following backbone hydrogen bonds: P1 NH with Lys³¹⁵⁵ carbonyl, P3 carbonyl with Ala³¹⁵⁷ NH, P3 NH with Ala³¹⁵⁷ carbonyl, and P4 carbonyl with NH of Cys³¹⁵⁷. In this binding mode, the P2 group of telaprevir was placed in the S2 pocket without any need to move the Lys³¹⁵⁵ side chain. The t-butyl and the cyclohexyl groups of telaprevir were placed in the S3 and S4 pockets, respectively. The inhibitor was energy-minimized in two stages. In the first stage, only the inhibitor and the side-chain atoms of Arg³¹³¹, Lys³¹⁵⁵, and Asp³¹⁶⁸ of the protease were allowed to move during energy minimization for 1000 steps. In the second stage, all of the side-chain atoms of the protease were allowed to move along with the inhibitor for 1000 additional steps. This modeled structure closely mimics the telaprevir model in the active site of the wild-type NS3 protease described previously (27). No significant shifts in the positions of Lys³¹⁵⁵ side chain or the other active site residue side chains were observed. The same procedure was repeated for docking telaprevir into the active site of the R155T vari-

| TABLE 1 | Demonstration of resistance in HCV replicon cell assays |
|----------|------------------------------------------------------|
| **Variants** | **Replicon IC_{50} of telaprevir** | **Change** |
| Wild type | 0.49 ± 0.11 | 1.0 ± 0.2 |
| R155K | 3.6 ± 0.3 | 7.4 ± 0.6 |
| R155T | 9.6 ± 0.9 | 19.8 ± 1.8 |
| R155S | 2.0 ± 0.2 | 4.1 ± 0.4 |
| R155I | 11.7 ± 2.5 | 24.0 ± 5.2 |
| R155M | 2.7 ± 0.2 | 5.5 ± 0.4 |
| R155G | 3.6 ± 0.2 | 7.4 ± 0.5 |

**FIGURE 1.** Amino acid sequence of the NS3 protease domain of HCV isolated from a patient who was enrolled in a phase 1b clinical trial with telaprevir alone. The amino acid sequences of the NS3 protease domain (residues 1–181) (GenBank accession number AM489456) are indicated with residue numbers. The extra residues from the expression vector (Met at the N terminus and IEGRIHHHHHH at the C terminus) are shown but not numbered.
ant of the NS3 protease domain. However, the enzyme structure in this case is not the x-ray crystal structure but a model built using the R155K variant crystal structure. The Lys155 residue was replaced with the Thr side chain and was minimized by holding all of the atoms of the enzyme fixed except for the Thr155 side chain. In this model, the hydroxyl group of the Thr155 side chain forms a hydrogen bond with the side chain of Asp81. All modeling and minimization procedures were carried out using the QUANTA molecular modeling software (Accelrys Inc., San Diego, CA).

RESULTS

Substitutions at Arg155 of the NS3 Protease Confer Low Level Resistance to Telaprevir in HCV Replicon Cells—To determine whether the observed substitutions of Arg155 of the HCV NS3 protease domain are sufficient to confer resistance to telaprevir (VX-950), several substitutions at the NS3 residue 155 (R155K, R155T, R155S, R155I, R155M, or R155G) were introduced into the Arg155 wild-type NS3 protease domain, both in a complex with the 5A/5B high pressure liquid chromatography substrate were determined in three independent experiments. For the R155S variant protease, only two independent experiments were done, and the average values were shown with two individual values in parentheses. The total concentration of HCV protease determined in the colorimetric Bradford assay was used for calculation of $V_{max}$.

### TABLE 2

| Variants | Enzymatic IC$_{50(1h)}$ of telaprevir | Change |
|----------|--------------------------------------|--------|
| Wild type (n = 5) | 0.050 ± 0.031 | 1.0 ± 0.6 |
| R155K (n = 4)  | 0.51 ± 0.23  | 10 ± 3  |
| R155T (n = 5)  | 0.49 ± 0.18  | 10 ± 4  |
| R155S (n = 3)  | 1.15 ± 0.48  | 23 ± 10 |
| R155I (n = 3)  | 0.88 ± 0.21  | 18 ± 4  |

### TABLE 3

Kinetic parameters of HCV NS3-4A variant proteases

The $V_{max}$ and $K_m$ values ± S.D. of the wild type and two variant HCV NS3 protease domains using the KK4A cofactor peptide and the 5A/5B high pressure liquid chromatography substrate were determined in three independent experiments. For the R155S variant protease, only two independent experiments were done, and the average values were shown with two individual values in parentheses. The total concentration of HCV protease determined in the colorimetric Bradford assay was used for calculation of $V_{max}$.

| Variants | $V_{max}$  | $K_m$  |
|----------|-------------|--------|
| Wild type (n = 3) | 0.067 ± 0.003 | 53 ± 14 |
| R155K (n = 3) | 0.080 ± 0.017 | 168 ± 25 |
| R155S (n = 3) | 0.047 ± 0.006 | 68 ± 18 |
| R155I (n = 2) | 0.079 (0.083, 0.075) | 552 (426, 677) |

### FIGURE 2

Superimposition of the x-ray structure of the Lys155 variant and the Arg155 wild-type NS3 protease domain, both in a complex with the NS4A co-factor. A, the main chain atoms of both the wild type (in green) and the R155K variant (in magenta) proteases are shown as lines. The residue 155 is highlighted with a stick model (Arg155 in green and Lys155 in magenta). B, a close-up view of residues 81, 123, 155, and 168. These four residues of the HCV NS3 protease are highlighted with the stick model for both the wild-type protease (in green) and the R155K variant (in magenta). All nitrogens are colored in blue, and oxygens are red. The distance between various atoms, indicated by a dashed line, is indicated.
replicons (Table 1). Similar levels of decreases in sensitivity to telaprevir were observed in HCV replicon cells containing the other four minor variants at Arg^{155}: R155S, R155I, R155M, or R155G (Table 1). These four variants were found at much lower frequency than R155K/T in the telaprevir monotherapy trial. The replicon 48 h IC_{50} values for these four variants were 2.0 ± 0.2 μM (R155S), 11.7 ± 2.5 μM (R155I), 2.7 ± 0.2 μM (R155M), and 3.6 ± 0.2 μM (R155G), which corresponds to a 4.1-, 24.0-, 5.5-, and 7.4-fold loss of sensitivity to telaprevir, respectively (Table 1). These results indicate that substitutions of NS3 Arg^{155} led to low level (<25-fold) resistance to telaprevir in HCV replicon cells, independent of the physical properties of the substituted residue, which include a positive charged residue (Lys), a hydrophilic residue (Thr or Ser), a hydrophobic residue (Ile or Met), or a residue that lacks a side chain (Gly).

Substitutions at Arg^{155} of the HCV NS3 Protease Resulted in Decreased Sensitivity to Telaprevir in Enzyme Assays—To confirm whether substitutions at Arg^{155} in the HCV NS3 protease domain are sufficient to cause a loss of sensitivity to telaprevir at the enzyme level, Arg^{155} was replaced with Lys, Thr, Ser, Ile, Met, or Gly in a genotype 1a NS3 protease domain protein. The sequence of this NS3 protease domain was derived from HCV samples in a patient prior to dosing with telaprevir (Fig. 1). All six protease variants with substitutions at Arg^{155} were expressed in E. coli. Unfortunately, no soluble protein was recovered for the R155M and R155G protease variants. The remaining four candidates yielded soluble protein, represented both major variants (R155K and R155T) and minor variants (R155S and R155I) observed in the telaprevir monotherapy trial, and were tested for resistance to telaprevir.

Resistance to telaprevir was defined as the fold change in IC_{50(1 h)}, which is the 50% inhibitory concentration after a 1-h preincubation with telaprevir, and is summarized in Table 2. The average
enzymatic IC<sub>50(1 h)</sub> value of telaprevir against the wild-type genotype 1a patient NS3 protease domain complexed with the KK4A peptide was 0.050 ± 0.031 μM after a 1-h preincubation. All four variants with substitutions at Arg<sup>155</sup> were approximately 1 order of magnitude less sensitive to telaprevir, which was statistically significantly different in Wilcoxon rank sum two-sided analyses (p < 0.05 for all pairwise comparisons with the wild-type enzyme, with a range of 0.008–0.036). The change in IC<sub>50(1 h)</sub> values for the variants compared with wild-type ranged from 10– to 23-fold. There was no significant difference (p > 0.05 for all pairwise comparisons) among the -fold resistances of these four variant proteases, which is not surprising given the standard variation of the enzymatic IC<sub>50(1 h)</sub> values. These data indicate that substitutions of Arg<sup>155</sup> with various amino acids, including a conservative substitution with Arg<sup>155</sup> N<sub>1</sub> of Lys<sup>155</sup> and the C<sub>α</sub> of Asp<sup>168</sup> in the wild-type protease are 5.4 and 6.7 Å (Fig. 2B), respectively, away from its Asp<sup>81</sup> Cβ. Thus, the terminal amino group of Lys<sup>155</sup> in the R155K protease is closer to the carboxyl group of Asp<sup>81</sup> than the comparative terminal azide group of Arg<sup>155</sup> (Fig. 2B). In addition, the distance between Lys<sup>155</sup> N<sub>η1</sub> and the carboxyl atom O<sub>62</sub> of Asp<sup>168</sup> is 6.5 Å in the R155K protease compared with 3.0 Å between the corresponding pair of the terminal N<sub>η2</sub> of Arg<sup>155</sup> and the O<sub>62</sub> of Asp<sup>168</sup> in the wild-type protease (Fig. 2B). Thus, the terminal amino group of Lys<sup>155</sup> in the R155K protease is further away from the carboxyl group of Asp<sup>168</sup> than the comparative terminal azide group of Arg<sup>155</sup>. Therefore, substitution of Arg with Lys at residue 155 alters the shape of the S2 binding pocket such that the positive charge at the N<sub>η</sub> atom of Lys<sup>155</sup> cannot be neutralized by the adjacent side chain of Asp<sup>166</sup>, as is the case of the wild-type Arg<sup>155</sup> and Asp<sup>168</sup> pair.

Mechanism of Resistance of R155K or R155T Variant to Telaprevir—A structural model of the interactions between telaprevir and NS3 protease has previously been described (27, 30). In a model of the co-complex of telaprevir with the R155K enzyme, the same interactions were maintained except for differences at the side chain of residue 155. In the wild-type protease structure, the Arg<sup>155</sup> side chain bends over the bicyclic P2 group of telaprevir to make several direct van der Waals contacts. Whereas the positively charged terminal guanidinium group of Arg<sup>155</sup> makes salt bridge interactions with the nega-
tively charged Asp<sup>168</sup> side chain, the aliphatic middle of the Arg<sup>155</sup> side chain provides a hydrophobic environment for the P2 group of telaprevir (Fig. 3A). However, the Lys<sup>155</sup> side chain of the R155K enzyme has a conformation that extends away from the P2 group of the inhibitor. It makes only one or two direct contacts with the P2 group, thus leaving the P2 group of telaprevir more exposed to the solvent (Fig. 3B). This observation is consistent with the R155K enzyme being less sensitive to telaprevir, as shown in enzyme assays. It is reasonable to assume that the R155M variant will also have an extended conformation of Met<sup>155</sup> side chain and therefore make similarly fewer interactions with the P2 group of telaprevir, consistent with the observed decrease in sensitivity to the inhibitor.

To understand the mechanism of binding of telaprevir to variants with residues with shorter side chains at position 155, we also built a computer model of R155T variant enzyme complexed with telaprevir. It is obvious from the model that the Thr<sup>155</sup> side chain is too short to provide a hydrophobic cover for the P2 group of the inhibitor (Fig. 3C). Other shorter side chains like Ile, Ser, and Gly are similar or even shorter in size and are expected to lose interactions with the P2 group and have a decreased binding affinity to the telaprevir.

**HCV Variant Replicons with Substitutions at Arg<sup>155</sup> of the NS3 Protease Remain Fully Sensitive to IFN-α**—We sought to determine whether the telaprevir-resistant variant replicon cells with substitution at NS3 residue 155 remain sensitive to IFN-α or ribavirin. As shown in Table 5, the replicon IC<sub>50</sub> of either IFN-α or ribavirin remained virtually the same for HCV replicon cells containing R155K, R155T, or R155M mutations compared with the wild-type replicon cells. These results suggest that combination with IFN-α with or without ribavirin could be a potential therapeutic strategy to suppress the emergence of HCV variants with substitutions at NS3 protease residue 155.

**DISCUSSION**

The *in vitro* selection of telaprevir-resistant protease variants in genotype 1b (Con1 strain) HCV replicon cells has previously been reported (27, 30). Substitutions at Ala<sup>156</sup> were found to confer low level (<25-fold; A156S) or high level (>50-fold; A156T or A156V) resistance to telaprevir (27, 30). All three mutations at Ala<sup>156</sup> were also observed in some genotype 1a and 1b HCV-infected patients who had been dosed with telaprevir alone for 14 days (35). This demonstrates that selection studies can be useful for identification of genetic variations that confer resistance to direct antiviral drugs, which might be selected in the clinical setting. However, additional telaprevir-resistant variants, such as substitutions at Arg<sup>155</sup>, were identified in the telaprevir-dosed patients (35) but were not found upon *in vitro* selection (27, 30). A BLAST search of the GenBank<sup>TM</sup> data showed that both Arg<sup>155</sup> and Ala<sup>156</sup> of the NS3 serine protease domain are absolutely conserved in over 500 HCV isolates of all six major genotypes, including subtypes 1a and 1b. Thus, the difference in selection *in vitro* and *in vivo* cannot be due to differences in conservation between Arg<sup>155</sup> and Ala<sup>156</sup> in HCV NS3 protease.

One possible explanation for the difference between *in vitro* and *in vivo* selection results is the pattern of nucleotide changes that are required for the amino acid substitutions to occur in genotype 1a versus 1b. Substitutions at Ala<sup>156</sup> (A156S, A156T, or A156V), which require only a one-nucleotide change in subtypes 1a and 1b, were observed in subtype 1b HCV replicon cells (27, 30) and in both subtype 1a and 1b HCV-infected patients dosed with telaprevir alone for 14 days (35). In contrast, substitutions at Arg<sup>155</sup> with Lys, Thr, Ser, or Ile, which require a two-nucleotide substitution in all subtype 1b HCV isolates deposited in GenBank<sup>TM</sup>, were not observed in subtype 1b HCV replicon cells (27, 30) or subtype 1b HCV-infected patients dosed with telaprevir alone (35). However, these R155K/T/S/M/I variants, which require a one-nucleotide change in all subtype 1a HCV isolates found in GenBank<sup>TM</sup>, were seen in several subtype 1a HCV-infected patients dosed with telaprevir alone for 14 days (35). The fact that we were able to generate subtype 1b HCV replicon cells harboring substitutions at Arg<sup>155</sup> provides further support that their absence in subtype 1b HCV-infected patients dosed with telaprevir alone is not due to a structural requirement of the HCV protease itself. If subtype 1a HCV replicon cells had been used for *in vitro* selection of telaprevir-resistant variants, it is possible that some of the R155K/T/S/M/I variants would have been detected.

In our opinion, the need for a two-nucleotide change for substitution of Arg<sup>155</sup> in genotype 1b could be the reason that these variants were not observed in genotype 1b HCV-infected patients dosed with telaprevir alone. In genotype 1b HCV, these substitutions could be generated through two paths. The first path is that both nucleotide substitutions at the Arg<sup>155</sup> codon of genotype 1b are generated simultaneously in a single RNA polymerase. The odds of this event (∼10<sup>10</sup>) are going to be much lower than a single-nucleotide substitution error at the Arg<sup>155</sup> codon by the genotype 1a HCV RNA polymerase (∼10<sup>−4</sup> to 10<sup>−5</sup>). The second path is through sequential generation of substitutions at both nucleotide positions by the viral polymerase, which is probably the path for generation of substitutions at two different codons (a single-nucleotide change at each codon) in the same genome. In the latter case, the introduction of the second nucleotide substitution is most likely dependent on the ability of the variant with the first substitution to grow in the presence of drugs, which is, in turn, dependent on both drug resistance and *in vivo* fitness of the first variant. For example, in order for the R155T substitution in genotype 1b HCV to occur, the original codon (CGG for Arg<sup>155</sup>) has to change to ACG for Thr via either one of the following intermediates: AGG for Arg or CCG for Pro. However, the HCV variant with Arg<sup>155</sup> (AGG)

**TABLE 5**

Lack of resistance to other anti-HCV agents in HCV replicon cells

The stable cell lines containing wild-type and variant HCV subgenomic replicons were generated using the T7 RNA run-off transcripts from the corresponding high efficiency Con1 replicon plasmids. The average replicon IC<sub>50</sub> values ± S.D. of IFN-α and ribavirin were determined for the HCV replicon cell lines in the 48-h assay in three independent experiments. Fold change was determined by dividing the replicon IC<sub>50</sub> of a given variant by that of the wild-type HCV replicon.

| Variants | Replicon IC<sub>50</sub> | IFN-α | Change | Ribavirin | Change |
|----------|--------------------------|-------|--------|-----------|--------|
| Wild type | 11.6 ± 1.1               | 1.0 ± 0.1 | 58 ± 18 | 1.0 ± 0.3 |
| R155K    | 15.2 ± 1.3               | 1.3 ± 1.1 | 37 ± 17 | 0.6 ± 0.3 |
| R155T    | 4.8 ± 3.3                | 0.4 ± 0.3 | 32 ± 18 | 0.6 ± 0.3 |
| R155M    | 4.9 ± 1.0                | 0.4 ± 0.1 | 39 ± 5 | 0.7 ± 0.1 |
is not resistant to telaprevir, and the one with Pro\(^{155}\) (CCG) probably has very little fitness to be able to grow \textit{in vivo}. Thus, the requirement of a two-nucleotide change at a single codon in subtype 1b HCV is probably the cause for the lack of selection of the R155K/T/S/M/I substitutions in subtype 1b HCV.

In general, the magnitude of resistance of the R155K/T/S/I variants seems to be similar between subtype 1a (enzymatic \(IC_{50(1h)}\)) and subtype 1b (replicon \(IC_{50}\)). However, multiple differences between these two assay systems, such as different genetic background (genotype 1a for enzyme \textit{versus} 1b for replicon), forms of proteases (protease domain plus an NS4A peptide for enzyme \textit{versus} replication complex with full-length NS3 and NS4A proteins for replicon), and assay temperatures (30 °C for enzyme \textit{versus} 37 °C for replicon), precluded a direct comparison of -fold resistance between these two sets of data. The observation that the \(V_{\text{max}}\) values of these variant proteases were comparable with that of the wild-type protease suggests that the mutations probably did not have drastic effects on solubility and stability of the variant proteins at 30 °C.

The lack of polymorphism at amino acid 155 or 156 of the NS3 serine protease suggests that substitution at either of these two positions might be unfavorable for viral replication. \textit{In vitro} studies from several laboratories demonstrated that HCV protease with A156T/V substitution had severely decreased replication capacity in HCV replicon cells (28, 30–32, 34). We recently calculated the \textit{in vivo} fitness of viral variants based on the changes in plasma viral load and the percentage of each variant between the end of telaprevir dosing and 7–10 days after the last dose. This analysis indicates that the A156T/V variants have significantly reduced \textit{in vivo} fitness in comparison with not only the wild-type HCV but also the R155K/T viral variant (35). In the current study, replication capacity of several variants with substitution at the Arg\(^{155}\) position of the NS3 protease was determined in Huh-7.5 cells. In general, the \textit{in vitro} results are in agreement with the \textit{in vivo} fitness of viral variants.

For example, R155I/M/G variants have significantly diminished \textit{in vitro} replication capacity, which is consistent with the rare appearance of these three variants in patients dosed with telaprevir alone, as compared with the R155K/T variant (35), although the \textit{in vitro} -fold resistance to telaprevir was similar among all of these five Arg\(^{155}\) variants. In addition, the \textit{in vitro} replication capacity of A156T/V variants was significantly lower than that of the R155K/T variants in Huh 7.5 cells,\(^4\) which is consistent with the much more rapid decline in the A156T/V viral variants, as compared with the R155K/T variants, in telaprevir-dosed patients after the cessation of dosing (35).

Substitution of Arg\(^{155}\) of the HCV NS3 protease domain with several different kinds of residues, including a hydrophilic residue (Thr or Ser), a hydrophobic residue (Ile or Met), a residue with no side chain (Gly), or even a positively charged residue (Lys), inevitably resulted in a low level loss of sensitivity to telaprevir. Analysis of the x-ray structure of the R155K protease indicates that substitutions of Arg\(^{155}\) with a different residue would result in two changes in the protease conformation: (i) loss of a salt bridge between Arg\(^{155}\) and Asp\(^{168}\) and (ii) shift of the side chain of residue 155. Computational modeling based on the x-ray structure of the R155K apoprotease suggests that the decrease in binding of R155K/T/S/M/I/G variant proteases with telaprevir is due to the loss of hydrophobic interactions of the P2 group of telaprevir with the mutated side chains at position 155. It is unlikely that the loss of salt bridge between Arg\(^{155}\) and Asp\(^{168}\) in R155K/T/S/M/I/G variants contributes to the resistance phenotype of these variants, because the salt bridge was also lost in D168A/V variants, which are not resistant to telaprevir (27).

The selection of variants that confer drug resistance is one of the potential factors that could limit the efficacy of virus-specific therapies against retroviruses and RNA viruses. These variants develop due to the error-prone nature of the reverse transcriptase of retroviruses or RNA-dependent RNA polymerase of RNA viruses. It is estimated that all possible single mutations can be randomly generated within 1 day in a human immunodeficiency virus- or HCV-infected patient. The replication rate of HCV in patients has been reported to be in the range of \(10^{10}\) to \(10^{12}\) viral particles/day, higher than the replication rate of human immunodeficiency virus in patients (43).

Coupled with the lack of proofreading of the HCV RNA-dependent RNA polymerase, the single variants of residue 36, 54, 155, or 156 of the NS3 protease domain are likely to be present at a very low frequency. The reduced replication capacity associated with most of the telaprevir-resistant variants suggests that their level in untreated patients should be below the detection limit of the highly sensitive, clonal sequencing methods, consistent with the analysis of base-line (untreated) HCV samples of patients in clinical trials with telaprevir alone (44). When the replication of wild-type HCV is significantly inhibited in patients dosed with telaprevir, the variants resistant to telaprevir can emerge due to their growth advantage in the presence of strong antiviral pressure.

Combinational therapies with multiple drugs against different targets are more effective than monotherapy in reducing human immunodeficiency virus load and suppressing the selection of drug-resistant variants. In this report, replicon cells containing the R155K, R155T, or R155M mutation were shown to remain fully sensitive to IFN-\(\alpha\), reminiscent of similar results of the A156T/V variants (28, 30). These data suggest that the addition of IFN-\(\alpha\) to a telaprevir-based regimen may raise the barrier for the emergence of telaprevir-resistant variants and increase the potency of anti-HCV therapy. The results from two recent clinical trials are consistent with this hypothesis. No viral rebound due to the development of resistance was observed either during 14 days of dosing with telaprevir plus peginterferon alfa-2a (45) or during 28 days of dosing with telaprevir in combination with peginterferon alfa-2a and ribavirin (46) in genotype 1 HCV-infected patients.

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