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Phenotypic Characterization of Resistant Val[36] Variants of Hepatitis C Virus NS3-4A Serine Protease[▽]

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In patients chronically infected with hepatitis C virus (HCV) strains of genotype 1, rapid and dramatic antiviral activity has been observed with telaprevir (VX-950), a highly selective and potent inhibitor of the HCV NS3-4A serine protease. HCV variants with substitutions in the NS3 protease domain were observed in some patients during telaprevir dosing. In this study, purified protease domain proteins and reconstituted HCV subgenomic replicons were used for phenotypic characterization of many of these substitutions. V36A/M or T54A substitutions conferred less than eightfold resistance to telaprevir. Variants with double substitutions at Val[36] plus Thr[54] had ~20-fold resistance to telaprevir, and variants with double substitutions at Val[36] plus Arg[155] or Ala[156] had >40-fold resistance to telaprevir. An X-ray structure of the HCV strain H protease domain containing the V36M substitution in a cocomplex with an NS4A cofactor peptide was solved at a 2.4-Å resolution. Except for the side chain of Met[36], the V36M variant structure is identical to that of the wild-type apoenzyme. The in vitro replication capacity of most variants was significantly lower than that of the wild-type replicon in cells, which is consistent with the impaired in vivo fitness estimated from telaprevir-dosed patients. Finally, the sensitivity of these replicon variants to alpha interferon or ribavirin remained unchanged compared to that of the wild-type.

It is estimated that about 170 million patients worldwide and ~1% of the population in developed countries are chronically infected with hepatitis C virus (HCV) (51). After an initial phase of acute infection, HCV infection becomes chronic in a majority of patients, which can lead to severe liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma (1, 14). HCV is classified into six genotypes (genotypes 1 to 6), and genotype 1 strains account for the majority of chronic HCV infections in developed countries. There are multiple subtypes (subtypes a, b, c, etc.) of each HCV genotype. For the difficult-to-treat genotype 1 HCV-infected patients, a sustained virologic response is achieved in only 40 to 50% of treated patients after a 48-week combination therapy with peginterferon alfa and ribavirin (8, 31) (for reviews, see references 5 and 44). Considerable adverse effects, such as depression, fatigue, and flu-like symptoms (associated with alpha interferon [IFN-α]) and hemolytic anemia (associated with ribavirin), may lead to a dose reduction or the discontinuation of treatment. Regimens with better efficacies, shorter treatment durations, or fewer side effects are needed to improve the therapeutic paradigm for HCV-infected patients.

The polyprotein precursor, encoded by the RNA genome of HCV, is ~3,000 amino acids in length and is proteolytically cleaved into four structural proteins, followed by six nonstructural (NS) proteins (for a review, see reference 2). The N terminus of the four nonstructural proteins (NS4A, NS4B, NS5A, and NS5B) is released by cleavage mediated by the NS3-4A serine protease, one of two HCV-encoded proteases (9, 10). The NS3-4A serine protease is a noncovalent heterodimer that contains a catalytic domain (the N-terminal 181-residue serine protease domain of the 631-residue NS3 protein) and a cofactor peptide (residues 21 to 30 of the 54-residue NS4A protein) (25, 26). The X-ray crystal structure of the HCV strain H NS3 serine protease domain in a complex with an NS4A cofactor was first determined in 1996 (15). Both NS3-4A serine protease and NS5B RNA-dependent RNA polymerase have been considered excellent targets for the discovery of specifically targeted antiviral therapies for hepatitis C (STAT-C).

The proof of concept for HCV NS3-4A serine protease inhibitors (PIs) was first achieved with BILN 2061 (ciluprevir) (11, 17) and was later confirmed with two other inhibitors, VX-950 (telaprevir) (38) and SCH 503034 (boceprevir) (41), in clinical trials. Telaprevir, a potent, reversible, and highly selective HCV PI, was discovered by using structure-based drug design techniques (23, 36). In a 14-day phase Ib monotherapy trial, genotype 1 HCV-infected patients dosed with 750 mg telaprevir every 8 h (2,250 mg/day) had a mean reduction of ~3.0 $\log_{10}$ in plasma HCV RNA levels after 2 days and a mean maximal reduction of 4.65 $\log_{10}$ during the 14-day dosing period (38). The plasma HCV RNA levels dropped by >4 $\log_{10}$ to below the limit of detection (<10 IU/ml) in some patients during the 14 days of telaprevir dosing. However, a breakthrough in the plasma HCV RNA levels was observed in some patients receiving telaprevir monotherapy (38).

Due to the error-prone character of the RNA-dependent RNA polymerase of RNA viruses, drug-resistant variants may exist at a low frequency in untreated patients as part of the viral quasispecies. In patients treated with potent direct antiviral drugs, which lead to a significant reduction in wild-type virus, drug-resistant

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       1          11         21         31         41         51
HCV-H  APITAYAQQT RGLLGCIITS LTGRDKNQVE GEVQIVSTAT QTFLATCING VCWTVYHGAG
3201   APITAYAQQT RGLLGCIITS LTGRDKNQVE GEVQIVSTAA QTFLATCING VCWTVYHGAG
HCV-BK APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG VCWTVYHGAG
3111   APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG VCWTVYHGAG

       61         71         81         91         101        111
HCV-H  TRTIASPKGP VIQMYTNVDQ DLVGWPAPQG SRSLTPCTCG SSDLYLVTRH ADVIPVRRRG
3201   TRTIASPKGP VIQMYTNVDQ DLVGWPAPQG ARSLTPCTCG SSDLYLVTRH ADVIPVRRRG
HCV-BK SKTLAAPKGP ITQMYTNVDQ DLVGWPKPPG ARSLTPCTCG SSDLYLVTRH ADVIPVRRRG
3111   SKTLAGPKGP ITQMYTNVDQ DLVGWQAPPG ARSLTPCTCG SSDLYLVTRH ADVIPVRRRG

       121        131        141        151        161        171
HCV-H  DSRGSLLSPR PISYLKGSSG GPLLCPAGHA VGLFRAAVCT RGVAKAVDFI PVENLETTMRS
3201   DSRGSLLSPR PISYLKGSSG GPLLCPAGHA VGIFRAAVCT RGVAKAVDFV PVESLETTMRS
HCV-BK DSRGSLLSPR PVSYLKGSSG GPLLCPFGHA VGIFRAAVCT RGVAKAVDFV PVESMETTMRS
3111   DGRGSLLSPR PVSYLKGSSG GPLLCPSGHA VGIFRAAVCT RGVAKAVDFV PVEAMETTMRS
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FIG. 1. Amino acid sequence of the NS3 protease domain of HCV isolates collected from patients who were enrolled in a 14-day clinical trial with telaprevir alone. The amino acid sequences of the NS3 protease domain (residues 1 to 181) of two genotype 1a isolates, HCV strains H and 3201 (GenBank accession number AM489456), and two genotype 1b isolates, HCV strains BK and 3111 (GenBank accession number AM489454), are aligned with the residue numbers indicated at the top. The four residues involved in telaprevir resistance are shown in boldface, and the catalytic triad residues of the HCV NS3 serine protease are underlined. The extra residues from the expression vector, a Met residue at the N terminus and an IEGRIHHHHHH sequence at the C terminus, are not shown.

variants may be selected. The selection of drug-resistant variants is probably dependent on at least three factors: the fold resistance conferred by the mutations, the in vivo fitness of the variants, and exposure of the drugs in target organs or tissues. In vitro-selected resistance mutations against PIs have been identified for several HCV NS3-4A PIs by using HCV genotype 1 replicon cell systems (22, 24, 30, 42, 48, 49, 52). These in vitro resistance mutations include A156S/T/V against telaprevir (22, 24); R155Q, A156T/V, and D168A/V against BILN 2061 (22, 24, 30); T54A, A156S/T, and V170A against SCH 503034 (48); R109K and A156T against SCH6 (52); and D168A/V/E/H/G/N, A156S/V, F43S, Q41R, S138T, and S489L of the NS3 protein and V23A of the NS4A protein against ITMN-191 (42). Although the A156T/V variants confer cross-resistance against multiple PIs, the HCV replicon containing these two mutations displayed a severely reduced replication capacity in replicon cells (22, 30, 33, 48, 52) and remained as sensitive to IFN-α and ribavirin as the wild-type replicon in cell-based assays (22, 30, 33).

A highly sensitive, clonal sequencing method was recently used to identify telaprevir-related variants in patients dosed with telaprevir alone (40). Substitutions at residue Val^{36} (V36A/M), Thr^{54} (T54A), Arg^{155} (R155K/T), or Ala^{156} (A156S/T/V) were observed in various 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 (40). The T54A substitution in the HCV NS3 protease was also identified in patients dosed with SCH 503034 by using a much less sensitive, population-based sequencing method (41).

In the study described in this report, we performed phenotypic analyses of several variants with single or double substitutions at Val^{36} or Thr^{54} plus either Arg^{155} or Ala^{156} of the HCV NS3 protease domain. Our data demonstrate that proteases with a single substitution, V36A/M or T54A, confer less than eightfold resistance to telaprevir. A double substitution at Val^{36} plus either Arg^{155} or Ala^{156} resulted in >40-fold resistance to telaprevir. When Val^{36} was replaced by Met, the change in the three-dimensional structure was subtle. In addition, these variants exhibited a decreased replication capacity in replicon cells, which is consistent with the reduced in vivo fitness shown previously in telaprevir-dosed HCV-infected patients (40).

MATERIALS AND METHODS

Plasmid construction. Single or double substitutions of Val^{36}, Thr^{54}, Arg^{155}, or Ala^{156} of the HCV NS3 protease domain were introduced into four sets of plasmids for characterization by replicon cell assays, enzymatic assays, or X-ray crystallography. For studies with stable replicon cells, substitutions at these NS3 residues were engineered by site-directed mutagenesis into an HCV genotype 1b subgenomic 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 (24) and was derived from a Con1 strain subgenomic replicon, I_{377}neo/NS3-3′/wt (GenBank accession number CAB46913) (28). The codon changes at these NS3 residues of the HCV genotype 1b replicon were as follows: Val^{36} (GTC) to Ala (GCC), Gly (GGC), Leu (CTC), or Met (ATG) and Thr^{54} (ACT) to Ala (GCT). Replicon constructs with substitutions at Arg^{155} (54) or Ala^{156} (24) have been described previously. Replicon plasmids with double substitutions at two of these four residues (V36A/M and R155K/T, V36A and T54A, or V36M and A156T) were generated from the plasmids with single substitutions by subcloning. The same set of single or double substitutions was then subcloned into pBR322-HCV-Luc-mADE, which was derived from pBR322-HCV-Neo-mADE by replacing the neomycin transferase gene with a firefly luciferase reporter gene (22), for determination of the replication capacity in transiently transfected cells. All constructs were confirmed by sequencing.

For expression of the protein used in the enzymatic studies, HCV cDNA was amplified by reverse transcription-PCR (RT-PCR) of the viral RNA isolated from an HCV genotype 1a-infected patient (isolate 3201) who was enrolled in the phase Ib 14-day telaprevir monotherapy trial (40). A cDNA fragment encoding HCV NS3 residues Ala^{1} to Ser^{181} from HCV isolate 3201 (GenBank accession number AM489456) (Fig. 1) was then subcloned into a pBEV10 expression vector containing a C-terminal six-histidine tag. In each expression construct, NS3 protease residue Leu^{13} (codon CTC) was replaced with Lys (codon AAG) to improve the solubility of the protein. Several variants with single substitutions (V36A, V36M, R155K, or A156T) were observed in this genotype 1a-infected patient either at the end of the 14-day telaprevir dosing period or in the 7- to 10-day follow-up period after the last dose. Other variants with single substitutions (V36G, V36L, T54A, or R155T) were seen in other patients (40). Variants with double substitutions (V36A/M and R155K or V36M and A156T) were also observed in this genotype 1a-infected patient in the 7- to 10-day follow-up period after the last dose, whereas other variants with double substitutions (V36A/M and R155T) were seen in other patients (40). The codon changes were as follows: Val^{36} (GTG) to Ala (GCG), Gly (GGG), Leu (CTG), or Met (ATG); Thr^{54} (ACC) to Ala (GCC); Arg^{155} (AGG) to Lys (AAG) or Thr (ACG); and Ala^{156} (GCC) to Thr (ACC).

For two of the variants with single substitutions (V36A or T54A), from which

no soluble proteins were recovered after the purification process, similar expression constructs were generated by RT-PCR of the viral RNA isolated from an HCV genotype 1b-infected patient (isolate 3111) who was enrolled in the 14-day telaprevir monotherapy study (40). A cDNA fragment encoding HCV NS3 residues Ala^{1} to Ser^{181} from this genotype 1b-infected patient (GenBank accession number AM489454) (Fig. 1) was then subcloned as described above. Variants with both the V36A and the T54A single substitutions were observed in this genotype 1b-infected patient at the end of the 14-day telaprevir dosing period and in the 7- to 10-day follow-up period after the last dose. The codon changes were as follows: Val^{36} (GTT) to Ala (GCT) and Thr^{54} (ACC) to Ala (GCC).

To solve the X-ray crystal structure of the V36M variant protease, the wild-type HCV strain H (genotype 1a) cDNA fragment encoding NS3 residues Ala^{1} to Ser^{181} 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 six-histidine tag at the C terminus, similar to what has previously been described for a pET-based expression plasmid (15). The substitution of Val^{36} (GTG) with Met (ATG) was generated by site-directed mutagenesis.

HCV replicon cell assays. The generation of stable HCV replicon cells and the determination of the concentrations of the antiviral agents effective at causing a 50% reduction (EC_{50}s) were carried out as described previously (24, 54). HCV subgenomic replicon RNA runoff transcripts were generated from a ScaI-linearized DNA template and then electroporated into naïve Huh-7 cells. G418-resistant HCV replicon cells were selected with 0.5 mg/ml G418 (Geneticin; Invitrogen, Carlsbad, CA) and were then maintained with 0.25 mg/ml G418, all in the appropriate medium. The RT-PCR products amplified from the total cellular RNA were sequenced to confirm the presence of mutations in the replicon cells. The EC_{50} values of the antiviral agents were determined in a 48-h assay with HCV replicon cells, as described before (24, 54). At least three independent assays were conducted for each viral variant, and the means and standard deviations (SDs) of the replicon EC_{50} values were calculated. The fold change in sensitivity to anti-HCV agents was calculated by dividing the mean EC_{50} of the agent against each variant by that against the wild-type (strain mADE) replicon cells.

T7 RNA runoff transcripts were generated from the ScaI-linearized pBR322-Luc-mADE plasmids and transfected into Huh-7.5 cells (3) by electroporation, as described previously (54). The transfected cells were plated in duplicate sets and incubated for 3 h (the first set) or 72 h (the second set). The cell lysates were kept frozen at −80°C until they were thawed prior to measurement of the luciferase activity by the use of a luciferase assay kit (Promega, Madison, WI). For any given replicon variant, a normalized luciferase signal was calculated by dividing the luciferase signal at 72 h postelectroporation by that at 3 h postelectroporation for the same replicon variant. The relative replication capacity of an NS3 protease variant is expressed as the percentage of the normalized luciferase signal of the mutant replicon compared with that of the wild-type replicon (as 100%) and that of an HCV polymerase null mutant (as 0%).

Expression and purification of HCV NS3 serine protease domain. The HCV NS3 serine protease domain containing the wild-type (baseline) sequence of isolate 3201 (Fig. 1) or mutations (V36M, V36L, or V36M and A156T) were expressed in *Escherichia coli* BL21(DE3) cells by induction for 5 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 25°C, as described before (24, 54). The HCV proteases were purified by using Talon affinity resin and a Hi-Load 16/60 Superdex 200 column at 4°C, as described previously (24, 54). The appropriate fractions of purified HCV proteins were pooled and stored at −80°C. The purities of these proteases were determined to be over 90% (wild type), 99% (V36M or V36M and R155K variants), or about 80% (V36L) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining.

The HCV strain H NS3 serine protease domain, which contains the V36M mutation and which is fused to a T7 tag at the N terminus and a six-histidine tag at the C terminus, was expressed from pBEV10/HCV-H/$NS3_{181}$-His_6 containing the V36M mutation in *E. coli* BL21(DE3) cells, as described before (54). The V36M protease variant was purified by using nickel-nitrilotriacetic acid resin and a Hi-Load 16/60 Superdex 200 column, as described previously (54). The pooled NS3 protease domain protein was bound with two equivalents of the NS4A peptide cofactor (15) and then loaded onto a Hi-Load Sephacryl S100 column. The pooled protein eluates were concentrated to 8.0 mg/ml for the crystallization experiments.

HCV NS3-4A serine protease enzyme assays. Substrate kinetic parameters were determined with a 5A/5B peptide substrate (EDVV-Abu-CSMSY) (45). Protease was preincubated with 5 μM cofactor peptide KK4A (KKGSVVIVG RIVLSGK) (18), as described previously (54). Briefly, the reaction was initiated by the addition of the 5A/5B substrate, and the reaction mixture was incubated for 20 min at 30°C and quenched. The reaction products were separated on a reverse-phase microbore high-performance liquid chromatography column with a linear gradient. The SMSY product peak was analyzed by using the data collected at 210 nM. The substrate kinetic parameters K_m and the maximum rate of velocity (V_{max}) were determined by fitting the data to the Michaelis-Menten equation with Prism software from GraphPad (San Diego, CA).

The sensitivities of the NS3 protease domain variants to telaprevir were determined in microtiter plates by using an internally quenched fluorogenic depsipeptide, RET-S1 {acetyl-DED(EDANS)EEαAbuψ[COO]ASK (DABCYL)-NH_2; Anaspec Incorporated, San Jose, CA}, as published previously (24, 54). Briefly, the NS3 protease domain was preincubated with 5 μM KK4A peptide for 10 min at 25°C and for another 10 min at 30°C. Then, the protease mixture was incubated with telaprevir for 60 min at 30°C and for another 20 min at 30°C with 5 μM RET-S1 substrate, and product release was monitored. The protease concentration was chosen such that 10 to 20% of the substrate was turned over during the course of the assay. The data were fit to a simple equation by using the Prism software for calculation of the values of the inhibitory concentration at a 50% reduction after a 1-h preincubation [$IC_{50(1\ h)}$].

Statistical analyses. The statistical analyses (both parametric and nonparametric) of the in vitro data (replicon EC_{50} or replication capacity) were performed by using SAS software (version 9.1). The parametric analysis consisted of a one-way analysis of variance (ANOVA) that accounted for within-group specific variances. The *P* values of the different comparisons of interest were adjusted by using Dunnett's adjustment for multiple comparisons of several groups to a "control." The nonparametric analysis consisted of Wilcoxon rank-sum tests, separately for each pair of treatments compared. Exact *P* values are reported for these tests.

X-ray structure of V36M variant protease. The HCV strain H protease domain of the V36M variant was purified in the presence of the NS4A peptide cofactor. The protein sample was concentrated to 9.0 mg/ml and was subsequently used for the crystallization trials. The conventional hanging-drop techniques were employed to obtain X-ray-quality crystals for further crystallographic experiments. A reservoir liquid of 0.1 M morpholineethanesulfonic acid (pH 6.2), 1.25 M NaCl, and 0.3 M KH_2PO_4 was used to equilibrate with the droplet in a solution, with the ratio of protein to reservoir being 1:1. Single crystals appeared after equilibration over a few days. A single crystal with dimensions of 0.175 by 0.175 by 0.35 mm was transferred into cryoprotectant solution of the mother liquid, with 25% glycerol added shortly before it was cooled to 100 K in the flush of a nitrogen gas stream. The diffraction images were collected by using a CCD4 image plate instrument mounted on an advanced light source (ALS) beam line 5,01. Data at a 2.4 Å resolution were indexed and integrated by using an HKL Incorporated (Charlottesville, VA) 2000 instrument and CCP4 software. The crystals belong to space group R32 with unit cell dimensions of *a* equal to 228.93 Å, *b* equal to 228.93 Å, *c* equal to 75.53 Å, α equal to 90.00°, β equal to 90.00°, and γ equal to 120.00°. Five percent of the data were assigned to testing of the free *R* factor in the later refinements. The crystals of the V36M variant studied here have a crystallographic lattice identical to that of the wild-type NS3-4A protease published previously (15). 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 identity of the side chain of Met^{36} instead of Val^{36} was confirmed in the electron density map. The protein molecule was visually inspected against the electron density map by using QUANTA programs (Accelrys Incorporated, San Diego, CA). Further inclusion of solvent molecules in the refinement and the individual *B*-factor refinement at a resolution range of 20.0 to 2.4 Å reduced the *R*-factor and the free *R*-factor values to 22.8% and 26.5%, respectively. The residues included in the refined model ranged from amino acids 1 to 181 of the NS3 protease domain and from residues 21 to 39 of the NS4A cofactor for the crystallographic independent molecule, two zinc metal ions, and 235 solvent molecules.

Computational modeling. A previously described method for the creation of a computational model of the NS3 protease variants in complex with telaprevir (24) was used to generate a model of the NS3 protease variants with substitutions at Val^{36}. Telaprevir was modeled into the X-ray crystal structure of the V36M protease variant by following the procedure described previously (24). The same procedure was used to dock telaprevir into the active site of the other Val^{36} variants (V36A/G/L) of the NS3 protease domain models. The modeled cocomplex of telaprevir with the crystal structure of the V36M protease variant was used to model other variants at this position. Met^{36} was replaced by Ala, Gly, or Leu to generate computer models of the structures of the V36A, V36G, and V36L proteases, respectively. The side chain of V36A/G/L in these structures was minimized by holding all the atoms of the enzyme except for those in the mutated side chain fixed. All modeling and minimization procedures were carried out by using the QUANTA molecular modeling software (Accelrys Incorporated).

TABLE 1. Characterization of HCV NS3 protease variants in replicon cells[a]

Variant	EC_{50} (μM) of telaprevir for replicons	Fold change
Wild type	0.49 ± 0.11	1.0 ± 0.2
V36M	3.4 ± 0.8	7.0 ± 1.6
V36A	3.6 ± 1.1	7.4 ± 2.2
V36L	1.1 ± 0.2	2.2 ± 0.4
V36G	5.4 ± 0.2	11.2 ± 0.4
T54A	3.0 ± 0.8	6.3 ± 1.7
V36M + R155K	~30	~62
V36A + R155K	~20	~40
V36M + R155T	>30	>62
V36A + R155T	>30	>62
V36A + T54A	9.7 ± 1.4	20.1 ± 2.9
V36M + A156T	>30	>62

[a] The stable wild-type (strain Con1-mADE) and variant HCV subgenomic replicon cell lines were generated by using the T7 RNA runoff transcripts from the corresponding high-efficiency Con1 replicon plasmids. The average replicon EC_{50} values of telaprevir ± SDs were determined for the HCV replicon cell lines in the 48-h assay in three independent experiments. The fold change was determined by dividing the replicon EC_{50} of a given variant by that of the wild-type HCV replicon.

Protein structure accession number. The coordinates of the V36M apoprotease have been deposited in the Protein Data Bank (PDB code 2QV1).

RESULTS

The single substitutions V36A/M and T54A confer less than eightfold resistance to telaprevir in HCV replicon cells. The NS3 protease substitutions V36A/G/L/M and T54A were generated in a high-efficiency subgenomic replicon plasmid (Con1-mADE). In the 14-day telaprevir monotherapy trial, V36A/M variants were observed more frequently among the variants with substitutions at Val^{36}, and the percentages of these two variants in each individual were much higher than those of the other two Val^{36} variants. In contrast, V36G/L variants were rarely detected (at a low percentage in only one or two of 28 telaprevir-dosed patients) (39, 40). Stable HCV replicon cells were generated for each of these variants, indicating that HCV RNA replication was not abolished by the replacement of NS3 Val^{36} or Thr^{54} with a different residue.

Telaprevir inhibits the wild-type HCV Con1-mADE replicon with an average 48-h EC_{50} of 0.49 ± 0.11 μM, which is slightly higher than the EC_{50} (0.35 μM) in Con1-based HCV replicon cells with a different set of adaptive mutations (24-2 replicon cells) (27, 36). The average 48-h replicon EC_{50} values of telaprevir against the V36M and V36A variants were 7.0- and 7.4-fold higher than those against the wild-type Con1-mADE replicons, respectively (Table 1). V36G variant, one of two rare variants, showed a slightly greater decrease (11.2-fold) in sensitivity to telaprevir in HCV replicon cells. V36L variant, the other rare variant, had little resistance to telaprevir, since the 48-h EC_{50} for this replicon variant was only about twofold higher than the EC_{50} for the wild-type replicon (Table 1). The 48-h EC_{50} values for the T54A variant in HCV replicon cells were 3.0 ± 0.8 μM, which corresponds to a 6.3-fold loss of sensitivity to telaprevir (Table 1). These results indicate that the major variants with substitutions at NS3 protease residue Val^{36} (V36A/M) or Thr^{54} (T54A) have less than eightfold resistance to telaprevir in HCV replicon cells. The V36A + T54A variant with both the V36A and the T54A substitutions, which was observed in about 1% of the cDNA clones in the telaprevir monotherapy trial, had a 20.1-fold loss of sensitivity to telaprevir (Table 1), which seems to be less than additive compared to the loss of sensitivity of the two single variants (7.4-fold for V36A and 6.3-fold for T54A).

The double substitutions V36A/M and R155K/T or V36M and R156T confer >40-fold resistance to telaprevir in HCV replicon cells. Besides the variant with the V36A and T54A double substitutions, several other variants with double substitutions were observed in the telaprevir monotherapy trial (40). The most common of these variants had double substitutions at residues Val^{36} and Arg^{155}, with V36M and R155K being the most frequently observed substitutions. In addition, a variant with the V36M and A156T double substitutions were seen at a very low percentage (<0.5% of total cDNA clones sequenced). It should be noted that all these variants with double substitutions (at residues 36 and 155 or residues 36 and 156) were observed only in HCV genotype 1a-infected patients dosed with telaprevir alone and not in HCV genotype 1b-infected patients (40).

For the variants with double substitutions at NS3 residues Val^{36} and Arg^{155}, the actual 48-h EC_{50} value could not be determined because the reductions in HCV RNA levels after the 48-h incubation with 30 μM telaprevir did not go beyond 90%. However, significant reductions (often 40% or more) were observed for two of the variants with double substitutions containing R155K (the V36M + R155K variants and the V36A + R155K variants), and estimates of the EC_{50} values for these variants are listed in Table 1. For variants with double substitutions containing R155T (the V36M + R155T variants and the V36A + R155T variants), no significant reduction in HCV replicon RNA levels was observed, even at a telaprevir concentration of 30 μM. These results indicate that three of the four variants with double substitutions at residues Val^{36} and Arg^{155} had a >60-fold resistance to telaprevir and that the R155T substitution resulted in a slightly higher level of resistance to telaprevir than the R155K substitution in both the variant with a single substitution (54) and the variant with a second substitution with V36M or V36A. The variant with the double substitutions V36M and A156T also showed a >60-fold resistance to telaprevir in replicon cells (Table 1).

A substitution at Val^{36}, Thr^{54}, Arg^{155}, or Ala^{156} of the HCV NS3 protease results in a diminished replication capacity in replicon cells. We used a transiently transfected cell system expressing a luciferase replicon to measure the in vitro replication capacity of the HCV replicon containing substitutions at NS3 protease residue Val^{36}, Thr^{54}, Arg^{155}, or Ala^{156}. As shown previously for other variants, these variants (except for V36A) had a significantly diminished replication capacity in Huh-7.5 cells (Table 2), with *P* values of <0.0001 by both ANOVA and the Wilcoxon rank-sum test. Among the variants with single substitutions, the V36M + T54A variant had similar in vitro replication capacities in Huh-7.5 cells (77% ± 12% and 65% ± 10%, respectively), and the in vitro replication capacities of both variants were significantly lower than from the replication capacity of the wild-type replicon (100% ± 6%). The V36G variant (59% ± 8%) had an even lower replication capacity, whereas that of the V36A variant (104% ± 26%) was virtually no different from that of the wild-type replicon, with a *P* value

TABLE 2. Relative replication capacities of HCV NS3 protease variants in replicon cells[a]

Variant	Relative replication capacity (%) in Huh-7.5 cells
Wild type ($n = 24$)	100 ± 6
V36M ($n = 6$)	77 ± 12
V36A ($n = 6$)	104 ± 26
V36G ($n = 5$)	59 ± 8
V36M + R155K ($n = 5$)	42 ± 6
V36A + R155K ($n = 5$)	57 ± 4
V36M + R155T ($n = 5$)	36 ± 8
V36A + R155T ($n = 5$)	23 ± 11
T54A ($n = 6$)	65 ± 10
V36A + T54A ($n = 6$)	8 ± 2
A156T ($n = 6$)	16 ± 2
V36M + A156T ($n = 6$)	9 ± 5

[a] T7 RNA runoff transcripts of the wild-type (strain Con1-mADE) and variant HCV subgenomic replicons, which contain luciferase-coding sequences, were transiently transfected into Huh-7.5 cells. The luciferase signal at 72 h posttransfection was normalized to that at 3 h posttransfection. The normalized luciferase signal of each variant was divided by that of the wild-type replicon to result in the relative replication capacity. The average relative replication capacities ± SDs from multiple independent determinations (indicated in parentheses) are shown.

of >0.05 by both ANOVA and the Wilcoxon rank-sum test (Table 2).

In general, the replication capacity of double variants with substitutions at both Val^{36} and Arg^{155} was similar to or slightly less than that of the corresponding variant with the single Arg^{155} substitution (54), but it was much lower than that of the variant with the single Val^{36} substitution (Table 2). For example, the in vitro replication capacity of the V36A + R155K variant was 57% ± 4%, whereas the replication capacities were 104% ± 26% for the V36A variant ($P < 0.005$) and 80% ± 16% for the R155K variant ($P < 0.01$) (54). The replication capacity was 23% ± 11% for the V36A + R155T variant, whereas the replication capacities were 41% ± 6% for the R155T variant ($P < 0.01$) and 104% ± 26% for the V36A variant ($P < 0.005$) (54). The same observation applied to the V36M + A156T variant, for which the in vitro replication capacity was 9% ± 5%, whereas the replication capacities were 16% ± 2% for the A156T variant ($P < 0.05$) and 77% ± 12% for the V36M variant ($P < 0.002$) (Table 2). These results suggest that the addition of a second substitution at Val^{36} to the Arg^{155} or Ala^{156} variant does not increase (or more often decrease) the in vitro replication capacity (Table 2), although it conferred a modest increase in resistance to telaprevir (Table 1).

One surprising observation was that the V36A + T54A variant had an in vitro replication capacity of 8% ± 2%, which was much lower than that of either variant with a single substitution ($P < 0.002$) (Table 2). These results demonstrate that V36A and T54A are synergistic with regard to their impairment of the replication capacity, and this may be due to their effects on the protease structure or protease activity (see the Discussion for more details).

HCV NS3-4A proteases with V36A/M substitutions have decreased sensitivities to telaprevir. To determine whether the resistance phenotype of the Val^{36} or Thr^{54} variants observed in replicon cells would also occur at the enzyme level, Val^{36} was replaced with Ala, Met, Leu, or Gly or Thr^{54} was replaced with Ala in an expression plasmid for an HCV genotype 1a (isolate 3201) NS3 protease domain protein (Fig. 1). All five protease variants with substitutions at Val^{36} or Thr^{54} were expressed in *E. coli*. However, despite repeated attempts, soluble protein was recovered only from the V36M or V36L variant and not from the V36A, V36G, or T54A variant. Several variants with double substitutions (V36A/M and R155K/T or V36M and A156T) were also generated in this genotype 1a (isolate 3201) protease expression plasmid, but soluble protein was recovered only for one variant, V36M + R155K.

TABLE 3. Characterization of NS3 protease variants in HCV enzyme assays[a]

Variant	Enzymatic $IC_{50(1\ h)}$ of telaprevir (μM)	Fold change
Wild type (genotype 1a; $n = 5$)	0.050 ± 0.031	1.0 ± 0.6
V36M ($n = 4$)	0.27 ± 0.11	5.4 ± 2.1
V36L ($n = 4$)	0.13 ± 0.03	2.6 ± 0.6
V36M + R155K ($n = 5$)	3.4 ± 1.2	69 ± 24
Wild type (genotype 1b; $n = 4$)	0.063 ± 0.051	1.0 ± 0.8
V36A ($n = 4$)	0.23 ± 0.12	3.6 ± 1.9

[a] The average enzymatic $IC_{50(1\ h)}$ values ± SDs of telaprevir were determined for the purified genotype 1a or 1b wild-type protease domains and for four variant HCV NS3 serine protease domains by using the KK4A cofactor peptide and the fluorescent resonance energy transfer substrate in three to five independent experiments. The fold change was determined by dividing the enzymatic $IC_{50(1\ h)}$ of a given variant by that of the corresponding wild-type protease (genotype 1a or 1b).

Resistance to telaprevir was defined as the fold change in $IC_{50(1\ h)}$ and is summarized in Table 3. Telaprevir had an average enzymatic $IC_{50(1\ h)}$ value of 0.050 ± 0.031 μM against the wild-type HCV genotype 1a (isolate 3201) NS3 protease domain plus the KK4A peptide after a 1-h preincubation. The changes in $IC_{50(1\ h)}$ values for the V36M or V36L variants compared to the value for the wild type were 5.4- and 2.6-fold, respectively (Table 3). These data indicate that the replacement of Val^{36} with Met results in less than sixfold resistance to telaprevir, whereas the Leu substitution at residue Val^{36} (V36L) confers little resistance to telaprevir. A variant with double substitutions, V36M and R155K, showed >60-fold resistance to telaprevir (Table 3).

The V36A or T54A single substitution was then introduced into an expression plasmid for an HCV genotype 1b (isolate 3111) NS3 protease domain protein (Fig. 1). In this case, soluble protein was recovered from the V36A protease variant but not the T54A protease variant, despite repeated attempts at purification. The average enzymatic $IC_{50(1\ h)}$ value of telaprevir for a wild-type genotype 1b isolate (isolate 3111) NS3 protease domain in complex with the KK4A peptide was 0.063 ± 0.051 μM after a 1-h preincubation, which is very similar to that for the wild-type HCV genotype 1a (isolate 3201) protease. The change in the $IC_{50(1\ h)}$ value for the V36A variant compared to the value for the wild-type enzyme was 3.6-fold (Table 3). All these enzymatic results are consistent with the fold resistance data determined with HCV replicon cells.

The kinetic parameters V_{max} and K_m were determined for these three variant proteases by using a peptide substrate corresponding to the HCV NS5A/5B cleavage site (Table 4). In general, the V_{max} values for all three variant proteases were similar (within threefold) to the value for the wild-type protease. The K_m values of variant proteases ranged from unaffected (V36M variant) to twofold lower (V36L variant) or

TABLE 4. Kinetic parameters of HCV NS3 protease variants[a]

Variant	V_{max} ($\mu M \cdot \mu g^{-1} \cdot s^{-1}$)	K_m (μM)
Wild type (genotype 1a; $n = 3$)	0.067 ± 0.003	53 ± 14
V36M ($n = 3$)	0.10 ± 0.02	51 ± 7
V36L ($n = 2$)	0.21 (0.25, 0.17)	29 (34, 24)
V36M + R155K ($n = 3$)	0.11 ± 0.08	180 ± 90
Wild type (genotype 1b; $n = 3$)	0.18 ± 0.06	290 ± 130
V36A ($n = 3$)	0.02 ± 0.01	380 ± 210

[a] The V_{max} and K_m values ± SDs of the genotype 1a or 1b wild-type protease domains and two variant HCV NS3 protease domains, using the KK4A cofactor peptide and the 5A/5B high-performance liquid chromatography substrate, were determined in three independent experiments. For the V36L variant protease, only two independent experiments were done, and the average values are shown, with the two individual values given in parentheses. The total concentration of HCV protease determined in the colorimetric Bradford assay was used for the calculation of V_{max}.

TABLE 5. Susceptibilities of HCV NS3 protease variants to other anti-HCV agents in replicon cells[a]

Variant	IFN-α		Ribavirin	
	EC_{50} (U/ml)	Fold change	EC_{50} (μM)	Fold change
Wild type	11.6 ± 1.1	1.0 ± 0.1	58 ± 18	1.0 ± 0.3
V36M	11.3 ± 5.9	1.0 ± 0.5	33 ± 18	0.6 ± 0.3
V36A	10.3 ± 6.0	0.9 ± 0.5	43 ± 21	0.8 ± 0.4
T54A	3.9 ± 0.5	0.3 ± 0.04	22 ± 11	0.4 ± 0.2
V36M + R155K	10.1 ± 5.9	0.9 ± 0.5	41 ± 6	0.7 ± 0.1
V36A + R155K	6.8 ± 0.5	0.6 ± 0.04	36 ± 2	0.6 ± 0.04
V36M + R155T	3.1 ± 0.2	0.3 ± 0.02	36 ± 1	0.6 ± 0.02
V36A + R155T	3.9 ± 2.1	0.3 ± 0.2	42 ± 22	0.7 ± 0.4

[a] Stable cell lines containing wild-type and variant HCV subgenomic replicons were generated by using the T7 RNA runoff transcripts from the corresponding high-efficiency Con1 replicon plasmids. The average replicon EC_{50} values of IFN-α and ribavirin ± SDs were determined for the HCV replicon cell lines in the 48-h assay in three independent experiments. The fold change was determined by dividing the replicon EC_{50} of a given variant by that of the wild-type HCV replicon.

threefold higher (V36M + R155K variant) (Table 4). In the genotype 1b protease background, the V36A variant had a K_m value that was very similar to that of the wild-type protease, although its V_{max} value was much lower than that of the wild-type protease (Table 4).

HCV variant replicons with a substitution at Val^{36}, Thr^{54}, or Arg^{155} of the NS3 protease remain fully sensitive to IFN-α. One question that we sought to answer is whether the replicons with single or double substitutions at NS3 residue Val^{36}, Thr^{54}, or Arg^{155} remain sensitive to IFN-α or ribavirin. The EC_{50} of either IFN-α or ribavirin remained virtually the same for HCV replicon cells containing the V36M, V36A, or T54A single mutation compared to the EC_{50}s for the wild-type replicon cells (Table 5). As shown in Table 5, little change in the replicon EC_{50} was observed with all four variants with double substitutions at both residues Val^{36} and Arg^{155}. These data are reminiscent of the similar results shown in previous studies that single substitutions, such as A156T/V and R155K/T, have no influence on susceptibility to IFN-α or ribavirin in replicon cells (22, 54).

X-ray structure of V36M HCV NS3 protease. We determined the X-ray crystal structure of the T7 tag-fused HCV strain H protease containing the V36M substitution in a cocomplex with an NS4A cofactor peptide in order to elucidate the mechanism of resistance conferred by this substitution. The 2.4-Å resolution X-ray structure of the V36M cocomplex is very similar to that of the wild-type HCV strain H protease complex described previously (15). In both structures, the globular entity, which consisted of one NS3 protease domain molecule (residues 1 to 181) and one molecule of the NS4A cofactor (residues 21 to 39), forms a homodimer with another globular entity in an asymmetrical unit. A superimposition of the V36M variant globular unit with that of the wild-type Val^{36} cocomplex is shown in Fig. 2A. Little difference in the structures of these two proteases was observed, which is reflected in the low root mean square deviation for the Cα atoms (0.291 Å).

The overall shift of the side chains of Met^{36} compared with that of Val^{36} is small, as shown in the close-up view of those residues surrounding the NS3 protease active site (Fig. 2B and C). In the V36M variant protease, the distance between the Cβ of Met^{36} and the benzyl ring of Phe^{43} is 3.7 Å (Cε1), 3.9 Å (Cδ1), and 4.2 Å (Cδ2). In the wild-type protease, the Cγ2 of Val^{36} is located slightly closer to Phe^{43}, as evidenced by the distance to Cε1 (3.4 Å), Cδ1 (3.5 Å), and Cδ2 (3.9 Å) of the benzyl ring of Phe^{43}. Thus, the side chain of Met^{36} in the V36M protease is farther away from its Phe^{43} than the Val^{36} in the wild-type protease. In contrast, Met^{36} made new van der Waals interactions with several residues of the NS3 protease domain (Ile^{64} and Trp^{85}) and the NS4A cofactor (Ile^{25}), which are located at the opposite side of Phe^{43}. For example, the Cε1 of Met^{36} is 3.5 Å away from the Cδ1 of Ile^{64} of the V36M variant protease domain, whereas the Cγ1 of Val^{36} is more than 4.8 Å away from the comparative Cδ1 of Ile^{64} of the wild-type protease. The distance between the same Cε1 of Met^{36} and the indole ring of Trp^{85} is 3.9 Å (Cζ3) and 4.5 Å (Cη2) in the V36M variant protease, whereas the Cγ2 of Val^{36} is about 5.8 Å away from the comparative Cη2 of Trp^{85} of the wild-type protease. In addition, the side chain of Met^{36} in the V36M protease moves closer to Ile^{25} of the NS4A cofactor. The distance between the sulfur atom of Met^{36} and Cγ1 of Ile^{25} of the NS4A cofactor in the V36M NS3-4A protease is 3.7 Å, whereas the distance is 4.1 Å between Cγ1 of Val^{36} and the comparative Cγ1 of Ile^{25} of the NS4A cofactor in the wild-type NS3-4A protease. Therefore, replacement of the Val^{36} of NS3 protease with Met alters the interaction of residue 36 with several surrounding residues of both the NS3 protease and the NS4A cofactor.

DISCUSSION

In general, the magnitude of resistance to telaprevir seems to be strikingly similar between enzyme assays (subtype 1a or 1b) and replicon cell assays (subtype 1b) for the V36A/M or V36M + R155K variants, despite multiple differences between these two assay systems, such as the use of different genetic backgrounds, forms of proteases (the use of a protease domain plus an NS4A peptide for the enzyme assay but the use of a replication complex with full-length NS3 and NS4A proteins for the replicon assay), and assay temperatures (30°C for the enzyme assay versus 37°C for the replicon assay).

Both Arg^{155} and Ala^{156} are conserved in over 500 HCV NS3 protease domain sequences from all six major genotypes de-

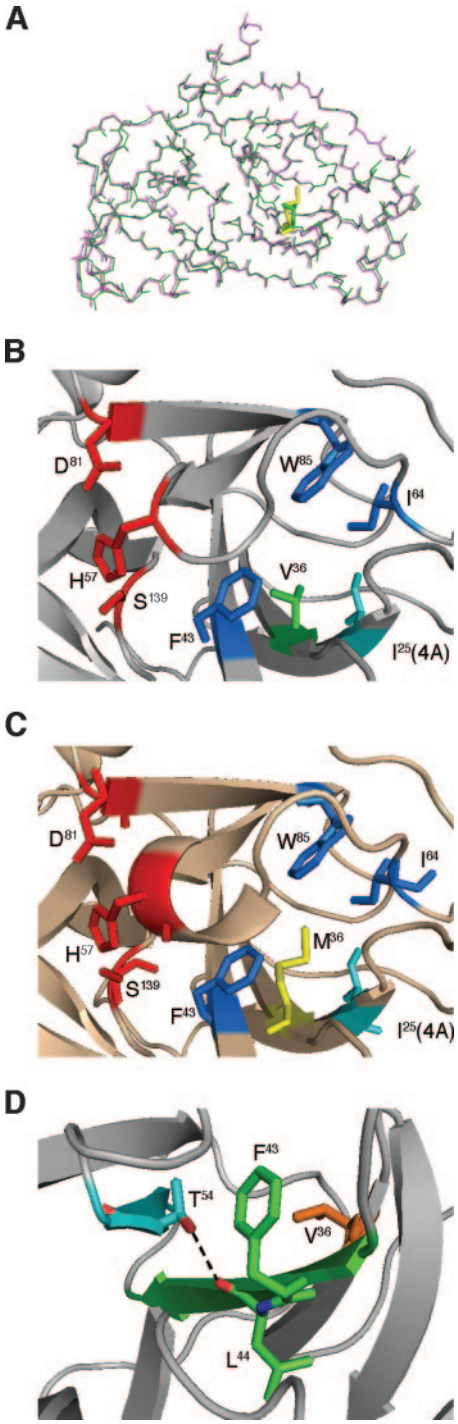

FIG. 2. (A) Superimposition of the X-ray structures of the wild-type and the V36M variant NS3 protease domains in a complex with the NS4A cofactor. The Cα atom traces of both the wild-type (in purple) and the V36M variant (in blue) proteases are shown as lines. Residue 36 is highlighted with a stick model (Val^{36} in green and Met^{36} in yellow). (B and C) Close-up view of the side chains of residue 36 and the other key residues in the wild-type NS3-4A protease (B) and the V36M variant protease (C). The catalytic triad (His^{57}, Asp^{81}, and Ser^{139}) is shown in red. Residue 36 is highlighted either in green (Val^{36} of the wild type) or in yellow (Met^{36} of the V36M variant). Other key NS3 protease residues (Phe^{43}, Ile^{64}, and Trp^{85}) are shown in blue, and Ile^{25} of the NS4A cofactor is shown in cyan. (D) Close-up view of the H bond between residues Thr^{54} and Leu^{44} in the wild-type NS3-4A protease. Thr^{54} of the C1 β strand (in cyan) forms an H bond (shown as a dashed line) with the main-chain carbonyl of Leu^{44} of the B1 β strand (in green). The locations of Phe^{43} (in green) and Val^{36} (in orange) are also shown. Nitrogen atoms are colored in blue, and oxygen atoms are colored in red.

posited in the GenBank database. However, intergenotype differences were found at residue 36. Whereas Val^{36} is highly conserved in HCV genotype 1NS3 proteases, Leu^{36} is found in HCV genotype 2, 3, and 4 NS3 proteases. In addition, about 1% of genotype 1 isolates have Leu^{36}. Our in vitro assay results for genotype 1 isolates showed that there was little change in sensitivity to telaprevir when Val^{36} was replaced with Leu in both the enzymatic and the replicon cell assays. These results suggest that Leu^{36} of HCV genotype 2, 3, or 4 may have a minimal impact on susceptibility to telaprevir.

The modeling analysis of telaprevir cocomplexes with the V36A/G/L/M proteases suggested that interactions between the active-site residues of the wild-type HCV NS3-4A protease and telaprevir were maintained in these variant proteases. As shown in the crystal structure of the V36M apoprotease, there is little change in the overall shape of the substrate/inhibitor-binding site when Val^{36} is replaced with Met, which is not surprising, since the Val^{36} side chain is not part of the substrate/inhibitor-binding site per se. Instead, the Val^{36} residue is located at the N-terminal A1 β strand of the protease, which interfaces with the NS4A cofactor peptide (Fig. 2B). It is buried in a hydrophobic environment and is not in direct contact with telaprevir. However, Val^{36} does make direct contact with Phe^{43}, which is part of the S1′ substrate-binding pocket of the HCV protease (Fig. 2B). In the crystal structure of the V36M apoenzyme, there is less interaction between Met^{36} and Phe^{43} (Fig. 2C), which would result in a less rigid conformation of the Phe^{43} side chain and which would therefore reduce its interaction with the cyclopropyl amine (P1′ side chain) of telaprevir. It is expected that V36A and V36G variants, which have smaller side chains, would also have a similar loss of direct contact with Phe^{43}, and consequently, the interaction between Phe^{43} and telaprevir would be reduced. On the other hand, the replacement of Val^{36} with Leu is expected to maintain the interaction between residue 36 and Phe^{43}, which is consistent with the lack of resistance to telaprevir by the V36L variant.

A polymorphism was also found at residue 54 of the HCV NS3 protease. Whereas Thr^{54} is found in most genotype 1 isolates and all of genotype 2, 3, and 4 isolates, about 3% of the genotype 1 isolates have Ser^{54}. Thr^{54} is located on the C1 β strand of the HCV protease, and its hydroxyl forms a hydrogen bond with the main-chain carbonyl of Leu^{44} of the adjacent B1 β strand (Fig. 2D). This interaction would keep the neighboring Phe^{43} in a more rigid conformation in the S1′ pocket. Substitution of Thr^{54} with Ser would allow this hydrogen bond to be maintained and therefore should have little effect on the conformation of the Phe^{43} side chain, as evidenced by the similar enzymatic IC_{50} values of the T54S variant and the wild-type enzyme (U. Müh, unpublished data). However, the replacement of Thr^{54} with Ala would lead to the loss of the hydrogen bond and, consequently, an increase in the flexibility of the Phe^{43} side chain. We have previously observed that the

F43A mutation caused a loss of binding to inhibitors with prime side interactions (like α-ketoamides) but had no effect on inhibitors that do not extend into the S1′ pocket (like carboxylates) (C. Gates and B.G. Rao, unpublished data). Therefore, it is reasonable to expect that a more flexible Phe^{43}, due to either a void on one side of the Phe^{43} side chain in the V36A/M variants or a loss of the hydrogen bond on another side of the Phe^{43} side chain in the T54A variant, would result in a reduced interaction with telaprevir.

The conservation of the HCV NS3 serine protease at residue 36 or 54 suggests that a substitution at either position could have a detrimental impact on viral replication. Previous studies with HCV replicon cells have demonstrated that the in vitro replication capacity of PI-resistant variants is reduced (R155K/T) (54) or significantly impaired (A156T/V) (22, 30, 33, 48, 52). In this study, we showed that the in vitro replication capacities of several variants with substitutions of Val^{36} with or without additional substitutions at one of following residues (Thr^{54}, Arg^{155}, or Ala^{156}) of the NS3 protease were also reduced in Huh-7.5 cells. Overall, a higher in vitro replication capacity was often seen with the viral variants that were more frequently observed in telaprevir-dosed patients. For example, the V36G variant has a significantly diminished in vitro replication capacity, which is consistent with the rare appearance of this variant in telaprevir-dosed patients, compared to the higher replication capacity in vitro and the frequent appearance of the V36A/M variants (40), even though the in vitro fold resistance to telaprevir was similar among these three Val^{36} variants. In addition, the in vitro replication capacities of the A156T/V variants were significantly lower than those of the V36A/M and the T54A variants (this report) as well as those of the R155K/T variants (54) in Huh-7.5 cells. These results are consistent with the much more rapid decline in the A156T/V viral variants (compared to that of the V36A/M, T54A, and R155K/T variants) and the rapid reemergence of wild-type HCV in telaprevir-dosed patients after the cessation of dosing (40). Finally, even though the R155K variants (those with R155K or V36M and R155K double substitutions) were slightly less resistant to telaprevir than the corresponding R155T variants, the R155K variants (with single or double substitutions) were more frequently detected than the corresponding R155T variants in the telaprevir monotherapy trial. One possible explanation for this phenomenon could be the higher replication capacity of variants with the R155K substitution than that in variants with the R155T substitution.

Surprisingly, the V36A + T54A variant has a much lower in vitro replication capacity than either of the variants with a single substitution. The synergistic loss of in vitro replication capacity could be due to the simultaneous loss of interactions with Phe^{43} at both sides of its side chain: Val^{36} at one side and Thr^{54} at the other side. The V36A + T54A variant would have a Phe^{43} side chain with a much less stable conformation. The Phe^{43} is a crucial residue for maintenance of overall conformation of the HCV NS3 serine protease.

We have recently calculated the in vivo fitness of viral variants in a 14-day telaprevir monotherapy trial using two different approaches (7, 40). The in vivo fitness of viral variants in the "plateau group" was estimated by using a so-called naïve approach, which is based on the plasma level change of the variants during the 7- to 10-day postdosing follow-up (40). More recently, a new method based on HCV dynamic models of antiviral effectiveness (34, 53) was developed for the calculation of the in vivo fitness of viral variants in the "continuous decline group" (7). The results of both approaches were strikingly similar to each other: all variants (V36A/M, T54A, R155K/T, and A156S/T/V) showed reduced in vivo fitness relative to that of wild-type HCV, and among these variants the A156T/V variants had the poorest in vivo fitness (7, 40). The similar rankings of these variants in the in vivo fitness estimates and the in vitro replication capacity determination suggest that the replication capacity plays a critical role in their ability to grow in vivo.

Substitutions at Ala^{156} were initially identified in the in vitro selection of telaprevir-resistant protease variants in HCV genotype 1b (Con1 strain) replicon cells (22, 24). All three mutations at Ala^{156} were also observed in some HCV genotype 1a- or 1b-infected patients who had been dosed with telaprevir alone for 14 days (40). However, other resistance mutations, such as V36A/M, T54A, or R155K/T, were selected in the telaprevir-dosed patients (40) but were not found during in vitro selection (22, 24). One possible explanation for the discrepancy between the in vitro and the in vivo selection results could be the number of nucleotide changes that are required for these amino acid substitutions to occur in subtype 1a compared with the number of changes required for these amino acid substitutions to occur in subtype 1b. For example, the A156S/T/V and V36A variants need only a single nucleotide change in both subtypes 1a and 1b (GTA/GTG in genotype 1a or GTC/GTT in genotype 1b for Val^{36} to GCN for Ala^{36}), and all these variants were observed in HCV subtype 1a- or 1b-infected patients dosed with telaprevir alone (40). In contrast, substitution of Val^{36} with Met (ATG) requires a double nucleotide substitution in genotype 1b (GTC/GTT for Val^{36}) but a single nucleotide substitution in some variants of HCV genotype 1a (GTG for Val^{36}). The V36M variant was not observed in HCV subtype 1b-infected patients dosed with telaprevir alone (40) or in HCV subtype 1b replicon cells incubated with telaprevir (22, 24). A similar phenomenon of a double nucleotide substitution versus a single nucleotide substitution was also observed for the R155K/T/S/M/I variants, which occurred in HCV subtype 1b variants but not in HCV subtype 1a variants (54). Constraints in the protein structure of the HCV protease are unlikely to be the explanation for the absence of V36M (this report) or R155K/T/S/M/I (54) in HCV subtype 1b-infected patients dosed with telaprevir alone, since HCV subtype 1b replicon cells containing these mutations can be generated. If HCV subtype 1a replicon cells had been used instead of HCV subtype 1b cells for the in vitro selection of telaprevir-resistant variants, it is probable that the V36M and R155K/T/S/M/I variants identified in vivo would have been selected in vitro.

One paradox that exists is why V36M and R155K/T/S/M/I were not observed in HCV genotype 1b, whereas double amino acid substitutions (such as the V36M and R155K double substitutions) were selected in HCV genotype 1a, given that both require a change of two nucleotides. In HCV genotype 1b, the V36M substitution can be generated through two possible paths. The first path is a simultaneous change at two nucleotides at the Val^{36} codon of genotype 1b in a single RNA genome by the error-prone HCV RNA-dependent RNA poly-

merase ("simultaneous selection"). The odds of this event (10^{-8} to 10^{-10}) are much lower than that of a single nucleotide substitution change (10^{-4} to 10^{-5}) by the HCV RNA polymerase. The second and more likely path is through the sequential generation of both nucleotide substitutions by the viral polymerase ("sequential selection"), which is probably the same path for substitutions at both amino acids (a single nucleotide change at each of two codons) in the same genome. In the case of a double amino acid substitution, the selection of a change at the second codon is dependent on the ability of the variant with a substitution at the first codon to grow in the presence of the drug. The growth of the first variant is dependent on its drug resistance and in vivo fitness. In order for the V36M variant to be selected sequentially in genotype 1b, the original codon (GTC/GTT for Val^{36}) has to go through one of the following intermediates: GTG (Val) or ATC/ATT (Ile) to change to ATG (Met). However, the Val^{36} (GTG) intermediate is not resistant to telaprevir, and the Ile^{36} intermediate is probably not resistant to telaprevir (as in the case of the V36L variant) and may not be sufficiently fit to grow in vivo in the presence of the drug. Thus, the requirement of a double nucleotide change at the Val^{36} codon is probably the cause for the lack of selection of the V36M substitution in HCV subtype 1b. This hypothesis of the sequential selection of variants that require a double nucleotide change could also explain the absence of the R155K/T/S/M/I variants(54) and the V36A/M variants in HCV subtype 1b-infected patients in the 14-day telaprevir monotherapy trial.

The other possible explanation for the difference between the in vitro and the in vivo selection results is the concentration of telaprevir used in the in vitro selection experiments. Relatively high concentrations of telaprevir (14 and 28 μM, or 40 and 80 times the 48-h EC_{50}, respectively) were used for the in vitro selection of resistant variants (22, 24). When 10 μM (~40 times the EC_{50}) of another HCV protease inhibitor, SCH 503034, was used in the in vitro selection experiments, only A156V and A156T variants were selected (48). However, when a lower concentration (2.5 μM, or ~10 times the EC_{50}) of SCH 503034 was used, the T54A substitution was observed in addition to the A156S/T/V substitution (48). Given the less than eightfold resistance conferred by the V36A or T54A substitution, it was probably suppressed by the high concentration of telaprevir used in our in vitro selection experiments (22, 24).

Besides the variations that confer resistance to the HCV NS3-4A protease inhibitors, numerous mutations that confer resistance to various nucleoside analog or nonnucleoside inhibitors of HCV RNA-dependent RNA polymerase have also been identified in HCV genotype 1 replicon cells. The mutations that confer resistance to nucleoside analog inhibitors include S282T against 2′-*C*-methyl-nucleoside analogs (NM107/NM283, MK-0608, PSI-6130/R7128) (20, 32) and S96T against 4′-azido-nucleoside analogs (R1479/R1626) (20). Many mutations that confer resistance in vitro have been identified for nonnucleoside inhibitors, which bind to one of three or four allosteric sites of HCV polymerase: (i) L419M, M423T/I, and I482L against thumb domain inhibitors (12, 21, 43); (ii) P495S/L/A, P496A/S, and V499A against benzimidazole or indole inhibitors (16, 46); (iii) H95Q/R, N411S, M414L/T, Y448H, C451R, and G558R against benzothiadiazine or palm domain inhibitors (21, 29, 33, 35, 47); and (iv) C316Y/F and S365A/T against a different palm domain inhibitor, HCV-796 (13). Mutations that confer resistance have also been found in HCV-infected chimpanzees dosed with HCV polymerase inhibitors, such as S282T against MK-0608 (4) and N411S, M414L/T, and Y448H against a nonnucleoside benzothiadiazine inhibitor (6). The resistance-conferring mutation C316Y was found by using the population sequencing method in ~70% of HCV-infected patients who had a viral rebound in a 14-day dosing trial with HCV-796 (50).

Combinational therapies that target multiple steps of the viral life cycle are much more effective than monotherapy in reducing the human immunodeficiency virus load and preventing the selection of drug-resistant variants. It is likely that more effective therapy for HCV infection may require the use of a combination of multiple anti-HCV agents, including various classes of STAT-C as well as IFN-α (with or without ribavirin). In the in vitro replicon studies, no change in susceptibility to IFN-α was observed in cells with various telaprevir-resistant variants, including many variants with a single substitution (V36A/M or T54A [this report], A156T/V [22, 30], or R155K/T [54]) or variants with the V36M and R155K double substitutions (this report), compared to the susceptibility of the HCV wild-type replicon. These data suggest that the addition of IFN-α to a telaprevir-based regimen could reduce the risk of the emergence of resistant variants and, therefore, improve the efficacy 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 (37) or during 28 days of dosing with telaprevir in combination with peginterferon alfa-2a and ribavirin (19) in HCV genotype 1-infected patients.

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