Drug resistance is a major issue in the development and use of specific antiviral therapies. Here we report the isolation and characterization of hepatitis C virus RNA replicons resistant to a novel ketoamide inhibitor of the NS3/4A protease, SCH6 (originally SCH446211). Resistant replicon RNAs were generated by G418 selection in the presence of SCH6 in a dose-dependent fashion, with the emergence of resistance reduced at higher SCH6 concentrations. Sequencing demonstrated remarkable consistency in the mutations conferring SCH6 resistance in genotype 1b replicons derived from two different strains of hepatitis C virus, A156T/A156V and R109K. R109K, a novel mutation not reported previously to cause resistance to NS3/4A inhibitors, conferred moderate resistance only to SCH6. Structural analysis indicated that this reflects unique interactions of SCH6 with P side residues in the protease active site. In contrast, A156T conferred high level resistance to SCH6 and a related ketoamide, SCH503034, as well as BILN 2061 and VX-950. Unlike R109K, which had minimal impact on NS3/4A enzymatic function, A156T significantly reduced NS3/4A catalytic efficiency, polyprotein processing, and replicon fitness. However, three separate second-site mutations, P89L, Q86R, and G162R, were capable of partially reversing A156T-associated defects in polyprotein processing and/or replicon fitness, without significantly reducing resistance to the protease inhibitor.

One of the major issues in the development of antiviral drugs is the emergence of drug-resistant viral variants. Although antiviral resistance occurs in DNA viruses (1), RNA viruses, such as hepatitis C virus (HCV), have higher mutation rates and typically exist as a complex population of genetically distinct but closely related viral variants, commonly referred to as quasi-species (2–4). During the emergence of escape variants, pre-existing minor viral species, resistant to the selecting drug, will gain a growth advantage over the existing wild-type viral population and rapidly become the dominant genotype. In human immunodeficiency virus (HIV)-infected patients treated with protease inhibitors, resistant viruses have been isolated and shown to correlate with breakthrough viral replication. Resistance can also be developed by selecting a virus with protease inhibitors in vitro, and in many cases the mutations that confer resistance in vitro are the same as those observed clinically (for review see Refs. 5 and 6).

Much research has been directed toward finding inhibitors of the nonstructural proteins of HCV that contribute to the viral RNA replication complex. The N-terminal third of NS3 is a serine protease involved in the cis-processing of the HCV polyprotein at the NS3-NS4A junction. NS3 assembles as a noncovalent complex with NS4A to form the mature protease responsible for the further processing of the nonstructural proteins at the NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions (for review see Ref. 7). The NS3 protease is essential for viral replication (8) and represents an important target for antiviral therapy. Recently, an NS3 protease inhibitor, BILN 2061, was reported to reduce viremia by 100–1000-fold in patients during a brief phase Ib clinical trial (9). Another protease inhibitor, VX-950, has also been shown to inhibit HCV replication in pre-clinical studies, and recently was reported to have clinical efficacy similar to that of BILN 2061 (10, 11). Mutations conferring resistance to both of these small molecule inhibitors of NS3/4A have been identified in the protease domain by G418 selection of cells containing HCV replicon RNAs expressing neomycin phosphotransferase in the presence of the compounds (10, 12, 13).

We have studied the development of resistance to a novel ketoamide, peptidomimetic protease inhibitor, SCH6 (originally SCH446211). SCH6 specifically inhibits NS3 proteolysis of the viral polyprotein, and also has been demonstrated to reverse the NS3/4A-mediated blockade of double-stranded RNA and virus infection-induced activation of interferon regulatory factor 3 (IRF-3) in cells containing HCV RNA replicons (14, 15). We report here the isolation and characterization of SCH6-resistant replicon variants derived from two genotype 1b strains of HCV, and we show the impact of these mutations on the catalytic activity of the protease as well as replicon fitness. We show that muta-
tion of Arg-109 leads to moderate resistance against SCH6 but not other inhibitors of the NS3 protease, reflecting novel interactions of SCH6 with P'-side residues in the protease active site. In contrast, mutation of Ala-156 results in broad resistance against NS3/4A inhibitors from multiple chemical classes but at a significant cost in terms of catalytic activity and replication fitness. This loss of fitness, however, is partially restored by second-site mutations within the NS3.

**EXPERIMENTAL PROCEDURES**

**Control Cells and Subgenomic HCV Replicon Lines**—The human hepatoma cell line Huh7 (16) was grown in Dulbecco’s minimal essential medium supplemented with 2 mM glutamine, nonessential amino acids, 10 mM HEPES, 0.075% sodium bicarbonate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. En5-3 cells, a clonal cell line derived from Huh7 cells by stable transformation with the plasmid pLTR-SEAP (17), were cultured in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum but without nonessential amino acids, additional sodium bicarbonate, or HEPES and with the addition of 2 μg/ml blasticidin (Invitrogen). Cell lines containing HCV replicons were cultured in 0.4–0.5 mg/ml G418, except for clone 16 for which 1 mg/ml G418 was used.

The HCV replicon cell lines used in these studies autonomously replicating, selectable dicistronic, and subgenomic RNAs (18) derived from two different genotype 1b strains of virus. The Con1 replicon used to select the Huh7-derived cell line, 2H8 1.3, was identical to that described by Lohmann et al. (18), except for the presence of a cell culture-adaptive mutation, S233L, within the NS5A sequence (19). The replicon in the stably selected 2H8 1.3 cell line also contains a spontaneously acquired adaptive mutation E176G in NS3, as discussed below. We also used an additional replicon cell line for selection of SCH6-resistant mutants, Ntat2ANeo/EG. This was established using En5-3 cells and contains a modified subgenomic replicon derived from the HCV-N strain of HCV that expresses the HIV Tat protein, in addition to neomycin phosphotransferase, from its upstream cistron (17). This replicon also carries the NS3 adaptive mutation E176G. The expression of Tat by Ntat2ANeo/EG drives the expression and secretion of secreted alkaline phosphatase (SEAP) in transfected En5-3 cells in a manner that correlates closely with the intracellular abundance of the viral RNA. Thus, measurement of SEAP activity in the supernatant fluids of Ntat2ANeo/EG cells allows real time estimates of the intracellular abundance of replicon RNA (17, 20). For transient replication assays, we used the replicon pNtat2ANeo/EG/SI, which is identical to pNtat2ANeo/EG but also contains the S233L adaptive mutation in NS5A.

**Selection of Replicons with Primary SCH6 Resistance Mutations and Second-site Compensatory Mutations**—To select replicon cell lines resistant to SCH6, replicon-bearing cells (1 × 10^6) were plated in a 10-cm dish and cultured with 2.5, 0.5, 0.1, or 0 μM SCH6 for 2 or 0 μM SCH6 (Ntat2ANeo/EG cells), in addition to G418. All cells were passed once at a 1:10 ratio once they became confluent. Surviving colonies resistant to both inhibitor and antibiotic were selected and expanded for further analysis.

To select additional mutations that might facilitate replication of the HCV-N A156T mutant (see “Results”), in vitro transcribed RNA (see below) derived from the Ntat2ANeo/EG/SI replicon engineered to contain the NS3 A156T mutation was electroporated into En5-3 cells, and cell colonies were selected in the presence of 400 μg/ml G418. RNA isolated from selected clonal cells was subjected to direct sequencing analysis, as described below.

**Evaluation of Inhibitor Resistance**—HCV-N replicons selected for resistance to SCH6 were tested for sensitivity to NS3/4A inhibitors, and the results were analyzed as described previously (20). For the Con1 replicons, cells were seeded at 4000 cells/well in 96-well collagen I-coated Biocoat plates (BD Biosciences). Twenty four hours post-seeding, protease inhibitors were added to replicon cells. The final concentration of Me_{2}SO was 0.5%, fetal bovine serum was 5%, and G418 was 500 μg/ml. Media and inhibitors were refreshed daily for 3 days, and cells were harvested by lysis in 1% cellysis buffer (Ambion catalog number 8721). The replicon RNA abundance was measured by quantitative, multiplex RT-PCR (Taqman) assay with the following NS5B PCR primers: 5B.2 forward, ATGGACAGGCCGCTCTGA, and 5B.2 reverse, TGTAGGCGACCTGGTTTC, with a 6-carboxyfluorescein-labeled probe, CACGCCATGCGGTTCG. As with the HCV-N replicons (20), the endogenous control was GAPDH mRNA. The real time RT-PCRs were run on the ABI PRISM 7900HT sequence detection system using the following program: 48 °C for 30 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The ΔCT values (CT_{5B} − CT_{GAPDH}) were plotted against inhibitor concentration and fitted to the sigmoid dose-response model using SAS (SAS Institute Inc.) or PRISM software (Graphpad Software Inc.). I_{50} was the inhibitor concentration necessary to achieve an increase of 1 in the ΔCT over the projected base line. I_{50} was the inhibitor concentration necessary to achieve an increase of 3.2 over the base line.

**Replicon Sequence Analysis**—To identify mutations that conferred resistance to SCH6, total cellular RNA was isolated from selected replicon cells and amplified by RT-PCR as described (21). The RT-PCR products were purified using the QiAquick PCR purification kit (Qiagen) and directly sequenced. Alternatively, replicon cell-derived RT-PCR products were cloned into TOPO TA vector (Invitrogen), and plasmid DNA from multiple bacterial colonies was sequenced. The sequences were aligned using Lasergene software (DNASTAR).

**Site-directed Mutagenesis**—To generate mutant protease expression vectors and mutant replicon constructs carrying individual resistance mutations, nucleotide changes were introduced into plasmid DNA using the QuikChange mutagenesis kit (Stratagene). The parental plasmid expressing His-tagged single chain NS3 protease domain, NS4A_{1–35}-GSGS-NS3_{3–181}, was described by Taremi et al. (22). For mutational analysis of HCV-N replicons in transient replication assays, the parental construct was pNtat2ANeo/EG/SI, identical to pNtat2ANeo/EG but containing the NS5A S233L mutation. All segments of the cDNA that were manipulated during site-directed mutagenesis were sequenced to confirm the presence of the desired mutation and to exclude adventitious changes.

**Evaluation of Replicon Colony Formation Efficiency (CFE)—**5 μg of each replicon RNA was transfected into 5 × 10^4 Huh7 cells in 400 μl of phosphate-buffered saline at room temperature. Electroporation conditions were 950 microfarads and 250 V in a 0.4-cm cuvette using a Gene Pulser system (Bio-Rad). Cells were selected with 500 μg/ml G418 for 2–3 weeks until cell colonies were established. One set of the dishes was stained with crystal violet (from Sigma, 0.48 mg/ml in 3% formaldehyde, 30% ethanol, 0.16 mg/ml NaCl), and the numbers of colonies were recorded. The CFE was reported as the number of colonies established/μg of input RNA. Cells from the duplicate set were pooled and expanded for further analysis.

**Transient Replication Assay**—RNA's representing the Ntat2ANeo/EG/SI, with and without SCH6 resistance mutations, were synthesized with T7 MEGaScript reagents (Ambion), after linearizing plasmids with XbaI. Following treatment with RNase-free DNase to remove template DNA and purification using the RNeasy RNA purification kit (Qi-
briefly, plasmid DNAs encoding mutant proteases were transformed into JM109 or BL21 (DE3) cells. Single colonies were used to initiate expression and purification protocol has been described in detail (22).

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Expression and Purification of Recombinant Mutant Proteases—The expression and purification protocol has been described in detail (22).

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Generation of HCV Replicon Cells Resistant to SCH6—SCH6-resistant subgenomic HCV RNA replicons were selected from two stably transduced G418-resistant cell lines harboring subgenomic and dicistronic replicon RNAs derived from two different genotype 1b strains of...
HCV (Table 1). The 2H8 1.3 cell line contains replicon RNA derived from the Con1 strain of HCV, whereas Ntat2ANeo/EG replicon cells contain RNA derived from the HCV-N strain (17). Both of these RNA replicons encode an E176G substitution near the C terminus of the NS3 protease domain that has been shown previously to promote the replication of HCV RNA in Huh7 hepatoma cells (29). Most interestingly, this cell culture-adaptive mutation was independently selected during the establishment of both of these replicon cell lines. HCV RNA replication was inhibited in both the 2H8 1.3 and Ntat2ANeo/EG cell lines by addition of SCH6 to the culture medium, with IC90 values of 0.4 and 1.6 μM (Table 1), respectively. In contrast, measurements of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide activity and RT-PCR assays for cellular GAPDH mRNA indicated that the CC50 for SCH6 was >10 μM. Thus, SCH6 selectively inhibits HCV RNA replication and has low toxicity for Huh7 cells.

In preliminary experiments, the 2H8 1.3 cells containing the Con1 replicon were cultured in the presence of 0.1–2.5 μM of SCH6 with continued G418 antibiotic selection in an effort to force the selection of variant replicons with resistance to the NS3/4A protease inhibitor. A majority of the cells was expected to die because of the loss of antibiotic resistance as a result of inhibition of RNA replication and the subsequent reduction in replicon copy number and neomycin phosphotransferase expression. Cells that survived were presumed to be able to maintain a minimal level of replication in the presence of the compound, and were evaluated for resistance to the compound. When cells were treated with SCH6 at a concentration equaling the IC90 of all the cells survived. On the other hand, when SCH6 was added to the media at ~6-fold the IC90 (2.5 μM), only 0.1% of the cells survived, suggesting that the compound had achieved nearly complete inhibition of virus RNA replication. Moreover, subsequent quantitative RT-PCR testing indicated that there was an ~33-fold increase in the SCH6 IC90 value for the surviving cells, suggesting that the surviving cells contained replicon RNAs selected for resistance to the inhibitor.

**Identification and Characterization of Mutations Associated with Resistance to SCH6**—By using the approach described above, we selected two independent colonies of 2H8 1.3 replicon cells that were resistant to SCH6. We also selected three colonies of SCH6-resistant replicon cells from the HCV-N replicon cell line Ntat2ANeo/EG. The nucleotide sequences of the NS3 protease domains in each of these five SCH6-resistant replicon cell lines were determined by direct sequencing of RT-PCR products and compared with the cognate base-line parental sequences (Table 2). Remarkably, the replicon RNA in each of the two Con1 cell lines and in two of the three HCV-N cell lines contained an R109K mutation. This was the only mutation identified within the NS3 protease domain of the two HCV-N cell lines, whereas in each of the Con1 cell lines this mutation was found in association with a second I153V substitution (Table 2). The third SCH6-resistant HCV-N cell line contained no substitutions at either of these residues but instead contained an A156T substitution (Table 2). Threonine or serine substitutions of the Ala-156 residue, which is broadly conserved across all HCV genotypes, have been reported previously to cause resistance to other small molecule inhibitors of the NS3/4A protease (10, 13). In contrast, I153V and R109K are novel mutations that have not been described previously.

In an effort to identify additional mutations that might possibly be associated with SCH6 resistance, RNA was extracted from a pool of 2H8 1.3 cells selected by growth in 2.5 μM SCH6, and replicon sequences amplified by RT-PCR were molecularly cloned followed by DNA sequencing. Most interestingly, four of seven cDNA clones contained the A156T mutation, although R109K was present in two others in association with either I153V or A156V. The remaining cDNA clone contained a solitary I153V substitution. No additional mutations were identified within the protease domain using this approach. Together, these data suggest a remarkable consistency in the selection of mutations associated with SCH6 resistance in replicon RNAs derived from two different genotype 1b viruses.

### Table 1

| Cell line          | HCV strain | SCH6 IC90 μM (n = 8) | SCH6 CC50 μM |
|--------------------|------------|----------------------|--------------|
| 2H8 1.3            | Con1       | 0.4 ± 0.2            | >10          |
| Ntat2ANeo/EG       | HCV-N      | 1.4 ± 0.01           | >20          |

**Identification and Characterization of Mutations Associated with Resistance to SCH6**—By using the approach described above, we selected two independent colonies of 2H8 1.3 replicon cells that were resistant to SCH6. We also selected three colonies of SCH6-resistant replicon cells from the HCV-N replicon cell line Ntat2ANeo/EG. The nucleotide sequences of the NS3 protease domains in each of these five SCH6-resistant replicon cell lines were determined by direct sequencing of RT-PCR products and compared with the cognate base-line parental sequences (Table 2). Remarkably, the replicon RNA in each of the two Con1 cell lines and in two of the three HCV-N cell lines contained an R109K mutation. This was the only mutation identified within the NS3 protease domain of the two HCV-N cell lines, whereas in each of the Con1 cell lines this mutation was found in association with a second I153V substitution (Table 2). The third SCH6-resistant HCV-N cell line contained no substitutions at either of these residues but instead contained an A156T substitution (Table 2). Threonine or serine substitutions of the Ala-156 residue, which is broadly conserved across all HCV genotypes, have been reported previously to cause resistance to other small molecule inhibitors of the NS3/4A protease (10, 13). In contrast, I153V and R109K are novel mutations that have not been described previously.

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To characterize better these mutations, the amino acid changes were introduced into a recombinant single-chain form of the wild-type protease domain (genotype 1b HCV-BK strain) (22) and analyzed for their impact on kinetic parameters as well as inhibitor binding. Individually, these mutations did not cause dramatic changes in the catalytic efficiency of the protease. The $k_{cat}$ values were all within the experimental error of the wild-type enzyme with the exception of I153V, which had a modest effect (~3-fold) (Table 3). $K_m$ values were also within experimental error except for A156T, which resulted in a decrease in substrate binding of ~5-fold. More importantly, however, the chromogenic substrate (23) used in these in vitro protease assays binds only to the P-side of the active site (30). Thus, any potential impact on protein substrates that also bind to the P’-side, such as the native viral polyprotein substrate, would not be detected in these assays. We addressed this limitation by carrying out polyprotein processing assays, which are described below.

In contrast to the relatively small effect on proteolysis, a significant increase (19-fold) in the SCH6 inhibition constant ($K_s^*$, see “Experimental Procedures”) was observed for the A156T mutation ($K_s^*$ was 7 nM for the wild-type protease and 130 nM for the A156T mutant; see Table 3). This is consistent with previous reports that Ala-156 is a major locus for susceptibility of the replicon harbored by each cell line to SCH6 was assessed by quantitative RT-PCR assays for replicon RNA abundance. These results correlated well with those from the in vitro protease assays (Table 3). The A156T mutation conferred a relatively high level of resistance (18-fold) to SCH6 in the Con1 replicon, whereas the R109K mutation gave rise to only a 3-fold increase in the SCH6 $IC_{50}$. Mutation I153V, although conferring a lower level of resistance in the in vitro assay, did not seem to affect SCH6 inhibition in the replicon assay. Similar results were obtained with the R109K and A156T mutations in the HCV-N replicon (Table 3). As in the cell-free protease assay, the two mutations appeared to work cooperatively when both were introduced into the Con1 replicon, as the double mutation R109K/A156T increased the $IC_{50}$ by at least 100-fold. In contrast, the R109K/I153V double mutant did not further reduce replicon susceptibility to SCH6 (Table 3).

Cross-resistance to SCH503034, BILN 2061, and VX-950—To investigate whether the mutations we identified as causing SCH6 resistance would also confer resistance to other published NS3/4A inhibitors, the mutant recombinant single-chain proteases described above (see Table 3) were assayed for their susceptibility to other small molecule inhibitors of NS3/4A, including the related ketoamide SCH503034, the macrocyclic peptidomimetic BILN 2061, and VX-950. An additional mutant protease was similarly assessed, bearing the D168V mutation that was reported previously to confer resistance to BILN 2061 (10, 13). As shown in Table 4, the resistance profiles of these compounds differed from that of SCH6. The rank order of the degree of resistance conferred by the mutations toward SCH6 was A156T $>$ R109K $>$ I153V, whereas the D168V mutation had no impact. On the other hand, for BILN 2061, the rank order was A156T $>$ D168V $>$ R109K and I153V had no impact. For both SCH503034 and VX-950, A156T was the only mutation that caused a significant decrease in potency. Thus, A156T was a major determinant of resistance to all four compounds, but SCH6 remained a relatively potent inhibitor of this mutant protease ($K_s^*$ = 0.13 μM compared with $K_s^*$ of 1, 12, and 13 μM for BILN 2061, SCH503034, and VX-950, respectively).

Impact of SCH6 Resistance Mutations on Replicon Fitness—The preceding results indicate that the R109K and A156T mutations lead to a loss of SCH6 inhibition of NS3 protease activity. To determine their

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**TABLE 3**

| Mutation | SCH6-resistant colonies | cDNA clones, 2H8 1.3 |
|----------|-------------------------|----------------------|
| 2H8 1.3  | Nat2TANeo/EG            |                      |
| R109K    | 0                       | 2                    |
| A156T    | 0                       | 1                    |
| R109K/A156V | 0   | 0                    |
| I153V    | 0                       | 0                    |
| R109K/I153V | 2  | 0                    |
| Total    | 2                       | 3                    |

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**Impact of NS3 mutations on protease activity and replicon resistance to SCH6**

| Mutation | $K_m$ (μM) | $K_m$ (μM) | SCH6 $K_s^*$ (μM) | Con1 $IC_{50}$ (μM) | HCV-N $IC_{50}$ (μM) |
|----------|------------|------------|------------------|---------------------|----------------------|
| Wild-type protease | 5 ± 1 (n = 4) | 25 ± 6 | 7 ± 4 (n = 8) | 0.4 ± 0.2 (n = 8) | 1.4 ± 0.01 (n = 6) |
| Mutations selected by passage of replicon cells in SCH6 | | | | | |
| R109K | 10 ± 1 (n = 2) | 15 ± 1 | 45 ± 30 (n = 2) | 1.2 ± 0.3 (n = 2) | 3.2 ± 0.36 (n = 2) |
| A156T | 26 ± 4 (n = 3) | 17 ± 7 | 130 ± 30 (n = 6) | 7.0 ± 0.3 (n = 3) | 20 ± 0.01 (n = 3) |
| R109K/A156T | 38 ± 3 (n = 2) | 15 ± 1 | 1500 (n = 1) | >40 | ND* |
| I153V | 4 ± 1 (n = 1) | 8 | 19 ± 1 (n = 3) | 0.28 ± 0.02 (n = 2) | ND |
| R109K/I153V | 3 (n = 2) | 3 ± 0.5 | 22 ± 2 (n = 2) | 1.4 ± 0.2 (n = 2) | ND |

*See Table 2 for definition of the inhibition constant, $K_s^*$.

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**TABLE 2**

| Mutation | SCH6-resistant colonies | cDNA clones, 2H8 1.3 |
|----------|-------------------------|----------------------|
| 2H8 1.3  | Nat2TANeo/EG            |                      |
| R109K    | 0                       | 2                    |
| A156T    | 0                       | 1                    |
| R109K/A156V | 0   | 0                    |
| I153V    | 0                       | 0                    |
| R109K/I153V | 2  | 0                    |
| Total    | 2                       | 3                    |

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* See Table 2 for definition of experimental procedures for the determination of the inhibition constant, $K_s^*$.

**HCV Replication Fitness and NS3/4A Inhibitor Resistance**

To further confirm the impact of these mutations on the inhibition of protease activity by SCH6, selected mutations were introduced into the Con1 and HCV-N replicon backgrounds, and stable G418-resistant replicon cell lines carrying the mutant proteases were established. The susceptibility of the replicon harbored by each cell line to SCH6 was assessed by quantitative RT-PCR assays for replicon RNA abundance. These results correlated well with those from the in vitro protease assays (Table 3). The A156T mutation conferred a relatively high level of resistance (18-fold) to SCH6 in the Con1 replicon, whereas the R109K mutation gave rise to only a 3-fold increase in the SCH6 $IC_{50}$. Mutation I153V, although conferring a lower level of resistance in the in vitro assay, did not seem to affect SCH6 inhibition in the replicon assay. Similar results were obtained with the R109K and A156T mutations in the HCV-N replicon (Table 3). As in the cell-free protease assay, the two mutations appeared to work cooperatively when both were introduced into the Con1 replicon, as the double mutation R109K/A156T increased the $IC_{50}$ by at least 100-fold. In contrast, the R109K/I153V double mutant did not further reduce replicon susceptibility to SCH6 (Table 3).
HCV Replication Fitness and NS3/4A Inhibitor Resistance

TABLE 4
Potency of inhibitors against wild-type protease and protease-containing resistance mutations

| Mutation | Inhibition constant \(K_i\) * | SCH6 | SCH503034 | BILN 2061 | VX-950 |
|----------|--------------------------------|------|------------|------------|--------|
| Wild type| 7 | 30 | 0.8 | 30 | |
| R109K    | 45 | 45 | 0.3 | 55 | |
| A156T    | 130 | 8300 | 900 | 13,000 | |
| I153V    | 19 | 19 | 0.7 | 24 | |
| D168V    | 4 | 20 | 190 | 12 | |

*See Table 3 for definitions of \(K_i\) values.

FIGURE 1. CFE of Con1 replicon RNAs bearing resistance mutations. Replicon RNAs containing resistance mutations were transfected into Huh7 cells followed by selection with 500 μg/ml G418 for 2–3 weeks until cell colonies were established. Bars represent the number of replicon colonies/μg of input RNA; the numbers above each bar show the efficiency normalized to the wild-type (wt) replicon.

Potential impact on viral replication fitness, two separate approaches were taken. First, we assessed the CFE of Con1 replicon RNAs bearing these resistance mutations. The number of stable G418-resistant cell colonies obtained per μg of transfected input replicon RNA reflects the efficiency with which the mutant replicon replicates in Huh7 cells and expresses the antibiotic selection marker. As shown in Fig. 1, the R109K mutation had no measurable effect on CFE compared with wild-type RNA, whereas the A156T mutation substantially reduced the number of colonies generated (6% of the wild-type Con1 replicon RNA). The double mutation R109K/A156T demonstrated an even greater reduction in CFE (0.7% of wild-type). These results thus suggested that the impact of these mutations on SCH6 potency was inversely correlated with their effect on replicon fitness.

Although the CFE provides an estimate of replication fitness, it does not account for the possible accumulation of additional mutations within the replicon RNA during the colony selection process (19). To measure more directly the impact of the A156T and R109K mutations on replication efficiency, we assessed the amplification of replicon RNA following its transfection into permissive cells in a transient replication assay. This was carried out by introducing the mutations into the subgenomic HCV-N replicon, Ntat2ANeo/EG/SI, which expresses the HIV Tat protein as well as neomycin phosphotransferase from the upstream cistron (17). This induces the synthesis and release of SEAP from transfected En5-3 cells in which SEAP expression has been placed under the control of the HIV long terminal repeat promoter. The amount of SEAP expressed correlates closely with the intracellular abundance of the viral RNA (17). This novel reporter function significantly facilitates measurement of the replication of transfected replicon RNA in transient assays in which the emergence of adaptive mutations is unlikely to play a significant role. Wild-type Ntat2ANeo/EG/SI and mutant replicon RNAs bearing either the A156T or R109K mutations were transfected into permissive En5-3 cells in parallel with a control, replication-defective NS5B GDDD mutant, and the SEAP activity expressed by the cells was monitored every 24 h.

As shown in Fig. 2, each of the RNAs induced comparable levels of SEAP activity during the first 24 h following transfection (~100-fold greater than nontransfected cells), reflecting translation of the transfected input RNA. Over the subsequent 72 h, SEAP expressed by cells transfected with the GDDD mutant returned to base line, although that induced by the wild-type Ntat2ANeo/EG/SI replicon demonstrated a sustained increase indicative of RNA replication (17). Although inducing demonstrably greater SEAP activity than the GDDD mutant, the A156T mutant was markedly impaired in SEAP induction and thus RNA replication compared with the parental Ntat2ANeo/EG/SI replicon. In contrast, the R109K mutant was only slightly less efficient in replicating and inducing SEAP expression than the parental Ntat2ANeo/EG/SI replicon. These results thus confirm that the A156T mutation is associated with very impaired replication fitness, consistent with the reduced CFE of replicons bearing this mutation in Fig. 1. Together, these data confirm the findings of Lu et al. (13) who reported recently that the A156T mutation substantially reduced the replication fitness of a luciferase-expressing HCV replicon.

Partial Rescue of Replicon Fitness by Second-site Mutations—A Q86R mutation within the protease domain of NS3 has been identified previously as one of the adaptive mutations associated with the enhanced replication activity of cell culture-adapted HCV replicons (19). It has also been identified recently in association with the A156T mutation following selection of replicons with a high concentration of an SCH6-related NS3/4A protease inhibitor, SCH503034.6 For this reason, we assessed its effect on NS3 protease activity, SCH6 resistance, and HCV replicon fitness alone and in association with the A156T mutation. As shown in Table 3, the Q86R mutation by itself had no effect on enzyme activity, as measured using the chromogenic assay, or sensitivity to SCH6 in vitro when introduced into the Con1 background. The SCH6 sensitivity of the double mutant Q86R/A156T was comparable with or only slightly less than that of the A156T mutant. However, the CFE of a Con1 replicon carrying the double mutation was significantly higher than the replicon containing the single A156T mutation (Fig. 1), suggesting that Q86R may partially rescue the loss in fitness of A156T.

To explore the possibility that second-site mutations within the NS3 protease domain might partially rescue the loss of replication fitness associated with the A156T mutation, and to ascertain the genetic stability of the A156T mutation, additional studies were carried out with the SEAP-expressing HCV-N A156T mutant. Huh7 cells were transfected with the Ntat2ANeo/EG/SI replicon RNA bearing the A156T mutation and selected for G418 resistance in the absence of the protease inhibitor SCH6. Under these conditions, the A156T mutant replicon could either retain the sequence of the transfected RNA, undergo reversion to the wild-type NS3 sequence in the absence of SCH6 pressure, or possibly develop second-site mutations that rescue the reduced fitness of the A156T mutant. Five stable G418-resistant, SEAP-expressing cell colonies were selected, and the NS3 region of HCV RNA isolated from these replicon cells was amplified by RT-PCR and directly sequenced. Replicons from three of the five colonies demonstrated no sequence changes within the protease domain but retained the A156T mutation. This is consistent with the reduced but still appreciable (6% of wild-type) CFE of the A156T Con1 replicon (Fig. 1). However, the protease

6 X. Tong, R. Chase, A. Skelton, T. Chen, J. Wright-Minoque, and B. A. Malcolm, submitted for publication.
HCV Replication Fitness and NS3/4A Inhibitor Resistance

Impact of Primary Resistance and Second-site NS3 Mutations on Polyprotein Processing—The results of the cell-free biochemical protease assays presented in Table 3 suggest that the A156T mutation causes a few-fold loss of proteolytic function, which correlates with the lower CFE activity (Fig. 1) and reduced fitness of HCV replicons (Fig. 2) containing this mutation. Such changes were minimal or not evident at all with the R109K and I153V mutants. Although more detailed biochemical analyses will be required to fully assess the impact of the resistance mutations on proteolysis of TRIF or IPS-1 by NS3/4A, the results from this experiment demonstrated equivalent disruption of IRF-3 signaling to the ISG56 promoter in cells expressing either the wild-type protease or mutant proteases containing the R109K or A156T mutation. This suggests that the loss of fitness associated with the A156T mutation is unlikely to be due to impaired evasion of host defense signaling pathways.

DISRUPTION OF INTERFERON SIGNALING BY PROTEASES CONTAINING RESISTANCE MUTATIONS—The NS3/4A protease cleaves at least two critical host cell signaling proteins, the Toll-like receptor 3 adaptor protein TRIF and interferon-β promoter stimulator 1 (IPS-1), also known as MAVS or Cardif, thereby disrupting virus activation of IRF-3, a transcription factor that controls the synthesis of interferon-β and numerous interferon-stimulated genes such as interferon-stimulated gene 56 (ISG56) (14, 15, 31, 32). Because these signaling pathways appear to control virus replication, it is possible that the reduction in viral fitness that we observed in association with the A156T resistance mutation could be due to impaired NS3/4A-mediated proteolysis of cellular targets involved in the activation of innate intracellular antiviral responses such as TRIF or IPS-1. To assess this possibility, we examined the ability of the Sendai virus to activate the ISG56 promoter in Huh7 cells ectopically expressing NS3/4A, either with or without the R109K or A156T resistance mutation (Fig. 3). Although more detailed biochemical analyses will be required to fully assess the impact of the resistance mutations on proteolysis of TRIF or IPS-1 by NS3/4A, the results from this experiment demonstrated equivalent disruption of IRF-3 signaling to the ISG56 promoter in cells expressing either the wild-type protease or mutant proteases containing the R109K or A156T mutation. This suggests that the loss of fitness associated with the A156T mutation is unlikely to be due to impaired evasion of host defense signaling pathways.

FIGURE 2. Transient replication profile of HCV-N replicons that express the HIV Tat protein, inducing SEAP secretion in transfected En5-3 cells. Shown are the SEAP activities present in supernatant culture fluids over 4 successive days following electroporation of cells with the parental WT replicon, mutated RNAAs containing the following SCH6 resistance mutations A156T, R109K, A156T/P89L, and A156T/G162R, and a replication- lethal mutant with a mutation in the active site of the N538 polymerase, AGDD. In this system, SEAP activities closely parallel the abundance of intracellular RNA (17). For each mutant, the data represent the SEAP expression at each time point as a percent of that of the AGDD control replicon at 24 h after transfection. The data shown are means from two independent experiments.

FIGURE 3. Wild-type and mutant NS3/4A proteases resistant to SCH6 disrupt Sendai virus infection-induced activation of the ISG56 promoter. Cells were transfected with vectors expressing the wild-type HCV-N NS3/4A segment, with or without the indicated resistance mutations. As a control, cells were transfected in parallel with a vector expressing an active-site NS3 mutant lacking protease activity (S138AA) or with empty vector. Cells were transfected subsequently with an ISG56 promoter luciferase reporter construct and then infected with Sendai virus. The ISG56 promoter stimulation index shown represents the ratio of luciferase activity present in Sendai-infected versus mock-infected cells. ± S.E. The results show that expression of the wild-type protease or proteases containing the A156T or R109K resistance mutations, but not the active-site S138AA mutant, block virus-induced activation of the ISG56 promoter through the IRF-3 pathway (14, 15, 31, 32).
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To determine the impact of these mutations on trans-cleavage activity, we synthesized the NS3-4A segment in similarly programmed in vitro translation reactions programmed with RNA encoding the NS3-4A segment of HCV-N and related SCH6 primary resistance and second-site mutations. Reactions were terminated after 20, 30, and 180 min. S138A indicates S138A/S139A, an enzymatically inactive NS3 protease mutant. See text for other details. C, PhosphorImager quantitation of the percent of the translation products shown in B that have undergone scission. See B for key to symbols.

FIGURE 4. SCH6 resistance mutations result in impaired cis-autoproteolysis of NS3-4A in vitro. A, autoproteolysis of an in vitro NS3-4A translation product results in the mature NS3/4A protease. B, SDS-PAGE of 35S-labeled products of in vitro translation reactions programmed with RNA encoding the NS3-4A segment of HCV-N and related SCH6 primary resistance and second-site mutations. Reactions were terminated after 20, 30, and 180 min. S138A indicates S138A/S139A, an enzymatically inactive NS3 protease mutant. See text for other details. C, PhosphorImager quantitation of the percent of the translation products shown in B that have undergone scission. See B for key to symbols.

FIGURE 5. SCH6 resistance mutations result in impaired trans-processing of the NS4B-5A polyprotein segment in vitro. A, autoproteolysis of a 35S-labeled in vitro NS4B-5A translation product by an unlabeled, mature NS3/4A protease results in NS4B and NS5A. B, SDS-PAGE of products of an in vitro cleavage reaction containing 35S-labeled translation reactions programmed with RNA encoding the NS4B-5A segment of HCV-N, to which unlabeled mature NS3/4A protease, both WT and related SCH6 primary resistance and second-site mutations, were added. Cleavage reactions were terminated at the times shown. See text for other details. C, PhosphorImager quantitation of the percent of the labeled NS4B-5A translation products shown in B that have undergone scission. See B for key to symbols.

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Development of drug resistance is a major cause for failure of antiviral therapy. Recent studies on HCV protease inhibitors suggest that variants resistant to drug treatment are readily generated by using subgenomic replicon systems (10, 12, 13). In this study, resistance mutations in the HCV protease domain were generated by culturing replicon cells in the presence of a novel protease inhibitor, SCH6, with continued G418 selection. Consistent with previous reports studying other NS3/4A inhibitors, we found that an A156T mutation conferred high levels of resistance to SCH6 (>14–18-fold) (Table 3). However, two novel mutations were also identified, I153V and R109K, that have not been reported previously in studies of NS3/4A inhibitor resistance. These were found to confer very low to moderate levels of resistance to SCH6 (<4-fold). We found a remarkable consistency in the resistance mutations selected in studies utilizing replicon RNAs derived from two quite distinct genotype 1b viruses, Con-1 and HCV-N. Certain combinations of the mutations (R109K/A156T and R109K/I153V) were also identified after selection with SCH6. Additional studies demonstrated that the double mutant A156T/R109K that substantially increased resistance to SCH6, reflecting the sum of the impact of each mutation on binding of the substrate and resulting in a multiplicative loss in the activity of SCH6.

Each of the three amino acid substitutions we identified required only a single nucleotide mutation. The base-line prevalence of these resistance mutations in natural HCV isolates may be important in predicting treatment outcome in the clinic. Ala-156 and Arg-109 are 100% conserved in ~250 HCV isolates analyzed, whereas significant polymorphism occurs at residue 153, with Ile-153, Leu-153, and Val-153 found in ~74, 17, and 9% of the sequences in the data base, respectively. These data may underestimate the true extent of amino acid variation at each of these residues, as minor quasi-species are likely to be underrepresented in the data base. Although the degree of SCH6 resistance conferred by I153V is low, and clearly demonstrated only in the cell-free protease assay (Table 3), the natural variation at this position raises the issue of potential differences in treatment responses based on patient isolates.

Although speculative, the cross-resistance studies we carried out using different inhibitor compounds suggest that SCH6 could be advantageous over two previously reported candidate HCV protease inhibitors, BILN 2061 and VX-950, in terms of the emergence of inhibitor resistance (Table 4). Mutation A156T drastically decreased binding of BILN 2061 and VX-950, raising the binding constants to the micromolar range (0.9 and 13 μM, respectively), whereas SCH6 remained relatively active (K_i = 0.13 μM). SCH6 was also not affected by a D168V substitution, which confers high level resistance to BILN 2061 (13). Independent of the pharmacodynamic properties of these inhibitors, such differences in the resistance profiles conferred by these mutations could be important considerations in designing future therapeutic regimens. More importantly, our results indicate that higher concentrations of SCH6, which result in more complete suppression of viral replication, significantly decrease the frequency of emergent colonies. Therefore, therapies designed to maximally suppress viral replication immediately should help to minimize emergence of resistant populations.

As has been shown in resistance studies of HIV protease inhibitors (34), most of the amino acid residues involved in HCV protease resistance are highly conserved, suggesting that the substitution of these residues may adversely affect protease activity and reduce viral replicative capacity. Indeed, A156T significantly reduced replicon fitness in the CFE assay (6% of wild-type), although R109K by itself had no effect on CFE of the Con1 replicon RNA (Fig. 1). These results are consistent with the results of transient replication assays using the HCV-N replicons, which demonstrated a substantial reduction in the fitness of the A156T mutant but little loss of replication fitness with R109K (Fig. 2). Most interestingly, the double mutant R109K/A156T demonstrated a greater loss of fitness than the single A156T mutant, as measured in the colony formation assay (0.7% of wild-type) (Fig. 1). Consistent with the loss of fitness imparted by the single A156T mutant, we also documented a decrease in protease enzymatic activity in vitro (Table 3 and Figs. 4 and 5) and in mutant replicon cells as evidenced by the accumulation of a polyprotein processing intermediate (Fig. 6). A similar correlation between fitness and enzymatic activity has been reported for HIV protease inhibitors. Certain mutations in the HCV protease domain have been shown to confer high levels of resistance with concomitant decrease in enzyme activity and viral fitness (35–37). Processing intermediates were also observed during infection by mutant viruses in those studies.

Modeling of the mutations into co-crystal structures of the NS3 protease domain with SCH6, BILN 2061, and VX-950 offers a logical rationale for the observed cross-resistance or lack thereof. Because of the anti-parallel binding of the three inhibitors, all make close contact with residue 156. The substitution of Ala-156 with residues carrying a larger side chain such as threonine would sterically interfere with inhibitor binding in all cases. Ile-153, although part of the P1-P3 pocket, interacts to varying extents with the three inhibitors. The mutation to valine may weaken enzyme interaction with SCH6, but not with BILN 2061 or VX-950, if less of their binding is dependent on this interaction. A novel feature of SCH6 is that it extends substantially toward the P'-side of the active site (Fig. 7, right panel). The phenylglycine at P’2 appears to interact with residue Arg-109. This interaction would be lost by mutation to lysine, consistent with the resistance of R109K mutants to SCH6 that we identified in this study. This feature is

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*a* P. Qiu, personal communication.

*a* A. Prongay et al., manuscript in preparation.
critical to understanding the differences in resistance profiles of the various inhibitors studied in this report. SCH503034, BILN 2061, and VX-950 do not interact with this residue and are therefore not affected by the R109K mutation. Although the activities of all three compounds are severely impacted by the A156T mutation on the P-side of the active site, SCH6 alone derives a significant part of its binding energy from contacts on both P- and P’-sides. Mutation of Ala-156 (P-side) or Arg-109 (P’-side) weakens but does not abolish SCH6 binding. The A156T/R109K double mutation severely impacts SCH6 activity but requires two mutational events and results in extremely poor replication fitness.

The partial rescue of the mutant A156T replicon by Q86R, P89L, or G162R (Table 3 and Figs. 2, 4, and 5) suggests that, as seen with highly resistant HIV strains, compensatory second-site mutations may arise in HCV mutants that are resistant to protease inhibitors. The fact that Q86R has been observed in replicons without selection suggests that it may offer a growth advantage to replicons in general. However, in addition to enhancing replication fitness, Q86R, like the other compensatory mutations we identified, partially rescues the processing defect that is evident in the A156T mutant. The P89L and G162R mutations both improved cis-autoproteolysis at the NS3/4A junction (Fig. 4), while P89L also improved the trans-processing activity of the A156T mutant significantly (Fig. 5). In addition, in the presence of the Q86R mutation, the aberrant p110 processing intermediate observed in the A156T mutant Con1 replicon cells was reduced in abundance, compared with replicon cells with A156T alone or A156T/R109K (Fig. 6). The lack of an impact of these mutations in the biochemical assay (Table 3) may simply reflect the nature of the substrate, a peptide ester binding almost exclusively to the P-side of the active site. The in vitro translation/proteolysis studies we carried out are more likely to reflect accurately the impact of the mutations on proteolysis, given the much larger size of the substrates and the potential for interactions with the protease outside of the active site.

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