Virologic Escape during Danoprevir (ITMN-191/RG7227) Monotherapy Is Hepatitis C Virus Subtype Dependent and Associated with R155K Substitution

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Danoprevir is a hepatitis C virus (HCV) NS3/4A protease inhibitor that promotes multi-log10 reductions in HCV RNA when administered as a 14-day monotherapy to patients with genotype 1 chronic HCV. Of these patients, 14/37 experienced a continuous decline in HCV RNA, 13/37 a plateau, and 10/37 a rebound. The rebound and continuous-decline groups experienced similar median declines in HCV RNA through day 7, but their results diverged notably at day 14. Plateau group patients experienced a lesser, but sustained, median HCV RNA decline. Baseline danoprevir susceptibility was similar across response groups but was reduced significantly at day 14 in the rebound group. Viral rebound in genotype 1b was uncommon (found in 2/23 patients). Population-based sequence analysis of NS3 and NS4A identified treatment-emergent substitutions at four amino acid positions in the protease domain of NS3 (positions 71, 155, 168, and 170), but only two (155 and 168) were in close proximity to the danoprevir binding site and carried substitutions that impacted danoprevir potency. R155K was the predominant route to reduced danoprevir susceptibility and was observed in virus isolated from all 10 rebound, 2/13 plateau, and 1/14 continuous-decline patients. Virus in one rebound patient additionally carried partial R155Q and D168E substitutions. Treatment-emergent substitutions in plateau patients were less frequently observed and more variable. Single-rebound patients carried virus with R155Q, D168V, or D168T. Clonal sequence analysis and drug susceptibility testing indicated that only a single patient displayed multiple resistance pathways. These data indicate the ascendant importance of R155K for viral escape during danoprevir treatment and may have implications for the clinical use of this agent.

Hepatitis C virus (HCV) is a positive-strand RNA virus that infects approximately 170 million people worldwide (39). It is a major cause of chronic liver disease, cirrhosis, and primary hepatocellular carcinoma. Therapy for chronic HCV infection is based on weekly injections of pegylated alpha interferon (PEG-IFN-α) and twice-daily orally administered ribavirin (RBV). This standard of care (SOC) is associated with significant side effects and achieves a sustained virologic response (SVR) in approximately half of treated patients (5, 28). Thus, novel therapeutic modalities are clearly needed.

The addition of one or more direct-acting antiviral agents (DAA) to the SOC has become a favored strategy to improve the treatment outcome of chronic HCV therapy. The two most clinically advanced DAAs, telaprevir (Incivek) and boceprevir (Victrelis), are linear peptidomimetic inhibitors of NS3/4A protease. These two NS3/4A protease inhibitors (PIs) have demonstrated significant improvements in SVR rates in genotype 1 patients when added to SOC (1, 14, 32, 46) and have recently been licensed for use in the treatment of chronic HCV infection in the United States and in Europe. While clearly a dramatic advance in the treatment of chronic HCV, both of these compounds are administered on a rigid three-times-daily (q8h) schedule and introduce significant side effects to the already poor tolerability profile of SOC (2, 5, 28).

PIs in late-stage clinical development may allow more convenient dosing and provide more favorable side effect profiles than initial PIs. Many of these compounds are being developed as components of multiple DAA regimens without SOC as well as in SOC-containing regimens (3, 6, 8, 13, 25, 27, 40, 47). Danoprevir (ITMN-191/RG7227), a noncovalent macrocyclic PI, is one such compound (37). Fourteen-day danoprevir monotherapy resulted in robust antiviral responses in treatment-naïve patients and patients who failed prior pegylated interferon therapy (4). This compound is currently in development as a twice-daily, ritonavir-boosted agent, where it has demonstrated a favorable side effect profile when administered with SOC for 12 weeks (7, 9, 43).

The high replication rate of HCV and the poor fidelity of the HCV RNA-dependent RNA polymerase result in the selection of drug-resistant HCV variants during treatment (20). In vitro and in vivo studies indicate that overlapping but distinct sets of NS3/4A variants are associated with reduced susceptibility to PIs (18, 23, 30, 38, 44, 49). In genotype 1a, resistance against both linear and macrocyclic PIs is principally achieved by substitution at position R155. In genotype 1b, resistance profiles for linear and macrocyclic inhibitors appear to be somewhat distinct. Resistance to linear PIs appears to depend on NS3 protease substitutions at A156 in genotype 1b (15, 35, 41, 42, 45), whereas resistance to macrocyclic
inhibitors in this genotype is typically associated with NS5 protease D168 substitutions (17, 21, 22, 26, 33, 34, 36). Importantly, however, the same substitution can have different effects on the potency of two PI s within the same class. For example, D168V and D168A reduce danoprevir potency less than 15-fold, whereas the same substitutions promote 140- and 63-fold changes, respectively, in susceptibility to the macrocyclic inhibitor BILN-2061 (10). Such differential effects on drug potency may impact resistance pathways and virologic outcome in vivo (10).

Here, virologic response patterns during danoprevir monotherapy are correlated with treatment-emergent amino acid substitutions in NS5 protease and the effect of these substitutions on danoprevir susceptibility is determined.

MATERIALS AND METHODS

Clinical trial. Patients were treated with danoprevir (ITMN-191/\(\text{RG7227}\)) monotherapy for 14 days (4). Patients received danoprevir at 100 mg twice daily (q12h), 100 mg q8h, 200 mg q12h, 200 mg q8h, or 300 mg q12h. The study was conducted in full accordance with the 1996 Declaration of Helsinki. The study protocol was reviewed and approved by the independent committee at each participating research facility, and written informed consent was obtained from each patient or legal guardian prior to study screening.

Classification of virologic response patterns. Virologic response patterns (rebound, plateau, and continuous decline) were determined in danoprevir-treated patients by comparison of end-of-treatment (EOT) viral load to nadir viral load using the following definitions. Rebound was defined as a \(\geq 1.0\) log\(_{10}\) IU ml\(^{-1}\) increase from nadir. Plateau was defined as a \(<1.0\) log\(_{10}\) IU ml\(^{-1}\) increase from nadir. All other virologic response profiles were classified as continuous decline.

Amplification of HCV NS3 protease from patient plasma. HCV viral RNA was extracted from patient plasma taken at screening or on day 1 (baseline), day 3, day 7, or day 14 (EOT). Viral RNA was extracted from patient plasma using the QiAamp Virus BioRobot 9604 kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was by random hexamer primers using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). HCV NS5 protease domain was amplified by nested PCR in triplicate. The first-round primer mix consisted of two forward primers, 5'-ATGGAGACCAAGATCATCACCTGGG-3' and 5'-ATGGA GACCAAGCTCATCACGTGGG-3', and two reverse primers, 5'-CGGT CGGCAAGGAACTTGCCATAGGTGGA-3' and 5'-CGGCAAGGAACTTGCC-3'. The study was conducted in full accordance with the 1996 Declaration of Helsinki. The study protocol was reviewed and approved by the independent committee at each participating research facility, and written informed consent was obtained from each patient or legal guardian prior to study screening.

Cloning of HCV NS3 protease into a reporter plasmid. Amplified NS3 protease was purified by the QiAquick gel extraction kit (Qiagen, Valencia, CA), digested with SwaI and NotI (New England BioLabs, Ipswich, MA), and cloned into a modified pCDNA3.1-based (Invitrogen, Carlsbad, CA) reporter vector that enabled both sequencing and phenotypic analysis. Following cloning, plasmids were isolated with a QIAwash 96 Ultra BioRobot kit (Qiagen, Valencia, CA) on a Hamilton STAR-12 robotic system platform (Hamilton Robotics, Reno, NV). For population-based analysis, a minimum of 88 individual clones from each patient time point were pooled prior to plasmid isolation.

Sequencing of HCV NS3 protease domain. Population sequencing was performed on an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA) using subtype-specific primers from the first round of nested PCR as appropriate based on results of a Trugene HCV genotype 5' NC assay (Siemens Healthcare Diagnostics Inc., Tarrytown, NY). Clonal sequencing was performed on an Applied Biosystems 3730 or 3700 DNA analyzer using a 'T7 primer or the subtype-specific reverse primers used in the second round of PCR amplification (see above).

Amplification, sequencing, and analysis of HCV NS3 helicase and NS4A. HCV viral RNA extraction from patient plasma, population sequencing, and analysis of the NS3 helicase domain and NS4A, including assignment of mixed bases, were performed by DDL Diagnostic Laboratory (Voorburg, Netherlands). Patient plasma samples were taken at day 1 (baseline) and day 14 (EOT).

Sequence alignment and analysis. The raw sequence files were imported into Sequencing Analysis Software v5.1.3 (Applied Biosystems, Foster City, CA). Low-quality sequences (i.e., incomplete coverage, poor signal, etc.) were excluded from further analysis. For clonal sequencing, sequences that showed mixed nucleotide bases were also excluded from further analysis.

For population sequencing, variable base substitutions at specific sequence positions were called when the minority peak(s) were at least 10% of the highest base peak. The amino acid and nucleic acid sequences were aligned using the MegaAlign suite of Lasergene 7 (DNASTAR, Inc., Madison, WI). Clonal sequence analysis was performed using Pipeline Pilot (Accelrys, San Diego, CA) and Lasergene 7. Empty vectors, nonsense mutations, or frameshift mutations were excluded from further analysis. An average of 69 clonal sequences per sample (median of 74 clones, range of 28 to 88 clones) were accepted and used for sequence analysis.

Definition of treatment-emergent amino acid substitutions. Amino acid positions were considered to display treatment-emergent substitution if they met the following two conditions: (i) a change in amino acid identity at EOT from the corresponding patient’s baseline was observed, and (ii) a position was substituted in two or more patients irrespective of the identity of the substituted amino acid. Treatment-emergent substitutions in NS3 protease were determined from available patients comprising rebound, plateau, and continuous-decline groups (10/10 rebound, 11/12 plateau, and 4/13 continuous-decline patients). Treatment-emergent substitutions in NS3 helicase and NS4A were determined from available patients comprising rebound and plateau groups (7/10 rebound and 11/13 plateau patients).

Secreted luciferase phenotyping assay. A cell-based NS3 protease phenotyping assay was used to determine the susceptibility of patient-derived NS3 protease to danoprevir (12). The phenotyping vector contained a cytomegalovirus (CMV) promoter, NS3 protease and helicase domains, NS4A, NS4B, and NS5A, the first six amino acids of NS5B, and a reference sequence (24) and unique restriction enzyme cleavage sites (SwaI and NotI) were incorporated for insertion of patient-derived NS3 protease sequences.

The assay was carried out on a Hamilton STAR-12 robotic system. FreeStyle 293-F cells (Invitrogen, Carlsbad, CA) were transfected with reporter vector using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN), plated in FreeStyle 293 Expression medium, and treated in duplicate with 10 or 11 concentrations of serially diluted danoprevir. After 24 h at 37°C, secreted luciferase substrate (Ready-To-Glow secreted luciferase system; Clontech, Mountain View, CA) was added and the mixture was allowed to incubate for 30 to 60 min prior to determination of luciferase activity on a VictorLight luminometer (Perkin-Elmer, Inc., Waltham, MA).

Duplicate determinations of luciferase activity versus danoprevir concentration were plotted on a semilog plot and fit to a four-parameter logistic function to determine a single 50% effective concentration (EC\(_{50}\)) (XLFit; IDBS, Guildford, United Kingdom). The mean EC\(_{50}\) for each clone was typically determined from at least three EC\(_{50}\)s. EC\(_{50}\) of >300
nM could not be reliably determined and were assigned a value of >300 nM (or 300 nM for inclusion in mean values).

HCV replicon. The HCV subgenomic replicon pFK I341 PI-Luc/NS3-3’/ET (wild type) (genotype 1b) and a Huh-cured cell line were licensed (Reblikon, Schriesheim, Germany). PCR-based site-directed mutagenesis was used to generate pFK I341 PI-Luc/NS3-3’/ET protease variants. Replicon RNA was synthesized using the MEGAscript T7 high-yield transcription kit, purified using the MEGAClear kit (Ambion, Austin, TX), and electroporated into Huh-7 cells with a Gene Pulser Xcell (Bio-Rad, Hercules, CA). Electroporated cells were plated and treated with 11 concentrations of serially diluted danoprevir and incubated for approximately 3 days. Following the removal of cell culture medium, luciferase signal was measured using the Bright-Glo luciferase assay (Promega, Madison, WI). Data from at least three independent experiments were plotted on a semilog plot and fitted to a four-parameter logistic function to determine EC_{50} (XLFit; IDBS, Guildford, United Kingdom). The relative replication capacity of replicon variants was determined by the luciferase measurements taken at approximately 4 h and 3 days posttransfection in untreated cells compared to the measurements taken from the wild-type replicon. The mean relative replication capacity for each variant was determined from at least three independent experiments.

RESULTS

Virologic response and danoprevir susceptibility during 14-day monotherapy. Patients treated with danoprevir monotherapy for 14 days were classified as experiencing a continuous decline, plateau, or rebound in HCV RNA irrespective of dosing cohort. Of 37 treatment-naive (TN) and nonresponder (NR) patients treated with danoprevir and available for analysis, 10/37 (27%) experienced a virologic rebound, 13/37 (35%) experienced a virologic plateau, and 14/37 (38%) experienced a virologic continuous decline. Median virologic response profiles for rebound, plateau, and continuous-decline patients were clearly distinct (Fig. 1A). Median profiles for rebound and continuous-decline groups were coincident through day 7 and diverged by 2.8 log_{10} IU ml^{-1} at end of treatment (EOT) (day 14). Plateau patients displayed a slower median first-phase decline in HCV RNA and reached virologic plateau by day 3.

A cell-based assay was used to quantify danoprevir susceptibility of patient-derived NS3 protease (12). Individual patient-derived NS3 protease sequences were cloned into a reporter plasmid, such that following transfection into mammalian cells, NS3/4A protease cleavage liberated secreted luciferase. An average of 69 (range, 28 to 88) individual sequences was expressed as a pool from each patient at each time point, and danoprevir susceptibility of the mixed population was determined. At day 1 (baseline), median danoprevir potency rates against rebound, plateau, and continuous-decline groups were similar (median EC_{50} = 3.1, 3.6, and 5.1 nM, respectively) (Fig. 1B). Hence, a population-based analysis did not suggest that susceptibility to danoprevir at baseline determined virologic fate.

The levels of susceptibility to danoprevir during treatment were largely similar in the three response groups until EOT, except in one rebound patient with reduced NS3 protease susceptibility to danoprevir at days 3 and 7 (Fig. 1B, arrows). In the rebound group, median susceptibility to danoprevir at EOT was reduced approximately 24-fold relative to baseline susceptibility. No significant differences in median danoprevir susceptibility were noted between baseline and EOT in plateau and continuous-decline groups. However, three plateau patients and one continuous-decline patient showed reduced NS3 protease susceptibility to danoprevir at EOT (Fig. 1B, daggers and double daggers, respectively). The reduced median susceptibility to danoprevir observed in the rebound group at day 14, but not at earlier time points, correlated with the median rebound in HCV RNA evidenced at day 14 in the rebound group.

Virologic response as a function of HCV genotype. Different HCV genotypes have differing nucleotide compositions in the regions encoding NS3 and NS4A that may contribute to different genetic barriers to drug-resistant viral variants. Therefore, the HCV genotype of patients comprising rebound, plateau, and continuous-decline groups was examined (Table 1). Genotype 1b was more prevalent than genotype 1a across all 37 danoprevir-treated patients in the study (23/37 [62%] versus 14/37 [8%], respectively). However, 8/10 rebound patients carried genotype 1a, compared to 2/10 who harbored genotype 1b (80% versus 20%, respectively). Thus, viral rebound was less frequent in genotype 1b than in genotype 1a patients despite the higher overall proportion of genotype 1b patients in the study.

![FIG 1 Plasma HCV RNA concentration and danoprevir susceptibility during monotherapy. (A) Median HCV RNA as a function of time in rebound, plateau, and continuous-decline groups. (B) Danoprevir susceptibility as a function of time in individual rebound, plateau, and continuous-decline patients as indicated. Potency determined in a cell-based assay by at least three independent experiments (12) against a pool of sequences representing the entire quasispecies population in the patient at that time. Median indicated by horizontal bar. Arrows emphasize single rebound patient with compromised danoprevir susceptibility at days 3 and 7. Daggers and double daggers indicate plateau and continuous-decline patients, respectively, with reduced danoprevir susceptibility at day 14 (EOT).](http://aac.asm.org/)

TABLE 1 Frequency of HCV genotypes 1a and 1b by virologic response group

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. in response group/no. in genotype</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rebound (n = 10)</td>
</tr>
<tr>
<td>1a (n = 14)</td>
<td>8/14</td>
</tr>
<tr>
<td>1b (n = 23)</td>
<td>2/23</td>
</tr>
</tbody>
</table>
TABLE 2 Prominent treatment-emergent NS3 substitutions in individual patients identified through population-based sequencing

<table>
<thead>
<tr>
<th>Virologic group</th>
<th>No. of patients with genotype</th>
<th>HCV 1a</th>
<th>HCV 1b</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rebound (n = 10)</td>
<td>8 1 1</td>
<td>R155K</td>
<td>V71I/V, R155R/K/Q, D168D/E, V170V/I</td>
<td></td>
</tr>
<tr>
<td>Plateau (n = 11)a</td>
<td>1 1</td>
<td>R155R/K</td>
<td>I/V71I, R155R/Kd</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 1</td>
<td>R155Q</td>
<td>D168D/V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 1</td>
<td>D168T</td>
<td>I/V71V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 1</td>
<td>V170V/T</td>
<td>WTc</td>
<td></td>
</tr>
<tr>
<td>Decline (n = 4)b</td>
<td>1 2</td>
<td>R155K, 1170I/V</td>
<td>WT</td>
<td></td>
</tr>
</tbody>
</table>

a Sequence information could not be obtained from 2 plateau patients at EOT.
b Sequence information could not be obtained from 10 continuous-decline patients at EOT.
c Clonal sequence analysis confirmed a mixed population of R, K, and Q at position 155.
d Clonal sequence analysis indicated a mixed population of R and K at position 155.

Prominent treatment-emergent amino acid substitutions at EOT. A population-based approach was used to identify prominent treatment-emergent amino acid substitutions associated with virologic response. Four amino acid positions in NS3 protease were identified as displaying treatment-emergent substitutions at EOT. Substitution at position 155 was most common (14 patients), followed by substitutions at positions 71 (3 patients), 168 (3 patients), and 170 (3 patients). No treatment-emergent substitutions were identified in the helicase domain of NS3 or in NS4A.

Rebound patients. Population-based sequence analysis indicated that the viral population of all 10 rebound patients displayed at least a partial replacement of arginine (R) with lysine (K) at NS3 amino acid position 155 (R155K) at EOT (Table 2). In the viral population of 9/10 rebound patients, including a patient harboring genotype 1b, R155K was the only treatment-emergent substitution observed. The second genotype 1b rebound patient also carried partial replacement with glutamine (R155R/K/Q) in the viral population. Additionally, the quasispecies population in this patient displayed partial substitution of glutamic acid (E) for aspartic acid (D) at position 168 (D168D/E), partial substitution of isoleucine (I) for valine (V) at position 71 (V71V/I), and partial substitution of I for V at position 170 (V170V/I).

Plateau patients. Population-based sequence information could be obtained for 11 of the 13 patients that experienced virologic plateau. Prominent treatment-emergent NS3 protease substitutions were observed in the viral population of seven of these patients, all of whom harbored genotype 1b (Table 2). In contrast to the rebound group, treatment-emergent substitutions in the plateau group were varied. Two patients carried viral populations with R155R/K, and in one of these patients a mixture of V and I at position 71 collapsed to I (V/I71I). Additionally, single patients were found to carry viral populations with R155Q, D168D/V, or D168T. The quasispecies population in these patients at EOT showed a reduced susceptibility to danoprevir (Fig. 1B, dagger). Finally, in the viral population of one patient, V/I at position 71 collapsed to V (V/I71V), and in a second patient, V170I/V was observed.

Continuous-decline patients. Sequence information at EOT could be obtained for only 4 of the 14 patients who experienced a continuous decline in HCV RNA (Table 2). Sequence changes in NS3 protease were detected in the viral population of a single patient. This patient harbored genotype 1a and the viral population carried what appeared to be a complete R155K substitution and development of a mixed viral population at position 170 (I710V/I). Notably, this patient experienced a 3.6 log10 IU ml⁻¹ reduction in HCV RNA at EOT, which was comparable to the median virologic response in this cohort (3.8 log10 IU ml⁻¹), with no evidence of virologic escape during danoprevir treatment (data not shown). However, the quasispecies population in this patient showed a reduced susceptibility to danoprevir at EOT (Fig. 1B, double dagger). The virologic continuous decline in this patient may reflect a higher danoprevir exposure, or this patient may have ultimately experienced a virologic failure with continuing mono-therapy.

Structural analysis. An X-ray crystallographic structure of danoprevir bound to NS3/4A (31) was used to investigate the potential mechanisms whereby treatment-emergent substitutions could impact danoprevir susceptibility. All four amino acid positions that displayed prominent treatment-emergent substitutions localized to the protease domain of NS3 (Fig. 2A). The amino acids at positions 155 and 168 (R and D, respectively) are highly conserved and through a bifurcated salt bridge interaction form a localized to the protease domain of NS3 (Fig. 2A). The amino acids at positions 155 and 168 (R and D, respectively) are highly conserved and through a bifurcated salt bridge interaction form a

FIG 2 Structural analysis of treatment-emergent substitutions. X-ray crystallographic structure of danoprevir (green) bound to genotype 1b NS3/4A (gray) at 2.6 Å resolution, with positions I71, R155, D168, and I170 indicated (yellow, orange, red, yellow). (A) Semitransparent surface representation showing relative positions of treatment-emergent substitutions. Note that R155 and D168 are solvent exposed whereas I71 and I170 are interior to the protein. (B) Interaction of R155, D168 with danoprevir, and I170. R155 and D168 engage in salt bridge interactions (dashed red lines) responsible for forming a plane on which the P2 group of danoprevir lies. I170 abuts R155 and forms hydrophobic interactions with its side chain on the face opposite that of danoprevir.
considerable portion of the P2 and P4 substrate binding region (Fig 2B). R155 and D168 undergo a conformational change upon danoprevir binding that enables the fluoro-isodindole P2 group of danoprevir to pack side-on with the side chain of R155 (31). Replacement of the guanidinium group at position 155 with an amino (R155K) or an amide (R155Q) is predicted to weaken interaction with the side chain carboxylate of D168. Similarly, lengthening the side chain length of D168 by a methyl group (D168E) or removal of the side chain carboxylate (D168T and D168V) would be expected to alter its interactions with the R155 side chain. Consequently, it is expected that substitution at either R155 or D168 would perturb the interaction of R155 and D168, which could profoundly impact danoprevir binding.

Position 170 is located on the opposite side of R155 from the P2 group of danoprevir (~7.7 Å away) and engages in hydrophobic packing interactions with the side chain of R155 (Fig 2B). No direct interaction between danoprevir and position 170 (I in the structure presented here) appeared likely. While the conservative change between I and V (single methyl group) was not predicted to significantly influence the position of R155, a small difference in R155 conformation in the context of V170 or I170 cannot be excluded. Examination of the Los Alamos National Laboratory HCV database indicated the amino acid identity at position 170 differed between genotype 1a (96% I, 4% V) and genotype 1b (35% I, 65% V). Position 71 is located in a small, surface-exposed hydrophobic cleft that is ~17 Å away from the P1’ of danoprevir (Fig 2A). Like that of position 170, the identity of the amino acid at position 71 differs between genotype 1a (100% V) and genotype 1b (32% V, 68% I). The structure described here contains an I at position 71. Thus, treatment-emergent substitutions were identified at two positions in direct contact with danoprevir (155 and 168) and at two more distal positions that have variable amino acid identity in genotype 1 (71 and 170).

Danoprevir susceptibility of NS3 protease carrying treatment-emergent substitutions. Treatment-emergent amino acid substitutions were introduced as site-directed mutations (SDMs) into con1b HCV replicon genetic background if Con-1 sequence differed from identified substitution.

### Table 3: Danoprevir potency against treatment-emergent NS3 substitutions

<table>
<thead>
<tr>
<th>NS3 protease variant</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; nM (mean ± SD)</th>
<th>Fold change&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R155K</td>
<td>45.1 ± 30.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>410</td>
</tr>
<tr>
<td>R155Q</td>
<td>6.3 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57</td>
</tr>
<tr>
<td>D168E</td>
<td>3.2 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29</td>
</tr>
<tr>
<td>D168V</td>
<td>10.4 ± 6.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>93</td>
</tr>
<tr>
<td>D168T</td>
<td>33.0 ± 16.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>300</td>
</tr>
<tr>
<td>I71V</td>
<td>0.06 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.57</td>
</tr>
<tr>
<td>V170I</td>
<td>0.12 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.05</td>
</tr>
<tr>
<td>Wild type</td>
<td>0.11 ± 0.05</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Introduced as site-directed mutations (SDMs) into con1b HCV replicon genetic background if Con-1 sequence differed from identified substitution.

<sup>b</sup> Fold change relative to parental Con-1 HCV replicon. N/A, not applicable.

<sup>d</sup> Relative replication capacity of variant replicon is greater than 50% of wild type.

<sup>e</sup> Relative replication capacity of R155Q variant is approximately 20% of wild type.
nearly equal V/I mixture at baseline and day 7 (Fig. 3B). R155K was observed only at day 14 and was exclusively associated with I at position 71. Danoprevir EC50 against the R155K/H11001 V/I71I class was 178 nM. This susceptibility is similar to that displayed by the R155K-bearing class isolated from the rebound patient described above who carried a V at position 71 (EC50 184 nM). Thus, the presence of an I or V at position 71 does not appear to impact danoprevir susceptibility when associated with R155K.

The final patient displaying a viral population with more than one treatment-emergent substitution at EOT by population-based analysis was a continuous-decline patient who carried R155K and I170I/V (Table 2) in the viral population. Clonal analysis indicated that the viral population of this patient carried R155K at a low abundance at baseline (1.3%) (Fig. 3C). The R155K-bearing quasispecies population at EOT was variably associated with I170V. However, association with I170V did not further reduce the potency of danoprevir (EC50s against NS3/4A carrying R155K and R155K/H11001 I170V were 336 nM and 239 nM, respectively). Thus, the presence of an I or V at position 170 does not appear to impact danoprevir susceptibility when associated with R155K.

DISCUSSION
Direct-acting antiviral agents are poised to revolutionize therapy for chronic HCV infection. The first such compounds, the NS3/4A protease inhibitors boceprevir and telaprevir, have recently been approved in the United States and Europe for the treatment of HCV genotype 1 chronic infection in combination with pegylated interferon and ribavirin. While both of these agents represent a tremendous advance in the treatment of HCV, both are administered on a strict q8h schedule, and both add significant toxicities to the current standard of care when used according to
patterns in genotype 1 patients treated with danoprevir mono-resistant viral variants. Clinical studies in order to minimize the development of drug-understanding of the reduced drug susceptibility of individual sequence dynamics in early-stage monotherapy studies and an understanding of the reduced drug susceptibility of individual variants are required for the optimal design of subsequent, larger clinical studies in order to minimize the development of drug-resistant viral variants.

In the current study, rebound, plateau, and continuous-decline response patterns were clearly evident by viral response patterns in genotype 1 patients treated with danoprevir monotherapy for 14 days. Continuous-decline and rebound patients had robust initial virologic responses which were similar until day 7, after which the HCV RNA concentration in the rebound group increased by \( \geq 1 \log_{10} \text{ IU ml}^{-1} \) but further decreased in the continuous-decline group. Plateau patients had comparatively lesser reductions in HCV RNA that were sustained to day 14. A cell-based assay examining danoprevir susceptibility of the quasi-species population from each patient indicated that reduced drug potency was primarily evident in the rebound group at day 14, in parallel to the rebound in viral kinetics evident at day 14. Thus, rebound of HCV RNA during danoprevir monotherapy is associated with a loss of drug susceptibility in the overall quasispecies population.

Population-based sequencing of the entire region encoding NS3 and NS4A identified prominent treatment-emergent substitutions at four amino acid positions in the viral populations of rebound and plateau groups. All of these positions localized to the NS3 protease domain (positions 71, 155, 168, 170). However, only two positions (155 and 168) are in direct contact with danoprevir. Observed substitutions at these two positions impact danoprevir potency when introduced into a heterologous replicon system. Among the identified variants that impact danoprevir potency, R155K represented the major variant associated with viral escape, as all patients who experienced a virologic rebound carried viral populations with R155K. Additionally, R155Q and D168E were present in the viral population of a single rebound patient. Treatment-emergent substitutions in the viral populations of plateau patients were less frequently observed and more heterogeneous in nature. R155K was observed in the viral populations of two plateau patients, and R155Q, D168V, and D168T were each found in the viral population of a single plateau patient. The relative lack of treatment-emergent substitutions in patients experiencing virologic plateau may reflect the lack of a strong pressure for drug-resistant variants in at least some plateau patients. The lesser virologic responses evidenced by plateau patients is consistent with this hypothesis, which has also been reported for patients experiencing less than a \( 2 \log_{10} \text{ IU ml}^{-1} \) decrease in HCV RNA when administered boceprevir (41, 42).

Interestingly, viral rebound was more common in genotype 1a patients than genotype 1b patients but when observed in genotype 1b was associated with R155K. Clonal analysis of baseline NS3 sequence found that HCV genotype 1b required 2 or more nucleotide changes to achieve R155K, whereas 99.8% of genotype 1a codons required only a single nucleotide substitution (data not shown). Substitution at position 168 was not a preferred route to viral rebound. Despite the identical genetic barrier to D168 substitution in genotypes 1a and 1b (1 nucleotide substitution; data not shown), treatment-emergent substitutions at position 168 were not frequently observed and when observed were restricted to genotype 1b patients. The lack of D168 variants in genotype 1a rebound patients and the limited number of genotype 1b patients who experienced a virologic rebound suggest that substitution at position 168 is not a robust route for viral rebound in either genotype during danoprevir treatment.

In addition to positions 155 and 168, prominent treatment-emergent substitutions were identified at positions 71 and 170. However, the observed substitutions were conservative in nature, were more distal to the danoprevir binding site, and varied between two amino acids commonly observed at these positions. Importantly, neither position converged to the same amino acid identity in multiple patients. At position 71, a mixture of V and I at baseline changed to an I in one patient (V/I71I) and to a V in another (V/I71V). In a third patient, V at baseline became a mixture of I and V at EOT (V/I71I). All three of these patients harbored HCV genotype 1b, which is known to carry either V or I at position 71, and danoprevir had equivalent potency against a genotype 1b HCV replicon carrying either a V or I at position 71. Similarly, V170I/V and I170I/V were observed as treatment-emergent substitutions. Danoprevir had equal potency against HCV replicon carrying either V or I at position 170, and patient-derived NS3 sequences carrying either D168E or R155K were equally susceptible to danoprevir whether V or I was present. Thus, while more significant substitution at position 170 impacts susceptibility to linear tetrapeptide inhibitors (17, 42), the current study does not suggest that danoprevir potency is impacted by the observed substitutions at position 71 or 170.

Interestingly, these studies did not identify several treatment-emergent substitutions associated with resistance to other PIs (e.g., positions 36, 54, and 156) (17, 42), which highlights the unique resistance profile of danoprevir in vivo can be rationalized by the in vitro finding that the relative replication capacity of different variants coupled with a compound’s potency against each variant predicts the preferred resistance pathways for individual compounds (10). Analysis of the differing replication capacities afforded by different NS3 substitutions and the overall level of replication supported under drug pressure has suggested that clinical resistance to danoprevir primarily would be associated with R155K (10). In contrast, the same model predicts that a larger variety of NS3/4A substitutions would be associated with clinical resistance to telaprevir and ciluprevir (10). Clinical studies confirm that several distinct substitutions in NS3/4A are associated with resistance to telaprevir (15, 35), whereas danoprevir resistance in the clinic is primarily associated with R155K (this work).

Further clinical experience with danoprevir highlights the
unique role of R155K in the development of resistance to this compound. When danoprevir is combined with either pegylated interferon and ribavirin or the NS5B inhibitor mericitabine, the emergence of viral variants with reduced danoprevir susceptibility is greatly reduced (7). However, when variants with reduced susceptibility are observed, they exclusively carry R155K (7). Substitution at position 168 was not reported in these studies (7). Thus, these studies suggest that the set of NS3 variants capable of promoting viral escape is compound specific and that certain PIs may allow viral escape only through a restricted number of NS3 substitutions. These findings may have implications for the clinical development of danoprevir and other direct-acting antiviral agents.

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