Characterization of Resistance to the Protease Inhibitor GS-9451 in Hepatitis C Virus-Infected Patients

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Running title: Resistance to the PI GS-9451 in HCV patients
Abbreviations


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Abstract

GS-9451, a novel NS3 protease inhibitor is highly active in HCV genotype 1 (GT 1) patients. The aim of this study is to characterize the clinical resistance profile of GS-9451 in GT 1 HCV infected patients in a phase 1, 3-day monotherapy study. The full-length NS3/4A gene was population sequenced at baseline, the final treatment day, and follow-up timepoints. NS3 protease domains from patients with emerging mutations were cloned into an NS3 shuttle vector and their susceptibilities to GS-9451 and other HCV inhibitors were determined using a transient replication assay.

No resistance mutations at NS3 positions 155, 156, or 168 were detected in any of the baseline samples or in patients treated with GS-9451 60 mg once daily. Among patients who received 200 mg and 400 mg of GS-9451, mutations at position D168 (D168E/G/V) and R155 (R155K) which confer high-level resistance to GS-9451 were detected in GT 1b and GT 1a patients, respectively. D168 mutations were no longer detected in any GT 1b patient at Day 14 and subsequent timepoints. In GT 1a patients, R155K was replaced by wild-type in 57% of patients at week 24. These NS3 clinical mutants were sensitive to NS5B and NS5A inhibitors, as well as IFN-α and ribavirin. Lack of cross resistance between GS-9451 and other classes of HCV inhibitors support the utility of combination therapy.
Introduction

Hepatitis C virus (HCV) infects an estimated 170 million people around the world (3, 20, 21). Infection can lead to cirrhosis and sometimes to hepatocellular carcinoma (1, 14). Prior to May 2011, when the two protease inhibitors (PIs) telaprevir and boceprevir were approved, treatment of chronic HCV infection included combination of pegylated (PEG) interferon (IFN) and ribavirin (RBV) (6, 14, 21). This treatment is associated with significant side effects such as fever, fatigue, anemia, leucopenia, thrombocytopenia, and depression (4, 15, 24) and sustained virologic response (SVR) rates in only 42% to 53% of patients with HCV genotypes 1 and 4, respectively, and up to 78% to 82% of patients infected with HCV genotypes 2 or 3 (6, 14).

Direct acting antivirals (DAAs), including the HCV nonstructural (NS) 3/4a serine protease inhibitors (NS3 PIs) have demonstrated antiviral activity in HCV infected patients (7)(9). Among NS3 PIs, telaprevir and boceprevir have recently been approved for genotype 1 infections. There are more than 9 other NS3 PIs in different stages of clinical development (TMC-435, danoprevir, vaniprevir, BI201335, narlaprevir, MK-5172, asunaprevir, BMS-791325, ABT-450, ACH-1625, GS-9451, and GS-9256). The approved NS3 PIs have demonstrated increased SVR rates in patients, when combined with the PEG + RBV. During the Phase 2b PROVE2 study, genotype 1 (GT 1) infected individuals treated with 12 weeks of telaprevir + PEG + RBV followed by 12 additional weeks of PEG + RBV had SVR rates of 60% compared to 46% in the SOC alone arm (7).

Similarly, the boceprevir SPRINT-1 trial reported a 75% SVR rate for patients treated...
with a 4 week PEG + RBV lead in followed by boceprevir + PEG + RBV for 44 weeks compared to 38% SVR in the PEG + RBV alone arm (9). Thus, proof-of-concept for the addtion of an HCV NS3 protease inhibitor to PEG + RBV for GT 1 HCV infected patients has been established. However, because of the short half-lives of telaprevir and boceprevir, these agents require frequent dosing (every 8 hours) with a large number of pills (6 and 12 per day, respectively) which may adversely impact adherence. Telaprevir and boceprevir have been associated with adverse events such as rash, pruritus, anemia, and dysgeusia. Furthermore, these PIs have also been found to select for viral resistance during monotherapy or combination studies in chronic HCV patients. Telaprevir selected multiple NS3 mutations in the clinic including V36A/M, T54A, R155K/T, and A156S/V/T (19). Boceprevir selected NS3 mutations of T54A and V170A during Phase 1 studies (28, 29). Viral variants with amino acids changes at one or more of the amino acids positions 80, 155, and/or 158 of NS3 were detected in each of the patients treated with the macrocyclic protease inhibitor, TMC435 (18). Furthermore, substitutions at NS3 positions 155 and 158 have been reported to be related to viral rebound in a 14-day multiple ascending dose trial of the HCV protease inhibitor ITMN-191 (danoprevir) (5).

GS-9451 (Figure 1) is a novel, reversible, non-covalent inhibitor of the HCV NS3 serine protease with an EC50 of 7-10 nM and CC50 of > 50,000 nM in replicon cell assays (2, 25). In biochemical assays, GS-9451 has a Ki of 0.41 nM against GT 1 NS3 protease. In clinical studies, GS-9451 was well-tolerated (10). In addition, a QD dosing of GS-9451 has shown highly effective antiviral activity in GT 1-infected patients in monotherapy.
(10) and is currently being evaluated in combination with other DAAs and RBV with or without PEG-IFN.

This study characterizes the resistance profile of GS-9451 in patients treated with multiple ascending doses of GS-9451 for three days.
Materials and Methods

Compounds

Interferon-αA human and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) were purchased from Sigma Aldrich (St. Louis, MO). Telaprevir and boceprevir were purchased from Acme Biosciences (Belmont, CA). All other compounds (GS-9451, GS-9256, GS-6620, GS-5885, GS-9190, GS-9669, danoprevir, and TMC-435) were synthesized by Gilead Sciences (Foster City, CA).

Patient Population and Study Design

Forty patients were enrolled into a randomized, double-blind, placebo controlled multiple ascending doses study designed to investigate the safety, tolerability, pharmacokinetics and antiviral activity of GS-9451 in 4 cohorts of HCV-infected patients: 3 cohorts with HCV genotype 1a and 1 cohort with genotype 1b. In all cohorts, oral tablets of GS-9451 or matching placebo were administered once daily on Days 1-3. Among these patients, GS-9451 was administered at 60 mg QD (n=8), 200 mg QD (n=9), and 400 mg QD (n=8) in GT 1a patients and at 200 mg QD (n=7) in GT 1b patients. All patients received a capsule formulation or matching placebo (n=8). Blood samples for determining plasma HCV RNA levels were collected prior to study drug dosing on Days 1, 2, and 3. Additional samples were collected at 12 (Day 3), 24 (Day 4), 48 (Day 5), and 96 (Day 7) hours after the last dose of study drug. Plasma HCV RNA was analyzed by real-time polymerase chain reaction (RT-PCR) using the COBAS TaqMan® RT-PCR HCV v2.0
with the High Pure System (Roche Molecular Systems, Inc., Branchburg, NJ; quantitation range, 25 IU/mL to $300 \times 10^6$ IU/mL).

All patients had a chronic infection with subtype 1a or 1b HCV with plasma HCV RNA levels of $\geq 10^5$ IU/mL, and no evidence of co-infection with hepatitis B virus (HBV), delta hepatitis virus (HDV), or human immunodeficiency virus (HIV). Written informed consent was obtained from each patient in accordance with the Declaration of Helsinki.

For NS3/4A sequencing and NS3 protease phenotypic analyses, plasma samples were collected and subsequently stored at –80º C from all patients before dosing on Day 1 (baseline), Day 4, and Day 14. Plasma samples were collected for those patients who returned for follow-up evaluation visits at Weeks 12 and 24.

**Amplification and Sequencing of the HCV NS3/4A**

Full-length HCV NS3/4A was amplified by RT-PCR and population sequenced using di-deoxy sequencing-based technology on the Applied Biosystems 3100 platform (Applied Biosystems, Foster City, CA). The QIAamp Viral RNA Mini Kit (Qiagen Inc, Valencia, CA) was used to isolate HCV RNA. Genotype specific primers (1a: 1a4a3’5735-5’ TTG GCT AGT GGT TAG TGG GCT GG-3’ or 1b:1b4a3’5650-5’ GTG GAC AAG CCT GCT AAG TAC TGT ATC CCG C-3’).

GCT AGT GGT TAG TGG GCT GG-3’ or 1b:1b4a3’5650-5’ GTG GAC AAG CCT

GCT AAG TAC TGT ATC CCG C-3’ were used to synthesize cDNAs. Reverse transcription using SuperScript III kit (Invitrogen, NY, USA) was run on a MJ Research PTC-100 thermal cycler (Bio-Rad Laboratories, Hercules, CA). A nested PCR strategy
was used with genotype-specific primers to amplify the NS3/4A gene which was subsequently used as a template for sequencing. GT 1a NS3/4A was amplified using primers 1a: 5'-ATC AAG TTA GGG GCG CTT ACT GGC AC-3' & 3'-5'TTG GCT AGT GGT TAG TGG GCT GG-3' for the first round PCR, and 1a: 5'-TTG GCT AGT GGT TAG TGG GCT GG-3' & 3'-5'ATG GAG ACC AAG CTC ATC ACG TG-3' & 1a4a-3'-CTG GTG ACA GCA GCT GTA AAA GCC ATC-3' for nested PCR. GT 1b NS3/4A was amplified using primers 1b: 5'-GTC GCT GGG GGT CAT TAT GTC CAA ATG G-3' & 3'-5'GTG GAC AAG CCT GCT AAG TAC TGT ATC CCG C-3' for the first round PCR, followed by primers 1b: 5'-GAG CCC GTC GTC TTC TCT GAC ATG G-3' & 3'-5'GTG TTC TGC AGC AAC CCG AGC GCC TTC TG-3' for nested PCR. PCR reaction parameters for both rounds of PCR were as follows: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds (s), 1a: 60°C for 30 s or 1b: 65°C for 30 s, 72°C for 3 minutes, 72°C for 7 minutes. An aliquot of each PCR product was run on an agarose gel to confirm amplification of target amplicons and the remainder of each reaction was purified using a QIAquick PCR Purification Kit.

Viral population sequencing was performed on plasma samples for all patients at baseline, Day 4, Day 14 and for those patients who returned for follow-up evaluation visits at Week 12 and Week 24. Sequence was not available due to sequencing failure from two subjects on Day 14 (one dosed with 60 mg and the other dosed with 400 mg), and 9 subjects on Day 4 (4 dosed with 200 mg and 5 dosed with 400 mg).
Sequence Alignment and Data Analysis

Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI) was used to assemble and analyze nucleotide sequences, and to translate into amino acid sequences. Full-length NS3/4A sequences were aligned against respective subtype references, 1a: H77-AF009606 or 1b: Con1-AJ238799, to identify differences between patient and reference amino acid sequences. Analyses of the emerging amino acid changes at Day 4 and Day 14 compared to Day 1 (baseline) were conducted and reported. In addition, amino acids substitutions were cross-referenced against an amino acid frequency database of 397 GT 1a and 541 1b HCV NS3 protease gene sequences obtained from the EU databases. Amino acid sequence analyses were performed for AA 1–631 of the NS3 gene and AA 1–54 of the NS4A gene for both GT 1a and GT 1b.

Generation of Chimeric Replicons Carrying the NS3 Protease Gene from Patient Isolates

HCV GT 1b-PI-luc, a bi-cistronic replicon and cured Huh7 cells (Huh-Lunet) were obtained from ReBLikon (Mainz, Germany) (12). To clone the NS3 protease gene from patient isolates, two unique restriction sites (ClaI and Ascl) were created in the 1b-PI-luc construct (17). PCR products generated for NS3 sequencing were used as template to generate gene cassettes encoding cloning sites at both ends. For GT 1b samples, the protease gene was amplified using the forward primer 1b PCR NS3-4A F2 (5’-ATTAGTCAATCGATCCATGGCGCCYATCACGGCCTACTCCCAACAGACGCG-3’) and the reverse primer 1b PCR Prot R2 (E1202G) (5’ATATGCTCAGGCGCGCCGTTGTCYGTGAAG-3’).
ACCGGRGACCGCATRGTRGTTCCCAT-3'). For GT 1a samples, the forward primer

1a PCR NS3-4A F2 (5'-ATTAGTCAATCGATACCATGGCGCCCATCACGGCGT

ACGCCCGACGAGAC-3') and the reverse primer 1a PCR Prot R2 (E1202G) (5’-ATA

tgctcaggccgcgttggtccgtgaacaccgggacccatggttgccct

agg-3’) were used. All nested PCR reactions were performed with a High Fidelity PCR

master kit (Roche Applied Science, Indianapolis, IN) as directed by the manufacturer.

Final PCR products were purified and digested with ClaI and Ascl. Shuttle vector DNA

was similarly digested and then ligated using DNA ligation kit (Takara Bio Inc, Madison,

WI), followed by transformation into E. coli by electroporation using XL-Gold

ultracompetent cells (Agilent Technologies, Santa Clara, CA). Ten percent of each

transformation mixture was plated on antibiotic selection plates to determine

transformation efficiency and the remaining 90% of each transformation mixture was

expanded in liquid culture to propagate pools of NS3 quasispecies. Plasmid DNA was

prepared for RNA transcription first by linearization with Scal and then RNA synthesized

using a T7 Megascript RNA synthesis kit (Ambion, Austin, TX).

**Transient Transfection of Replicon RNA into Huh7 Cells and EC₅₀ Determination**

Replicon RNA was transfected into Huh7-lunet cells following the method of R.

Bartenschlager (12). Briefly, cells were trypsinized and washed twice with PBS. A

suspension of 4 × 10⁶ cells in 400 μL of PBS was mixed with 1-5 μg of replicon RNA

and subjected to electroporation using settings of 960 μF and 270 V. Cells were quickly

transferred into 25 mL of culture medium and seeded into 96-well plates at 100 μL/well

and allowed to attach overnight. For EC₅₀ determinations, compounds were serially
diluted in 100% DMSO and then added to the cells at a 1:200 dilution, achieving final concentrations of 0.5% DMSO in total volumes of 200 μL per well. Cells were cultured for three days at 37°C, after which culture media were removed and Renilla luciferase activity was measured using the Renilla Luciferase assay system (Promega, Madison, WI) with a Victor Luminometer (PerkinElmer, Waltham, MA).

**Data Analysis**

EC₅₀ values were calculated as the compound concentration at which a 50% reduction in the level of Renila reporter activity was observed when compared with control samples with DMSO. Dose response curves and EC₅₀ values were generated using GraphPad Prism software package (GraphPad Software, La Jolla, CA) by nonlinear regression analysis. The replication level of either reference strains (1b-Con1 or 1a-H77) or chimera replicons derived transiently from clinical isolates was determined as the ratio of the Renila luciferase signal at day 4 to that at 4 h post-electroporation, to normalize for transfection efficiency. The replication capacity of each chimera replicon derived from clinical isolates was expressed as their normalized replication efficiency when compared with that of the reference strain (1b-Con1 or 1a-H77) within the same experiment. For the relationship between resistance emergence and viral response, the maximal viral load reduction derived from each individual during treatment and emergent resistance were compared in a statistical analysis using a two-tailed unpaired t test at the 95% confidence interval.
Results

Antiviral Response to GS-9451

The samples analyzed in this phase 1 study were obtained from 25 GT 1a patients and 7 GT 1b patients who were dosed with GS-9451 for 3 days as well as 8 patients (7 GT 1a and 1 GT 1b) who received placebo for 3 days. The GS-9451 60 mg QD dose for 3 days resulted in a mean maximal HCV RNA reduction of -0.91 log_{10} IU/mL in HCV GT 1a patients (Table 1). Mean maximal reductions in HCV RNA were -3.16 log_{10} IU/mL for 200 mg QD GT 1a, -3.26 log_{10} IU/mL for 200 mg QD GT 1b and -3.77 log_{10} IU/mL for 400 mg QD GT 1a compared to -0.2 log_{10} IU/mL reduction for placebo patients. Comparison of the maximum viral reduction between the two subtypes at 200 mg QD dose showed that there was not a statistically significant difference (p = 0.7).

Population Sequencing Analysis

Population sequencing of HCV NS3 did not detect any of the previously identified HCV PI-resistance mutation at positions 155, 156 and 168 in any of the patients at baseline. Drug resistance mutations were also not detected in patients receiving placebo or 60 mg of GS-9451 for 3 days by population sequencing (Table 2). However, NS3 substitutions at residue R155 (R155K or R155K/R) were observed in 13/17 GT 1a patients who received multiple doses of GS-9451, 200 mg QD or 400 mg QD, at Day 4 or Day 14 or Week 12 (Table 2). By week 24, most of the R155K variant was replaced by wild-type (WT) and was detected in 43% (3/7) of patients who came for follow-up visits at Week
Similarly, NS3 substitutions at residue D168 (D168G, D168D/E or D168V) were observed at Day 4 in 4/7 GT 1b patients who received 200 mg doses of GS-9451 QD (Table 2). For these GT 1b patients, D168E/V/G were no longer detected by population sequencing in any patient at Day 14 or the Week 12 and Week 24 follow-up time points (Table 3).

Other Substitutions in the NS3 Protease Gene (AA 1-181)

In addition to the resistance mutations at amino acids 155, 156, and 168, there were 12 positions in the NS3 protease with amino acid changes at Day 4 and/or 14 (positions: 14, 18, 54, 72, 80, 86, 87, 91, 114, 147, 170 and 174). Seven of these twelve substitutions were observed with R155 or D168 mutants (Table 1, supplementary). Cross referencing the amino acid substitutions at these residues against an amino acid frequency database of 938 NS3 protease amino acid sequences of GT 1a and 1b collected from the EU databases revealed that these substitutions appear to be at highly polymorphic sites and may therefore be natural variation (>1%) of the WT HCV population. Substitutions at positions 80 and 147 that were observed as a mixtures of 2-4 amino acids (K/Q80I/K/L/Q and A/S147A/L/S/V) do not appear to be associated with GS-9451 selection and most of these amino acids are possible substitutions observed in WT HCV. One substitution at position 170 (I170T) was changed to an amino acid observed with <1% frequency in the databases (I170I/T, I=58%, T=0.1%, V=42%). This substitution was observed in only one patient.
Phenotypic Analysis

To determine if the sequence changes described above are associated with reduced susceptibility, phenotypic analyses were performed for samples from patients with amino acid substitutions detected at R155 or D168 by population sequencing. Given the sensitivity limitation of the phenotypic assay in detection of WT/mutant mixtures, patients with full mutants detected were selected for phenotypic analysis. The phenotypic analyses were also performed for the corresponding baseline samples for use as individual comparators. GS-9451 EC$_{50}$ values were obtained for six patients at both baseline and either Day 4 or Day 14. The results for GS-9451 susceptibility are summarized in Table 4. Samples from GT 1a patients with full R155K mutants (patients BE, BJ, CD and CF) had at least an increase in EC$_{50}$ of >595 fold to GS-9451 compared to baseline (Table 4b). As with replicon single mutants (Table 4a), all patient isolates had reduced replication capacities compared to the wild-type replicon. Both patient isolates and replicon R155K showed high levels of resistance to GS-9451.

Amino acid changes at position D168 were observed in four GT 1b patients. Patients with full D168V (patient DH) and D168G (DD) were analyzed for their susceptibility to GS-9451. The Day 4 samples from both patients DH and DD displayed >152-fold reduced susceptibility to GS-9451 as compared to their baseline samples (Table 4b). Interestingly, patient DH with full D168V displayed higher levels of resistance to GS-9451 compare to patient DD with full D168G, which correlates with the resistance levels of the replicon mutants (Table 4a).
Cross-resistance Analyses

It is anticipated that GS-9451 may be used in combination with PEG-IFN and RBV as well as other DAAs in the future. Therefore, the susceptibilities of GS-9451 resistant variants to other HCV inhibitors were determined using a transient replication assay. Chimeric replicons carrying the NS3 protease gene from patients determined to carry known PI resistant mutations together with their corresponding baseline samples were used for cross-resistance analyses. Table 5 shows cross-resistance data from a set of patient isolates harboring full mutations at positions 155 or 168 to represent the spectrum of HCV mutants observed. Baseline isolates for all these patients (BE, BJ, CD, CF, DH and DD) were sensitive to all nine HCV DAAs tested. Day 4 or Day 14 GT 1a patient isolates with mutations at R155 in HCV protease displayed moderate to high levels of resistance to the HCV PIs GS-9256, danoprevir and TMC-435, and low to moderate levels to telaprevir and boceprevir. The D168V or D168G mutants displayed a high level of resistance to the protease inhibitor GS-9256, however they were fully susceptible to telaprevir and boceprevir. One GT 1b patient isolate (DH) with a full D168V mutation displayed high levels of cross-resistance to TMC-435 (>174-fold). However, an isolate with a full D168G mutation from patient DD was susceptible to TMC-435. All of these GS-9451- resistant mutants were fully susceptible to the non-nucleoside polymerase inhibitors GS-9190 and GS-9669, the nucleoside polymerase inhibitor GS-6620, the NS5A inhibitor GS-5885, as well as IFN-α, and RBV, indicating no cross-resistance of GS-9451 resistance mutations with these inhibitors (Table 5).
There were 32 patients that received GS-9451. HCV NS3 sequence data were obtained from 23 out of 32 patients on Day 4, but not from the remaining 9 patients most likely because of low viral load. Out of these 23 patients, 12 had a known PI resistance mutation detected. The mean maximal viral load reduction for patients with identified resistance mutations was -3.33 log\(_{10}\) compared to -1.37 log\(_{10}\) for those patients without resistance mutations observed (Figure 2). The \(P\) value comparing viral load reductions between the two groups was < 0.0001 (two-tailed t test).

Discussion

This study analyzed drug-resistant HCV variants selected in patients dosed with GS-9451, an HCV protease inhibitor in clinical development. The NS3 mutation R155K is the predominant mutation observed in GT 1a patients while NS3 substitutions at residue D168 (D168G, D168E, or D168V) were commonly observed in GT 1b patients who received GS-9451. No patient had double mutants at both positions 155 and 168. The R155K isolates confer high-levels of resistance (> 50-fold) to GS-9451 and GS-9256, moderate to high levels to TMC-435 and danoprevir, and low to moderate levels to telaprevir and boceprevir, in vitro. Interestingly, one patient isolate with a full D168V mutation displayed high levels of cross-resistance to TMC-435 compare to another patient isolate with a full D168G mutation. Our observations support previously reported data that showed that D168V confer higher levels of resistance to TMC435 compare to D168G (11). The D168 isolates were fully susceptible to telaprevir and boceprevir, suggesting the option of treatment with these approved PIs for these patients. These
Results are in agreement with other previous in vitro resistance studies and clinical observations. For examples, R155 in GT 1a and D168 in GT 1b conferring cross resistance to other macrocyclic PIs are also commonly seen in patients treated with vaniprevir, danoprevir, BI 201335 and TMC-435 (8, 13, 16, 26). Clinical studies with telaprevir identified V36A/M, T54A, R155T/K and A156S/V/T mutations in NS3. Phenotypic analysis showed low- to intermediate-level (V36, T54, R155, A156S) and high-level (A156V/T) resistance to telaprevir. Double mutants that were detected on the same isolate (V36A/M + R155K/T) conferred high levels of resistance (19). In patients treated with boceprevir, mutations that were observed were V36M/A, T54A/S, R155K/T, A156S, V170A and V55A (less common). Genotypic analysis showed a largely overlapping cross-resistance profile of boceprevir and telaprevir. However, V170A and V55A variants conferred higher level resistance to boceprevir in comparison with telaprevir (22). Variants at positions 36, 54, 55, 156 and 170 were not detected in this study by population sequencing after three days administration of GS-9451. In vitro studies showed that T54A/S, V36M, and R155T are sensitive to GS-9451, while mutations at A156 were cross-resistant to GS-9451 (data not shown). Interestingly, no NS3 mutations were observed by population sequencing in patients who received GS-9451 at 60 mg QD for three days. We observed that the emergence of resistant variants in this study correlated with the degree of GS-9451 selective pressure, higher dose, and greater HCV viral load reductions. This observation suggests that the mutant viruses pre-exist at low levels prior to treatment, and once the WT population was sufficiently inhibited by GS-9451, the resistant variants were detectable by population sequencing. The levels of pre-existing mutations at baseline are too low to be detected by
population sequencing (detection limit is 20%) or deep sequencing (detection limit of
1%), however, it has been proposed that single mutants (at positions 155, 156 and 168)
pre-exist in infected subjects with an estimated average of 0.025% and 0.015% for
genotype 1a and genotype 1b, respectively, using a back-calculation estimate from
mutant frequencies observed at Day 2 and/or 4 (23). A more substantial suppression of
WT virus consequently resulted in more frequent detection of resistance mutations. In
this case, the rapid emergence of the resistant variants indicates a greater inhibition of
WT HCV variants.

Sequence analysis of the follow-up timepoints demonstrated that mutations at position
D168 were no longer detected at Day 14 compared to the R155K that was detected at
Week 24 in 43% of the patients (3/7). The D168 resistant variants (D168G, D168E or
D168V) are replaced more rapidly by WT virus, compared to the R155K variants. In
addition, the D168 mutants were not observed in GT 1a patients although these mutants
also confer high levels of resistance to GS-9451, and require only one nucleotide change
similar to the R155K in GT 1a. The difference in the persistence of the R155K and
D168G/E/V mutants may indicate that the in vivo replication fitness of R155K is higher
than D168 mutants. In addition, two nucleotide changes are required to generate an
amino acid change in position 155 in subtype 1b isolates R155K (CGG-AAG), while
only one change (AGG-AAG) is needed for subtype 1a. That can result in lower
frequency of the pre-existing R155K mutant in 1b patients compare to GT 1a patients and
hence its lack of detection upon suppression of WT virus in 1b patients. The duration of
the persistence of mutants may also depend on the degree of the WT clearance and the
duration of treatment.

The replication capacity of the R155K replicon was slightly lower than the D168
mutants, however, the backbone of the R155K (1a replicon) and D168 (1b replicon)
mutants were different. For all patient isolates the replication capacities were lower than
the 1b replicon which suggests incompatibility between the patient isolates and the
replicon subtype backbone or other accumulated mutations that affect RNA replication.

Interestingly, the replication capacities at Day 4 or 14 were comparable or lower than the
baseline of the same subject. That may also suggest that most of the virus populations at
these timepoints are mutants with lower replication capacities.

Previous in vitro replicon studies identified the A156T/G resistant mutant for GS-9451
(27). The reason that this variant was not detected in patients could be due to a lower
frequency of the pre-existing A156 mutants as compared to the R155K mutant in GT 1a
and D168 mutants in GT 1b at baseline and/or reduced fitness of the virus in patients.

Substitutions at positions 80 and 170 that previously were shown to confer resistance to
other PIs (11) were observed in only one patient for each substitution, and will be further
investigated.

Sequences of the C-terminal helicase domain of NS3 and the co-factor NS4A were also
analyzed for their change from baseline. Substitutions detected in this region appear to
be natural variations of the HCV WT population that are present at >1% in the EU
databases. In this study the GS-9451-associated resistant mutants all mapped to the NS3
protease domain.
It is clear that monotherapy with the majority of DAAs is ineffective in curing HCV because resistance mutants are detected after a few days of treatment resulting in virologic rebound and treatment failure. Combination therapy would be required for sustained viral suppression and prevention of viral resistance. Cross-resistance analysis showed that patient isolates with reduced susceptibility to GS-9451 maintained WT sensitivity to IFN-α, RBV, GS-9190 (non-nucleoside site III/IV inhibitor), GS-9669 (non-nucleoside site II inhibitor), GS-6620 (nucleoside inhibitor) and GS-5885 (NS5A inhibitor). These results support the use of GS-9451 in combination with these anti-HCV agents in GT-1 infected patients.

In summary, highly effective inhibition of WT HCV by the NS3 protease inhibitor GS-9451 revealed the resistance mutant R155K in GT 1a and D168E/G/V in GT 1b patients. The R155K mutants persisted longer than the D168 mutants suggesting their greater relative fitness. Lack of cross-resistance between GS-9451 and other classes of DAAs, IFN, and RBV supports the combination of GS-9451 with these agents.

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### Table 1. Antiviral Response to GS-9451 Monotherapy

<table>
<thead>
<tr>
<th>GS-9451 Dose</th>
<th>n</th>
<th>Mean Maximal HCV RNA Reduction$^a$ ± SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>8</td>
<td>-0.20 ± 0.16 (0 to -0.45)</td>
</tr>
<tr>
<td>60 mg QD GT 1a</td>
<td>8</td>
<td>-0.91 ± 0.41 (-0.28 to -1.54)</td>
</tr>
<tr>
<td>200 mg QD GT 1a</td>
<td>9</td>
<td>-3.16 ± 0.51 (-2.51 to -4.15)</td>
</tr>
<tr>
<td>400 mg QD GT 1a</td>
<td>8</td>
<td>-3.77 ± 0.53 (-2.97 to -4.66)</td>
</tr>
<tr>
<td>200 mg QD GT 1b</td>
<td>7</td>
<td>-3.26 ± 0.46 (-2.29 to -3.58)</td>
</tr>
</tbody>
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$^a$ Mean maximal viral load reductions at any timepoint during first 7 days ± SD.
Table 2. Summary of NS3 Drug Resistance Mutations Detected*

<table>
<thead>
<tr>
<th>Mutation in NS3 at Day 4 or Day 14 or Week 12</th>
<th>Placebo (n=8)</th>
<th>60 mg QD GT 1a (n=8)</th>
<th>200 mg QD GT 1a (n=9)</th>
<th>400 mg QD GT 1a (n=8)</th>
<th>200 mg QD GT 1b (n=7)</th>
<th>Reference Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>R155K</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>R155 (99% in 1a, 99% in 1b)</td>
</tr>
<tr>
<td>D168E</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>D168 (99% in 1a, 99% in 1b)</td>
</tr>
<tr>
<td>D168V</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>D168G</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

* If patient had mutations at Day 4 or/and Day 14 or/and Day 12, he was counted once.

1 Frequency analyses includes a total of 397 GT 1a and 541 1b HCV NS3 protease gene sequences obtained from the EU databases.

2 Number of placebo dosed patients from all 4 cohorts.

3 Number of dosed patients in each cohort.

4 Detected as a full or mixture mutation.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Dose (mg)</th>
<th>Day 4</th>
<th>Day 14</th>
<th>week 12</th>
<th>week 24</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH</td>
<td>1a</td>
<td>200</td>
<td>Unable to sequence</td>
<td>R155K</td>
<td>R155K/R</td>
<td>R155K/R</td>
</tr>
<tr>
<td>CG</td>
<td>1a</td>
<td>400</td>
<td>Unable to sequence</td>
<td>Unable to sequence</td>
<td>R155K/R S/T343S K/R469R</td>
<td>A68A/S T87A/T R155K/R K213K/R</td>
</tr>
<tr>
<td>BB</td>
<td>1a</td>
<td>200</td>
<td>Unable to sequence</td>
<td>No change from baseline</td>
<td>V71I/V R155K/R K/R213R G314G/R</td>
<td>No change from baseline</td>
</tr>
<tr>
<td>CD</td>
<td>1a</td>
<td>400</td>
<td>R155K E/V183V V609I/V</td>
<td>R155K/R E/V183V</td>
<td>R155K/R E/V183V</td>
<td>-</td>
</tr>
<tr>
<td>CK</td>
<td>1a</td>
<td>400</td>
<td>A/S147A/L/S/V R155K/R L/P574P</td>
<td>R155K/R A/S147A/L/S/V A/S147A/L/S/V</td>
<td>A/S147A/L/S/V</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1a</td>
<td>400</td>
<td>Unable to sequence</td>
<td>R155K/R I/V329I I386I/V A/T459T I/T586T</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF</td>
<td>1a</td>
<td>400</td>
<td>Unable to sequence</td>
<td>T72I/T R155K</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BE</td>
<td>1a</td>
<td>200</td>
<td>R155K</td>
<td>No change from baseline</td>
<td>No change from baseline</td>
<td>-</td>
</tr>
<tr>
<td>BF</td>
<td>1a</td>
<td>200</td>
<td>R155K/R</td>
<td>No change from baseline</td>
<td>No change from baseline</td>
<td>-</td>
</tr>
<tr>
<td>BK</td>
<td>1a</td>
<td>200</td>
<td>R155K/R</td>
<td>G237A/G</td>
<td>I248I/V</td>
<td>A/V306V</td>
</tr>
<tr>
<td>CB</td>
<td>1a</td>
<td>400</td>
<td>K/Q80I/K/L/Q</td>
<td>R155K/R</td>
<td>A87A/S</td>
<td>K/Q80Q</td>
</tr>
<tr>
<td>DJ</td>
<td>1b</td>
<td>200</td>
<td>F/L14F</td>
<td>D/E357D</td>
<td>T358I</td>
<td>T574L/P</td>
</tr>
<tr>
<td>DD</td>
<td>1b</td>
<td>200</td>
<td>D168G</td>
<td>A/T358T</td>
<td>A/T358T</td>
<td>A/T358T</td>
</tr>
<tr>
<td>DH</td>
<td>1b</td>
<td>200</td>
<td>D168V</td>
<td>T358A/T</td>
<td>T358A/T</td>
<td>-</td>
</tr>
<tr>
<td>DF</td>
<td>1b</td>
<td>200</td>
<td>D168D/E</td>
<td>T402S/T</td>
<td>No change from baseline</td>
<td>-</td>
</tr>
</tbody>
</table>

- Plasma sample was not available due to loss to follow-up.
Table 4. Summary of the GS-9451 Susceptibility of Patient Isolates and Replicons

a. Replication capacities and susceptibilities of NS3 mutations in the replicon backbone to GS-9451

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Replication capacity</th>
<th>Fold shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>R155K (1a)</td>
<td>56.9 ± 13.44</td>
<td>&gt;150</td>
</tr>
<tr>
<td>D168E (1b)</td>
<td>86.1 ± 53.5</td>
<td>82±28.3</td>
</tr>
<tr>
<td>D168G (1b)</td>
<td>64.6 ± 15.3</td>
<td>85±35.2</td>
</tr>
<tr>
<td>D168V (1b)</td>
<td>85.6 ± 33.2</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

1 Replication capacity relative to WT GT 1a or GT 1b replicon was determined as the ratio of luciferase activity in untreated cells at 96 hr relative to the 4 hr input timepoint.

2 Mean average (± SD) of at least 2 independent experiments
b. GS-9451 Susceptibility and replication capacities of patients isolates with resistance mutations.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Dose (mg)</th>
<th>in NS3 Protease&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Timepoint</th>
<th>Replication capacity&lt;sup&gt;1,2&lt;/sup&gt;</th>
<th>EC50(nM)</th>
<th>Mean GS-9451 EC&lt;sub&gt;50&lt;/sub&gt; Fold Change from Baseline&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE</td>
<td>1a</td>
<td>200</td>
<td>R155K</td>
<td>BL&lt;sup&gt;3&lt;/sup&gt;</td>
<td>45.3±17.2</td>
<td>2.6±1.1</td>
<td>&gt;595</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D4&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.7±1.5</td>
<td>&gt;1458</td>
<td></td>
</tr>
<tr>
<td>BJ</td>
<td>1a</td>
<td>200</td>
<td>R155K</td>
<td>BL</td>
<td>2.8±0.2</td>
<td>3.9±3.6</td>
<td>&gt;1279</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D4</td>
<td>2.7±1.5</td>
<td>&gt;5000</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>1a</td>
<td>400</td>
<td>R155K, E/V183V, V609I/V</td>
<td>BL</td>
<td>56.0±12.7</td>
<td>1.7±0.4</td>
<td>&gt;3007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D4</td>
<td>8.9±2.9</td>
<td>&gt;5000</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>1a</td>
<td>400</td>
<td>T7211/T, R155K</td>
<td>BL</td>
<td>12.7±1.1</td>
<td>1.4±1.0</td>
<td>&gt;874</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D14&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5.5±1.44</td>
<td>&gt;1250</td>
<td></td>
</tr>
<tr>
<td>DH</td>
<td>1b</td>
<td>200</td>
<td>D168V, N357D, T358I</td>
<td>BL</td>
<td>37.8±1.6</td>
<td>2.5±0.34</td>
<td>&gt;2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D4</td>
<td>30.6±2.9</td>
<td>&gt;5000</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>1b</td>
<td>200</td>
<td>D168G, A/T358T</td>
<td>BL</td>
<td>5.3±0.4</td>
<td>2.2±0.2</td>
<td>&gt;152</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D4</td>
<td>7.7±2.6</td>
<td>&gt;336</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Bold font indicates GS-9451 resistance mutation

<sup>1</sup> Replication capacity of clinical isolates was determined as the ratio of luciferase activity in untreated cells at 96 hr relative to the 4 hr input timepoint.

<sup>2</sup> Mean average (± SD) of at least 2 independent experiments

<sup>3</sup> BL: baseline; D4: Day 4; D14: Day 14.
Table 5. Susceptibility of GS-9451 Resistant Isolates to Other HCV Inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target protein</th>
<th>Fold Change from Baseline&lt;sup&gt;d&lt;/sup&gt;</th>
<th>BE&lt;sup&gt;a&lt;/sup&gt; (D4&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>BJ (D4)</th>
<th>CD (D4)</th>
<th>CF (D14)</th>
<th>DH (D4)</th>
<th>DD (D4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-9256</td>
<td>NS3 protease</td>
<td></td>
<td>&gt;265</td>
<td>&gt;147</td>
<td>&gt;688</td>
<td>&gt;758</td>
<td>&gt;114</td>
<td>&gt;268</td>
</tr>
<tr>
<td>Danoprevir</td>
<td>NS3 protease</td>
<td></td>
<td>118.1</td>
<td>65.6</td>
<td>64.2</td>
<td>97.2</td>
<td>8.9</td>
<td>9.1</td>
</tr>
<tr>
<td>TMC-435</td>
<td>NS3 protease</td>
<td></td>
<td>16.9</td>
<td>11.8</td>
<td>39.2</td>
<td>8.5</td>
<td>174.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Telaprevir</td>
<td>NS3 protease</td>
<td></td>
<td>10.3</td>
<td>46.7</td>
<td>23.8</td>
<td>4.5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Boceprevir</td>
<td>NS3 protease</td>
<td></td>
<td>4.7</td>
<td>3.9</td>
<td>3.2</td>
<td>2.9</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>GS-9190</td>
<td>NS5B NNI</td>
<td></td>
<td>1.7</td>
<td>1.4</td>
<td>1.1</td>
<td>1.5</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>GS-9669</td>
<td>NS5B NNI</td>
<td></td>
<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>GS-6620</td>
<td>NS5B NI</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
<td>0.8</td>
<td>1.0</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>GS-5885</td>
<td>NS5A</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
<td>1.1</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Interferon-α</td>
<td>SOC</td>
<td></td>
<td>2.1</td>
<td>0.4</td>
<td>1.1</td>
<td>0.9</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>SOC</td>
<td></td>
<td>1.2</td>
<td>0.9</td>
<td>1.5</td>
<td>1.3</td>
<td>1.0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

NA = not available; NNI = non-nucleoside inhibitor; NI = nucleoside inhibitor; SOC = standard of care.

<sup>a</sup> Patient identification code

<sup>b</sup> D4: Day 4.

<sup>c</sup> NS3 resistance mutations.

<sup>d</sup> Mean of at least 2 independent experiments are shown and calculated as a fold change in compound EC<sub>50</sub> on Day 4 or 14 relative to compound EC<sub>50</sub> at baseline.
Figure legends

Figure 1. GS-9451 structure
Figure 2. Maximal viral load reduction in patients with and without resistance mutations. Maximal viral load reduction of 23 patients who received GS-9451 and had sequence data on Day 4 (GT 1a and GT 1b). Mean ± SD is shown. P value compares viral load reductions between the two groups (two-tailed t test).

- Patients with resistance mutation as detected by population sequencing on Day 4.
- Colors for different doses: green for 60mg, pink for 200 mg, and purple for 400mg.