Clinical and *In Vitro* Resistance to GS-9669, a Thumb Site II Nonnucleoside Inhibitor of the Hepatitis C Virus NS5B Polymerase

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Treatment with GS-9669, a novel nonnucleoside inhibitor (site II) of hepatitis C virus (HCV) nonstructural 5B (NS5B) polymerase, resulted in significant antiviral activity in HCV genotype (GT) 1 patients dosed at 50 and 500 mg once daily (QD) and at 50, 100, and 500 mg twice daily (BID) for 3 days. This report characterizes the virologic resistance to GS-9669 *in vitro* and in GT1 HCV-infected patients from a phase I clinical study. An *in vitro* resistance selection study with GS-9669 revealed substitutions at several NS5B residues that conferred resistance. The M423 variants were selected at low drug concentrations (5× the 50% effective concentration [EC₅₀]), and the L419, R422, and I482 variants were selected at higher drug concentrations (20× the EC₅₀). During the phase I clinical study, substitutions at NS5B residues 419, 422, and 486 were the predominant changes associated with GS-9669 monotherapy. Substitutions at position 423 were observed only in GT1a patients in the low-dose groups (50 and 100 mg BID). Interestingly, four HCV patients had substitutions at position 423 at baseline. Consistent with the low resistance level at this position, three patients with M423I or M423V at baseline achieved >2-log₁₀ reductions of HCV RNA when treated with 100 mg BID or with 500 mg QD or BID of GS-9669. The fourth patient, who had the M423V substitution at baseline, had a 4.4-log₁₀ reduction of HCV RNA with 500 mg BID of GS-9669. Phenotypic analyses demonstrated that the viral isolates with multiple GS-9669 resistance-associated variants have reduced susceptibility to GS-9669 and lomitubuvir (VX-222) but are not cross-resistant to other classes of HCV inhibitors. (This study has been registered at ClinicalTrials.gov under registration no. NCT01431898.)

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide (1). HCV infection can lead to cirrhosis, hepatocellular carcinoma, or other complications. Until recently, the standard of care for the treatment of chronic HCV infection consisted of 24 to 48 weeks of pegylated interferon (PEG-IFN) and ribavirin (RBV) (2), which are associated with significant side effects, including fever, fatigue, anemia, leukopenia, thrombocytopenia, and depression (3, 4). A sustained virologic response (SVR) occurs in only 42% to 53% of patients with genotype (GT) 1 or GT4 HCV and up to 78% to 82% of patients infected with GT2 or GT3 HCV (5, 6). Novel direct-acting antiviral agents (DAAs) are being developed in combination with PEG-IFN-RBV and are also being pursued as components of IFN-free and IFN- and RBV-free regimens to improve efficacy and shorten treatment duration. Two protease inhibitors (PIs) approved for the treatment of HCV, telaprevir and boceprevir, have demonstrated significantly improved SVR rates when given in combination with PEG-IFN-RBV in GT1 patients (60 to 75% for combination compared with 38 to 46% for PEG-IFN-RBV only) (7, 8). However, these new agents require thrice-daily dosing and are associated with more frequent occurrences of and severe anemia and rash (9, 10). Two HCV drugs received FDA approval at the end of 2013, simeprevir (Olysio), a nonstructural 3/4A (NS3/4A) protease inhibitor in combination with PEG-IFN-RBV, and sofosbuvir (Sovaldi), a nucleoside inhibitor, which is the first drug that has demonstrated safety and efficacy for treating non-genotype-1 HCV infection without the need to coadminister PEG-IFN.

GS-9669 (Fig. 1) is a novel thumb site II nonnucleoside inhibitor (NNI) of the HCV NS5B RNA polymerase, with a binding affinity of 1.4 nM for the GT1b NS5B protein. It is a selective inhibitor of HCV RNA replication, with a mean 50% effective concentration (EC₅₀) of ≤11 nM in GT1 and GT5 replicon assays

(11). Other NNIs currently in phase II clinical studies include BI-207127 and BMS-791325 (binding to thumb site I), filibuvir and lomitubuvir (binding to thumb site II), setrobuvir, ABT-072, and ABT-333 (binding to palm site I), and tegobuvir (also binding in the palm) (12). In a phase Ib study of filibuvir, resistance-associated variants (RAVs) at NS5B residue M423 (M423I/T/V) were observed in 76% of the patients following treatment (13). The frequencies of RAVs at this residue were similar between the subtype 1a and 1b viruses. RAVs at NS5B residues R422 (R422K), M426 (M426A), and V494 (V494A) were also detected in a small number of patients at baseline or the end of therapy and were found to mediate reductions in filibuvir susceptibility (13). GS-9669 has reduced *in vitro* activity against known resistance variants associated with thumb site II inhibitors (L419M, R422K, F429L, and I482L in GT1b, and L419M and I482L in GT1a) (11). To further investigate the resistance profile of GS-9669, *in vitro* resistance selections were performed, and NS5B gene sequencing and phenotypic assessments were conducted for HCV patients treated with GS-9669 at multiple doses during a 3-day phase I

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FIG 1 GS-9669 structure.

clinical study (registered at [ClinicalTrials.gov](https://clinicaltrials.gov) under registration no. NCT01431898).

MATERIALS AND METHODS

Compounds. Human alpha interferon (IFN- α) and RBV (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) were purchased from Sigma-Aldrich (St. Louis, MO). All other compounds (GS-9451 [vedroprevir], GS-5885 [ledipasvir], GS-9190, GS-9669, sofosbuvir, filibuvir, and VX-222 [lombibuvir]) were synthesized by Gilead Sciences (Foster City, CA).

In vitro resistance selection in replicons. Resistance selections were performed as previously described (14). Briefly, GT1a- or GT1b-containing replicon cells were cultured in the presence of $5\times$ or $20\times$ the EC_{50} of GS-9669 until small colonies formed. These colonies were expanded and characterized by sequence analysis.

Transient transfection of replicon RNA into Huh7 cells and EC_{50} determination. Resistance mutations were introduced into the GT1a (15) or GT1b replicon (16) by site-directed mutagenesis and tested in transient-transfection assays, as previously described (14). Briefly, NS5B mutations were introduced into a plasmid carrying the gene encoding the PI-hRluc replicon using a QuikChange II XL mutagenesis kit, according to the manufacturer's instructions (Stratagene, La Jolla, CA). The mutations were confirmed by DNA sequencing. The replicon RNAs were transcribed *in vitro* from plasmids carrying replicon-encoding genes using a MEGAscript kit (Ambion, Austin, TX). RNA was transfected into Huh-Lunet cells using the method of Lohmann et al. (16). Briefly, the cells were trypsinized and washed twice with phosphate-buffered saline (PBS). A suspension of 4×10^6 cells in 400 μ l of PBS was mixed with 5 μ g of RNA and subjected to electroporation using settings of 960 μ F and 270 V. The cells were transferred into 40 ml of prewarmed culture medium and then seeded into 96-well plates (100 μ l/well). The compounds were 3-fold serially diluted in 100% dimethyl sulfoxide (DMSO) and added to the cells at a 1:200 dilution, achieving a final DMSO concentration of 0.5% in a total volume of 200 μ l/well. The cells were treated for 3 days, after which the culture media were removed, the cells were lysed, and *Renilla* luciferase activity was quantified using a commercially available assay (Promega, Madison, WI) and a TopCount instrument (PerkinElmer, Waltham, MA). The EC_{50} s were calculated as the compound concentration at which a 50% reduction in the level of *Renilla* reporter activity was observed compared with that of the control samples with DMSO. Dose-response curves were generated and EC_{50} s were determined using the GraphPad Prism software package (GraphPad Software, La Jolla, CA) by nonlinear regression analysis. The replication levels of either the reference strains (1b-Con1 or 1a-H77) or chimera replicons derived transiently from the clinical isolates were determined as the ratio of the *Renilla* luciferase signal at day 4 to that at 4 h postelectroporation in order to normalize for transfection efficiency. The replication capacity of each replicon was expressed as their normalized replication efficiency compared with that of the reference strain (1b-Con1 or 1a-H77) within the same experiment.

Colony reduction assays. Colony reduction assays were performed by incubating GT1b cells at different multiples of the EC_{50} as indicated for 21 days in the presence of 0.5 mg/ml G418 and drug in 6-well plates. The surviving colonies were stained with crystal violet and counted using a colony counter (Bio-Rad, Hercules, CA).

Clinical trial population and study design. A total of 70 patients were enrolled in 1 of 7 cohorts of 10 patients, each randomized 8:2 to treatment with GS-9669 or matching placebo for 3 days. All patients completed dosing with the study drug. GS-9669 was administered once daily (QD) at 50 mg and 500 mg in the GT1a patients and at 500 mg in the GT1b patients, or twice daily (BID) at 50 mg, 100 mg, and 500 mg in the GT1a patients and 100 mg in the GT1b patients. The patients had plasma HCV RNA levels of $>5 \log_{10}$ IU/ml at screening. Of the 70 patients in the study, 49 had HCV GT1a, 20 had HCV GT1b, and 1 had HCV GT3a (placebo). The study was conducted in compliance with the Declaration of Helsinki. The study protocol and informed consent documents were reviewed and approved by the institutional review board of the participating institution, and informed consent was obtained from all patients before any study-specific procedures were conducted.

Plasma samples were collected from all patients before dosing on day 1 (baseline), day 4 (or earlier time point if the viral load was $<1,000$ IU/ml), and day 17 and stored at -80°C for NS5B sequencing and phenotypic analyses.

Antiviral activity. Plasma samples were obtained at baseline, day 1 (6 and 12 h postdose), 2, 3, and 5 (a.m. and p.m.), and 7, 10, and 17. The HCV RNA levels were quantified using the TaqMan version 2.0 assay (Roche Molecular Systems, Inc., Branchburg, NJ; lower limit of quantification, 25 IU/ml). The change from baseline in HCV RNA was determined for each time point.

Amplification and population sequencing of the HCV NS5B gene. The HCV genotypic analyses were performed at Monogram Biosciences, Inc. (South San Francisco, CA). Briefly, virus particles were disrupted by adding lysis buffer, and genomic viral RNA (vRNA) was extracted. Purified vRNA was used as a template for cDNA synthesis in a reverse transcriptase reaction and then was used as the template for the first round of a two-round nested-PCR that resulted in the amplification of the entire NS5B region. The inner (nested) primers contained restriction endonuclease recognition/cleavage sites that enabled the cloning of NS5B amplification products into an HCV replicon test vector for phenotypic drug susceptibility analysis. The PCR products were purified and used for the template in each of 12 sequencing reactions using either subtype 1a- or 1b-specific sequencing primers. The sequencing assay was validated and detected quasiespecies in 20% and 10% of the mixtures in 100% and 75% of the assays, respectively (17).

HCV NS5B phenotypic assay. The NS5B amplification products were ligated into a bacterial plasmid cloning vector and then used to transform competent *Escherichia coli* organisms. After ligation and transformation to competent *E. coli* cells, plasmid DNA was purified from the bacterial cultures and then linearized by restriction endonuclease digestion. *In vitro*-transcribed RNA was then electroporated into a Huh7 cell line, and the electroporated cells were incubated in the absence and presence of serially diluted inhibitors. Luciferase activity was expressed as relative light units. Inhibitor susceptibility was determined by evaluating the ability of the patient isolates to replicate in the absence and presence of inhibitor at 72 to 96 h postelectroporation. The percent inhibition at each serially diluted inhibitor concentration was derived as follows: $(1 - [\text{luciferase activity in the presence of inhibitor} / \text{luciferase activity in the absence of inhibitor}]) \times 100$. Inhibitor susceptibility profiles (curves) were derived from these values, and the inhibition data (e.g., the inhibitor concentration required to reduce virus replication by 50% [EC_{50}]) were extrapolated from the fitted curves. The inhibition data were reported as the fold change relative to that of a reference vector (e.g., $EC_{50 \text{ sample}} / EC_{50 \text{ reference}}$) processed in the same assay batch (e.g., EC_{50} fold change from reference).

To assess the effect of baseline polymorphisms on antiviral activity, the fold changes in EC_{50} s were plotted against the change in HCV RNA from baseline to day 17.

Nucleotide sequence accession numbers. The sequences were automatically trimmed and assembled based on homology to a subtype-specific reference sequence (H77 for 1a and Con1 for 1b). The final consensus

TABLE 1 Summary of *in vitro* resistance selection with GS-9669 in GT1a and GT1b replicon systems^a

Amino acid substitution	Clonal frequency (%) of amino acid substitutions in presence of GS-9669 ^b			
	GT1a for concn (×EC ₅₀ [nM]) of:		GT1b for concn (×EC ₅₀ [nM]) of:	
	5 (45)	20 (180)	5 (25)	20 (100)
WT ^c	20		10	6
L419 M		10		32
L419W				12
R422K		10		50
M423I	30			
M423T	50		60	
M423V			30	
I482L		80		

^a GT, genotype.^b HCV replicon cells were incubated with compound for 30 to 40 days (GT1a) and 20 to 30 days (GT1b). At least 25 isolated colonies were sequenced from each selection condition.^c WT, wild type.

sequences were exported, along with a list of the amino acid differences compared to the reference. All produced NS5B sequences were submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank/index.html>) and assigned accession no. [KM215799](#) to [KM216009](#).

RESULTS

Selection of *in vitro* resistance to GS-9669 using HCV replicons.

To characterize the resistance profile of GS-9669, we selected resistance in GT1 HCV using replicon cell lines. Specifically, GT1a and GT1b replicon cell lines were treated with GS-9669 at 5× or 20× the EC₅₀ and G418 for several weeks until resistant colonies formed. Approximately 25 individual colonies from each selection condition were isolated, expanded, and analyzed for changes in the HCV NS5B gene. At the lower concentration of GS-9669 (5× the EC₅₀), resistant variants were detected only at residue 423 of NS5B (M423I/T/V) (Table 1). M423T was the dominant vari-

ant (50% in GT1a and 60% in GT1b). In addition, M423I was found in GT1a and M423V in GT1b. At the higher concentration of GS-9669 (20× the EC₅₀), changes at residues 419, 422, and 482 were detected, with I482L and R422K being the most frequently observed variants in GT1a and GT1b, respectively (Table 1). Phenotypic analyses of the variants observed during *in vitro* resistance selections confirmed that they reduced GS-9669 susceptibility *in vitro* (Table 2). In both genotypes, the M423 RAVs conferred low to moderate resistance levels (4.6- to 19.3-fold), while L419M, R422K, and I482L conferred higher levels of resistance (26- to 815-fold).

Replicon colony reduction assay. The *in vitro* resistance barrier for an HCV inhibitor is influenced by various factors: first, the genetic barrier to resistance, which is the number of amino acid substitutions needed for a viral variant to confer resistance to the drug (if a single substitution is sufficient to confer high-level resistance, the drug is generally considered to have a low genetic barrier to resistance), and second, the fitness of the resistance viral variant, which enables it to grow in a replicative environment (18).

Resistance barriers can be assessed using the HCV replicon system in colony formation assays; upon treatment of a replicon cell culture with an inhibitor and G418, the number of surviving replicon colonies at different drug conditions will reflect the resistance barrier of the inhibitor. GS-9669 was thus tested in a colony formation assay in GT1b replicon cells at 1×, 5×, 10×, 20×, and 40× the EC₅₀ (Fig. 2). After 21 days, the DMSO-treated control wells contained confluent monolayers of cells. The number of colonies present in the GS-9669-treated wells decreased with increasing drug concentration (Fig. 2). Only a few colonies in the highest dose treatment group (40× the EC₅₀) remained. GS-9669 treatment resulted in fewer colonies at 5× and 10× the EC₅₀ (25 nM and 50 nM, respectively) compared with filibuvir and lomibuvir. This result suggests that GS-9669 presents a higher barrier to resistance in HCV GT1b replicon cells at the low multiples of EC₅₀ (5× and 10× the EC₅₀) than that from filibuvir and lomibuvir.

TABLE 2 Replicon resistance of NS5B site II RAVs to GS-9669^a

NS5B site II RAV	GT1a ^b			GT1b		
	GS-9669 fold change ^c	Lomibuvir fold change ^c	Replication capacity ^d	GS-9669 fold change ^c	Lomibuvir fold change ^c	Replication capacity ^d
L419 M	87.3 ± 25.2	50.9 ± 17.5	115 ± 27.5	123.4 ± 37.6	127.7 ± 49.7	77.2 ± 20.6
L419S	197 ± 95.9	134.6 ± 44.3	0.95 ± 0.46	789.8 ± 354.3	346.9 ± 262	11.4 ± 1.9
R422K	144.7 ± 56.5	97.8 ± 5.7	2.53 ± 1.16	814.6 ± 476.9	545.8 ± 305	44.0 ± 14.6
M423V	8.5 ± 3.8	17.9 ± 9.0	51.9 ± 30.5	7.0 ± 3.5	18.0 ± 8.9	37.1 ± 13.4
M423T	15.8 ± 4.8	28.3 ± 9.1	82.4 ± 19.5	19.3 ± 4.0	49.6 ± 6.7	56.8 ± 21.9
M423I	10.6 ± 2.5	10.5 ± 1.6	106 ± 19.8	4.6 ± 0.4	5.6 ± 1.4	59.3 ± 26.1
M426L	1.1 ± 0.1	ND ^e	71.6 ± 17.3	ND	ND	ND
I482L	26.1 ± 5.3	34.2 ± 9.0	127 ± 41.0	51.4 ± 8.5	101.2 ± 21.0	75.6 ± 23.0
A486I	ND	ND	ND	48.7 ± 15.5	102.1 ± 31.3	23.2 ± 9.2
A486T	ND	ND	ND	31.1 ± 8.4	55.7 ± 16.2	57.6 ± 24.3
A486V	39.6 ± 11.8	48.5 ± 17.2	84.6 ± 27.8	49.8 ± 19.3	77.5 ± 31.5	83.7 ± 17.8
V494A	17.4 ± 4.2	27.2 ± 11.1	32.1 ± 15.6	18.1 ± 5.1	32.5 ± 4.0	51.5 ± 16.0
V494I	0.55 ± 0.05	ND	6.8 ± 2.1	ND	ND	ND

^a Values are the results from ≥2 independent experiments.^b GT, genotype.^c Fold change values are the means ± standard deviation (SD) in EC₅₀ for the mutant replicon compared with the wild-type replicon determined in each experiment.^d The replication capacity was normalized with that of the reference strain (1b-Con1 or 1a-H77) within the same experiment and expressed as the percentage ± SD.^e ND, not determined.

FIG 2 Influence of NNI site II on the formation of replicon colonies. A total of 75,000 GT1b replicon cells were treated for 18 to 21 days in the presence of drug in 6-well plates. The cell monolayers were then stained with crystal violet to visualize the resistant colonies (top). Colonies were counted using a Bio-Rad colony counter (bottom). The assays were performed at least three times for each compound. The average numbers of colonies per plate ± standard deviation (SD) are presented.

Antiviral response to GS-9669 in GT1 HCV-infected patients. The samples analyzed in the phase I study were obtained from 49 GT1a and 20 GT1b HCV patients who were dosed with GS-9669 or placebo for 3 days. Among the QD-dosed cohorts, the mean maximum reductions in HCV RNA levels through day 4 were -2.18 , -3.36 , and -3.45 log₁₀ IU/ml for the 50 mg GT1a, 500 mg GT1a, and 500 mg GT1b GS-9669 groups, respectively (Table 3 and Fig. 3). Among the BID-dosed cohorts, the median maximum reductions in HCV RNA levels through day 4 were -3.35 , -3.40 , -4.02 , and -3.47 log₁₀ IU/ml in the 50 mg GT1a, 100 mg GT1a, 500 mg GT1a, and 100 mg GT1b GS-9669 groups, respectively (Table 3 and Fig. 3). The maximum reductions in HCV RNA levels were comparable among patients with GT1a and GT1b HCV infection dosed with 500 mg QD or 100 mg BID ($P = 0.75$ and 0.8 , respectively).

TABLE 3 Antiviral response to GS-9669 monotherapy

GS-9669 dose (mg), HCV genotype of patients (<i>n</i> = 8 for each) ^a	Mean maximal HCV reduction ± SD (range) (log ₁₀ IU/ml) ^b
50 QD, GT1a	−2.18 ± 0.60 (−2.97 to −1.22)
500 QD, GT1a	−3.36 ± 0.69 (−4.04 to −2.20)
500 QD, GT1b	−3.45 ± 0.47 (−4.06 to −2.89)
50 BID, GT1a	−3.35 ± 0.37 (−3.79 to −2.87)
100 BID, GT1a	−3.40 ± 0.59 (−3.98 to −2.19)
500 BID, GT1a	−4.02 ± 0.33 (−4.44 to −3.31)
100 BID, GT1b	−3.47 ± 0.37 (−4.11 to −3.03)

^a QD, once daily; GT, genotype; BID, twice daily.

^b Mean maximal viral load reduction at any time point during the first 7 days.

FIG 3 (A) Maximum change from baseline in HCV RNA. The maximum mean ± SD viral load reductions in patients dosed with GS-9669 for 3 days are shown. The P values compare the viral load reductions between the 500 mg QD GT1a and 500 mg QD GT1b groups and between 100 mg BID GT1a and 100 mg BID GT1b groups (two-tailed t test). The patients had the following resistance-associated variants (RAVs) at baseline: AH, M423V; EB, M423I; EH, M423I; and BC, M423V. (B) *In vitro* susceptibility of HCV genotype 1a and 1b clinical isolates to GS-9669 at baseline. The mean ± SD of the EC₅₀s for GS-9669 inhibition of 30 GT1a and 16 GT1b treatment-naïve patient isolates that were cloned in HCV replicons and tested *in vitro* are shown. The patients had the following RAVs detected at baseline: AH, M423V; EB, M423I; and BC, M423V.

NS5B polymerase polymorphisms present at baseline. NS5B polymorphisms present at baseline included variants associated with reduced susceptibility to GS-9669. Substitutions at positions previously shown to have reduced susceptibilities to NNIs were observed in four patients dosed with GS-9669 (Table 4 and Fig. 3). Four patients had RAVs at amino acid residue 423 (M423V and M423I) that conferred low-level reduced susceptibility to GS-9669 *in vitro* (Table 2). Despite the presence of M423V/I, three of the four patients showed partial antiviral responses, with >2 log₁₀ reductions in HCV RNA, after receiving 100 mg BID or 500 mg QD of GS-9669, and the remaining patient had a 4.44-log₁₀ HCV RNA reduction after receiving 500 mg BID of GS-9669. Other NS5B variants at positions M426, Y448, and V494 previously shown to affect susceptibility to site II and III NNIs (19–21) were observed at baseline either alone or in combination with M423 substitutions (M426L, $n = 8$; Y448H, $n = 1$; V494I, $n = 3$). A previous study found that Y448H does not confer cross-resistance to GS-9669 (22), and the drug susceptibility assays conducted here indicated that neither M426L nor V494I confers reduced susceptibility to GS-9669 (Table 2). The patients with these variants showed a similar response to that of the other patients in the same treatment group. These three positions are highly polymorphic in

TABLE 4 Patients with NS5B NNI RAVs detected at baseline

Patient ID ^a	Patient genotype	Treatment group (mg) ^b	Known NNI RAVs ^c	Max VL reduction (log ₁₀ IU/ml) ^d	Mean max VL reduction in treatment group (log ₁₀ IU/ml)
EH	1a	500 QD	M423I	−2.20	−3.36
EB	1a	500 QD	M423I	−2.30	−3.36
AH	1a	100 BID	M423V	−2.19	−3.41
BC	1a	500 BID	M423V	−4.44	−4.02

^a ID, identification.^b QD, once daily; BID, twice daily.^c Frequency analyses from a total of 639 GT1a and 405 GT1b NS5B gene sequences obtained from Gilead databases showed that at position M423, 98% of the sequences contain the wild-type amino acid residue Met, 0.6% contain Ile, and 0.5% contain Val. NNI, nonnucleoside inhibitor; RAVs, resistance-associated variants.^d Max, maximum; VL, viral load.

NS5B, and the substitutions observed at baseline do not appear to affect GS-9669 susceptibility *in vitro* or *in vivo*. A mixture of the variant S556S/G that was previously shown to reduce susceptibility to site III NNIs was detected in one patient at baseline. This patient had a 3.38-log₁₀ reduction in HCV RNA after receiving 500 mg QD of GS-9669, suggesting this variant did not affect the antiviral response to GS-9669; NS5B residue 556 also appears to be highly polymorphic.

The effect of baseline polymorphisms on GS-9669 activity *in vitro* was further assessed in a transient replicon assay in which the NS5B regions derived from the patient isolates were introduced into a GT1b replicon backbone. The average $\pm$ standard deviation (SD) EC₅₀s for GS-9669 at baseline for GT1a and GT1b were 3.8 $\pm$ 1.3 and 6.8 $\pm$ 2.7 nM, respectively (Fig. 3b). The only patient isolates with significantly decreased susceptibility to GS-9669 at baseline were from patients AH and EB, who had M423V or M423I at baseline (4.5- and 9.3-fold reduced, respectively, compared with the reference wild-type replicon). The other patient (BC) with M423V at baseline had only a 1.4-fold change from the reference. This patient had a 4.44-log₁₀ reduction in HCV RNA after receiving 500 mg BID of GS-9669. No phenotypic data were obtained for the fourth patient, who had M423I at baseline (EH). No changes in GS-9669 activity were observed when the baseline isolates with the M426L or S556S/G polymorphisms were tested (<2.5-fold change from reference).

Substitutions selected in HCV RNA of patients following GS-9669 treatment. HCV NS5B amino acid substitutions were identified by comparing the sequences obtained during or after com-

pletion of dosing with the baseline population sequence for each patient. Amino acid changes at positions not known to be associated with resistance to HCV NNIs were considered potential resistance mutations if they developed in multiple patients at conserved sites. NS5B conserved sites were defined as amino acid positions that are >99% conserved in Gilead's virology database, containing 639 GT1a and 406 GT1b sequences from treatment-naïve patients. The conservation of these amino acids was also confirmed using publically available sequences from the European Hepatitis C Virus Database (euHCVdb). The NS5B positions 419, 422, 482, 486, and 494 were >99% conserved in GT1a and GT1b. M423 was also conserved in GT1b, whereas M423I was found in 2% of the GT1a HCV sequences.

Analyses of on-treatment and posttreatment samples when the viral load was >1,000 IU/ml on day 4 (day 5 or 7) indicated that drug RAVs were present in 23/32 (71.9%) GT1a patients and 14/16 (87.5%) GT1b patients who received BID or >50 mg QD of GS-9669 (Table 5). The patients treated with the lowest dose of GS-9669, 50 mg QD, had the least resistance detected (1/8 [12.5%]). HCV from most patients had multiple NNI RAVs detected that appeared as mixtures with the wild type at each position. Substitutions at residues 419, 422, and 486 were the predominant changes associated with GS-9669 therapy and were observed in 22/48 (46%), 22/48 (46%), and 31/48 (65%) patients, respectively, following 3 days of treatment with >50 mg QD of GS-9669. Substitutions at position 423 were observed only in GT1a patients who received lower doses of GS-9669 (50 mg and 100 mg BID).

TABLE 5 NS5B drug-resistant variants at days 2 to 7^a

RAVs ^b	No. of patients by genotype and dosage (n = 8 for each) ^c								No. (%) of patients with RAVs for group (n):		
	GT1a					GT1b			GT1a (32) ^d	GT1b (16)	Total ^d
	50 mg QD	50 mg BID	100 mg BID	500 mg BID	500 mg QD	500 mg QD	100 mg BID				
V494V/A				1					1 (3.1)		1 (2.1)
I482I/L		1					1		1 (3.1)	1 (7.7)	2 (4.2)
M423 M/A/I/T/V		2	4						6 (18.75)		6 (12.5)
L419L/S/M/P/T/V/I		3	3	3	3	4	6		12 (37.5)	10 (62.5)	22 (45.8)
R422R/K		4	4	2	1	7	4		11 (34.4)	11 (68.75)	22 (45.8)
A486A/V/I/T/M	1	7	4	4	2	8	5		17 (53.1)	13 (81.2)	30 (62.5)
No. (%) of patients with RAVs	1 (12.5)	8 (100)	6 (86)	5 (62.5)	4 (50)	8 (100)	6 (75)		23 (71.9)	14 (87.5)	37 (77.1)

^a Any patient who had mutant virus at day 2, 3, 4, 5, and/or 7 was counted.^b Resistance-associated variants (RAVs) detected as full mutant or mixture with wild type.^c GT, genotype; QD, once daily; BID, twice daily.^d Patients from the 50-mg-QD cohort were not included because of the suboptimal antiviral response in this treatment group.

TABLE 6 NS5B drug-resistant variants observed at day 17

RAVs ^a	No. of patients by genotype and dosage (<i>n</i>)							No. (%) of patients with RAVs
	GT1a					GT1b		
	50 mg QD (8)	50 mg BID (8)	100 mg BID (8)	500 mg BID (8)	500 mg QD (8)	500 mg QD (8)	100 mg BID (5)	
V494V/A								
I482I/L								
M423 M/A/I/T/V								
L419L/S/M/P/T V/I					1	1	3	5 (11.1)
R422R/K						3	1	4 (8.9)
A486A/V/I/T/M						6	3	9 (20.0)
No. (%) of patients with RAVs in each arm	0	0	0	0	1 (12.5)	6 (75)	3 (60)	

^a RAVs, resistance-associated variants.
GT, genotype; QD, once daily; BID, twice daily.

NNI RAVs were not detected in the HCV samples of any placebo patients.

The stability of RAVs after the cessation of treatment was also assessed. Except for one GT1a patient in the 500 mg QD cohort, the NNI RAVs were no longer detected by population sequencing in any of GT1a patients at day 17 (Table 6). For the GT1b patients with RAVs detected at earlier time points, they were still detected in 9 of 13 patients (69%) on day 17. No other substitutions were observed in multiple patients across all dosage groups.

Phenotypic and cross-resistance analyses. To determine whether the sequence changes described above are associated with reduced susceptibility, phenotypic analyses were performed for the samples from patients with amino substitutions detected at NS5B position 419, 422, 423, 482, 486, or 494. Phenotypic analyses were also performed for the corresponding baseline samples for use as individual comparators. GS-9669 EC₅₀s were obtained for viruses from 39 patients at both baseline and either day 4 or 17 (Fig. 4; see also Table S1 in the supplemental material).

Phenotypic analyses demonstrated that 26/39 samples analyzed with multiple RAVs at positions A486, L419, R422, and/or M423 had reduced susceptibilities to GS-9669, with >3-fold EC₅₀ changes from baseline. Thirteen of 39 patient isolates had RAVs detected but did not have a change in susceptibility (fold change from baseline, <3); this is most likely due to the recombinant mutant virus analyzed having a mixture with the wild type and the more efficient replication of the wild-type replicon compared with that of the variants during the assay. Only two patients, BB and EG, had full single substitutions detected (L419M or L419S), and these had 48- and >317-fold changes from baseline, respectively, for GS-9669. All other substitutions were detected as mixtures alone or with other mutations (mixtures of 2 or 3 positions) and showed low to high levels of resistance to GS-9669. Almost all patient isolates with GS-9669 RAVs also had reduced susceptibility to lomitubuvir, another site II NNI. All GS-9669-resistant mutants maintained wild-type susceptibility to other tested classes of HCV inhibitors, including sofosbuvir (NI), GS-9451 (PI), ledipasvir (NS5A), and ribavirin. In addition, phenotypic analyses for 3 patients with no RAVs but other NS5B substitutions detected (M173I, V329V/I, G/S543G, G66D/G, V/I116V, and R300R/Q) showed that these isolates were fully susceptible to GS-9669 and the other tested HCV inhibitors.

To evaluate the resistance levels of single RAVs that were ob-

served as mixtures alone or with other RAVs in the clinical samples, the predominant RAVs detected in the GT1a or GT1b isolates were introduced into the wild-type replicon by site-directed mutagenesis and were phenotyped (Table 2). All substitutions at positions 419 and 422 conferred high levels of resistance to GS-9669 (>90-fold). The V482I, A486V, and V494V variants conferred more moderate levels of resistance to GS-9669 in GT1a and GT1b (20- to 50-fold, respectively). The M423 substitutions conferred the lowest levels of resistance to GS-9669 (5- to 20-fold). Most GS-9669 RAVs conferred comparable resistance levels to lomitubuvir.

DISCUSSION

Treatment with GS-9669, a novel NNI site II NS5B inhibitor, resulted in a significant antiviral effect in GT1 HCV-infected patients in a phase I monotherapy study (12). Our current study analyzed drug-resistant HCV variants selected *in vitro*, using the

FIG 4 *In vitro* susceptibility of patient isolates to GS-9669 and other HCV inhibitors. Phenotypic analysis of NS5B clinical isolates selected from those who had amino substitutions detected at 419, 423, 422, 482, 486, and 494 by population sequencing (*n* = 39) and the corresponding baseline samples for use as individual comparators are shown. The isolates were cloned into HCV replicons, and drug susceptibility was tested. The mean $\pm$ SD EC₅₀ fold changes from baseline are shown. The *P* values compare the EC₅₀ fold change from baseline between GS-9669 and lomitubuvir (two-tailed *t* test). The EC₅₀s for the baseline GT1a and GT1b isolates were as follows: GS-9669, 4.9 $\pm$ 2.4; lomitubuvir, 10.4 $\pm$ 9.5; sofosbuvir, 77.3 $\pm$ 29.8; vedroprevir, 14.4 $\pm$ 6.7; ledipasvir, 0.005 $\pm$ 0.0016; ribavirin, 19.8 $\pm$ 5.1; and interferon, 1.6 $\pm$ 1.1.

replicon system, and in patients from the phase I study. Our *in vitro* results indicate that NS5B residues L419, R422, M423, and I482 are major resistance loci for GS-9669, with M423 RAVs selected at low drug concentrations and L419, R422, and I482 RAVs selected at higher drug concentrations. In agreement with these genotypic observations, phenotypic analyses of the RAVs indicated that M423 RAVs conferred low to moderate levels of GS-9669 resistance, whereas the remaining variants conferred higher degrees of resistance. The colony reduction assays indicated that GS-9669 selects fewer resistant colonies at higher doses, suggesting that variants conferring low-level resistance may be suppressed at high doses. We noted that at 5 $\times$ and 10 $\times$ the EC₅₀, there were significantly fewer colonies selected by GS-9669 than those by fildabuvir and lomitabuvir, potentially due to the enhanced suppression of M423V/T mutants by GS-9669 versus those of these other inhibitors. Interestingly, it has been shown that fildabuvir has high levels of resistance to the M423 mutants (>560-fold) and R422K (>340-fold) (13) compared with 8.5- to 15.8- and 144.7-fold for GS-9669 for the M423V/T/I and R422K mutants, respectively.

Analysis of GS-9669 susceptibility of the baseline clinical NS5B isolates indicated potent (low-nanomolar) antiviral activities against both GT1a and GT1b in the vast majority of the patient isolates. In general, there was limited variation in baseline susceptibility (mean EC₅₀s, 3.8 $\pm$ 1.3 and 6.8 $\pm$ 2.7 nM for GT1a and GT1b, respectively) of the different GT1 patient isolates to GS-9669. This limited variation was consistent with the limited variability in the virologic response to GS-9669 in patients during the study. However, there were a few outlier isolates with lower *in vitro* susceptibilities to GS-9669. These patients had known NS5B NNI RAVs at position M423 prior to treatment with GS-9669. Overall, four analyzed patients had NS5B NNI RAVs at baseline associated with reduced *in vitro* susceptibility to GS-9669. HCV RNA reductions of >2 log₁₀ were observed for three of these patients, but these antiviral responses were reduced relative to those of the patients without the M423 mutant in the same dosing group; no reduction in response was observed for the fourth patient with M423V, who received the highest dose of GS-9669 (>4-log₁₀ reductions in HCV RNA). No other NS5B variants observed at baseline showed reduced susceptibility *in vitro* or reduced responses *in vivo* to GS-9669.

Similar to other NNIs, RAVs were detected shortly after the suppression of the wild-type virus by GS-9669. Resistance variants were detected in patients at NS5B positions 419, 422, 423, 482, 486, and 494. These results are in good agreement with findings from the *in vitro* resistance selections, in which substitutions were also detected at positions 419, 422, 423, and 482. The variety of amino acid substitutions observed *in vivo* at position 419 was greater than that observed *in vitro*, with L419 substitutions to M/S/P/T/V/I observed *in vivo*. Although we did not observe resistance at positions 486 and 494 during the *in vitro* resistance selections, this may be due to the limited sequence diversity represented by the lab HCV strains compared with that of the quasispecies present in different HCV patients. A486V, R422K, and L419M were the predominant NS5B RAVs observed in viruses from GT1a and GT1b patients. Interestingly, M423 variants were only observed at lower GS-9669 doses (50 mg and 100 mg BID), agreeing with our *in vitro* resistance selections in which M423 RAVs were observed with the 5 $\times$ EC₅₀ treatment but not at the higher 20 $\times$ EC₅₀ treatment. The lack of clinical appearance of M423 RAVs at higher

GS-9669 doses also is in agreement with the low to moderate (<20-fold) resistance levels that M423 variants confer to GS-9669; collectively, these data imply clinical suppression of M423 variants by higher doses of GS-9669, as also evidenced by the antiviral responses observed among the four patients with these mutants at baseline. However, we note that the results from ongoing deep sequencing analysis of representative samples showed that M423 variants were detected at low frequencies (<3.1%) in about 40% of the patients treated with doses of 500 mg QD and BID in the GT1a patients and 500 mg QD in the GT1b patients (other groups were not tested). Nine of 12 GT1a patients and 7 of 7 GT1b patients still had detectable variants on day 17 (23), and L419I/M, M423T, and A486T/V appeared to persist longer as minority variants. R422K, with the lowest *in vitro* replication capacity, was not detected on weeks 24 and 48 in most patients.

RAVs at positions 419 and 422 that were selected with higher concentrations of GS-9669 *in vitro* were a good predictor of the major variants *in vivo* and were observed in GT1a and GT1b patients dosed with GS-9669. In contrast, in a monotherapy study with fildabuvir, RAVs at residue M423 were the predominant change associated with fildabuvir therapy (76% of patients treated), and a small number of patients had RAVs at positions 422 and 426 (13). Overall, most substitutions at positions 423 conferred a low level of resistance to GS-9669 and lomitabuvir, while higher levels of resistance to all three NNIs (GS-9669, fildabuvir, and lomitabuvir) were conferred by RAVs at positions 419, 422, and 486.

NS5B RAVs were observed by population sequencing in only one patient who received 50 mg QD of GS-9669 but in the majority of patients who received higher doses. This finding is likely explained by the degree of antiviral suppression at different GS-9669 doses. More substantial suppression of wild-type virus consequently resulted in more frequent detection of resistance variants (higher dose and greater wild-type HCV viral suppression). Consistent with other phase I HCV monotherapy studies, this observation suggests that RAVs preexist at low levels prior to treatment and become detectable once the wild-type population is sufficiently inhibited.

Drug-resistant variants were no longer detected by population sequencing in any of the GT1a patients at day 17, except for in one GT1a patient in the 500 QD cohort. For the GT1b patients with RAVs detected at earlier time points, RAVs were either no longer detectable in 4/13 patients at day 17 or were detected at a significantly lower percentage of the viral population in 9/13 patients. These results suggest a decreased fitness of these mutants *in vivo*, which is more readily observed in GT1a. Interestingly, the *in vitro* replication capacity of these mutants did not suggest any fitness differences between GT1a and GT1b RAVs, perhaps illustrating the limitations of assessing viral fitness *in vitro* using replicons.

Phenotypic analyses demonstrated that most viral isolates with multiple RAVs had reduced susceptibility to GS-9669 and lomitabuvir but wild-type susceptibility to other classes of HCV inhibitors, including sofosbuvir, GS-9451, ledipasvir, and ribavirin. These results are consistent with phenotypic analyses of site-directed recombinant replicons at positions 419, 422, 423, 482, 486, and 494, which displayed low to high levels of resistance to GS-9669 and lomitabuvir but remained sensitive to other classes of HCV inhibitors. Previous data from our group also showed similar results using different assays (11).

In summary, the highly effective inhibition of wild-type HCV by the site II NNI GS-9669 revealed variants that confer resistance

at NS5B positions 419, 422, 423, 482, 486, and 494. The patients with substitutions at position 423 at baseline achieved a $>2\text{-log}_{10}$ reduction in HCV RNA after 100 mg to 500 mg BID of GS-9669 treatment. This result is consistent with *in vitro* phenotypic analyses indicating that substitutions at position 423 confer only low to moderate levels of resistance to GS-9669, and it is also consistent with the selection of these mutants at low but not high levels of GS-9669 *in vitro*. The frequency of GS-9669 RAVs declined over 14 days off treatment, indicating reduced fitness of these RAVs compared with that of the wild type. The lack of cross-resistance between the GS-9669-resistant mutants and sofosbuvir, ledipasvir, GS-9451, and ribavirin makes GS-9669 a candidate for use in combination with these inhibitors in GT1 HCV-infected patients.

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