MAJOR ARTICLE

RG7128 Alone or in Combination with Pegylated Interferon-α2a and Ribavirin Prevents Hepatitis C Virus (HCV) Replication and Selection of Resistant Variants in HCV-Infected Patients

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Introduction. RG7128 (prodrug of PSI-6130) shows potent antiviral efficacy in patients infected with hepatitis C virus (HCV) genotypes 1, 2, or 3, with mean viral load decreases of 2.7 and 5 $\log_{10}$ IU/mL, respectively, associated with 1500-mg doses twice daily after monotherapy for 2 weeks and with 1000-mg and 1500-mg doses twice daily after treatment in combination with the standard of care (SOC) for 4 weeks.

Results. From 32 patients treated with RG7128 monotherapy for 2 weeks, marginal viral load rebound was observed in 3 HCV genotype 1–infected patients, whereas partial response was observed in 2 genotype 1–infected patients. From 85 patients receiving RG7128 in combination with SOC, 1 HCV genotype 1–infected patient experienced a viral rebound, and 2 genotype 3–infected patients experienced a transient rebound. Five genotype 1–infected patients had an HCV load of >1000 IU/mL at the end of 4-week treatment. No viral resistance was observed, per NS5B sequencing and phenotypic studies. PSI-6130 resistance substitution S282T needs to be present at levels of ⩾90% within a patient's quasispecies to confer low-level resistance. No evidence of S282T was found by population or clonal sequence analyses.

Conclusions. The requirement for a predominant S282T mutant quasispecies, its low replication capacity, and the low-level resistance it confers probably contribute to the lack of RG7128 resistance observed in HCV-infected patients.

Hepatitis C virus (HCV), a positive-strand RNA virus, is a member of the genus *Hepacivirus* and the leading cause of liver disease worldwide, with >170 million infected individuals [1]. The current standard of care (SOC), pegylated interferon-α in combination with ribavirin, has shown good clinical efficacy in patients infected with HCV genotypes 2 or 3, with ~80% of treated patients achieving a sustained viral response, but it is less efficacious in genotype 1–infected patients, with a sustained viral response in ~50% of cases. Thus, there is a clear need for more effective antiviral therapy for HCV infection [2, 3].

Direct-acting antivirals (DAAs) are currently being evaluated in the clinic, mainly directed against 2 essential viral enzymes, the NS3/4A protease protein and the NS5B polymerase protein [4, 5]. The most advanced HCV inhibitors are the NS3/4A protease inhibitors telaprevir and boceprevir, currently in phase III trials.

Among NS5B polymerase inhibitors, the nucleoside inhibitor, RG7128, a diisobutyl ester prodrug of β-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine (PSI-6130) [6] is the most advanced. It has shown efficacy in phase I trials in patients infected with HCV genotypes 1, 2, or 3, with maximal mean viral load decreases of 2.7 $\log_{10}$ IU/mL for 2-week monotherapy (1500 mg twice-daily) and 5 or 5.1 $\log_{10}$ IU/mL (1000 or 1500 mg twice-daily,

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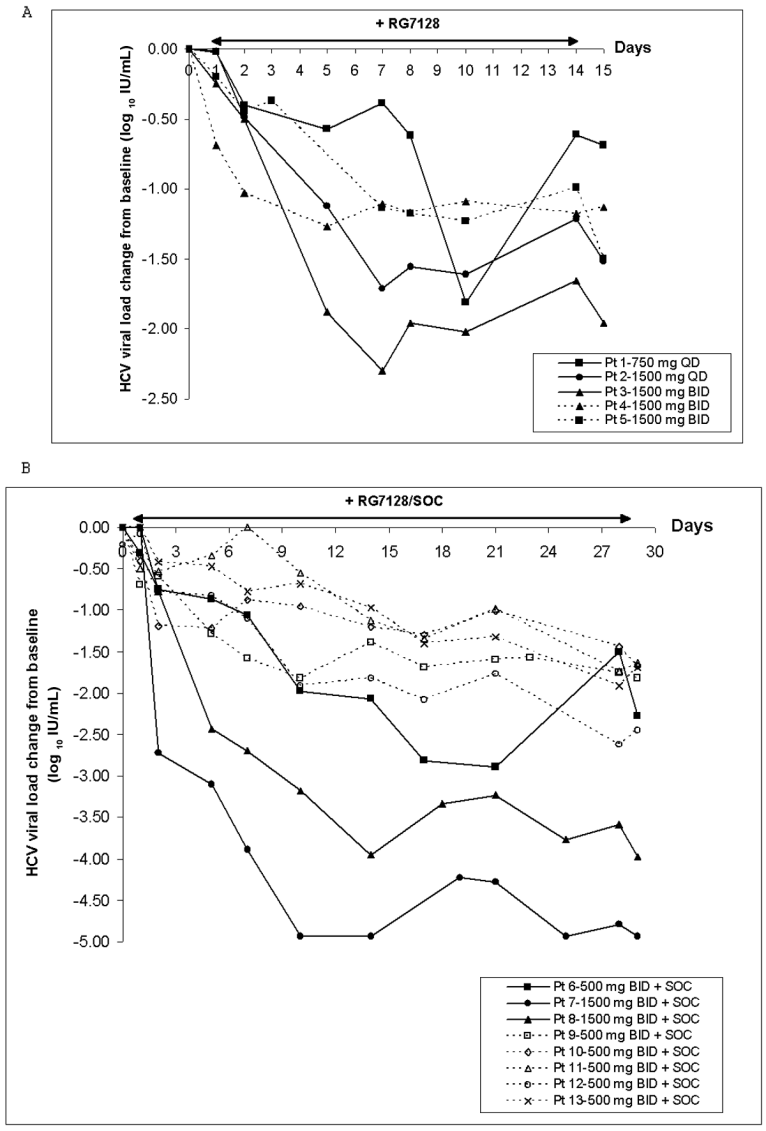

Figure 1. Hepatitis C virus (HCV) load change from baseline in patients monitored for viral drug resistance. *A,* In the RG7128 monotherapy study, 3 patients (patients 1–3, *solid lines*) showed a viral load rebound phenotype, and 2 (patients 4 and 5, *dotted lines*) showed an initial viral load decrease followed by a stabilization at ~1 $\log_{10}$ IU/mL below baseline during the last 8 and 10 days of treatment, respectively. *B,* In the RG7128 combination therapy study, 3 patients (patients 6–8, *solid lines*) showed a viral load rebound phenotype, and 5 (patients 9–13, *dotted lines*) showed a viral load of >1000 IU/mL at the end of treatment. BID, twice daily; Pt, Patient; QD, once daily. SOC, standard of care.

respectively) for 4-week combination therapy with the SOC; RG7128 is currently in phase II clinical development.

Because of the "quasispecies" nature of HCV [7], resistant variants to DAAs are likely to exist and to become rapidly selected during therapy if they have a replication advantage [8, 9]. Indeed, the emergence of resistance to DAAs has been observed in clinical trials for protease inhibitors telaprevir [10, 11], boceprevir [12], TMC435 [13], BI 201335 [14], and ITMN-191 [15] and for nonnucleoside polymerase inhibitors HCV-796 [16], VCH-916 [17], and filibuvir [18]. In contrast, no selection of resistance was observed in HCV-infected patients treated with the nucleoside analogs NM283 [19] or R1626 after 2-week monotherapy [20] or 4-week treatment in combination with the SOC [21], suggesting an apparent higher barrier to resistance for nucleoside inhibitors than for HCV protease and nonnucleoside inhibitors.

In vitro, low-level resistance to RG7128 is conferred by the selection of a serine-to-threonine substitution at position 282 in the NS5B polymerase gene [22], and it has been shown that in vitro emergence of resistance to nucleoside analogs, such as R1626 or RG7128, is slower than to other classes of DAAs [8, 23].

Here we report the studies performed to monitor for the potential development of RG7128 resistance in vivo duringthe

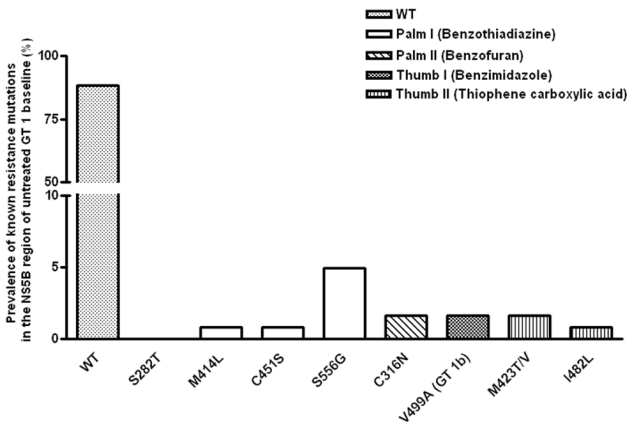

Figure 2. Prevalence of nonnucleoside inhibitor and RG7128 resistance mutations among the NS5B baseline population of patients with hepatitis C virus genotype 1 infection enrolled in RG7128 clinical trial P7081-5101. White bars, palm I–related amino acid residues; hatched bar, palm II–related amino acid residues; cross-hatched bar, thumb I–related amino acid residues; vertically lined bars, thumb II–related amino acid residues; dotted bar, wild type (all NS5B isolates without any NNI resistance mutations). GT, genotype; WT, wild type.

phase 1b study P7081–5101 after 2-week monotherapy in genotype 1–infected patients or after 4-week combination therapy with the SOC in HCV genotype 1–, genotype 2–, or genotype 3–infected patients. Population and clonal sequence analyses were performed, as well as phenotypic testing of NS5B from clinical isolates. In addition, studies to fully characterize the RG7128-associated resistance amino acid substitution S282T were performed, with results reported herein.

MATERIALS AND METHODS

Study design. The RG7128 clinical study was a multicenter, observer-blinded, randomized, placebo-controlled study in patients chronically infected with HCV genotypes 1, 2, or 3. The study was conducted in the United States (52 genotype 1a–, 20 genotype 1b–, 3 genotype 2–, and 5 genotype 3–infected patients), New Zealand (20 genotype 1a–, 6 genotype 1b–, 1 genotype 2–, and 10 genotype 3–infected patients), and Puerto Rico (17 genotype 1a–, 6 genotype 1b–, and 6 genotype 2–infected patients). All patients provided informed consent, and the study protocol conformed to the 1975 Declaration of Helsinki.

Clinical isolates obtained from other sources. Serum samples from untreated individuals infected with HCV genotypes 2, 3, or 4 were obtained from the American Red Cross (BBI Diagnostics). Untreated genotype 1 isolates (other than those from study P7081-5101) have been described elsewhere [8].

HCV RNA extraction, NS5B amplification, and cloning of NS5B clinical isolates into replicon vectors. HCV RNA extraction, reverse transcription, NS5B amplification, and cloning were carried out as described elsewhere [8].

Population and clonal sequencing of NS5B polymerase gene from clinical isolates. Population sequencing spanning the entire NS5B polymerase coding region was performed based on direct polymerase chain reaction products, as described elsewhere [8].

Plasmid constructions. Genotype 1a and 1b transient replicons that include restriction sites flanking the start and the end of the NS5B gene have been described elsewhere [8]. Amino acid substitution S282T was introduced by site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene), as described elsewhere [22]. All constructs were confirmed by double-stranded DNA sequencing.

Compounds. Compound PSI-6130, β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine was synthesized at Pharmasset. Stocks of 10 mmol/L were prepared in 100% dimethyl sulfoxide and stored at −20°C.

Determination of half-maximal effective concentrations. Briefly, 4 million cured Huh7 cells were transfected with 10 μg of in vitro transcribed replicon RNA from genotype 1b or genotype 1a reference strains or from clinical isolate–derived transient replicons, as described elsewhere [8].

RESULTS

Continuous viral decline observed in RG7128 monotherapy or combination therapy. Continuous viral load decline was observed in 27 (84%) of the 32 genotype 1–infected patients who received RG7128 monotherapy at doses of 750 mg once daily, 1500 mg once daily, 750 mg twice daily, or 1500 mg twice daily [24]. Only 3 experienced a viral load rebound (defined as a consistent increase in viral load $\geqslant$0.5 $\log_{10}$ IU/mL above nadir before the end of RG7128 treatment, with the nadir defined as a >0.5 $\log_{10}$ IU/mL decrease from baseline during treatment); patient 1 (750 mg once daily) experienced a viral load rebound of 1.2 $\log_{10}$ IU/mL, whereas patients 2 and 3 (both 1500 mg once daily) experienced apparent viral load rebounds of 0.5 and 0.6 $\log_{10}$ IU/mL, respectively, on the last day of treatment (Figure 1*A*). Two patients with genotype 1 infection (patients 4 and 5; 1500 mg twice daily) experienced a partial response, with an initial viral load reduction followed by stabilization of viral load at ~1 $\log_{10}$ IU/mL below baseline during the last 8 and 10 days of treatment, respectively (Figure 1*A*).

Of the 65 treatment-naive HCV genotype 1–infected patients receiving RG7128 plus the SOC for 4 weeks at doses of 500, 1000, or 1500 twice daily, only 1 patient (patient 6; 500 mg twice daily) experienced viral load rebounds (1.4 and 0.6 $\log_{10}$ IU/mL above nadir on the second-to-last and last days of treatment, respectively) (Figure 1*B*) [25, 26]. Two of the 20 genotype 2– or genotype 3–infected patients in the 1500 mg plus SOC dose group (patients 7 and 8, both genotype 3 infected) experienced a transient viral load rebound of 0.7 $\log_{10}$ IU/mL above nadir, although they achieved a reduction in viral load

Table 1. Sequence Comparison between Baseline and Treatment Time Points in NS5B Clinical Isolates from Patients Selected for Viral Resistance Monitoring in an RG7128 Clinical Study

Patient	HCV genotype	NS5B amino acid residue changes from baseline
1 (day 14)	1a	No change
2 (day 14)	1a	M423I
3 (day 14)	1a	No change
4 (day 14)	1b	F162F/Y, S218A/S, K254K/R, T267I/T, A/V400A, A442A/T, I585I/V
5 (day 14)	1b	K/M426M, H/Y452Y
6 (day 28)	1a	A/S15A, A73A/V, K/Q206Q, Q/R300R
6 (day 29)	1a	I/V11V, A/S15A, C/S110S, K/Q206Q, Q/R300R, D444A
7 (day 19)	3a	I432V
8 (day 21)	3a	A/V67V, V147M, A/V150A, I/T184I, S376N
9 (day 29)	1a	A16T, T235M, Q/R309R, A/V327V, S/T389T, I/V405V, A/T552T, F/S/Y588Y
10 (day 29)	1a	N/S130S, F/Y162F, S180G, S189N/S, N/S231S, K/R270R, A/V421A
11 (day 29)	1a	K510R, F/L572L
12 (day 29)	1a	E/K77K, C/S110S, A/V178V, S556G
13 (day 29)	1a	S470G/S, K523K/R, S543G/S, A553A/T

NOTE. For each patient, the sequences obtained at the treated time points were compared with the baseline sequence. HCV, hepatitis C virus.

of >5.0 $\log_{10}$ IU/mL (Figure 1*B*), with patient 7 reaching an undetectable viral load (<15 IU/mL) and patient 8 having a viral load of 169 IU/mL at the end of treatment. Five genotype 1–infected patients from the 500 mg twice daily plus SOC dose group (patients 9–13) experienced a slower decline in HCV load, with maximal HCV RNA reductions of 1.6–2.5 $\log_{10}$ IU/mL compared with baseline levels, and an HCV load of >1000 IU/mL at the end of treatment (Figure 1*B*). Development of viral resistance was monitored by performing NS5B sequencing and phenotypic analysis of samples from the patients described above, as well as sequencing for all patients at baseline to assess for potential markers of prediction of treatment response or failure.

NS5B population sequence analysis shows no S282T in samples from untreated and treated patients. To assess the potential existence of RG7128-associated resistance mutations before treatment, the population sequence spanning the entire NS5B polymerase-coding region was obtained for all patients at baseline, except for 1 HCV genotype 3–infected patient for whom the NS5B region could not be amplified by polymerase chain reaction; these included 121 genotype 1–, 10 genotype 2–, and 14 genotype 3–infected patients.

There was no preexisting NS5B S282T, the in vitro selected PSI-6130 resistance amino acid substitution [22], nor any common amino acid changes within the NS5B sequences of patients, at baseline. In contrast, 11% of the 121 genotype 1 isolates (Figure 2) and all of the genotype 2 and genotype 3 isolates contained ⩾1 amino acid substitution associated with resistance to nonnucleoside inhibitors [27].

The NS5B population sequences of on-treatment samples from RG7128-treated patients that were selected for resistance monitoring were analyzed and compared with sequences for their respective baseline samples (Table 1). No selection of NS5B S282T substitution was observed in any of the patients. With a single exception, all observed amino acid changes were present in only 1 patient and were unlikely to be treatment induced. The on-treatment NS5B sequence in 2 patients showed polymorphic changes at position 162 (F162F/Y and F/Y162F). Among the baseline sequences, 111 patients with genotype 1 infection carried F162, 7 carried Y162, and 3 had a mixture of both residues (F/Y162). These well-known polymorphisms at position 162 confer no resistance to RG7128.

Clonal sequence analysis shows no S282T within quasispecies of HCV-infected patients. To investigate whether RG7128-resistant variants could be detected in the quasispecies of patients before or after RG7128 treatment at low levels (and below the 20% limit of detection of population sequence), 2415 NS5B molecular clones (~90 clones/sample) were sequenced: 1165 from the 13 baseline isolates (11 genotype 1 isolates and 2 genotype 3 isolates) and 1250 from the RG7128-treated isolates (12 genotype 1 isolates and 2 genotype 3 isolates). None of the clones carried the S282T substitution. One of 85 sequences from the day 29 sample for patient 6 contained the amino acid substitution S282R (1.2%). Single clones found in 4 samples carried an S282G amino acid substitution; these included patient 4 on day 14 (1 [1.1%] of 94 clones), patient 5 on day 14 (1 [1.0%] of 96 clones), patient 7 on day 19 (1 [1.1%] of 89 clones), and patient 12 on day 29 (1 [1.2%] of 83 clones).

In contrast, and in agreement with data published else-

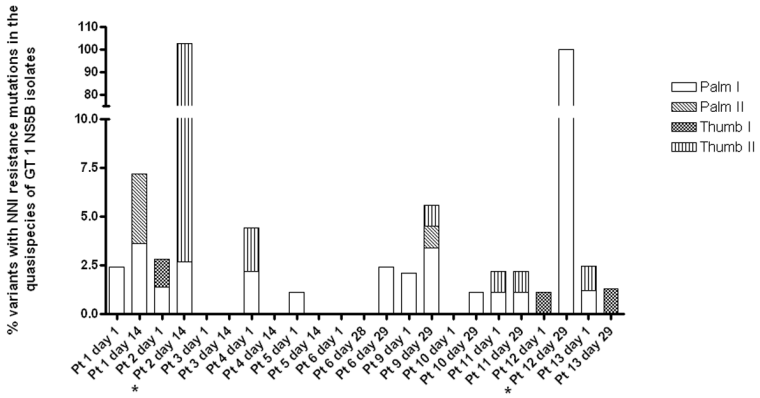

Figure 3. Prevalence of nonnucleoside inhibitor resistance mutations in the quasispecies of patients monitored for viral resistance (baseline and treatment time points). White bars, palm I–related amino acid residues; hatched bars, palm II–related amino acid residues; cross-hatched bars, thumb I–related amino acid residues; vertically lined bars, thumb II–related amino acid residues. Asterisks denote NNI resistance mutations (M423I and S556G) present at the population level. GT, genotype; Pt, patient.

where [8], variants containing nonnucleoside inhibitor resistance mutations were found in 8 of 11 genotype 1 baseline samples and in 8 of 12 RG7128-treated samples, at low frequencies (1%–4%) in most isolates but also as majority species in some patients (Figure 3; Table 1).

No phenotypic resistance to RG7128 after up to 4 weeks of treatment. Phenotypic analyses were performed on the baseline and on-treatment samples of the patients included in the viral resistance monitoring study to evaluate their susceptibility to PSI-6130, as described elsewhere [8]. All NS5B isolates were able to replicate in the transient system (data not shown) and were susceptible to PSI-6130, with similar half-maximal effective concentration (EC_{50}) values for on-treatment and baseline samples and reference replicons Con1 and H77 (Table 2).

Replication incompetence of NS5B variants containing S282R or S282G. To investigate whether variants containing S282R and S282G, observed at low frequency in some RG7128-treated patients, could help reduce susceptibility to PSI-6130, replicon clones containing S282R or S282G were characterized from the quasispecies of 4 samples: S282R from patient 6 at day 29 and S282G from patients 4 and 5 at day 14 and patient 7 at day 19. Three of the clones (S282R, patient 6 at day 29; S282G, patient 5 at day 14 and patient 7 at day 19) differed from the consensus sequence only at position 282 of the NS5B protein, but one (S282G, patient 4 at day 14) contained an additional amino acid change at position 162 (Y to F). The amino acid substitution S282G was also engineered into the Con1 reference transient replicon by site-directed mutagenesis. All mutant replicons were replication incompetent, whereas all clones containing the consensus sequence replicated and were susceptible to PSI-6130, with EC_{50} values similar to those obtained for the population sequence (data not shown). The S282R recombinant NS5B enzyme has been reported to have weak activity [28].

Effects of S282T engineered into clinical isolates on replication capacity and resistance to PSI-6130. The effect of the S282T substitution on 7 genotype 1a, 2 genotype 2b, 2 genotype 3a, and 3 genotype 4a NS5B clinical isolates was evaluated. Introduction of S282T resulted in a severe reduction in the replication capacity in all genotype 1a, 3, and 4 isolates (85%–99% reduction relative to the parent replicon) (Figure 4*A*) and completely abolished the replication in genotype 2 chimeric replicons. A 3–8-fold reduction in sensitivity to PSI-6130 was observed for all S282T mutant replicons (Figure 4*B*), whereas (as described elsewhere) S282T conferred a larger decrease in sensitivity to 2′-C-methylcytidine (60–90-fold EC_{50} shift; data not shown) [29].

High frequency of mutant S282T in viral population necessary for decreased sensitivity to PSI-6130. Given the low replication capacity and the low-level resistance conferred by the S282T amino acid substitution, the minimum frequency at which S282T variants need to be present in a viral population to cause a significant decrease in susceptibility to PSI-6130 was determined in vitro. Different ratios of each wild-type NS5B isolate and its corresponding S282T mutant were used to determine the EC_{50} values for PSI-6130. The study was performed using 10 genetically diverse isolates, 4 genotype 1b and 6 genotype 1a. As seen in Figure 5*A* for the genotype 1b isolates, a significant shift (>3-fold) was seen only when the S282T variant was present at frequencies of >90% for isolate RO-2 and >95% for isolates RO-3 and RO-11. For the Con1 sequence, a 100% frequency of S282T was necessary to produce a low-level effect (3.7-fold shift) on susceptibility to RG7128. In all genotype 1a isolates (Figure 5*B*), the S282T variant had to be

Table 2. Phenotypic Analysis of Replicons Containing NS5B Clinical Isolates from Patients Selected for Viral Resistance Monitoring in RG7128 Clinical Study

RG7128 dosage group and NS5B origin	HCV genotype	VL increase relative to nadir	Resistance to PSI-6130, EC_{50} ± SEM, μmol/L (no. of experiments)	EC_{50} relative to day 1
Reference replicon				
H77	1a	…	0.30 ± 0.02 (26)	…
Con1	1b	…	0.27 ± 0.03 (10)	…
750 mg once daily				
Patient 1 (day 1)	1a	…	0.25 ± 0.03 (3)	…
Patient 1 (day 14)	1a	1.2	0.33 ± 0.08 (3)	1.32
1500 mg once daily				
Patient 2 (day 1)	1a	…	0.15 ± 0.02 (3)	…
Patient 2 (day 14)	1a	0.5	0.20 ± 0.01 (2)	1.33
1500 mg twice daily				
Patient 3 (day 1)	1a	…	0.16 ± 0.01 (2)	…
Patient 3 (day 14)	1a	0.6	0.16 ± 0.01 (3)	1.00
Patient 4 (day 1)	1b	…	0.24 ± 0.04 (3)	…
Patient 4 (day 14)	1b	0.1[a]	0.31 ± 0.03 (3)	1.29
Patient 5 (day 1)	1b	…	0.33 ± 0.05 (4)	…
Patient 5 (day 14)	1b	0.24[a]	0.39 ± 0.06 (3)	1.18
500 mg twice daily plus SOC				
Patient 6 (day 1)	1a	…	0.31 ± 0.04 (5)	…
Patient 6 (day 28)	1a	1.4	0.35 ± 0.04 (4)	1.12
Patient 6 (day 29)	1a	0.6	0.23 ± 0.02 (5)	0.74
1500 mg twice daily plus SOC				
Patient 7 (day 1)	3a	…	0.54 ± 0.07 (5)	…
Patient 7 (day 19)	3a	0.7	0.57 ± 0.05 (3)	1.05
Patient 8 (day 1)	3a	…	0.54 ± 0.05 (6)	…
Patient 8 (day 21)	3a	0.7	0.71 ± 0.04 (4)	1.31
500 mg twice daily plus SOC				
Patient 9 (day 1)	1a	…	0.34 ± 0.06 (4)	…
Patient 9 (day 29)[b]	1a	0[c]	0.30 ± 0.06 (3)	0.88
Patient 10 (day 1)	1a	…	0.25 ± 0.04 (4)	…
Patient 10 (day 29)[b]	1a	0[c]	0.22 ± 0.02 (4)	0.88
Patient 11 (day 1)	1a	…	0.29 ± 0.02 (4)	…
Patient 11 (day 29)[b]	1a	0.11	0.27 ± 0.04 (3)	0.93
Patient 12 (day 1)	1a	…	0.57 ± 0.03	…
Patient 12 (day 29)[b]	1a	0.16	0.60 ± 0.002	1.05
Patient 13 (day 1)	1a	…	0.54 ± 0.04	…
Patient 13 (day 29)[b]	1a	0.22	0.41 ± 0.07	0.76

NOTE. EC_{50}, half-maximal effective concentration; HCV, hepatitis C virus; SOC, standard of care; SEM, standard error of the mean; VL, viral load.

[a] These patients were selected for viral resistance monitoring because they demonstrated initial viral load reduction followed by stabilization at ~1 $\log_{10}$ IU/mL below baseline during the last 8 or 10 days of treatment.

[b] Patients with an HCV load measurement >1000 IU/mL at the end of the dosing period.

[c] The nadir was observed on the last day of the dosing period.

present at 100% in the replicon population to produce a >3-fold reduction of sensitivity in a 3-day EC_{50} determination assay.

DISCUSSION

RG7128, a diisobutyl ester prodrug of PSI-6130 (β-D-2′-deoxy-2′-fluoro-2′-C-methylcytidine), has shown good antiviral efficacy in patients infected with HCV genotype 1, 2, or 3. Most patients (89%) experienced a continuous viral load decline, and only 6 (5%) experienced a marginal viral load rebound of ≤1.2 $\log_{10}$ IU/mL. These results highlight the potency of the nucleoside analog RG7128 across genotypes 1–3. In contrast, most of the nonnucleoside polymerase inhibitors, especially those

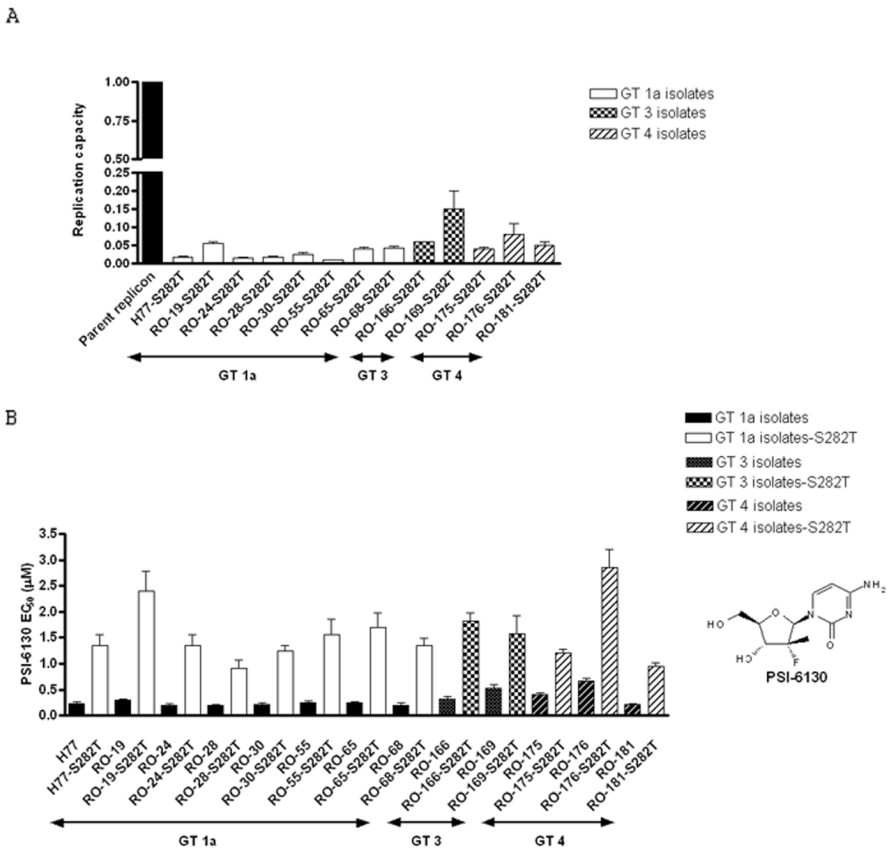

Figure 4. S282T mutation introduced in genotypes (GTs) 1a, 2, 3, and 4 confers low-level replication capacity and low-level resistance to PSI-6130. *A,* Replication capacity of chimeric replicons containing NS5B sequences from GT 1a, 3, and 4 clinical isolates with the S282T mutation. The 2 GT 2b isolates used in this study were found to be replication defective (not shown). *B,* Determination of half-maximal effective concentration (EC_{50}) for PSI-6130 using chimeric replicons containing NS5B sequences from GT 1a, 3, and 4 clinical isolates with the S282T mutation. A 3–8-fold reduction in sensitivity to PSI-6130 was observed for all S282T mutant replicons.

binding to the palm I or the thumb II sites of the NS5B, lack inhibitory activity in non–genotype 1 polymerases due to the existence of resistance polymorphisms at baseline [30, 31].

No evidence for the development of RG7128 resistance was observed in any sample from patients who experienced a viral load rebound ($n = 6$) or a partial response ($n = 2$) after either 2 weeks of monotherapy or 4 weeks of combination treatment with the SOC or who had a viral load of ⩾1000 IU/mL at the end of 4-week treatment ($n = 5$). No S282T mutation or any common amino acid change was found at the population (Table 1) or quasispecies level, and no decrease in phenotypic sensitivity to PSI-6130 was observed in any of the samples, highlighting the lack of genotypic and phenotypic resistance to RG7128 in vivo (Table 2). The only common change across patients, at position 162, has been observed in the public database, with 94.35% of the 672 samples having F162, 5.5% Y162, and 0.15% S162. The serine at position 282 is well conserved among all genotypes, and only a few isolates with a substitution at this position have been recorded in the public European HCV database (1 genotype 1b isolate with S282T [0.2%] and 1 genotype 1a isolate with S282R [0.4%]) [32].

Clonal sequence analysis of the NS5B coding region of a total of 2415 molecular clones (~90 clones per sample) showed no preexisting S282T amino acid substitution among the quasispecies of untreated patients, within the sequencing detection limit of minority variants present at a frequency of 1% [8]. Furthermore, no S282T mutation was found among samples from RG7128-treated patients, indicating the lack of selection and subsequent replication of RG7128-resistant variants (even at low levels) during treatment.

Amino acid substitutions S282R and S282G were found at very low frequencies (~1%) in the quasispecies of 5 RG7128-treated patients. Both substitutions were found to be replication defective in the replicon system, in agreement with findings of a previous study that observed weak polymerase activity of the S282R recombinant NS5B enzyme [28]. Furthermore, the samples that contained these variants within the quasispecies were fully susceptible to RG7128, suggesting that those changes are

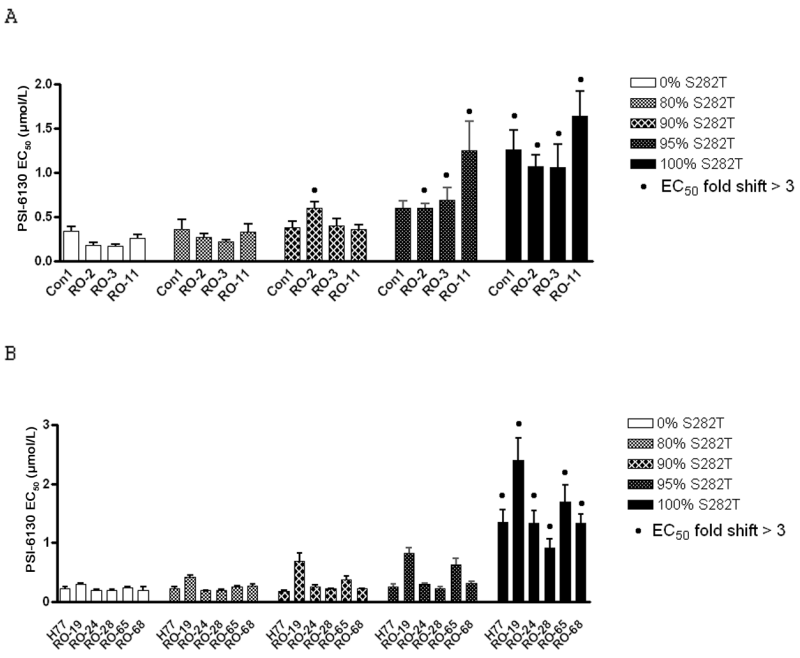

Figure 5. Decreased PSI-6130 sensitivity correlates with high frequency of S282T-resistant variant in replicon population. Different ratios of wild-type and S282T mutant transient replicons were transfected, and cells were incubated in the presence of PSI-6130. The half-maximal effective concentration (EC_{50}) was determined from ⩾2 independent experiments, as described in Materials and Methods. Four isolates of GT 1b origin (*A*) and 6 isolates of GT 1a origin (*B*) were used.

likely to be random mutations and unlikely to become predominant in the quasispecies. Recent sequencing studies have shown the high level of conservation at position 282: S282N substitution was found in 1 genotype 1a isolate (0.14%) among 729 genotype 1 samples, and S282R substitution in 1 (0.9%) of 108 genotype 3 isolates [27, 33]. The prevalence of substitutions at amino acid residue 282 in genotypes 2 and 4 has been observed at low levels (<1%).

The reason for the minimal viral load rebound observed in some patients is unclear; it could be due to variability of the HCV load quantification procedure. The variability in viral load measurements was calculated for the 8 placebo-treated patients in the monotherapy arm. An average maximal variation in viral load quantification ± 1 SD of 1 $\log_{10}$ IU/mL (confidence interval, 0.87–1.17) was calculated. This indicates that the use of a 0.5 $\log_{10}$ UI/mL increase above the nadir as the criterion for the classification of patients experiencing viral load rebound is within the variability of the assay and therefore that some of the observed minimal increases in viral load could be due to this variability.

The short duration of combination therapy (4 weeks) could explain why 5 of the 85 patients who received the triple therapy did not achieve an HCV load of <1000 IU/mL at the end of the RG7128 treatment. Variability in exposure may also contribute to viral load rebounds of small amplitude, especially transient rebounds.

To understand the underlying mechanism for the lack of selection of RG7128 resistance in the clinic, the replication capacity and the level of resistance that the S282T mutant confers in diverse genetic contexts were evaluated in vitro. When introduced into clinical isolates from genotypes 1–4, the S282T mutation showed a significant decrease in replication in genotype 1, 3, and 4 isolates and abolished the replication of genotype 2 isolates. Low-level resistance to PSI-6130 (3–8-fold EC_{50} shift) was observed in S282T-containing genotype 1a, 3, and 4 chimeric replicons, confirming the effect of S282T observed in genotype 1b isolates (Figure 4*B*) [22]. It has been shown in the transient replicon system that a resistance mutation that confers high replication capacity and a high level of resistance (40-fold EC_{50} shift) needs to be present in ⩾25% of the population to produce an observable decreased sensitivity to the compound [8]. Due to the low-level replication and low-level resistance to PSI-6130 conferred by the S282T mutation in both genotype 1a and 1b isolates, a decrease in susceptibility to PSI-6130 was observed only when the S282T mutation was present in the population at a high proportion (>90%) in genotype 1b isolates (Figure 5*A*). In all genotype 1a isolates (Figure 5*B*), 100% of the mutant population was needed to cause an observable decrease in PSI-6130 potency, which would therefore be easily detectable by standard population sequencing.

The observed low replication capacity and level of resistance conferred by the S282T substitution probably accounts for

the lack of selection in vivo, even at low frequencies among RG7128-treated patients. However, we cannot exclude the possibility of selection in vivo of compensatory mutations in the NS5B gene that could increase the viral replication of variants carrying S282T and facilitate their emergence during prolonged therapy, as seen in the in vitro resistance selection studies [22]. None of the NS5B sequences (baseline or treated) contained the combination of amino acid substitutions observed during the in vitro resistance selection, which was shown to partially rescue the replication of the S282T variant in vitro.

The short duration of the monotherapy phase (14 days) and the combination with the SOC might not be adequate to observe the emergence of RG7128 resistance. Indeed, in the NM283 clinical trial, resistance mutation S282T appeared only in ~2% of the patients and after prolonged monotherapy (~6 months) [19].

In conclusion, RG7128 leads to a high rate of rapid virological response, with no selection of drug resistance after up to 4 weeks of treatment in combination with the SOC. Additional clinical studies are underway to assess the efficacy and safety of longer-term combination treatment.

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