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Sofosbuvir susceptibility of genotype 1 to 6 HCV from DAA-naïve subjects

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ABSTRACT

High sequence diversity of HCV may lead to variation in susceptibility to antiviral agents amongst different genotypes and subtypes of the virus. We assessed the susceptibility to sofosbuvir of chimeric replicons carrying the full length NS5B coding region from 479 HCV infected, treatment-naïve patients, including 15 subtypes in 6 genotypes. NS5B replicon vectors with subtype 1b, subtype 4a and subtype 6a backbone were modified to support testing of patient samples. We also evaluated sofosbuvir susceptibility in a panel of 331 replicons containing engineered NS5B inhibitor resistance-associated substitutions. The mean 50% effective sofosbuvir concentration (EC_{50}) amongst different genotypes ranged from 32 (subtype 2a) to 130 nM (genotype 4); while some variation in susceptibility amongst patient isolates was observed, the 95th percentile for any genotype did not exceed 189 nM. Levels of resistance to sofosbuvir in replicons containing S282T were between 2.4 and 18 fold-change in EC_{50}; no other single NS5B resistance-associated substitution demonstrated reduced sofosbuvir susceptibility. These data suggest that S282T is the only known substitution that confers detectable resistance to sofosbuvir in vitro. Sofosbuvir displayed potent antiviral activity across a diverse range of NS5B mutants and HCV clinical isolates in multiple subtypes of genotypes 1 to 6.

1. Introduction

Recent studies report an estimated global prevalence of viremic HCV of 1% in 2015, corresponding to 71.1 million viremic infections (Mohd Hanafiah et al., 2013; Polaris Observatory HCV Collaborators, 2017). Of those affected, an average of 80% develop chronic hepatitis, which can lead to steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (Tong et al., 1995). HCV is a heterogeneous virus comprised of eight genotypes and over 67 assigned subtypes (Borgia et al., 2018; Smith et al., 2014). Extensive genetic variation exists between different HCV genotypes and subtypes: genotypes differ from each other by 31%–33% at the nucleotide level, and subtypes differ from each other up to 25%. Furthermore, 5–8% sequence divergence was observed between individual strains of HCV within a given subtype (Simmonds et al., 2005; Zein, 2000). Such a high degree of natural sequence variation may have an impact on the susceptibility of patient isolates to HCV inhibitors, possibly influencing the clinical response to antiviral agents. Therefore, it is of interest to evaluate the susceptibility of a variety of circulating HCV strains to new and approved inhibitors.

Inhibitors of the HCV NS5B RNA polymerase are classified into nucleoside/nucleotide inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (Ma et al., 2005; Sarisky, 2004; Tomei et al., 2005). The NIs mimic natural polymerase substrates and bind to the NS5B active site, causing chain termination. These inhibitors tend to have similar efficacy across all HCV genotypes, because they bind to a well-conserved site. NNIs bind to one of four less conserved allosteric sites, inhibiting important conformational changes in the polyprotein replication complex that are necessary for the catalytic efficiency of the enzyme's active site (Kwong et al., 2008).

Sofosbuvir (SOF), is a NS5B polymerase NI with a high barrier to resistance and antiviral activity across all genotypes (Gane et al., 2013; Jacobson et al., 2013; Kowdley et al., 2013; Lawitz et al., 2013a, 2013b; Poveda et al., 2014; Svarovskaia et al., 2012). SOF is approved for use with other agents for the treatment of chronic hepatitis C infection in genotype (GT) 1–6 and has demonstrated clinical efficacy in combination with other indirect (Doss et al., 2015; Jacobson et al., 2013; Kowdley et al., 2013; Lawitz et al., 2013a; Rodriguez-Torres et al., 2013) and direct-acting antivirals (DAA)s (Bourliere et al., 2017; Feld et al., 2015; Foster et al., 2015; Gane et al., 2016; Jackson and Everson, 2017; Lawitz et al., 2017). SOF has EC_{50} values of 14–110 nM against genotype 1a, 1b, 2a, 2b, 3a, 4a, 5a, and 6a in laboratory replicons (Dvory-Sobol et al., 2014a).

Resistance to SOF is conferred by the S282T substitution in NS5B (Lam et al., 2012); S282T was first described as the major resistance-

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associated variant for other NIs (Dutartre et al., 2006; Ludmerer et al., 2005; Migliaccio et al., 2003; Stuyver et al., 2006). Other substitutions have also been implicated in resistance to NIs: the combination of S96T and N142T was observed following in vitro selection with the investigational NI, R1479 (Le Pogam et al., 2006), M289I/L/V was selected in vitro by various NIs (Cheng et al., 2011; Lam et al., 2012), a combination of L159F and L320F was observed in one patient who had a partial response during treatment with mericitabine, interferon and ribavirin (Tong et al., 2012), and L159F and V321I were observed in a few (15% and 5%) patients with virologic failure in SOF clinical studies (Svarovskaia et al., 2016). NS5B NI resistance-associated substitutions (RASs) are observed at very low frequencies in untreated patients, compared to RASs for NS5B NNI, NS3 protease and NS5A inhibitors (Dietz et al., 2018; Sarrazin et al., 2016; Welzel et al., 2017; Zeuzem et al., 2017). Amongst 2144 patients who participated phase 2 and 3 clinical studies, 2.5% had NS5B NI RASs at baseline, though none had S282T. In contrast, 16% and 54% of the patients had baseline NS5A and NS3 RASs, respectively. All of the patients who had NS5B NI RASs at baseline achieved sustained virological response (Sarrazin et al., 2016).

In the present study, we evaluated SOF susceptibility of HCV from genotypes 1 to 6 in 479 untreated patients using three NS5B replicon vectors in a transient transfection susceptibility assay. We also evaluated SOF susceptibility in a large panel of NS5B NI mutants selected based on NS5B polymorphisms that emerged during in vitro SOF selection studies and in patients who received NI treatments in clinical studies.

2. Materials and methods

2.1. Clinical samples

All patients included in these analyses provided written informed consent for enrollment in clinical studies run by Gilead Sciences (Foster City, CA). The samples tested in this study were collected at baseline from DAA treatment-naïve patients enrolled in Gilead-sponsored studies GS-US-257-0102 (Dvory-Sobol et al., 2014b), GS-US-334-0107 (Jacobson et al., 2013), GS-US-334-0108 (Jacobson et al., 2013), GS-US-334-0110 (Lawitz et al., 2013b), GS-US-334-1119 (Naggie et al., 2015), GS-US-342-1138 (Feld et al., 2015), GS-US-367-1171 (Bourliere et al., 2017), GS-US-367-1172 (Jacobson et al., 2017), P2938-0721 (Svarovskaia et al., 2012, 2014), P7977-0221 (Rodriguez-Torres et al., 2013), P7977-0523 (Gane et al., 2014), P7977-0724 (Kowdley et al., 2013), P7977-1231 (Lawitz et al., 2013b). The study protocols followed the ethical guidelines set in place by the 1975 Declaration of Helsinki and were approved by the relevant institutional review board committees.

2.2. Cell lines

The Huh 7 1C cell line was generated from a lunet-based genotype 1a replicon clone (Robinson et al., 2010). The clone was cured by treatment with interferon, BILN2061 and VX222 to generate the 1C cell line. 1C cells have been intensively validated for a complete cure and no surviving colonies have been observed after G418 treatment (Peng et al., 2013). 1C cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Carlsbad, CA) supplemented with fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were maintained at 37 °C in humidified incubators (85% humidity) and 5% CO_2.

2.3. Compounds

Sofosbuvir (SOF; GS-7977) was synthesized by Gilead Sciences, Inc. (Foster City, CA, USA).

2.4. Sequencing of the NS5B coding region from patient samples

Total RNA isolation, cDNA synthesis, and PCR amplification of NS5B were performed by DDL Diagnostic Laboratory (Rijswijk, The Netherlands).

Reverse transcription polymerase chain reaction (RT-PCR) products spanning the entire NS5B coding region from patient plasma were generated and sequenced using a variety of standard (Sanger) and next-generation sequencing (Illumina, Inc., San Diego, CA) methods at DDL Diagnostic Laboratory (Rijswijk, The Netherlands), WuXi AppTec (Shanghai, China) or Monogram Biosciences (San Francisco, CA). Internally-developed software (Gilead Sciences) was used to process and align sequencing data to identify the substitutions present at more than 1% of reads. The presence of variants in GT1 to 6 samples was established by comparison with the wild-type reference sequence: H77 for subtype 1a (Genbank accession ID NC_004102); Con1 for subtype 1b (AJ238799), JFH-1 for subtype 2a (AB047639), S52 for subtype 3a (GU814263), ED43 for subtype 4a (GU814265), SA13 for subtype 4a (AF064490), and EUHK2 for subtype 6a (Y12083).

NS5B NI resistance associated substitutions (RASs) were defined as any substitution that confers > 2.5-fold reduced susceptibility to any NS5B NI in vitro in one or more genotypes of HCV, or that emerged in patients who failed SOF-based regimens, using Sanger-based sequencing or deep sequencing with a threshold of 15%: S96T, N142T, L159F, E/N237G, any change at position 282, C/F/M289I/L, L320F/I/V, and V321A/I.

2.5. Site-directed mutagenesis

Replicons with specific amino acid substitutions were generated by site-directed mutagenesis in genotype 1 to 6 replicons (see Supplemental Methods). Mutants were generated by using a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), following the manufacturer's instructions, and mutations were confirmed by DNA sequencing. The NS5B region containing the introduced mutant was subcloned back into a wild-type replicon plasmid to avoid unwanted extraneous mutations.

2.6. Transient transfection and susceptibility assay

In order to characterize patient isolates, restriction sites were added to existing NS5B replicon (see Supplementary Materials). A subtype 1b vector was used for patient samples of all genotypes except 4 and 6, for which a subtype 4a or 6a vector, respectively, was used.

A second amplification of NS5B PCR products used for sequencing was performed using PCR primers for each subtype and the High Fidelity PCR Master kit (Roche Diagnostics, Dallas, TX, USA) at Gilead Sciences. The sequences of the PCR primers for the second PCR are listed in the Supplementary Materials. PCR products were purified, and transferred to the replicon vector using 200 ng of the vector DNA and 50 ng of insert DNA with the In-Fusion HD EcoDry Cloning kit (Clontech, Mountview, CA) according the supplier's instructions. Plasmid DNA was isolated and RNA was synthesized from the linearized DNA using the T7 RiboMAX Express Large Scale RNA Production system (Promega, Madison, WI) according to the supplier's instructions.

Replicon RNA was transfected into 1C cells as described (Lohmann et al., 1999). Briefly, cells were mixed with 10 µg of replicon RNA and subjected to electroporation using settings of 960 µF and 270V. Cells were seeded into 96-well plates at 100 µL/well. Antiviral compounds were serially diluted in 100% DMSO and then added to the cells at a 1:200 dilution 24 h post-transfection, achieving final concentrations of 0.5% DMSO in total volumes of 200 µL per well. Cells were cultured for 3 days at 37 °C in the presence of compounds, 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). EC_{50} values were

Table 1
RAS detected in untreated patient samples.

Subtype	RAS[a]	mean EC_{50} (nM) ± SD (N)	mean EC_{50} (nM) ± SD, no RASs[b] (N)
1b	L159F	106 ± 35.8 (4)	92 ± 33 (78)
1b	V321I	129 ± 35.1 (5)	
2a	M289L	30 (1)	32 ± 9.4 (14)
2b	M289I	54 ± 20 (3)	42 ± 20 (19)
4a	E237G	163 ± 0.4 (2)	130 ± 29 (31)[c]
4r	V321I	129 (1)	
6e	M289L	101 (1)	85 ± 16 (6)[d]

[a] Data are shown for patient samples with the indicated RAS if it was present at > 90% in deep sequencing data or unmixed in Sanger sequencing data.
[b] From pre-treatment samples.
[c] Genotype 4, all subtypes.
[d] Genotype 6, all subtypes.

calculated using a GraphPad Prism software package (La Jolla, CA) by nonlinear regression analysis. Mean EC_{50} was calculated from at least 2 experiments for most samples. Fold-change in EC_{50} for site-directed mutants was calculated as the ratio of mutant to wild-type replicon.

3. Results

3.1. NS5B replicon compatibility for testing of clinical isolates

To evaluate the susceptibility to SOF of NS5B from HCV infected patients, we used subtype 1b, 4a or 6a NS5B replicon vectors into which to transfer NS5B from GT 1 to 6 patient samples. To enable reliable susceptibility determination, luciferase activity in the transient transfection assay should be at least 25-fold over background; background activity is typically about 100 relative light units (RLU). The subtype 1b vector containing patient-derived NS5B sequences from all tested subtypes met this condition, except subtypes 4r, 4t, 6a, and 6e, which generated only 450 to 1500 RLU. However, replicons derived from genotype-matched backbones (subtype 4a vector for subtype 4r and 4t patient sequences, and subtype 6a for subtype 6a and 6e patient sequences) did replicate well (luciferase activity ranged from 10^5 to 10^6 RLU).

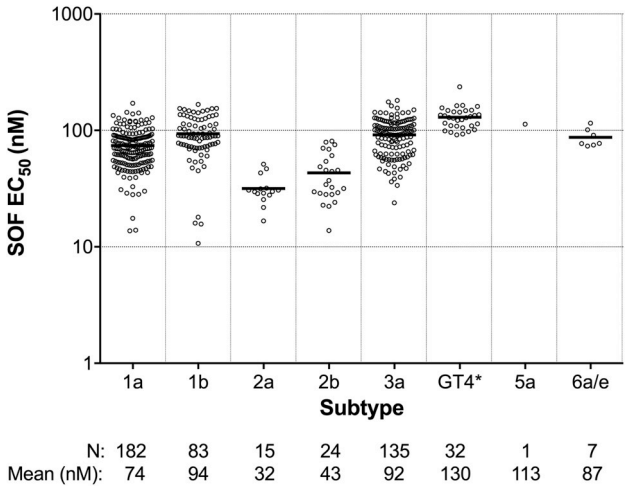

Fig. 1. Susceptibility to SOF of patient-derived NS5B. Horizontal bars indicate the mean for each group. *For genotype 4, subtypes 4a, 4b, 4d, 4k, 4m, 4r and 4t were represented at least once.

3.2. SOF susceptibility and NS5B RASs in clinical isolates

We evaluated the SOF susceptibility of clinical isolates from treatment-naïve patients by transferring the NS5B polymerase coding region from HCV in plasma samples into a compatible replicon-based vector. The resulting pool of replicon variants was used for susceptibility testing in a transient transfection replication assay. NS5B from 479 patient isolates belonging to GT1 to 6 were tested. SOF EC_{50} values (mean ± SD) ranged from 32 ± 9.0 nM for subtype 2a (n = 15) to 130 ± 29 nM for genotype 4 (subtypes a/b/d/k/m/r/t, n = 32) (Fig. 1). Low to moderate variation in susceptibility within genotype was observed, with differences between the 95th and 5th percentiles ranging from 1.6- to 6.5-fold (data not shown). The 95th percentile was never higher than 189 nM, and the highest individual patient sample EC_{50} was 237 nM.

The NS5B coding region from the clinical isolates was sequenced to determine whether any pre-existing RASs were present. Overall, 3.8% (18/479) of the sequences contained one or more RASs. No patient isolates were identified with S96T, N142T, S282T, L320F/I/V or V321A. No RASs were detected in subtype 1a, 3a, or 5a patient samples, while between one and five samples with RASs were identified for the other genotypes: one each for subtypes 2a (M289L), 4r (V321I), and 6e (M289L), two for subtype 4a (E237G), four for subtype 2b (all with M289I), and nine for subtype 1b (four with L159F and five with V321I). The SOF susceptibilities of samples in which the RASs were present as the predominant variant (i.e. no wild-type detected by Sanger sequencing, or > 90% mutant by deep sequencing, 17 of the 18 patients) are shown in Table 1. SOF EC_{50} values for the samples with RAS were less than 2-fold different than the mean EC_{50} of samples from the same genotype that lacked detectable RASs (Table 1).

3.3. In vitro resistance analysis of NS5B with RAS

To complement the susceptibility results from patient isolates, and to determine the SOF susceptibility of NS5B NI RASs and other substitutions of interest not observed in patient samples, each substitution was introduced by site-directed mutagenesis in replicons from 8 subtypes belonging to genotypes 1 to 6. S282T conferred a reduction in SOF susceptibility between 2.4 (subtype 2a) and 18 (subtype 5a) in the different subtypes tested (Table 2). No other RAS conferred greater than 2.3-fold reduction in SOF susceptibility in any subtype (Table 2). In addition to the 12 RASs shown in Table 2, a larger panel of mutants was prepared in GT1 to 6 replicons, that included NS5B substitutions that were observed in (1) treatment-naïve subjects, at resistance-associated positions (positions 96, 142, 159, 237, 282, 289, 320 and 321), (2) substitutions that were observed in patients with genotype 1 and were tested in other genotypes, or (3) substitutions that were observed in

Table 2
Effect of Selected NS5B RASs on SOF Susceptibility in vitro.

	Mean fold-change in SOF EC_{50} ± SD[a]											
Subtype	96T	142T	159F	237G	282T	289C	289F	289I	289L	289M	320F	321A
1a	0.3 ± 0.04	0.6 ± 0.25	1.2 ± 0.01	1.3 ± 0.2	**8.4** ± 2			0.8	1.2	0.5 ± 0.19	1.8 ± 0.2	1.2 ± 0.07
1b	0.8 ± 0.12	1.0 ± 0.17	1.3 ± 0.3	1.0 ± 0.01	**8.8** ± 1.9			1.2	1.3	1.5 ± 0.06	1.7 ± 0.17	1.4 ± 0.07
2a			1.1 ± 0.17	1.3 ± 0.13	2.4 ± 0.1			1.5 ± 0.09	1.9 ± 0.08		0.9 ± 0.15	0.9 ± 0.06
2b	0.9 ± 0.03	0.9 ± 0.05	1.6 ± 0.5	1.1 ± 0.09	**16** ± 0.8	1.7 ± 0.39	1.2 ± 0.01	1.1 ± 0.27	1.5 ± 1.22			
3a	1.0 ± 0.04	0.7 ± 0.06	1.3 ± 0.02	1.1 ± 0.05	**3.5** ± 0.3				0.5 ± 0.3	0.7 ± 0.24		1.3 ± 0.3
4a				0.9 ± 0.28	**6.1** ± 0.3				1.6 ± 0.75	1.6 ± 0.13	0.79	1.8 ± 0.72
5a		1.2 ± 0.11	1.4 ± 0.03	1.5 ± 0.08	**18** ± 3.1			1.5 ± 0.23	2.3 ± 0.13		1.7 ± 0.07	1.8 ± 0.05
6a			1.6 ± 0.34	1.1 ± 0.07	**9.1** ± 0.07	0.8 ± 0.07	0.98		1.1 ± 0.03		2.1 ± 0.11	1.9 ± 0.33

Values greater than 2.5 are highlighted inbold type.
[a] Means are derived from two independent experiments, unless no SD is shown (these values are from a single experiment).

patients with virologic failure at baseline or at the time of the virologic failure (Supplementary Material Tables S1 and S2). Of the additional 186 single mutants (158 different RASs in one or more of 8 different subtype replicons) that replicated sufficiently well to enable susceptibility determination, none showed reduced susceptibility to SOF (fold change in EC_{50} compared to original replicon < 2.5-fold; Table S1). Amongst the 25 mutants with two or more RASs that replicated, 13 showed no reduction in SOF susceptibility, while amongst the 12 double mutants that contained S282T in combination with another NS5B substitution, EC_{50} fold change ranged from 3.0 to 15 (Table S1). The SOF fold change for these double mutants was not significantly increased compared to the single S282T mutant.

4. Discussion

SOF demonstrated potent antiviral activity in a large collection of samples from DAA-naïve patients. While small differences in mean EC_{50} between genotypes were observed, they are unlikely to be clinically meaningful, since no difference in response to SOF-containing regimens has been reported in patients infected with any particular genotype. For example, the mean EC_{50} measured in vitro was highest for genotype 4 (130 nM), but response rates in patients infected with genotype 4 HCV are high (Ajlan et al., 2016; Doss et al., 2015; Lawitz et al., 2013b), and reduced response rates in such patients compared to other subtypes have not been reported.

The only NS5B RAS that conferred reduced susceptibility to SOF in the transient transfection assay was S282T. In patients treated with SOF with or without ribavirin on pegylated interferon, one patient out of 1662 treated (0.06%) or out of 300 (0.33%) with virologic failure had S282T (Svarovskaia et al., 2014). In patients treated with SOF or SOF and ledipasvir (LDV) with or without ribavirin, 10 patients out of 12,012 treated (0.08%) or out of 978 (1.02%) with virologic failure had S282T (Gane et al., 2017). None of the 20 virologic failures (1.1% of the 1778 treated patients) treated with SOF and velpatasvir had S282T (Hezode et al., 2018). S282T is not observed in patients who have not been treated with NIs, even when sensitive methods are used with detection thresholds as low as 1% (Gane et al., 2017). Our results are similar to previous reports of the impact of S282T on susceptibility to SOF (Gane et al., 2017; Lam et al., 2012; Pham et al., 2018; Ramirez et al., 2016; Svarovskaia et al., 2014) and other NIs (Klumpp et al., 2006; Lam et al., 2010; Le Pogam et al., 2006; Ludmerer et al., 2005; Migliaccio et al., 2003).

Other RAS and combinations of RASs not containing S282T have been reported to be associated with failure of NI-containing regimens, including N142T, L159F, M289I/L (Svarovskaia et al., 2016), L159F + V320F (Tong et al., 2014), L159F + C316N (Donaldson et al., 2015; Svarovskaia et al., 2016), V321A (Donaldson et al., 2015; Svarovskaia et al., 2016) and E237G (Charlton et al., 2016; Sarrazin et al., 2014). A 2.6- (subtype 1b) to 4.3-fold (subtype 1a) increase in SOF EC_{50} was reported for replicons containing L159F + V320F (Tong et al., 2014); while the results reported here are similar for subtype 1b (EC_{50} fold change: 2.0), for subtype 1a in our system no reduction in susceptibility was observed (EC_{50} fold change: 1.2). Differences in vitro susceptibility measurements could be related to variation in cell lines, replicon vectors, or assay conditions. (Paquet et al., 2012). L159F, when present at baseline, has no impact on response to SOF/LDV (Sarrazin et al., 2016). Our results showing no impact of RAS other than S282T on susceptibility to SOF are in agreement with another recent report for L159F, L320I, V321A, and E237G (Wyles et al., 2017).

In summary, SOF had potent antiviral activity across a diverse range of HCV clinical isolates belonging to all six genotypes. Of 160 NS5B substitutions that were evaluated, only S282T conferred reduced susceptibility to SOF.

Conflicts of interest

All authors are employees of Gilead Sciences.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104574.

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