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NS5A Sequence Heterogeneity and Mechanisms of Daclatasvir Resistance in Hepatitis C Virus Genotype 4 Infection

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Background. Daclatasvir is an NS5A inhibitor approved for treatment of infection due to hepatitis C virus (HCV) genotypes (GTs) 1–4. To support daclatasvir use in HCV genotype 4 infection, we examined a diverse genotype 4–infected population for HCV genotype 4 subtype prevalence, NS5A polymorphisms at residues associated with daclatasvir resistance (positions 28, 30, 31, or 93), and their effects on daclatasvir activity in vitro and clinically.

Methods. We performed phylogenetic analysis of genotype 4 NS5A sequences from 186 clinical trial patients and 43 sequences from the European HCV database, and susceptibility analyses of NS5A polymorphisms and patient-derived NS5A sequences by using genotype 4 NS5A hybrid genotype 2a replicons.

Results. The clinical trial patients represented 14 genotype 4 subtypes; most prevalent were genotype 4a (55%) and genotype 4d (27%). Daclatasvir 50% effective concentrations for 10 patient-derived NS5A sequences representing diverse phylogenetic clusters were ≤0.080 nM. Most baseline sequences had ≥1 NS5A polymorphism at residues associated with daclatasvir resistance; however, only 3 patients (1.6%) had polymorphisms conferring ≥1000-fold daclatasvir resistance in vitro. Among 46 patients enrolled in daclatasvir trials, all 20 with baseline resistance polymorphisms achieved a sustained virologic response.

Conclusions. Circulating genotype 4 subtypes are genetically diverse. Polymorphisms conferring high-level daclatasvir resistance in vitro are uncommon before therapy, and clinical data suggest that genotype 4 subtype and baseline polymorphisms have minimal impact on responses to daclatasvir-containing regimens.

Keywords. NS5A; HCV; resistance; polymorphism; daclatasvir.

Hepatitis C virus (HCV) is genetically diverse, with 7 recognized genotypes [1]. HCV genotype 4 represents approximately 8% of chronic HCV infections worldwide; it is most prevalent in the Middle East and North and Central Africa, representing the most common HCV genotype in many countries in these areas [2–4]. Recently, genotype 4 prevalence has increased in Europe and has been reported in Asia and North and South America [2, 4–6]. Genotype 4 is highly heterogeneous, with 17 recognized subtypes and other subtypes awaiting assignment. The relative prevalence of genotype 4 subtypes varies geographically [7].

The introduction of direct-acting antiviral (DAA) agents has markedly improved therapeutic outcomes for patients with chronic HCV infection [8]. However, most studies of new HCV therapies have focused on genotype 1, in part because

of its predominance in North America, eastern Asia, and most European countries [2]. Despite its substantial global prevalence, genotype 4 has received limited attention with respect to basic virologic and therapeutic research. Recently, several DAA-based regimens have been evaluated in genotype 4 infection, including combinations of a DAA with pegylated interferon alfa and ribavirin (pegIFN/RBV) [9–12] and more recently, all-oral, pegIFN-free regimens [13–16]. Consistent with results in genotype 1 infection, DAA-based regimens have demonstrated greater antiviral efficacy than pegIFN/RBV in genotype 4 infection.

With genotype 1 infection, there is extensive evidence that genotype 1 subtype (1a or 1b) influences the efficacy and barrier to resistance of some DAAs and DAA-based HCV therapeutic regimens [11, 17–20]. However, the potential influence of genotype 4 subtype on the efficacy and resistance profiles of DAA-based therapeutic regimens has not been reported. Owing to the heterogeneity of genotype 4 and the marked geographic differences in the prevalence of genotype 4 subtypes, the potential impact of these differences on antiviral therapies needs to be evaluated because they may affect treatment strategies for patients with genotype 4 infection.

Daclatasvir (DCV) is a potent, pan-genotypic inhibitor of the HCV NS5A protein [21]. DCV has been evaluated in multiple

clinical studies and has demonstrated robust antiviral responses and a good safety and tolerability profile in combination with other agents [22]. Studies in patients with genotype 4 infection have investigated DCV combined with pegIFN/RBV, as well as the all-oral combination of DCV with the NS3 protease inhibitor asunaprevir and the nonnucleoside NS5B polymerase inhibitor beclabuvir [11, 15].

The objective of this study was to investigate the prevalence and geographic distribution of genotype 4 subtypes with which patients were infected, preexisting polymorphisms at NS5A amino acid positions associated with DCV resistance (28, 30, 31, or 93) in relation to genotype 4 subtype, the effect of these polymorphisms on DCV susceptibility in vitro, the relationship of baseline polymorphisms to therapeutic outcome, and resistance polymorphisms that emerged in patients who experienced virologic failure.

METHODS

Clinical Samples

Samples for phylogenetic analysis were obtained at baseline from 186 of 203 patients with genotype 4 infection who were enrolled in 5 clinical studies of DAA-containing regimens. Studies contributing patients to this analysis included AI444-010 (clinical trials identifier NCT01125189; 28 of 31 patients with genotype 4 infection) [11], AI443-014 (NCT01455090; 21 of 21 patients) [15], AI447-016 (NCT01030432; 24 of 25 patients) [12], AI444-042 (NCT01448044; 79 of 82 patients) [23], and AI447-029 (NCT01573351; 34 of 44 patients) [24]. Two of these studies provided virologic outcome data with DCV-based regimens that are included in this report. AI444-010 was a randomized, placebo-controlled study of DCV in combination with pegIFN/RBV; 31 patients with genotype 4 infection from North America, Europe, and Egypt were enrolled, of whom 12, 13, and 6 were treated for 24 weeks with pegIFN/RBV + DCV 20 mg/day, DCV 60 mg/day, or placebo, respectively [11]. AI443-014 was a randomized, open-label study of the twice-daily combination of DCV 30 mg, asunaprevir 200 mg (NS3 protease inhibitor), and beclabuvir 75 mg or 150 mg (nonnucleoside NS5B inhibitor). The study included 21 treatment-naive patients with genotype 4 infection who received therapy for 12 weeks [15].

Phylogenetic Analysis

The phylogenetic analysis included NS5A sequences (amino acid positions 9–213) obtained at baseline from 186 patients with genotype 4 infection enrolled in clinical studies and 43 sequences (Supplementary Table 2) from the European HCV Database (euHCVdb) [25]. Sequences, including the ED43 reference strain [26], were aligned using the Align X program of Vector NTI (Invitrogen, Carlsbad, California) with the ClustalW algorithm. A neighbor-joining tree was created from the alignments by using the PHYLIP package, version 3.695,

and was drawn using Mega, version 6. Genotype 4 NS5A sequences were assigned subtypes based on phylogenetic comparison of patient-derived sequences with euHCVdb sequences that had confirmed genotype subtype information.

Phylogenetic analyses of NS3 sequences (amino acid positions 1–218) and NS5B sequences (positions 116–333) were derived from the same patients in studies AI443014 (NS3 and NS5B sequences from 21 patients) and AI444042 (NS5B sequences from 6 patients) used to derive NS5A sequences. Analyses of 31 full-length HCV sequences comprising NS3, NS5A, and NS5B from the euHCVdb were also performed as described above.

### Genotypic and Phenotypic Analysis of Clinical Samples

Viral RNA purification, complementary DNA (cDNA) synthesis, and NS5A amplification were performed as described previously [27]. The NS5A coding region was amplified and sequenced with genotype-specific primers (Supplementary Table 1). DNA sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California). Sequences were compared with the reference strain genotype 4a (ED43; accession GU814265). DCV resistance–associated amino acid positions monitored included L28, L30, M31, H54, P58, D62, A92, and Y93 [28, 29].

For phenotypic analyses, the genotype 4 reference strain was represented by the baseline sequence from a study patient with 99% sequence homology to the consensus genotype 4 NS5A domain-1 sequence (residues 1–213), calculated from 13 baseline sequences from study AI444-010 and 15 sequences from the Los Alamos HCV sequence database (Supplementary Table 2). The truncated NS5A region (residues 3–427) from the reference strain was introduced into the GT2a JFH-1 replicon backbone [30], producing a hybrid replicon. To evaluate the potential susceptibility of baseline genotype 4–NS5A polymorphisms to NS5A inhibitors, substitutions of interest were introduced into the genotype 4a-NS5A hybrid replicon by site-directed mutagenesis and were confirmed by sequence analysis. Patient-derived NS5A sequences were amplified by polymerase chain reaction (PCR), using patient-specific primers [31]. PCR products were infused into JFH-1 replicon cDNA to replace the NS5A coding region representing NS5A amino acids 3–427, using the In-Fusion Cloning kit according to the manufacturer's protocol (Clontech Laboratories, Mountain View, California). The HCV-luciferase transient replication assay has been described previously [29]. The 50% effective concentration (EC_{50}) was calculated as the concentration of inhibitor required to reduce luciferase activity by 50%.

RESULTS

Phylogenetic Analysis

Genotype 4 NS5A sequences (186 from patients in clinical studies and 43 from euHCVdb) grouped in distinct clusters on the
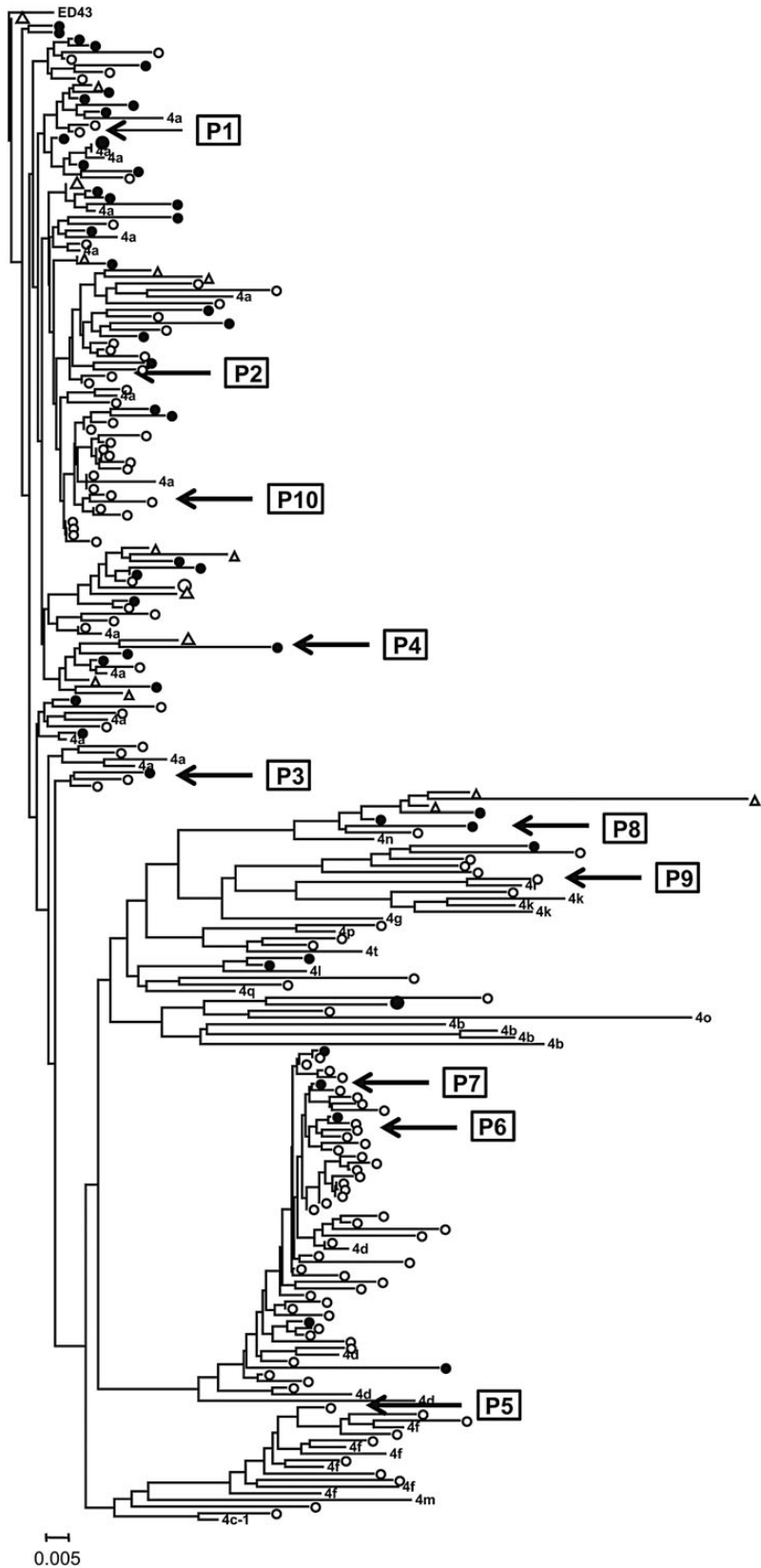
Figure 1. Phylogenetic analysis of hepatitis C virus genotype 4 NS5A sequences. A total of 186 patient-derived baseline genotype 4 NS5A sequences (amino acid positions 9–213) and 43 from the European HCV database were analyzed. Closed circles represent NS5A sequences from patients enrolled from sites in the Americas, open circles represent sequences from patients enrolled in Europe, and open triangles represent sequences from patients enrolled in Africa. NS5A sequences from the European HCV database are labeled with genotype 4 subtypes. Sequences P1–P10 were chosen from different clusters and used for phenotypic testing (Figure 2*B*).

Table 1. Geographic Distribution of Hepatitis C Virus Genotype 4 Subtypes

Region	Genotype 4 Subtype															Total
	a	b	c	d	f	g	k	l	m	n	o	p	q	r	t	
Americas																
Overall	44 (63)	1 (1)	1 (1)	7 (10)	0	2 (3)	1 (1)	3 (4)	1 (1)	4 (6)	2 (3)	1 (1)	1 (1)	1 (1)	1 (1)	70 (100)
US	34	0	0	2	0	1	0	2	0	3	1	0	0	0	0	43
Canada	8	1	1	2	0	1	1	1	1	1	1	1	1	1	1	22
Puerto Rico	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Argentina	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	3
Europe																
Overall	54 (42)	3 (2)	2 (2)	46 (36)	10 (8)	2 (2)	3 (2)	0	0	1 (1)	1 (1)	1 (1)	2 (2)	1 (1)	2 (2)	128 (100)
France	41	0	2	17	10	2	3	0	0	1	0	1	2	0	2	81
Germany	3	0	0	3	0	0	0	0	0	0	1	0	0	0	0	7
Denmark	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
United Kingdom	3	0	0	1	0	0	0	0	0	0	0	0	0	1	0	5
Italy	1	0	0	6	0	0	0	0	0	0	0	0	0	0	0	7
Spain	6	0	0	18	0	0	0	0	0	0	0	0	0	0	0	24
Portugal	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Africa																
Overall	20 (77)	0	0	0	2 (8)	0	0	0	0	3 (12)	1 (4)	0	0	0	0	26 (100)
Egypt	20	0	0	0	0	0	0	0	0	3	1	0	0	0	0	24
Cameroon	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2
Unknown[a]	0	0	0	1 (20)	2 (40)	0	2 (40)	0	0	0	0	0	0	0	0	5 (100)
Total	118	4	3	54	14	4	6	3	1	8	4	2	3	2	3	229

Data are no. or no. (%) of patients with the specified genotype 4 subtype. A total of 186 patient-derived baseline genotype 4 NS5A sequences and 43 genotype 4 NS5A sequences from the European HCV Database are included. The genotype 4 subtype for each patient-derived baseline NS5A sequence was identified by phylogenetic analysis.

[a] The country was not stated for 5 NS5A sequences from the European HCV Database.

phylogenetic tree (Figure 1). NS5A sequences were heterogeneous; overall, 15 genotype 4 subtypes were identified by phylogenetic grouping of patient-derived sequences, with 43 euHCVdb sequences having confirmed subtypes (Table 1). The most common subtype was genotype 4a (118 sequences [52%]). A further 54 sequences (24%) were genotype 4d, with the remaining 57 sequences (25%) distributed across 13 genotype 4 subtypes. Genotype 4a predominated in North America and Egypt, representing 66% and 83% of patients in these regions, respectively. In Europe, genotype 4a (42% of patients) and genotype 4d (36%) were most common.

Analysis of genotype 4 subtypes by line-probe assay (LiPA) was less accurate and less specific than sequencing (Supplementary Table 3). All 186 samples had specific subtypes assigned by sequencing analysis. In contrast, 86 of 186 samples had no subtype assigned by LiPA, and 20 of 186 were assigned incorrectly. Of the 83 of 186 samples assigned as genotype 4a/c/d by LiPA, 11 were determined by sequencing to be neither a, c, nor d. Analysis of a sample subset indicated that genotype 4 subtype assignments were comparable whether using NS5A, NS3, or NS5B sequences (Supplementary Figure 1).

NS5A Polymorphisms at Amino Acid Positions Associated With Resistance

Frequencies of NS5A polymorphisms in the 229 genotype 4 sequences at residues 28, 30, 31, or 93, previously shown to confer

DCV resistance against genotype 1a and genotype 1b, were determined (Table 2) [21, 29]. NS5A-L30 polymorphisms, predominantly L30R, were the most common (93 of 229 [41%]). The predominance of certain NS5A polymorphisms depended on the genotype 4 subtype. Thus, 52 of 54 genotype 4d NS5A sequences harbored L30R, while L30Q was observed only in genotype 4f NS5A sequences. NS5A-L30 polymorphisms were present in all non–genotype 4a sequences and in 11 of 102 genotype 4a sequences. NS5A-L28M was observed in 13 of 118 genotype 4a sequences but in none of the 54 genotype 4d sequences. NS5A polymorphisms at Y93 (Y93H/S/T) were observed in 4 of 4 genotype 4b sequences, while only 1 of 188 genotype 4a (Y93W) and none of 54 genotype 4d sequences harbored Y93 polymorphisms.

Susceptibilities of genotype 4 NS5A substitutions at residues previously associated with DCV resistance were assessed using genotype 4a NS5A hybrid replicons (Table 3). DCV exhibited subnanomolar anti-HCV activity against substitutions observed in 94% of baseline NS5A sequences (126 of 134; EC_{50} values, 0.002–0.7 nM). DCV EC_{50} values against single substitutions identified in this study ranged from 0.004 nM to 3.5 nM (unpublished data). The most frequent polymorphism, L30R, conferred a 10-fold decrease in DCV susceptibility in our in vitro susceptibility assay, with an increase in EC_{50} of 0.002 nM to 0.02 nM. Preexisting NS5A-Y93H has been associated with reduced efficacy in patients infected with genotype 1b [32, 35]. The

Table 2. Baseline NS5A Polymorphisms, by Hepatitis C Virus Genotype 4 Subtype

| NS5A Polymorphism[a] | DCV EC$_{50}$, nM[b] | Polymorphism Frequency,% of Patients (No.), by Source | | No. of Patients With NS5A Polymorphisms, by Genotype 4 Subtype | | | | | | | | | | | | | | |
		Patients (n = 186)	euHCVdb (n = 43)	a	b	c	d	f	g	k	l	m	n	o	p	q	r	t
None	0.002	44.1 (82)	30.2 (13)	95	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L28M	0.02	4.8 (9)	2.3 (1)	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L30H	1.2	0.5 (1)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L30Q	0.02	0.5 (1)	4.7 (2)	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
L30R	0.02	41.4 (77)	37.2 (16)	8	0	2	52	10	1	1	3	0	8	0	2	3	0	3
L30S	0.3	0.5 (1)	2.3 (1)	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Y93H	0.09	0	2.3 (1)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
L28M-L30A	4.9	0.5 (1)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
L28M-L30R	0.7	1.1 (2)	2.3 (1)	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0
L28M-L30T	0.4	0	2.3 (1)	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
L28M-L30V	0.2	0.5 (1)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
L30A-Y93T	35	0	2.3 (1)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
L30C-M31L	0.002	1.6 (3)	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0
L30Q-M31L	0.003	0.5 (1)	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
L30R-M31L	0.003	0.5 (1)	4.7 (2)	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
L30R-M31V	0.03	1.1 (2)	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
L30S-M31L	0.003	0.5 (1)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
L28I-L30R-M31L	0.01	0	2.3 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
L28M-L30H-Y93W	2459	0.5 (1)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L28M-L30R-M31L	0.009	0.5 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
L28M-L30S-M31V	221	0.5 (1)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
L28M-L30S-Y93S	2436	0	4.7 (2)	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
L30S-A92T-Y93H	ND	0	2.3 (1)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total with NS5A polymorphisms	NA	55.9 (104)	69.8 (30)	23	4	3	54	14	4	6	3	1	8	4	2	3	2	3
Total NS5A sequences	NA	100 (186)	100 (43)	118	4	3	54	14	4	6	3	1	8	4	2	3	2	3

Abbreviations: DCV, daclatasvir; EC$_{50}$, 50% effective concentration; euHCVdb, European HCV Database; NA, not applicable; ND, not determined (the genotype 4 NS5A hybrid replicon harboring L30S-A92T-Y93H did not replicate in a transient replication assay).

[a] NS5A polymorphisms at amino acid positions L28, L30, L31, or Y93 were examined because these positions have been associated with DCV resistance.

[b] Data were determined against a genotype 4 NS5A hybrid replicon harboring the respective substitution and are the average of 3 independent experiments.

NS5A substitutions Y93T, Y93H, Y93S, and Y93W, identified in genotype 4 sequences, exhibited EC$_{50}$ values in vitro of 0.004 nM, 0.09 nM, 0.1 nM, and 3.5 nM, respectively (unpublished data). Combinations of NS5A substitutions at amino acid positions previously associated with DCV resistance exhibited EC$_{50}$ values ranging from 0.002 nM to 2459 nM. Six NS5A sequences had substitutions resulting in >1000-fold reduction in DCV susceptibility in vitro; 4 of 6 were from the euHCVdb, for which treatment histories are unknown. In patients known to be treatment naive, NS5A substitutions conferring >1000-fold resistance in vitro were observed in 2 of 186 NS5A sequences (1.1%); however, only 1 of 186 (0.5%) had an EC$_{50}$ value higher than the mean steady-state

Table 3. Effect of NS5A Resistance Polymorphisms on Daclatasvir (DCV) Potency in Hepatitis C Virus Genotype 1a, Genotype 1b, and Genotype 4

| NS5A aa Position | DCV Resistance, by Fold-Change and Genotype | | | | | | | | |
| | ≤10-fold | | | >10- to ≤1000-fold | | | >1000-fold | | |
	4	1b	1a	4	1b	1a	4	1b	1a
28	L, M	I, L, M	I, M, V	. . .	. . .	. . .	. . .	. . .	T
30	C, L, Q, R	H, Q, R	Q	A, H, S	. . .	. . .	. . .	. . .	H, R
31	L, M	I, L, M	L	V	V	M	. . .	. . .	V
93	T	C, F, S	. . .	H, S	H	. . .	W	. . .	C, N, S, H

Polymorphisms represent those identified in 186 patients with genotype 4 and 43 genotype 4 sequences from the European HCV Database, as well as published genotype 1a and genotype 1b polymorphisms [11, 32–34]. Data indicate categories of fold-resistance of each NS5A amino acid (aa) polymorphism in genotype 1a, 1b, and 4 relative to the genotype 4a NS5A reference sequence described in "Methods" section. The fold-change in susceptibility of genotype 1a and 1b polymorphisms to inhibition by DCV was determined using H77 and Con1 replicons, respectively. DCV 50% effective concentrations (mean ± standard deviation) against H77 and Con1 replicons, and the JFH-1 replicon harboring the genotype 4a NS5A reference sequence, were 0.006 ± 0.004 nM, 0.003 ± 0.001 nM, and 0.002 ± 0.001 nM, respectively, and represent data from >3 independent experiments.

DCV trough concentration observed in patients [36]. The genotype 4a–infected patient with this polymorphism at baseline achieved sustained virologic response (described below).

Comparative Effect of NS5A Polymorphisms on DCV Potency Against Genotypes 1a, 1b, and 4

The effect of genotype 4 NS5A substitutions at amino acid positions associated with DCV resistance on anti-HCV activity in vitro was compared with previous data for genotype 1a and genotype 1b (Table 3) [11, 37]. Among the single NS5A substitutions at positions 28, 30, 31, and 93 that were detected in pretreatment genotype 4 clinical samples, all but Y93W (detected in a single individual) had low-to-moderate effects on DCV potency when introduced into a genotype 4 NS5A hybrid replicon, similar to the pattern seen with genotype 1b. In contrast, some substitutions at NS5A residues 30, 31, and 93 were associated with high-level resistance in genotype 1a.

NS5A Polymorphisms and Virologic Response

Sustained virologic response data have been reported for 2 studies of DCV-based regimens in genotype 4 infection: AI444-010 (DCV 20 mg or 60 mg + pegIFN/RBV) and AI443-014 (DCV + asunaprevir + beclabuvir) [11, 15]. In both studies, NS5A polymorphisms associated with DCV resistance were observed at
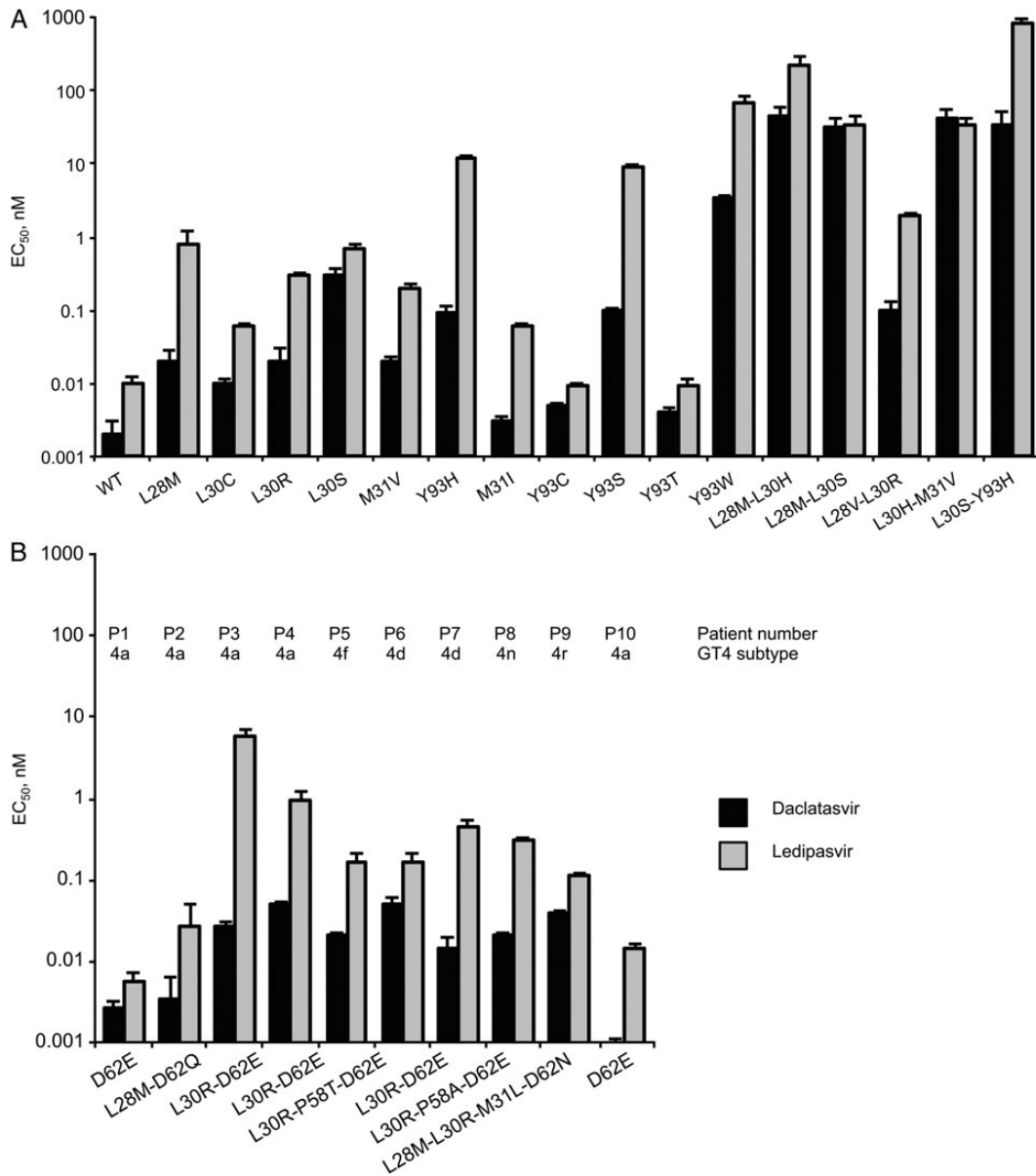
Figure 2. Comparison of daclatasvir (DCV) and ledipasvir (LDV) anti–hepatitis C virus (HCV) genotype 4 activity. Bars indicate the 50% effective concentration (EC_{50}) ± the standard deviation of DCV (black) and LDV (gray) against HCV genotype 4 NS5A hybrid genotype 2a replicons harboring the indicated NS5A substitutions. A, Substitutions in the genotype 4a NS5A reference sequence (see "Methods" section). B, Patient-derived NS5A sequence with observed substitutions at positions of interest. For each pair of bars, patient numbers reflecting the NS5A sequence clusters identified in the phylogenetic analysis (Figure 1) and genotype 4 subtypes are shown.

baseline in some patients but had no apparent effect on virologic outcome.

In study AI444-010, polymorphisms associated with DCV resistance were detected at baseline in 10 of 25 DCV recipients with genotype 4, including L28L/M (n = 2), L30R/S (n = 7), and L28L/M-L30R/S-M31V (n = 1). All of these patients achieved sustained virologic response. This included one patient (DCV 20 mg, a dose subsequently found to be suboptimal) with L28M-L30S-M31V at baseline who experienced a rapid virologic response and achieved sustained virologic response at posttreatment week 12; DCV potency against a genotype 4a NS5A hybrid replicon containing L28M-L30S-M31V was 221 nM.

Four DCV recipients with genotype 4 infection in the AI444-010 study experienced virologic failure, all in the DCV 20 mg dose group, including 2 patients with virologic breakthrough during treatment and 2 patients who experienced relapse after treatment. None of these 4 patients had polymorphisms associated with DCV resistance before treatment; 3 of 4 had HCV RNA levels sufficient for analysis following virologic failure. NS5A-L28M-L30H emerged in 1 patient, and 2 patients had L28M-L30S; these combinations of NS5A substitutions confer high-level DCV resistance in vitro (Figure 2A). By posttreatment week 48, partial replacement or change in the NS5A resistance variants was observed in 2 of 3 patients: in one, L28M-L30S progressively changed to L28M-L30H and then to L28L/M-L30S; in the other, L28M-L30S progressively changed to L28M-L30P/S. In the third patient, L28M-L30H persisted through posttreatment week 48.

In the AI443-014 study, none of the baseline samples from 21 patients with genotype 4 infection had detectable NS3 or NS5B polymorphisms at residues previously identified with drug resistance. Ten patients had NS5A polymorphisms at amino acid positions associated with DCV resistance, including 8 patients with single polymorphisms (L28M [n = 2], L30R [n = 4], and L30H [n = 1]). Two patients had multiple NS5A polymorphisms (L28M-L30A and L28L/M-L30L/H-Y93Y/W); these combinations conferred high-level DCV resistance in vitro. However, following 12 weeks of treatment, all 21 patients with genotype 4 infection achieved sustained virologic response.

Comparison of NS5A Inhibitor Activity in HCV With Genotype 4 NS5A Substitutions

NS5A single and linked substitutions that were frequently identified in genotype 4–infected patients were examined using HCV genotype 4 NS5A hybrid replicons to determine differences in susceptibilities to DCV and ledipasvir (LDV), another NS5A inhibitor [38]. DCV was ≥5-fold more potent than LDV against 11 of the 16 substitutions and similarly potent against the remaining 5 (Figure 2A). DCV was 15-fold more potent than LDV against L30R, the most frequent genotype 4 substitution, and 133-fold more potent against Y93H, an important

DCV-resistant substitution that influences sustained virologic response rates in genotype 1b–infected patients.

DCV and LDV antiviral activities were also evaluated against 10 NS5A sequences derived from genotype 4–infected patients, representing different phylogenetic clusters, that were introduced into a genotype 2a replicon. DCV EC_{50} values against these 10 sequences were 0.001–0.08 nM, and no association between DCV potency and specific phylogenetic clusters was observed (Figures 1 and 2B). DCV was ≥10-fold more potent than LDV against 7 of 10 patient-derived sequences and 2–4 fold more potent for the remaining 3 sequences. NS5A substitutions at resistance-associated positions (L28M, L30R, and M31L) decreased susceptibility of the patient-derived NS5A sequences to both DCV and LDV. In addition to these substitutions, other NS5A substitutions may also affect susceptibility to DCV and LDV. DCV was 20-fold more potent than LDV against the replicon harboring the patient 4–derived NS5A sequence, while it was only 2-fold more potent than LDV against the replicon harboring the patient 2–derived NS5A sequence. Sequence analysis revealed that T56M was present at baseline in patient 4 but not in patient 2. The NS5A-T56I substitution had previously been shown to increase DCV susceptibility against patient-derived genotype 1b NS5A sequences [39]. Similarly, DCV susceptibility increased 4-fold in a genotype 4 NS5A hybrid replicon harboring the T56M substitution, whereas this substitution was 2-fold less susceptible to LDV. In the patient 1–derived NS5A sequence, DCV was 400-fold more potent than LDV, while L28M in the reference sequence demonstrated only a 40-fold difference in potency. Sequence analysis of the NS5A region from patient 3 identified a polymorphism at position 62 (D62Q); NS5A substitutions at position 62 alone exert no effect on DCV activity but can modulate potency when combined with signature resistance substitutions in genotype 1a [40].

DISCUSSION

The results of the phylogenetic analysis are consistent with those of previous epidemiologic studies, demonstrating a diversity of genotype 4 subtypes among patients enrolled in HCV clinical trials in North and South America, Europe, and Egypt. In our analysis of HCV NS5A sequences from clinical trials and the euHCVdb, genotype 4a and genotype 4d were the most common subtypes, representing 52% and 24% of patients, respectively, but 25% of patients were represented by 13 other genotype 4 subtypes. This genotypic diversity contrasts with epidemiologic results for genotype 1, in which 99% of patients with subtype data were identified as either genotype 1a or genotype 1b [4]. Comparison of results from genotyping methods indicate that LiPA is less specific than sequencing and sometimes unreliable for genotype 4 subtyping; accordingly, sequencing is the best method for this purpose.

Genotype 4a was the predominant subtype found in North America, whereas both genotype 4a and 4d were common in

Europe, consistent with previous surveys [41, 42]. This may reflect epidemiologic variables, which together suggest a recent increase in genotype 4 infections outside Africa and the Middle East. European studies have associated genotype 4a infection with immigrants from Egypt and the Middle East, while genotype 4d infection has been associated with high-risk behaviors such as injection drug use. Although this study was not a formal epidemiologic analysis, the results suggest that a diversity of genotype 4 subtypes is likely to be encountered in clinical practice in North America and Europe.

Baseline polymorphisms that conferred high-level DCV resistance in our genotype 4 NS5A hybrid replicon assay were uncommon; only 5 of 229 NS5A sequences (2%) had polymorphisms that significantly attenuated DCV potency in vitro (EC_{50} values, >5 nM; Table 2). Of these 5 NS5A sequences, 2 were from clinical trial samples and 3 were from euHCVdb NS5A sequences, for which treatment histories were unknown. The L30R polymorphism was present in nearly all NS5A sequences of subtypes other than genotype 4a, suggesting that it may be wild type for non–genotype 4a subtypes. The L30R substitution reduces DCV potency by approximately 10-fold, based on the genotype 4a NS5A hybrid replicon assay; however, the resulting EC_{50} of 0.02 nM remains >10 000-fold lower than the mean steady-state trough concentration of DCV observed in HCV-infected patients [36]. Y93H is one of the major signature resistance substitutions in genotype 1b, with an overall prevalence of approximately 8% observed in a Japanese study [43]. However, in this study Y93H was not detected in 186 patient-derived baseline NS5A sequences and was detected in only 1 euHCVdb sequence, suggesting a lower prevalence of Y93H in genotype 4 than in genotype 1b.

DCV susceptibility analyses against 10 patient-derived NS5A sequences representing diverse phylogenetic clusters were consistent with analyses of substitutions in the genotype 4a NS5A reference sequence. This suggests that DCV potency may not vary significantly according to genotype 4 subtype and that NS5A polymorphisms have similar influences on DCV potency across genotype 4 subtypes. This is an important finding in view of the diversity of genotype 4 subtypes circulating worldwide and pertains to the possible value of genotype 4 subtyping in the context of DAA-based therapy. As yet, genotype 4 subtype has shown no discernible effect on virologic response with DCV-containing regimens, suggesting that determination of genotype 4 subtype may be unnecessary in the context of therapeutic decision making. However, the potential contribution of genotype 4 subtype to therapeutic outcomes requires additional data, particularly for DAA regimens, which have not yet been evaluated in this regard.

DCV is intrinsically about 5-fold more potent than LDV against the genotype 4a NS5A hybrid reference replicon; moreover, most of the commonly observed resistance substitutions, as well as patient-derived NS5A sequences representing different phylogenetic clusters, had a relatively greater adverse effect on the potency of LDV, compared with DCV. Further study is needed to determine the potential relevance of these differences to the potencies of combination regimens containing these agents and to the usefulness of assessing subtype-related effects for other DAAs being developed for treatment of genotype 4 infection.

It should be noted that all susceptibility assays used a genotype 2a JFH-1 replicon harboring genotype 4 NS5A sequences (see "Methods" section), as opposed to a genotype 4 subgenomic NS3-NS5B replicon [44, 45] or patient-derived full-length HCV. Therefore, our reported EC_{50} values may vary from those generated if using a genotype 4 subgenomic replicon, although trends in EC_{50} values for the NS5A substitutions would be expected to be comparable. We found that polymorphisms influencing DCV activity are located in the first 100 amino acids of the NS5A region [21, 29] and, correspondingly, that polymorphisms beyond the first 100 amino acids exert minimal effects on DCV potency (unpublished results). In this study, potential associations between observed EC_{50} values for DCV against tested NS5A substitutions and virologic outcome could not be assessed, since all genotype 4–infected patients who received DCV at the recommended dose (60 mg once daily) achieved sustained virologic response at posttreatment week 12.

Overall, the resistance profile of DCV in genotype 4 infection appears to be more similar to that observed previously in genotype 1b infection, compared with genotype 1a infection [37]. Generally, a combination of residue changes are required for high-level DCV resistance against genotype 1b and genotype 4, and baseline polymorphisms with potentially high-level DCV resistance are infrequent with these genotypes. Baseline genotype 4 NS5A polymorphisms that confer low-level DCV resistance have as yet shown no effect on therapeutic outcomes. Such polymorphisms can potentially lower the barrier to subsequent acquisition of high-level resistance and virologic failure, although there is no evidence that this has occurred in clinical outcome data currently available. In the 46 patients with virologic outcome data, there was no apparent effect of baseline polymorphisms on sustained virologic response: all 20 patients with NS5A RAVs at baseline achieved sustained virologic response, and no NS5A RAVs were present at baseline in the 4 patients who experienced virologic failure following treatment with a suboptimal DCV dose in study AI444-010. However, multiple factors can affect the emergence of resistance variants, particularly the overall potency and barrier to resistance of the combination regimen. The all-oral DAA combination that provided some of the clinical outcome data in this analysis comprises 3 highly potent DAAs with different antiviral mechanisms; highly potent combination regimens are more likely to retain adequate antiviral effect even if the potency of a regimen component is attenuated due to the presence of RAVs at

baseline. With less potent DAA combinations, RAVs at baseline may have a greater effect. Ongoing phase 3 studies with DCV-based regimens in genotype 4 infection will provide more-detailed information concerning the impact of baseline polymorphisms on clinical outcome.

In summary, this analysis confirms the substantial diversity of circulating genotype 4 subtypes. The potency of DCV appears to be comparable across subtype sequences derived from genotype 4–infected patients, and baseline NS5A polymorphisms are generally associated with subnanomolar DCV potencies in our in vitro susceptibility assays. Baseline NS5A polymorphisms that confer high-level DCV resistance are uncommon; however, neither genotype 4 subtype nor the presence of NS5A polymorphisms associated with DCV resistance appears to have a clinically significant impact on the usefulness of DCV as part of potent antiviral regimens.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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