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Susceptibilities of Genotype 1a, 1b, and 3 Hepatitis C Virus Variants to the NS5A Inhibitor Elbasvir

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Elbasvir is an investigational NS5A inhibitor with *in vitro* activity against multiple HCV genotypes. Antiviral activity of elbasvir was measured in replicons derived from wild-type or resistant variants of genotypes 1a, 1b, and 3. The barrier to resistance was assessed by the number of resistant colonies selected by exposure to various elbasvir concentrations. In a phase 1b dose-escalating study, virologic responses were determined in 48 noncirrhotic adult men with chronic genotype 1 or 3 infections randomized to placebo or elbasvir from 5 to 50 mg (genotype 1) or 10 to 100 mg (genotype 3) once daily for 5 days. The NS5A gene was sequenced from plasma specimens obtained before, during, and after treatment. Elbasvir suppressed the emergence of resistance-associated variants (RAVs) *in vitro* in a dose-dependent manner. Variants selected by exposure to high elbasvir concentrations typically encoded multiple amino acid substitutions (most commonly involving loci 30, 31, and 93), conferring high-level elbasvir resistance. In the monotherapy study, patients with genotype 1b had greater reductions in HCV RNA levels than patients with genotype 1a at all elbasvir doses; responses in patients with genotype 3 were generally less pronounced than for genotype 1, particularly at lower elbasvir doses. M28T, Q30R, L31V, and Y93H in genotype 1a, L31V and Y93H in genotype 1b, and A30K, L31F, and Y93H in genotype 3 were the predominant RAVs selected by elbasvir monotherapy. Virologic findings in patients were consistent with the preclinical observations. NS5A-RAVs emerged most often at amino acid positions 28, 30, 31, and 93 in both the laboratory and clinical trial. (The MK-8742 P002 trial has been registered at ClinicalTrials.gov under identifier NCT01532973.)

Elbasvir (MK-8742) is a small-molecule inhibitor of nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) being developed as a component of treatment regimens for chronic HCV infection (1–4). Elbasvir possesses activity against genotype 1a, 1b, and 3 *in vitro*, including against some viral variants resistant to other NS5A inhibitors (4). In a phase 1b dose escalation study, elbasvir once daily for 5 days resulted in mean reductions in HCV RNA levels of 3.7 to 5.1 $\log_{10}$ IU/ml in patients with genotype 1a or 1b infections given 5 to 50 mg/day and of ~3 $\log_{10}$ IU/ml in patients with genotype 3 given 50 or 100 mg/day (3). In later phase 2 trials, treatment with elbasvir combined with grazoprevir (MK-5172, an investigational once-daily NS3/4A protease inhibitor) with or without ribavirin for 12 weeks produced rates of sustained virological response at week 12 (SVR_{12}) of 87 to 98% for patients with genotype 1 infections, including in historically difficult-to-treat subgroups (1, 2).

Drug resistance poses a challenge to interferon-sparing regimens for chronic HCV infection (5–12). Variants associated with decreased drug susceptibility exist at low levels in most patients before any exposure to direct-acting antiviral agents. Preexistent resistance-associated variants (RAVs) may then emerge as a dominant species under selective drug pressure during or after direct-acting antiviral therapy. The current report compares and contrasts RAVs identified in preclinical studies with baseline and emerging variants encountered during a phase 1b dose-escalating trial using elbasvir monotherapy for 5 days in patients chronically infected by HCV genotypes 1 and 3 (3).

MATERIALS AND METHODS

Preclinical studies. HCV replicons were used to determine the effective concentrations (EC) of elbasvir necessary to inhibit HCV RNA levels by a specified percentage (50% or 90%) for genotypes 1a, 1b, and 3 variants compared to no treatment (13). Replicons maintained in 0.5 mg/ml of G418 (HyClone, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) to select replicating cells were seeded on 384-well plates in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum. Twofold dilutions of elbasvir concentrations from 1 μM down to 0.002 μM were added to the medium the next day in the presence of 0.5% dimethyl sulfoxide (DMSO). After 72 h of incubation, cells were harvested and subjected to real-time reverse transcriptase PCR (RT-PCR) (14). For each variant, threshold cycle numbers were plotted against the log of elbasvir concentrations and fitted to a sigmoid dose-response curve using Prism (GraphPad Software, San Diego, CA, USA) to obtain the EC_{50} and EC_{90} (the drug concentrations needed to achieve 50% and 90% inhibition, respectively, relative to a DMSO control without drug). For reference, the steady-state minimum concentration (C_{min}) for once-daily 50 mg elbasvir dosing is about 22 nM (3).

To select cell lines with decreased elbasvir susceptibility, subconfluent monolayers of replicon cells were cultured in the presence of various drug concentrations at multiples of EC_{90}. Plates were prepared at 2×10^5 cells

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per 60-mm plate and passaged only once at a 1:10 ratio when the cells reached 95% confluence. Colonies surviving selection were first counted and then pooled and expanded for analysis. The colony count in the presence of elbasvir was divided by the number of cells seeded to calculate resistance frequency. Total cellular RNA was isolated from pooled colonies and amplified by RT-PCR. The RT-PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germantown, MD, USA), and the full-length NS5A gene was sequenced. Additionally, the RT-PCR products were cloned into the TOPO TA vector (Invitrogen), and the plasmid DNA from bacterial colonies was sequenced to look for linked variations. The replicative capacity ("fitness") of RAVs was evaluated in a replicon colony formation assay (13).

Phase 1b randomized clinical trial design. MK-8742 P002 was a randomized, double-blind, placebo-controlled, sequential dose-escalating phase 1b study of elbasvir monotherapy to assess safety, pharmacokinetics, and viral responses in adult men with chronic HCV-1 or HCV-3 infection (3). Patients between 18 and 60 years of age (up to 65 years old at the discretion of the investigator) with HCV RNA levels of >100,000 IU/ml were eligible. Elbasvir doses were 5, 10, and 50 mg once daily for patients infected with genotype 1a or 1b, and 10, 50, and 100 mg once daily for patients infected with genotype 3. A total of 6 patients were to be enrolled at each dosing level, including 5 patients to receive elbasvir and 1 patient to receive a matching placebo orally for 5 consecutive days, starting at the lowest dose for the infecting genotype. Doses were escalated stepwise once adequate safety data had been reviewed from the previous dosing group. Viral load and resistance testing was to be performed daily and every other day, respectively, for the first 10 days of the study and then at 2 weeks, 3 weeks, 1 month, and 2 months after the last dose of elbasvir. The study was conducted in accordance with good clinical practice guidelines. All participants provided written informed consent.

Viral quantification, sequencing, and resistance analyses. In the phase 1b study (3), HCV RNA levels were measured in plasma specimens obtained at baseline, during and at the end of elbasvir monotherapy, and at periodic follow-up visits by the TaqMan 2.0 assay (Roche Diagnostics, Branchburg, NJ, USA) with lower limits of quantification and detection of 25 and 9.3 IU/ml, respectively. Blood samples were also collected for viral resistance testing at prespecified time points, including prior to the first elbasvir dose, near the nadir of the HCV RNA level, and up to 2 months after the last elbasvir dose provided that the HCV RNA level remained >1,000 IU/ml.

The full-length NS5A gene was amplified from plasma samples using RT-PCR followed by population and selective clonal sequencing (15). Due to the sensitivity of the assay, resistance analyses were routinely performed only on samples with HCV RNA levels of >1,000 IU/ml. The resultant amino acid sequences were compared to genotype 1a (H77; GenBank no. NC_004102), genotype 1b (con1; GenBank no. AJ238799), or genotype 3 (S52; GenBank no. GU814263) referents. The limit of variant detection in population sequencing was presumed to be ~20 to 25% of the viral quasispecies (16). Polymorphisms identified in ≥10% of patients were selected for more detailed analysis. For clonal sequencing, amino acids 1 to 448 of NS5A were amplified by RT-PCR, and the resultant amplicons were cloned into a TOPO TA vector (Invitrogen). Approximately 40 clones were sequenced at each time point. Polymorphisms detected in more than a single clone were included in the analysis.

In phenotypic analyses to determine the antiviral potency of elbasvir against detected variants, genotype-specific replicons were constructed to incorporate NS5A polymorphisms. The shift (*n*-fold) in elbasvir EC for each variant replicon was expressed relative to the EC for the corresponding wild-type replicon. The fitness of resistant variants was evaluated by comparing colony counts to a wild-type referent.

RESULTS

Preclinical results. (i) Activity of elbasvir against genotype 1a and 1b replicons. In the 3-day replicon assay, the EC_{90} of elbasvir was 0.006 nM for both the wild-type 1a_H77 and 1b_con1 replicons (Table 1). The elbasvir EC_{90} against genotype 1a shifted 13-fold to 0.08 nM when 40% human serum was added to the assay medium in addition to the standard 5% fetal calf serum. To assess elbasvir activity against a range of genetically diverse clinical isolates, more than 200 independent genotype 1 sequences collected from clinical databases were subjected to phylogenetic analysis. The full-length NS5A sequences from 5 genotype 1a and 4 genotype 1b patients were then synthesized and cloned into a con1 subgenomic replicon. The EC_{90} of elbasvir ranged from 0.01 to 0.02 nM for genotype 1a and 0.01 to 0.03 nM for genotype 1b.

TABLE 1 Activity of elbasvir against HCV genotype 1 NS5A variant replicons[a]

Sequence identification[b]	Effective inhibitory concn (nM)[c]	
	50%	90%
1a_H77	0.004 ± 0.002	0.006 ± 0.002
1a_H77 in 40% NHS	0.040 ± 0.013	0.082 ± 0.027
1a DQ889262	0.009 ± 0.007	0.019 ± 0.012
1a DQ889305	0.005 ± 0.000	0.011 ± 0.004
1a DQ889320	0.003 ± 0.000	0.009 ± 0.005
1a EU155348	0.003 ± 0.002	0.007 ± 0.001
1a EU155380	0.006 ± 0.001	0.015 ± 0.004
1b_con1	0.003 ± 0.001	0.006 ± 0.004
1b AF033358	0.005 ± 0.003	0.012 ± 0.006
1b AJ132996	0.009 ± 0.005	0.019 ± 0.011
1b B9016	0.014 ± 0.006	0.029 ± 0.009
1b EU482860	0.010 ± 0.004	0.026 ± 0.013

[a] A convenience sample of genotype 1 isolates available in the public domain was used to test susceptibility to elbasvir in the replicon assay. The table summarizes the results from cell lines that were able to replicate in cell culture.
[b] Numbers are GenBank accession numbers except for 1b B9016. H77 and con1 are wild-type referents for genotypes 1a and 1b, respectively. NHS, normal human serum.
[c] Values are averages and ranges from duplicate independent experiments.

(ii) Activity of elbasvir against genotype 3 replicons. To assess the potency of elbasvir against genotype 3, a subgenomic replicon harboring the genotype 3 NS5A sequence was first constructed by cloning the full-length NS5A gene from a patient isolate (GenBank accession no. NC_009824) into a con1 replicon background, yielding a cell line with an elbasvir EC_{90} of 0.12 nM (Table 2). Subsequently, more than 100 independent genotype 3 sequences collected from clinical databases were subjected to phylogenetic analysis, from which 11 unique and nonclustered sequences were selected to ensure genetic diversity and cloned into con1 and JFH replicons for further evaluation. In all cases, replicons containing the JFH backbone yielded higher colony counts than the con1 constructs. Whereas con1 and JFH replicon backbones differentially impacted replication fitness, the potency of elbasvir remained nearly constant irrespective of the replicon backbone. The elbasvir EC_{90} stayed in the subnanomolar range for all the tested variants.

(iii) Resistance selection in genotype 1 and genotype 3 replicons. To examine the resistant barrier for elbasvir, genotype 1a, 1b, and 3 replicons were treated with elbasvir in the presence of G418. Surviving colonies were counted, and pooled colonies were subjected to phenotypic and genotypic analysis (Table 3). Elbasvir demonstrated dose-dependent suppression of resistant genotype 1a replicons, illustrated by the reductions in colony counts at higher doses. The numbers of surviving colonies were 204, 56, and 4, respectively, in the presence of 0.06 nM, 0.6 nM, and 6 nM

TABLE 2 Activity of elbasvir against HCV genotype 3 replicons[a]

Sequence identification[b]	Con1 backbone		JFH backbone	
	No. of replicon colonies	Mean[c] EC_{90} ± SD (nM)	No. of replicon colonies	Mean[c] EC_{90} ± SD (nM)
Parental replicon	300	0.006 ± 0.004	TMTC	0.019 ± 0.010
GU814823 (S52)	9	0.042 ± 0.011	503	0.065 ± 0.026
GQ356207	3	0.016 ± 0.008	472	0.035 ± 0.027
AM493639	0	ND	0	ND
EU826291	21	0.005 ± 0.003	TMTC	0.096 ± 0.117
EU826299	0	ND	0	ND
GQ300882	0	ND	482	0.613 ± 0.855
GQ356215	0	ND	242	0.016
HM042077	2	0.011 ± 0.010	598	0.034 ± 0.037
HM042078	4	0.007 ± 0.006	412	0.031 ± 0.022
HQ912953	2	0.007 ± 0.004	51	0.016
AF320799	0	0.119	43	0.387 ± 0.380
NC_009824	ND	0.120 ± 0.060	ND	ND

[a] EC, effective (inhibitory) concentration; TMTC, too many to count; ND, not done.
[b] Numbers are GenBank accession numbers. Parental replicons of con1 (genotype 1b) and JFH (genotype 2a) were used as controls.
[c] Average from ≥3 independent experiments.

elbasvir (ranging from 10× to 1,000× EC_{90} against the control genotype 1a replicon). Cells emerged as loosely interconnected colonies at 1× EC_{90} which were too numerous to count. Replicons selected by treatment with high-dose elbasvir were highly resistant to elbasvir, as measured by fold shifts in EC_{90} from values determined in cells treated with DMSO in the absence of drug. The resistance frequency was estimated to be 0.002% at 6 nM elbasvir. Population sequencing of the full length of the NS5A gene identified Y93N as the only substitution selected by exposure to 0.6 nM elbasvir. After exposure to 6 nM elbasvir, Q30D was detected in all colonies, usually associated with Y93N. Clonal analysis confirmed the Q30D-Y93N linkage in cells treated with 6 nM elbasvir. Y93N conferred high-level elbasvir resistance *in vitro*.

For genotype 1b replicons, dose-dependent suppression of resistance was also observed. The respective numbers of surviving colonies selected were 122, 38, 5, and 3 in the presence of 0.006 nM, 0.06 nM, 0.6 nM, and 6 nM elbasvir (ranging from 1× to 1,000× EC_{90} for the control genotype 1b replicon). Compared with genotype 1a, fewer colonies emerged in genotype 1b at 1×, 10×, and 100× EC_{90} under identical experimental conditions. Y93H was the majority variation in the population treated with 0.006 nM elbasvir. Y93H and V121I were detected in cells treated with 0.06 nM elbasvir, and Y93H-V121I linkage was confirmed with clonal analysis. Cells exposed to 0.6 nM elbasvir failed to expand and thus were not available for testing. L31F, Y93H, and V121I were detected in cells treated with 6 nM elbasvir, and L31F-Y93H-V121I linkage was identified in 80% of tested clones. Y93H was associated with high-level elbasvir resistance.

A subgenomic replicon cell line harboring the genotype 3 NS5A sequence from a patient isolate (NC_009824) cloned into a con1 background with an elbasvir EC_{90} of 0.12 nM was used for the genotype 3 resistance selection experiments. As for genotype 1a and genotype 1b, genotype 3 replicons were treated with elbasvir at multiples of the EC_{90}. Innumerable colonies grew in the presence of 0.12 nM and 1.2 nM elbasvir (corresponding to 1× and 10× EC_{90}). Population sequencing detected low levels of the Y93H and E92K variants. Increasing the elbasvir concentrations to

TABLE 3 *In vitro* selection of RAVs in genotype 1a, 1b, and 3a replicons after exposure to varying concentrations of elbasvir[a]

Replicon cell type	Elbasvir treatment (×EC_{90})[b]	Recovered colony counts	Elbasvir susceptibility			Observed amino acid substitution(s)[e]
			EC_{50} (nM)[c]	EC_{90} (nM)[c]	Fold shift in EC_{90}[d]	
Genotype 1a_H77	1,000	4	135	526	90,000	Q30D, Q30D-Y93N
	100	56	5	15	3,000	Y93N
	10	204	2	11	2,000	Not detected
	1	TMTC	<2	<2	<300	Not detected
	DMSO	NA	0.008	0.015	<3	Not detected
Genotype 1b_con1	1,000	3	27	120	20,000	Y93H, L31F-Y93H-V121I
	100	5	NA	NA	NA	NA
	10	38	0.6	12	2,000	Y93H, V121I, Y93H-V121I
	1	122	0.2	1	200	Y93H
	DMSO	NA	0.008	0.011	<3	Not detected
Genotype 3_NC_009824[f]	1,000	15	328	959	10,000	E92K, Y93H
	100	23	245	518	5,000	E92K, Y93H
	10	TMTC	122	704	7,000	E92K, Y93H
	1	TMTC	157	725	7,000	Y93H
	DMSO	NA	0.2	0.8	6	Not detected

[a] EC, effective (inhibitory) concentration; TMTC, too many to count (as the result of a lawn of cells); NA, not available (because cells failed to expand).
[b] Elbasvir concentration (in multiples of EC_{90}) used to select RAVs.
[c] Values represent single experiments.
[d] Calculated as the EC_{90} for selected cell line/EC_{90} for the corresponding wild-type genotype 1a_H77 or genotype 1b_con1 referent (EC_{90} = 0.006 nM for both wild-type genotype 1 replicons).
[e] Based on population sequencing. Because the lower limit of reliable detection is >20% with population sequencing (16), the failure to detect polymorphisms by this method does not necessarily imply that minority variants were not present in low concentrations.
[f] Replicons were constructed using genotype 3 NS5A sequences in a con1 background (EC_{90} = 0.12 nM for the wild-type genotype 3 replicon).

12 nM and 120 nM (corresponding to 100× and 1,000× EC_{90}) significantly reduced the number of resistant colonies to 23 and 15, respectively. Analysis of 40 individual clones did not identify a linkage between the Y93H and E92K variations. Y93H conferred high-level elbasvir resistance. Establishing a genotype 3 replicon with E92K alone was not successful despite many attempts, suggesting that the isolated E92K substitution rendered the variant unfit.

(iv) Activity of elbasvir against variants selected by other NS5A inhibitors. NS5A RAVs previously identified in clinical trials with other NS5A inhibitors were included among the resistant variants selected in our experiments. Substitutions arose principally at amino acid residues 28 (genotype 1a and 1b), 30 (genotype 1a), 31 (genotypes 1a and 1b), and 93 (genotypes 1a, 1b, and 3) (17). In replicons containing individual NS5A substitutions, the elbasvir EC_{90} against 9 of the 13 genotype 1a variants tested had increases in EC_{90} of >10× (range, 12× to 1,333×), whereas EC_{90}s for only 2 of 6 tested genotype 1b variants increased by >10× over that for wild type [L31F (44×) and Y93H (67×)] (Table 4). All 3 tested genotype 3a variants had increases in EC_{90} of >20×.

(v) Fitness of RAVs in a replicon assay. The fitness of resistant variants was assessed in a replicon formation assay, in which the replicon RNA from resistant variation were transfected into Huh7 cells and replicon colonies were selected and counted. In this assay, substitutions at amino acid positions 30 and 31 in genotype 1a replicons were well tolerated (replicative capacity ranging from 41% to 100% of that of the wild type), whereas variations at position 93 reduced replicon fitness by ≥85% (Table 5). In particular, Y93H dramatically reduced replication to <1% compared to that of the wild-type genotype 1a replicon. Genotype 1b replication was sensitive to changes at amino acids 31 and 93. L31V/F and Y93H/C caused a dramatic decrease in genotype 1b fitness, reducing the colony number to <2% of that of the wild type. In contrast, Y93H/C in genotype 3 replicons modestly reduced replicative capacity to 50% of that of the wild type.

Phase 1b clinical trial results. (i) Subject accounting. A total of 48 patients received elbasvir or placebo in an elbasvir dose-ranging study (3), including 17 patients with genotype 1a, 13 patients with genotype 1b, and 18 with genotype 3. All patients completed the 5-day course of therapy. Mislabeling of 7 samples from 3 patients (1 each infected with genotype 1a, 1b, or 3) occurred at the sequencing facility; these samples were therefore excluded from the analysis. Baseline virus from 1 additional genotype 3 patient could not be amplified. Consequently, only sequence data from the other 44 patients (including 16 with genotype 1a, 12 with genotype 1b, and 16 with genotype 3) were used for the resistance analyses.

Variants with NS5A substitutions at positions 28, 30, 58, and 93 (which had been previously identified as potential RAVs for NS5A inhibitors) were detected at baseline in 7 of 44 patients (15.9%) (Table 6). Except for possibly 1 patient infected with genotype 3 in the 10-mg elbasvir dose group, baseline NS5A variants did not appear to impact the magnitude of viral load reduction during treatment. In particular, M28V or Q30R in genotype 1a and Y93H in genotype 1b at baseline had little impact on the magnitude of viral load reduction during treatment.

Postbaseline sequencing was performed for 35/36 elbasvir recipients (97.2%) during follow-up visits. The sole exception was a genotype 1b patient who had HCV RNA levels of <1,000 IU/ml at all follow-up visits. These postbaseline sequences were compared to pretreatment results to identify treatment-emergent variants selected by elbasvir. The most common postbaseline variants (present in >10% of patients) encoded M28T, Q30R, L31V, or Y93H in genotype 1a, L31V or Y93H in genotype 1b, and A30K, L31F, and Y93H in genotype 3 (Fig. 1). Of substitutions at the 4 amino acid positions indicated in the graph, Y93H/C/N and L31V/M/I/F were the most prevalent, occurring in 83% (29/35) and 54% (19/35) of the postbaseline sequences obtained from the 35 patients. Postbaseline variations at position 30 were observed in 37% (12/35) of the patients, including 11 genotype 1a sequences with Q30R and 1 genotype 3a sequence with A/E/K/T30K. M28T, L28M, or V28A was noted in 20% (7/35) of the patients.

For genotype 1a patients, 10/11 and 8/11, respectively, with variants at positions 30 and 31 still had changes at these loci at the last follow-up. For genotype 1b patients, 8/9 with variants at position 93 had persistent changes at this locus at the last follow-up.

TABLE 4 Effective inhibitory concentrations of elbasvir against variant replicons[a]

Replicon	EC_{50}		EC_{90}	
	Mean ± SD (nM)	Fold shift[b]	Mean ± SD (nM)	Fold shift[b]
1a_H77 (WT)	0.007 ± 0.004		0.017 ± 0.009	
1a_M28T	0.108 ± 0.035	15	0.378 ± 0.180	22
1a_M28V	0.009 ± 0.006	1	0.016 ± 0.012	1
1a_M28A	0.427 ± 0.220	61	1.527 ± 0.688	91
1a_Q30D[c]	3.7 ± 1.7	925	8.6 ± 3.5	1,433
1a_Q30H	0.045 ± 0.033	6	0.108 ± 0.067	6
1a_Q30R	0.114 ± 0.087	16	0.407 ± 0.212	24
1a_L31V	0.431 ± 0.130	61	2.143 ± 0.737	127
1a_L31M	0.070 ± 0.031	10	0.245 ± 0.162	15
1a_L31F	0.673 ± 0.384	96	2.203 ± 1.605	131
1a_H58D	0.041 ± 0.017	6	0.168 ± 0.066	10
1a_A92P	0.007 ± 0.003	1	0.017 ± 0.005	1
1a_Y93H	1.543 ± 0.782	220	5.930 ± 2.900	351
1a_Y93N	6.552 ± 1.253	929	22.50 ± 8.544	1,333
1a_Y93C	0.078 ± 0.004	11	0.208 ± 0.038	12
1a_Q30D_Y93N[c]	86.4 ± 60.1	21,600	257.7 ± 105.7	42,950
1b_con1 (WT)	0.003 ± 0.002		0.006 ± 0.004	
1b_L28M	0.006 ± 0.003	2	0.020 ± 0.006	3
1b_L31M	0.003 ± 0.001	1	0.010 ± 0.004	2
1b_L31F	0.046 ± 0.015	15	0.265 ± 0.206	44
1b_L31V	0.013 ± 0.008	4	0.057 ± 0.029	10
1b_Y93H	0.050 ± 0.030	17	0.400 ± 0.200	67
1b_V121I	0.0005 ± 0.0004	0.2	0.0010 ± 0.0007	0.2
GT3_S52 (WT)	0.14 ± 0.09		0.49 ± 0.19	
3a_A30K	7.0 ± 1.7	50	20 ± 6.5	41
3a_L31F	20 ± 2	143	45 ± 8	92
3a_E92K[d]				
3a_Y93H	68 ± 40	485	159 ± 56	324

[a] RAV, resistance-associated variant; WT, wild type; EC_{50}, effective concentration to inhibit growth by 50% compared to WT growth; EC_{90}, effective concentration to inhibit growth by 90% compared to WT growth. The genotype 1a results were generated with a transient replicon system (except where indicated), whereas the genotype 1b and genotype 3a data were generated with a stable replicon system.
[b] Relative to WT referent.
[c] Results were generated with a stable replicon system where genotype 1a_H77 (WT referent) yielded an EC_{50} of 0.004 nM and an EC_{90} of 0.006 nM.
[d] The replicon was unable to be generated due to poor fitness.

TABLE 5 Replicative capacity (fitness) of variants in genotype 1a_H77 or genotype 1b_con1 replicons

Genotype	Replicon variation	Fitness (% of wild-type control replication)
1a	H77 (WT)	100
	Q30D	70
	Q30E	57
	Q30K	100
	L31V	41
	Y93N	6
	Y93H	1
	Q30D_Y93N	15
1b	con1 (WT)	100
	L31F	0.1
	L31V	1.7
	Y93C	0.5
	Y93H	0.5
3a	S52 (WT)	100
	Y93H	50

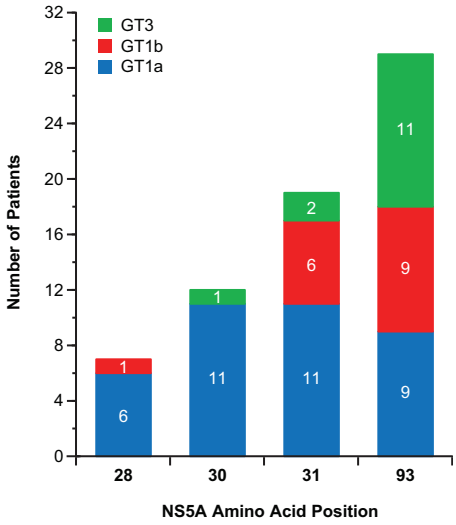

FIG 1 Prevalence of specific amino acid substitutions at established NS5A resistance loci detected by population sequencing after monotherapy with elbasvir in the phase 1b study. The column graph displays the most common postbaseline variants identified in patients infected with genotype (GT) 1a, 1b, or 3 in the phase 1b study. Among the polymorphisms examined, substitutions at amino acids 28, 30, 31, and 93 were each observed in more than 10% of evaluable patients.

For genotype 3 patients, 11/11 with variants at position 93 had persistent changes at this locus at the last follow-up.

(ii) Genotype 1 infections. All doses of elbasvir led to rapid HCV RNA reductions of 3.7 to 5.1 $\log_{10}$ IU/ml in genotype 1 infections (3). At the same dose, larger viral load reductions were achieved in genotype 1b infections than in infections with genotype 1a. The viral load decline after discontinuation of 5-day elbasvir monotherapy was more sustained in genotype 1b patients than in genotype 1a patients at the same elbasvir dose. In general, the types and prevalence of postbaseline RAVs selected within each subgenotype were similar across dosing levels.

In the 2 genotype 1a patients with pretreatment M28V or Q30R polymorphisms, >3-log viral load reductions were achieved with 5 mg and 50 mg dosing of elbasvir, respectively. In the patient with baseline M28V (which did not confer elbasvir resistance *in vitro*) treated with the 5-mg dose of elbasvir, Q30H/Q and L31L/V were additionally detected during the posttreatment follow-up period along with M128V/A. Clonal analysis identified linkages between M28Vand L31V as well as between M28A and Q30H, but no linkage was found between L31V and Q30H. Only M28V was detectable at the last follow-up on day 61 by population sequencing. In the genotype 1a patient with baseline Q30R (associated with a 24-fold increase in elbasvir EC_{90} *in vitro*) treated with the 50-mg dose of elbasvir, Q30R was detected with L31V from the end of treatment until the last follow-up on day 56. Clonal sequencing was not performed for this patient. One genotype 1b patient with Y93H/Y and A92T/A mixtures at baseline treated with the 50-mg dose of elbasvir achieved a >4-log viral load reduction (even though Y93H was associated with a 67-fold increase in elbasvir EC_{90} *in vitro*). After treatment cessation, clonal sequencing identified L28M linked to Y93H and L31V linked to A92K, without linkage between Y93H and either L31V or A92K. At the last visit

TABLE 6 Patients harboring baseline NS5A variants at positions 28, 30, 58, and 93, detected by population sequencing in the phase 1b trial[a]

Identification no. of patients with key baseline NS5A variant(s)	No. of evaluable patients in the corresponding full cohort[b]	Cohort specification		Baseline NS5A polymorphism(s) by population sequencing	Variation(s) and % prevalence by clonal sequencing	Baseline HCV RNA level (viral load) (IU/ml)	Max viral load reduction ($\log_{10}$ IU/ml) in:	
		HCV subgenotype	Elbasvir dose (mg)				Patient	Cohort[c]
1	4	1a	5	M28V	M28V, 94	2.77×10^5	−4.04	−4.09
2	5	1a	50	Q30R	Not tested	1.29×10^7	−3.92	−4.17
3	1	1b	5	P58S	Not tested	6.71×10^6	−4.11	Not applicable
4	5	1b	50	Y93H/Y	Y93H, 64	1.19×10^7	−4.46	−4.82
5	3	3	10	A30A/E/K/T	A30K, 44; Y93H, 15	4.29×10^6	<1	−1.43
6	5	3	50	P58S	Not tested	8.17×10^6	−2.62	−3.24
7	2	3	0 (placebo)	A30A/L/S/V Y93H	Not tested	3.99×10^6	<1	−0.28

[a] Sequence data from 44 patients were used for the resistance analysis. Variants with NS5A substitutions at positions 28, 30, 58, and 93 were detected at baseline in 7 of 44 patients (15.9%) by population sequencing. Only data for patients in dosing cohorts where at least 1 patient had a NS5A polymorphism detected at baseline are shown. Seven cohorts enrolled 1 patient each with baseline variants and overall included a total of 25 patients. The last 2 columns allow comparison of the maximum viral load reduction in the patient with baseline variants to the other patients in the same cohort. All patients completed the 5-day course of monotherapy, including elbasvir in patients 1 through 6 and placebo in patient 7.

[b] All evaluable patients with the same subgenotype infection treated with the same dose of elbasvir.

[c] The geometric mean reduction in HCV RNA levels for the entire cohort of patients with the same subgenotype treated with the same dose of elbasvir exclusive of the 1 patient with the specified NS5A variant was computed for comparison.

on day 59, only the Y93H/Y and L31V/L mixtures were detected by population sequencing.

(iii) Genotype 3 infections. Antiviral responses were less robust for genotype 3 than for genotype 1 infections in the 10-mg elbasvir dosing group, but mean HCV RNA reductions of ~3 log were achieved at the 50- and 100-mg doses (3). Posttreatment Y93H was found in all 10 patients in the 50- and 100-mg elbasvir dose groups and persisted through the last follow-up in each case. L31F was also detected in 2 of these patients.

One of the 3 evaluated patients with genotype 3 infection in the 10-mg dose group harbored a baseline A30A/E/K/T mixture and had a $<$1-$\log_{10}$ IU/ml reduction in the level of HCV RNA at nadir compared to a mean 1.43-$\log_{10}$ drop in viremia in the other 2 patients in the 10-mg dosing group without detectable RAVs at baseline. Population sequencing showed that A30A/E/K/T converted to A30K (which conferred a 41-fold increase in elbasvir EC_{90} *in vitro*) from treatment discontinuation through the last follow-up on day 61.

DISCUSSION

In preclinical experiments, elbasvir exhibited more potent antiviral activity against genotype 1a and 1b (EC_{90}, 0.006 nM) than against genotype 3 replicons (EC_{90}, 0.12 nM). In *de novo* resistance selection assays, elbasvir suppressed the emergence of resistant genotype 1 and genotype 3 colonies in a dose-dependent manner. Under the same selection pressure (expressed as multiples of the elbasvir EC_{90}), resistant colonies emerged less frequently in genotype 1b than genotype 1a at 10× or 100× EC_{90}. At 1,000× EC_{90}, the frequencies of resistant colonies were similar in genotype 1a and 1b replicons; most of these RAVs involved more than one resistance locus, suggesting that an elbasvir dose of 1,000× EC_{90} can suppress RAVs involving only a single amino acid change. In genotype 3, increasing the selection pressure from 10× EC_{90} to 100× EC_{90} significantly reduced the number of resistant colonies, although a further increase to 1,000× EC_{90} did not lead to an incremental reduction. Increased doses selected cells that were highly resistant and contained single and double amino acid substitutions. Although elbasvir retained meaningful activity against some clinically relevant NS5A variants, susceptibility *in vitro* was significantly reduced against genotype 1a and 3 variants harboring particular substitutions at certain loci, especially at position 93 (Y93N and Y93H, respectively). In contrast, substitutions in genotype 1b (including Y93H) caused much less loss of potency. The sequence context of NS5A might impact the level of resistance beyond what can be attributed to individual RAVs (17). Cross-resistance to NS3 protease inhibitors would not be expected (18).

In a subsequent small dose-escalating study of elbasvir given as 5-day monotherapy, viral load reductions were greater for genotype 1 than genotype 3 infections, especially at lower elbasvir doses (3). The decreases in HCV RNA levels were generally more pronounced and longer lasting in infections caused by genotype 1b than by genotype 1a. Robust antiviral responses were observed in the presence of baseline M28V or Q30R variants in genotype 1a infection or the baseline Y93H variant in genotype 1b infections. Variations at resistance loci common to other NS5A inhibitors emerged following exposure to elbasvir monotherapy. Population sequencing detected NS5A RAVs less frequently in genotype 1b than in genotype 1a infections. Viral rebound following cessation of therapy was generally slower with genotype 1b than with genotype 1a. With clonal sequencing, polymorphisms could be found in a large proportion of the viral population irrespective of genotype. The evolution of variants was dynamic, with the linkage of substitutions changing over time.

The antiviral activity and resistance profile of elbasvir observed in patients were generally predictable from the preclinical findings. Similar RAVs were selected *in vitro* and *in vivo*. Population sequencing of viruses from patients enrolled in the phase 1b trial confirmed M28T, Q30R, L31V, and Y93H in genotype 1a, L31V and Y93H in genotype 1b, and A30K, L31F, and Y93H in genotype 3 as the predominant RAVs selected by elbasvir monotherapy. Variants containing these substitutions had reduced susceptibility to elbasvir in HCV replicons with variable replicative capacity. The phenotypic impact of minority variants has not yet been established in most cases.

The preclinical genotypic and phenotypic analyses presaged the clinical findings in the phase 1b study (3). Fewer resistant colonies were selected by elbasvir among genotype 1b than genotype 1a or 3 quasispecies across the dosing range tested in the laboratory. These *in vitro* observations were consistent with the greater antiviral effect seen in patients with genotype 1b infections relative to genotype 1a or 3 infections during the clinical trial. In contrast, *in vitro* replicative capacity ("viral fitness") did not reliably explain viral-load kinetics *in vivo*. Variants that impaired fitness in the colony formation assay (illustrated by Y93 substitutions in genotype 1a) often rebounded as major species, persisting after cessation of elbasvir monotherapy.

The intriguing observation that some RAVs with impaired fitness in the colony formation assay rebounded as major species in patients after cessation of elbasvir monotherapy may reflect assay or biological factors. Since *in vitro* and *in vivo* measurements have different targets, discordance might be expected in some cases. While the replicon assay yields the number of cells that support HCV replication in the laboratory, viral load measurements give the actual number of circulating viruses in the patient. The impact of a substitution within NS5A may to some degree depend on the surrounding context, including the degree of phosphorylation (19). Adaptive amino acid changes elsewhere in and/or outside NS5A in the virus not incorporated into the replicon could enhance replication of certain RAVs *in vivo*. Most known drug-associated substitutions have been located in domain I, and much less is known about the impact of variants in domains II and III (20). Substitutions in domains II and III typically do not directly cause a shift in replicon EC_{50}; however, the effect of these substitutions on viral replication in the setting of polymorphisms in domain I has not been systematically investigated.

The differential impact of RAVs on the outcomes of genotype 1a and 1b infections in the clinical trials was consistent with the lower degree of resistance observed *in vitro* with substitutions in genotype 1b compared to genotype 1a replicons. In the pivotal C-EDGE study of treatment-naive patients (21), SVR_{12} was attained in 2 of 9 (22%) evaluable patients infected with genotype 1a with baseline NS5A RAVs conferring $>$5-fold-decreased susceptibility to elbasvir, as opposed to 9 of 10 (90%) patients with baseline NS5A RAVs conferring $\leq$5-fold-decreased susceptibility and 133 of 135 (99%) patients without baseline NS5A RAVs. In contrast, 16 of 17 (94%) evaluable patients with genotype 1b infections harboring baseline NS5A RAVs conferring $>$5-fold-decreased susceptibility achieved SVR_{12}.

Results with other NS5A inhibitors suggest the generalizability of this observation (22). For example, in patients with baseline

NS5A RAVs treated with the NS5A inhibitor daclatasvir combined with asunaprevir (a NS3 protease inhibitor) and beclabuvir (a nonnucleoside NS5B polymerase inhibitor), SVR_{12} was achieved in 25 (74%) of the 34 patients with genotype 1a infection versus all 17 (100%) patients with genotype 1b infection (23). There was no evident association between baseline NS3 or NS5B variants and SVR_{12}. As found in the C-EDGE trials (21, 24), the UNITY-1 findings again indicated a clinically meaningful impact of baseline NS5A RAVs on the outcome of interferon-sparing treatment exclusively in genotype 1a as opposed to genotype 1b infections.

Elbasvir plus grazoprevir (an investigational NS3/4A protease inhibitor) as a once-daily, oral, single fixed-dose combination tablet is currently being developed for treatment of chronic HCV infection (1, 2, 21, 24–26). Further analyses of the phase 3 trials of grazoprevir-elbasvir will soon provide more critical data concerning the safety and efficacy of this novel double direct-acting antiviral combination.

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