In Vitro Activity of Daclatasvir on Hepatitis C Virus Genotype 3 NS5A

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The NS5A replication complex inhibitor daclatasvir (DCV; BMS-790052) inhibits hybrid replicons containing hepatitis C virus (HCV) genotype 3a (HCV3a) NS5A genes with 50% effective concentrations (EC_{50}s) ranging from 120 to 870 pM. Selection studies with a hybrid HCV3a replicon identified NS5A residues 31 and 93 as sites for DCV-selected resistance. Our results support the potential use of DCV as a component in combination therapies for HCV3a chronic infection.

epatitis C virus genotype 3 (HCV3) is distributed worldwide and is prevalent in India, Southeast Asia, and Australia (1). Daclatasvir (DCV) potently inhibits HCV RNA replication by targeting the essential replication factor NS5A (2). The antiviral activity of DCV has been confirmed in clinical trials with subjects chronically infected with HCV genotype 1 (HCV1) (3–6). In both *in vitro* and *in vivo* studies, mutations that confer resistance to DCV have mapped to the N-terminal 93 amino acids of NS5A, with residues 28, 30, 31, and 93 being particularly prominent resistance-associated sites (2, 6–9).

With the promise of all-oral, interferon-free HCV treatment regimens becoming an ever more attainable goal (3, 10), the ability of direct-acting antivirals (DAA) to exhibit broad HCV genotype coverage is increasingly important. We previously reported on the *in vitro* activity of DCV toward HCV genotypes 1, 2, and 4 (7, 11, 12). Here, we examined the effectiveness of DCV for hybrid replicons containing NS5A sequences derived from three HCV3a isolates. NS5A cDNA was isolated by reverse transcription-PCR (RT-PCR) from HCV3a-positive sera (lot numbers 10650533, 10650577, and 9990964; ProMedDx, Norton, MA) and inserted into BspEI sites in a JFH1 subgenomic replicon (11) such that the resulting replicons encoded hybrid NS5A proteins with amino acids 1 to 429 derived from the HCV3a isolates and amino acids 430 to 471 derived from the parental HCV2a JFH1 strain (Fig. 1). Since residues within the first 100 amino acids of NS5A are largely responsible for mediating sensitivity to DCV and related NS5A inhibitors (2, 13, 14), an alignment of this region of NS5A from the HCV3a isolates (HCV3a1, HCV3a2, and HCV3a4) and four HCV3a reference sequences (15–17) is shown in Fig. 1B. Over NS5A amino acids 1 to 429, HCV3a1, HCV3a2, and HCV3a4 were 94 to 96% identical to the four reference strains, indicating that they are representative HCV3a strains. In replicon transient assays (7), DCV inhibited the JFH-HCV3a hybrid replicons with 50% effective concentrations (EC_{50}s) ranging from 120 to 870 pM (Table 1). Similar DCV potencies were also observed with replicon cell lines (EC_{50}s = 0.14 to 1.25 nM) (Table 1) that were established by neomycin selection as previously described (18). Sequence analysis of NS5A cDNA isolated by RT-PCR from the established replicon cell lines revealed no changes within the NS5A coding region compared to input sequences, consistent with the ability of JFH1-based replicons to replicate efficiently without cell culture-adaptive mutations (19). To identify DCV resistance-associated mutations, the
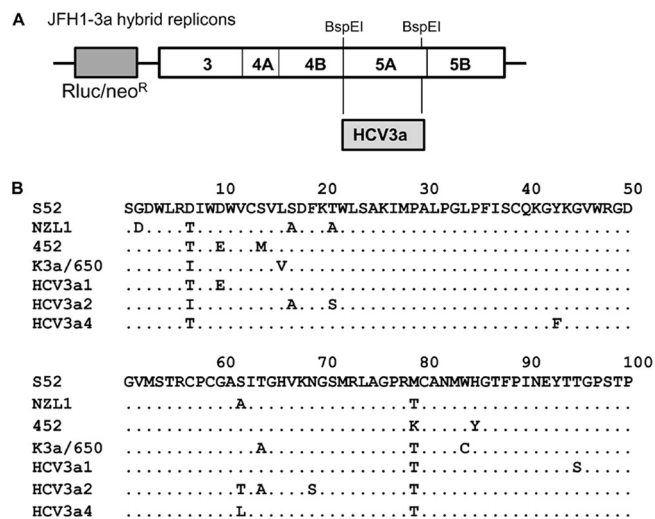

FIG 1 (A) Schematic diagram of a bicistronic JFH1 replicon with Renilla luciferase (Rluc) and neomycin resistance (neoR) genes. The 5′ untranslated region (UTR) is derived from the HCV1b Con1 strain, and the nonstructural genes and 3′ UTR are from the HCV2a JFH1 strain. Hybrid replicons were constructed by replacing the indicated BspEI restriction fragment with sequences from HCV3a1, HCV3a2, and HCV3a4 clinical isolates. (B) Alignment of the N-terminal 100 amino acids of NS5A from the HCV3a1, HCV3a2, and HCV3a4 isolates and four genotype 3a reference sequences (S52, GenBank accession number GU814263; NZL1, GenBank accession number D17763; 452, GenBank accession number DQ437509; K3a/650, GenBank accession number D28917). Amino acid identities are indicated with dots.

HCV3a1 replicon cell line was treated with 10 or 100 nM DCV until resistant cell populations emerged as previously described (7). At both selection concentrations, the selected replicon cells were highly resistant to DCV ($EC_{50} > 1 \mu M$) (Table 2) but still

TABLE 1 Susceptibility of HCV3a hybrid replicons to DCV

Replicon	Replication capacity[a]	EC50 (nM)[b] Transient assay	EC50 (nM)[b] Replicon cells
JFH1	100	0.051 ± 0.025	0.021 ± 0.004
HCV3a1	74 ± 44	0.25 ± 0.10	0.53 ± 0.15
HCV3a2	19 ± 17	0.12 ± 0.09	0.14 ± 0.04
HCV3a4	98 ± 52	0.87 ± 0.45	1.25 ± 0.5

[a] Relative to the parental JFH1 replicon. Values are means and standard deviations.
[b] Half-maximal effective concentrations. Values are means and standard deviations ($n \geq 3$).

TABLE 3 Susceptibility of HCV3a1 variants to DCV[a]

NS5A variant	Replication capacity	EC50 (nM) Transient assay	EC50 (nM) Cell line
HCV3a1	100	0.25 ± 0.10	0.53 ± 0.15
A30K	13.8 ± 2.0	15.40 ± 7.3	29.6 ± 4.4
A30T	103.3 ± 4.5	0.11 ± 0.05	0.57 ± 0.013
L31F	152.8 ± 4.1	80.00 ± 35	320 ± 5.9
S62L	88.1 ± 3.1	0.53 ± 0.039	0.93 ± 0.17
Y93H	29.3 ± 5.3	688.00 ± 253	1,451 ± 344

[a] Values are means and standard deviations ($n \geq 3$).

sensitive to other DAA (data not shown). NS5A cDNA, isolated by RT-PCR from resistant cells as previously described (7), revealed the presence of two amino acid changes (L31F and Y93H) that were not observed in cDNA isolated from dimethyl sulfoxide (DMSO)-treated control cells. Based on visual examination of the population sequence traces, we estimated that the L31F and Y93H amino acid substitutions were present in 30 to 40% and 60 to 70% of the DCV-selected replicon populations, respectively (Table 2). The L31F and Y93H amino acid substitutions were introduced into the parental HCV3a1 hybrid replicon by standard cloning techniques. DCV inhibited these mutant replicons with EC50s of 80 nM (L31F) and 688 nM (Y93H) in transient assays and 320 nM (L31F) and 1.4 μM (Y93H) in assays with neomycin-selected replicon cell lines (Table 3). The L31F mutant replicon replicated as well as or better than the parental replicon, but the replication capacity of the Y93H replicon was ~29% of that of the parental replicon (Table 3).

The naturally occurring variability at NS5A positions 31 and 93 in HCV3a was assessed by examining sequences in the European HCV database (20) (Table 4). DCV resistance-associated NS5A residues (28, 30, 62, and 92) identified from previous studies with HCV1a and HCV2a were also included in this analysis (7, 8, 21). A leucine was present at NS5A position 31 in all 454 HCV3a sequences examined; however, 1.3% (6/454) of the sequences had a histidine at NS5A position 93. Variability was also observed at NS5A positions 30 and 62, while very little or no variability was observed at positions 28 and 92 (Table 4). At position 30, alanine was the predominant residue, but threonine and lysine residues were also commonly observed (5.5% and 2.6%, respectively). An A30T amino acid substitution in the HCV3a1 hybrid replicon did not negatively impact DCV sensitivity, but a replicon with an A30K substitution was ~62-fold more resistant to DCV than was

the parental replicon (Table 3). A lysine at NS5A position 30 (Q30K) was also previously found to be associated with DCV resistance in an HCV1a replicon (9). At position 62, most database sequences had a serine residue (67.6%), but threonine (18.9%) and leucine (10.6%) residues were also commonly observed. Each of these amino acids was represented in one of the HCV3a hybrid replicons described in this study (Fig. 1). DCV exhibited similar potencies toward these replicons (Table 1), suggesting that the variations at position 62 did not substantially impact DCV potency. In agreement with this assessment, the DCV potency toward an HCV3a1 hybrid replicon with an S62L amino acid substitution was only slightly less than that toward the parental replicon (~2-fold less) (Table 3).

In this report, we showed that DCV is a potent inhibitor of hybrid replicons with NS5A sequences derived from three HCV3a strains. Resistance selection and site-directed mutagenesis studies with one of the HCV3a hybrid replicons identified resistance-associated amino acid substitutions at positions 30 (A30K), 31 (L31F), and 93 (Y93H), residues also associated with DCV resistance in HCV1 (7). Previous studies have demonstrated a good correlation between DCV resistance-associated sites *in vitro* and *in vivo* (6, 8, 12, 21), indicating that A30, L31, and Y93 are probable sites for resistance development in DCV-treated HCV3a-infected subjects. Amino acid substitutions at these positions resulted in substantial resistance to DCV (Table 3), possibly indicating a low resistance barrier of DCV in HCV3a strains. On the whole, however, our results indicate that DCV is an effective HCV3a inhibitor with the potential to be a valuable component of future combination therapies.

Nucleotide sequence accession numbers. HCV3a1, HCV3a2, and HCV3a4 NS5A sequences were deposited in GenBank under accession numbers JX944789, JX944790, and JX944791.

TABLE 2 Amino acid substitutions in NS5A cDNA isolated from DCV-treated HCV3a1 replicon cells

DCV selection conc (nM)	DCV EC50 (nM) toward selected cells[a]	NS5A amino acid substitutions[b]
0[c]	0.47 ± 0.08	None
10	>1,000	L31F (40%), Y93H (60%)
100	>1,000	L31F (30%), Y93H (70%)

[a] Values are means and standard deviations (where applicable) ($n \geq 3$).
[b] Values in parentheses are estimates of likelihood deduced from bulk population sequence traces.
[c] DMSO control.

TABLE 4 Variability at HCV3a NS5A amino acid positions associated with DCV resistance

NS5A position	No. of sequences in the European HCV database with the indicated amino acid M	V	I	A	T	K	L	S	P	E	Y	H	X	Other
28	451	2	1											
30	1			414	25	12	2							
31							454							
62		5			86		48	307	3					5
92										454				
93											447	6	1	

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