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TOPIC HIGHLIGHT

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Hepatitis C virus protease inhibitor-resistance mutations: Our experience and review

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Abstract

Direct-acting antiviral agents (DAAs) for hepatitis C virus (HCV) infection are one of the major advances in its medical treatment. The HCV protease inhibitors boceprevir and telaprevir were the first approved DAAs in the United States, Europe, and Japan. When combined with peginterferon plus ribavirin, these agents increase sustained virologic response rates to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection. Without peginterferon plus ribavirin, DAA monotherapies increased DAA-resistance mutations. Several new DAAs for HCV are now in clinical development and are likely to be approved in the near future. However, it has been reported that the use of these drugs also led to the emergence of DAA-resistance mutations in certain cases. Furthermore, these mutations exhibit cross-resistance to multiple drugs. The prevalence of DAA-resistance mutations in HCV-infected patients who were not treated with DAAs is unknown, and it is as yet uncertain whether such variants are sensitive to DAAs. We performed a population sequence analysis to assess the frequency of such variants in the sera of HCV genotype 1-infected patients not treated with HCV protease inhibitors. Here, we reviewed the literature on resistance variants of HCV protease inhibitors in treatment naïve patients with chronic HCV genotype 1, as well as our experience.

Key words: Direct-acting antiviral agent; Hepatitis C virus; Protease inhibitor; Resistance mutation; Sequence analysis

Core tip: The standard of care for the treatment of hepatitis C virus (HCV) infection was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies. These HCV protease inhibitors occasionally cause HCV variants with resistance mutations. We reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment-naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some cases. The combination of direct-acting antiviral agents against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single-stranded RNA virus, approximately 9600 nt in length, that belongs to the *Flaviridae* family. Globally, HCV infects 170 million people and approximately 120-140 million chronic HCV carriers exist[1,2]. HCV infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3,4]. HCV is classified into six major genotypes and > 100 subtypes[5]. HCV genotype 1 (subgenotypes 1a and 1b) is the most common genotype in western countries and Japan[5]. Treatment of HCV is complicated by the existence of different HCV genotypes. The standard of care was peginterferon plus ribavirin until the recent approval of telaprevir- and boceprevir-containing combination therapies[6-14]. Combination of peginterferon plus ribavirin results in sustained virological response (SVR) in nearly 70%-80% of patients with HCV genotype 2 or 3, but in only approximately 50% of those with HCV genotype 1[15,16]. Thus, treatment response is dependent on HCV genotypes and viral loads[17], viral sequence[18-21], host factors such as IL28B genotypes[22-35], drug adherence[36], and adverse events induced by therapeutic drugs[36].

Pharmaceutical companies are actively investigating and developing direct-acting anti-viral agents (DAAs) against HCV, which directly target specific HCV proteins such as NS3/4A protease[6-14], NS5A protein[37-39], and NS5B polymerase[40], which are important for HCV replication in hepatocytes. Two first-generation HCV protease inhibitors, boceprevir and telaprevir, were approved in combination with peginterferon plus ribavirin for treatment of chronic HCV genotype 1 in 2011[6-14]. Both protease inhibitors combined with peginterferon plus ribavirin increased SVR rates up to 70%-80% in treatment-naïve patients and previous-treatment relapsers with chronic HCV genotype 1 infection[6-14]. Next-generation HCV protease inhibitors will be available in clinics in the near future (Table 1)[41]. For example, simeprevir[42,43], faldaprevir[44,45], and vaniprevir[46-48] are currently in phase 3 trials. HCV protease inhibitors primarily are specific agents for HCV genotype 1. However, studies have demonstrated that simeprevir is fairly active against most HCV genotypes with the exception of HCV genotype 3a[42], and recently, in a phase 2 trial, the novel protease inhibitor MK-5172 showed even broader activity across HCV genotypes compared to simeprevir[49].

The low fidelity of HCV NS5B polymerase, high replication rate, and strong selective pressures on this virus lead to emergence of viral quasispecies. The quasispecies nature exists in a mixed population of viruses, with the fittest viruses being the predominant viral populations, as observed by sequence analysis[50,51]. In addition, new populations with every potential substitution are likely created and lost each day, some of which convey various degrees of resistance to DAAs[52-54]. Due to the high sequence diversity of HCV, naturally occurring pre-existing resistance mutations have been found at a low prevalence in HCV-infected, treatment-naïve patients[55,56].

In a previous study[56], 9% of the HCV genotype 1a-infected patients who were not treated with HCV protease inhibitors had at least one pre-existing dominant protease inhibitor-resistant variant, as observed by population sequencing. In another report[57], although the number of patients was small, the prevalence of protease inhibitor-resistance mutations was high (28%) in 53 genotype 1a samples, while no mutations were found in only 5 patients infected with HCV genotype 1b. In HCV genotype 1b treatment-naïve patients, the percentage of naturally occurring pre-existing resistance mutations appears to be lower[58]. Recently, next generation sequencing technology, with a detection limit as low as < 0.1%, have shown the ability to detect most resistance variants (including high resistance variants, *i.e.*, 155, 156 and 168) in patients infected with protease inhibitor-untreated HCV genotype 1[59]. Thus, the prevalence of naturally occurring pre-existing resistance mutations in patients infected with HCV genotype 1 who were not treated with HCV protease inhibitors remains unclear.

NATURALLY OCCURRING PREEXISTING RESISTANCE MUTATIONS IN PATIENTS INFECTED WITH HCV GENOTYPE 1B

We estimated a 98%-99% prevalence of HCV subgenotype 1b among patients infected with HCV genotype 1 in Japan[60]. Sera from 88 Japanese patients infected with HCV genotype 1b who were not treated with HCV protease inhibitors were examined. Some patients had been included in a previous study[60]. We investigated the naturally occurring pre-existing resistance mutations in these patients by using a direct-sequencing method. The study protocol was approved by the Ethics Committee of Chiba University School of Medicine.

The clinical background of the 88 patients is shown in Table 2. All but one patient had high viral loads. Population sequencing of the HCV NS3 region was performed in these 88 patients, and then the amino acid sequences were compared to the HCV NS3 amino acid sequence corresponding to the HCV genotype 1b Con1 strain. The prevalence of pre-existing variations in the HCV genotype 1b samples was 39% (34/88). Among these mutations, the resistance mutations T54S and D168N were found in 6.8% (6/88) and 1.1% (1/88) of the patients, respectively. Other resistance mutations Q80L, V170Y, V170N and V170L were found in 22% (19/88), 4.5% (4/88), 3.4% (3/88) and 1.1% (1/88) of the patients, respectively. In 5.7% (5/88) of the patients, more than one mutation was identified: four patients had T54S and Q80L, and one patient had T54S, Q80L and

Table 1 Overview of representative clinical trials of hepatitis C virus NS3/4A protease inhibitors

Name of drug (other name)	G	Trial phase	Features of clinical trials (ClinicalTrials.gov Identifier)
Telaprevir (VX-950)	1	FDA approved	Telaprevir, PEG-IFN alpha-2a, RBV
	1b	3	Telaprevir, Daclatasvir (NS5A inhibitor), PEG-IFN alpha-2a, RBV (COMMAND-3) (NCT01492426)
	1	3	Telaprevir, PEG-IFN lambda-1a, RBV (NCT01598090)
	4	2	Telaprevir, PEG-IFN alpha-2a, RBV (NCT0050801)
Boceprevir	1	FDA approved	Boceprevir, PEG-IFN alpha-2a, RBV
(SCH 503034)	1	3	Simeprevir, PEG-IFN alpha-2a, RBV (NCT01290731)
Simeprevir (TMC435)	1b/4	2	Simeprevir, IDEX719 (NS5A inhibitor), RBV (NCT01852604)
Faldaprevir (BI201335)	1	3	Faldaprevir, PEG-IFN alpha-2a, RBV
	1a	2	Faldaprevir, PPI-668 (NS5A inhibitor), BI207127 (non-nucleoside NS5B inhibitor), (+ RBV) (NCT01859962)
	1b	2	Faldaprevir, BI207127, RBV (NCT01858961)
Danoprevir (ITMN-191)	1	2	Danoprevir, PEG-IFN alpha-2a, RBV (NCT00963885)
	1/4	2	Danoprevir, Ritonavir, PEG-IFN alpha-2a, RBV (NCT01220947)
	1	2	Danoprevir, Ritonavir, RO5024048 (NS5B inhibitor), RBV, (± PEG-IFN alpha-2a) (NCT01331850)
Vaniprevir (MK-7900)	1	3	Vaniprevir, PEG-IFN alpha-2b, RBV (NCT01405937)
Asunaprevir (BMS-650032)	1	3	Asunaprevir, Daclatasvir (NCT01497834)
	1	2	Asunaprevir, PEG-IFN lambda, RBV (NCT01309932)
	1/4	2	Asunaprevir, PEG-IFN alpha-2a, RBV (NCT01030432)
	1a/1b/4	2	Asunaprevir, Daclatasvir, BMS-791325 (NS5B inhibitor) (NCT01455090)

Data from http://www.clinicaltrials.gov accessed on September 8, 2013. FDA: United States Food and Drug Administration; G: Genotype; HCV: Hepatitis C virus; PEG-IFN: Peginterferon; RBV: Ribavirin.

Table 2 Clinical characteristics of hepatitis C virus genotype 1b-infected patients in sequence analysis study of the hepatitis C virus NS3 region

No. of patients (men/women)	88 (43/45)
Age (yr)	55 ± 14
HCV RNA levels (low/high)	1/87
ALT (IU/L)	67 ± 44
WBC (x 10^3/mcL)	5.2 ± 1.5
Hemoglobin (g/dL)	14 ± 1.2
Platelet counts (x 10^4/mcL)	20 ± 18
IL28B rs8099917, TT/TG/GG/unknown	45/29/0/14

HCV RNA levels, low: less than 5 log IU/mL; HCV RNA levels, high: equal to or more than 5 log IU/mL; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; WBC: White blood cell.

V170N (Table 3). We did not identify high resistance variants at 155 and 156 in protease inhibitor-untreated HCV genotype 1b-infected patients (Table 3). Suzuki *et al*[58] reported that amino acid substitutions conferring resistance to protease inhibitors (V36A, T54S, Q80H, and D168E) were detected in 15 of 307 (4.9%) patients infected with HCV genotype 1b who had not received DAAs previously, and T54S (3.3%) predominated over V36A (0.3%), Q80R (0.7%) and D168E (0.7%), similar to our results. Leggewie *et al*[61] measured the prevalence of natural resistance polymorphisms in 38 acutely human immunodeficiency virus (HIV)-HCV co-infected treatment-naïve patients by using direct and deep sequencing. They found that 26% of patients (10/38) had a majority variant resistance mutation (in order of frequency: Q80K-16%, V36M-5%, T54S-3%, V55A-3% and D168A-3%). Low-frequency mutations were detected in all samples.

RESISTANCE MUTATIONS AND VIROLOGIC FAILURE

Despite extensive efforts to develop more potent next-generation protease inhibitors, the long-term efficacy of this drug class is challenged by the rapid emergence of resistance[62,63], which could result in treatment failures such as viral breakthrough or relapse. Our identified mutations associated with resistance to protease inhibitors are shown in Figure 1. In the Protease Inhibitor for Viral Evaluation (PROVE) 1 and 2 clinical trials[8,9] of telaprevir in combination with peginterferon plus ribavirin, viral breakthrough occurred in approximately 7% of patients with HCV genotype 1a infection, compared with about 2% of those with HCV genotype 1b infection; approximately 10% of patients with either subgenotype 1a or 1b suffered a relapse after cessation of HCV protease inhibitor-treatment. In both ADVANCE and Illustrating the Effects of Combination Therapy with Telaprevir (ILLUMINATE) trials[11,13], about 60% of patients treated with telaprevir-based triple therapy achieved an extended rapid virologic response (eRVR), with no virus detected at weeks 4 and 12.

HCV variants associated with on-treatment virologic failure or relapse were evaluated by using site-directed mutagenesis in HCV replicon assay[62,64]. Variants V36A/M, T54A/S, R155K/T, and A156S conferred lower levels of *in vitro* resistance to telaprevir (three- to 25-fold increase in telaprevir IC_{50}), and A156V/T and V36M + R155K variants conferred higher levels of *in vitro* resistance to telaprevir (> 25-fold increase in telaprevir IC_{50}). HCV replicon variants generated from patient-derived

Table 3 Naturally occurring pre-existing resistance amino acid mutations in the hepatitis C virus NS3 regions of 28 protease inhibitor-naive patients infected with hepatitis C virus genotype 1

Patient No.	V36	T54	V55	Q80	R155	A156	D168	V170Y/N/L		
	A/M	S	A	L	K/T/Q	S/T/V	N	Y	N	L
31		S		L						
95				L						
15				L						
17				L						
24				L						
26		S		L						
29		S								
81		S		L						
61								Y		
72								Y		
11				L						
12				L						
2								Y		
53				L						
55				L						
66				L						
84				L						
85				L						
110				L						
112								Y		
114		S		L						
100				L						
101									N	
107							N			
111									N	
99										L
92				L						
97		S		L					N	

sequences showed similar results. The *in vitro* replication capacity of telaprevir-resistant variants was lower than that of wild-type virus in the HCV genotype 1b Con1 replicon system[64-67]. When telaprevir-resistant variants were tested for cross-resistance against representative protease inhibitors in the HCV replicon system, HCV replicons with single substitutions at position 155 or 156 and double variants with substitutions at residues 36 and 155 showed cross-resistance to all protease inhibitors tested with a wide range of sensitivities. All telaprevir-resistant variants studied remained fully sensitive to interferon-alpha, ribavirin, and representative HCV nucleoside and non-nucleoside polymerase inhibitors in the replicon system. There are limited clinical data regarding re-treating patients who have failed an HCV NS3-4A protease inhibitor-based therapy such as telaprevir monotherapy, suggesting that re-treatment with triple therapy might be useful for certain patients.

In the boceprevir Serine Protease Inhibitor Therapy 2 (SPRINT-2) trial[6], patients showing a decrease in HCV viral load $\geqslant$ 1 log10 IU/mL during the four-week lead-in period of peginterferon plus ribavirin therapy had very low rates of emergence of boceprevir-resistant mutants (4%-6%) during subsequent triple therapy, whereas those with a < 1 log10 IU/mL decrease in HCV RNA had higher rates (40%-52%) of boceprevir-resistance-associated variants (genotypic mutations of the protease conferring reduced sensitivity to boceprevir). The majority of boceprevir-treated subjects not achieving SVR had one or more specific treatment-emergent NS3 amino acid substitutions, most of which were previously shown to reduce the anti-HCV activity of boceprevir. These substitutions included V36A, V36M, T54A, T54S, V55A, V107I, R155K, A156S, A156T, A156V, V158I, D168N, I/V170A, and I/V170T. Detection of these substitutions was most common among subjects who experienced virologic breakthrough or incomplete virologic response[68].

COMBINATIONS OF DAAS FOR HCV STRAINS WITH RESISTANCE MUTATIONS

Protease inhibitors are used in combination with peginterferon plus ribavirin because monotherapy with protease inhibitors results in the early emergence of drug-resistance mutations[62,63]. As peginterferon plus ribavirin treatment is frequently associated with serious adverse events, an interferon-free DAA combination therapy such as protease inhibitors with an NS5A inhibitor and/or NS5B inhibitor would offer an ideal treatment option for patients with chronic HCV infection. However, combinations of DAA-resistant variants both in a single target protein and across multiple targets have been reported following failure of single and combination DAA regimens[55,69-71]. HCV population sequences of the complete HCV NS3 and 4A regions obtained from 2,111 HCV subgenotype 1a and HCV subgenotype 1b DAA-naïve patients were analyzed by Bartels *et al*[72]. It was reported that the strongest association was the combination of variants at NS3 V55, with lower-level resistance to boceprevir, and NS3 T54, with lower-level resistance to boceprevir and telaprevir[73]. The complete HCV NS3 study dataset showed that 69% (33/48) of patients with HCV NS3 V55I also had T54S. An association was also observed between HCV NS3 positions 54 and 155, with 17% (3/18) of the patients with the HCV NS3 T54S substitution also having R155K. The HCV NS3 T54S and R155K combination appeared in boceprevir and telaprevir trials. The study[73] also reported that treatment-naïve patients with viral populations containing the telaprevir-resistant variants HCV NS3 V36M, T54S or R155K at baseline achieved a 74% SVR rate with DAAs, similar to that (76%) in patients without resistant variants detected prior to treatment. Further studies are needed to confirm these findings.

DIFFERENCES IN RESISTANCE MUTATION SELECTION BETWEEN HCV GENOTYPE 1A AND HCV GENOTYPE 1B

It is possible that a different pattern of nucleotide changes is required for the resistance amino acid mutations between HCV genotypes 1a and 1b[67]. Substitutions at

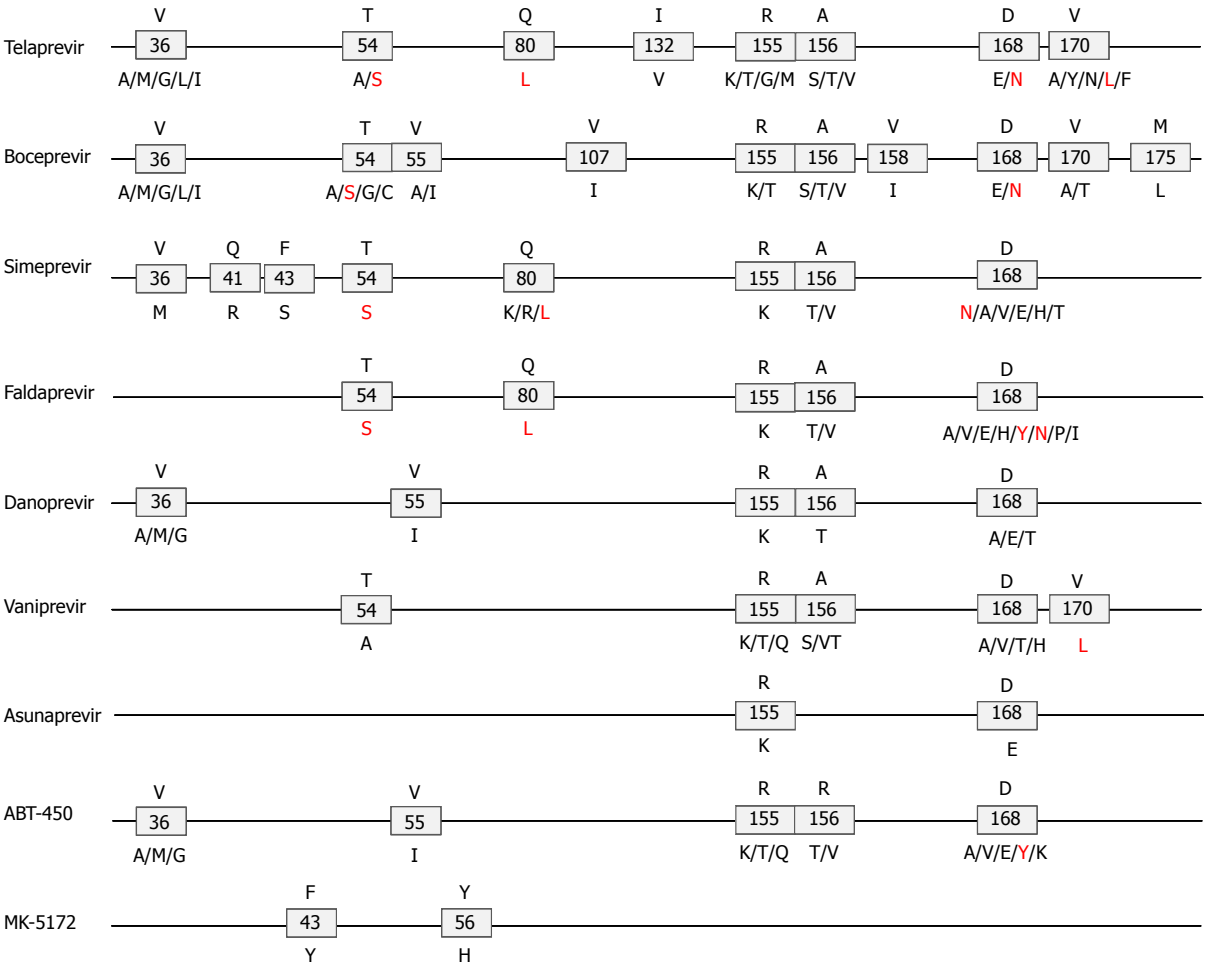

Figure 1 Mutations in hepatitis C virus NS3/4A serine protease that impact susceptibility to hepatitis C virus drugs approved by the United States Food and Drug Administration and investigated in phase 2 or 3 clinical trials. The numbers indicate the positions of the amino acids of the hepatitis C virus genotype 1 Con 1 strain. The amino acids above and below the numbers indicate wild-type amino acids and their substitutions, respectively. The red color indicates the mutations detected among the population using sequencing in the present study.

Table 4 Nucleotide changes were required for amino acid substitutions at position 155 of hepatitis C virus NS3 among hepatitis C virus genotype 1 samples

Amino acid at position 155	HCV genotype 1a	HCV genotype 1b
R	AGG	CGG
K	AAG	**AAG**
T	ACG	**ACG**
S	AGC	**AGC**
I	ATC	**ATC**

Bold-faced nucleotides nucleotides were required for amino acid substitutions at position 155. HCV: Hepatitis C virus.

A156 (A156S, A156T or A156V) require only a one-nucleotide change in HCV genotype 1a and HCV genotype 1b. In contrast, substitutions at R155 with K, T, S, M or I require a two-nucleotide substitution in HCV genotype 1b isolates. However, R155K/T/S/M/I substitutions require a one-nucleotide substitution in HCV genotype 1a isolates. The need for a two-nucleotide change for substitution R155 in HCV genotype 1b could be one of the reasons that HCV genotype 1a is more resistant to protease inhibitors than HCV genotype 1b (Table 4). In the ELECTRON study[74] of NS5B inhibitor sofosbuvir, no differential resistance was observed between genotypes 1a and 1b despite 89% of the subjects being in the HCV genotype 1a population, suggesting that combination DAAs targeting other HCV regions with next-generation HCV protease inhibitors could overcome the challenges of resistance mutations. In the near future, although mutation analysis was previously performed with population sequencing using Sanger methods, ultra-deep sequencing technology should provide new information[60,75-78]. Ligand bioactive conformation also plays a critical role in the design of HCV NS3 protease inhibitors and may allow for a large variety of HCV protease drug candidates to be designed[79].

CONCLUSION

In summary, we reviewed the literature reports of resistance variants of HCV protease inhibitors in treatment naïve patients with chronic HCV genotype 1, as well as our experience. Even in treatment-naïve patients with

chronic HCV genotype 1, naturally occurring HCV protease inhibitor-resistance mutations exist in some. Monotherapy with HCV protease inhibitors should be absolutely avoided. Regarding HCV protease inhibitor-resistance mutations, attention should also be paid to DAA-treatment-experienced patients, who previously used HCV protease inhibitor-monotherapies or combination therapies with HCV protease inhibitors. HCV genotype 1a is more resistant to protease inhibitors than HCV genotype 1b, and it is easier for HCV genotype 1a strains to be resistant to the currently available HCV protease inhibitors. At present, patients should be treated according to the recommendations of several HCV clinical practice guidelines[80-86]. However, it may also be possible that the combination of DAAs against regions other than HCV NS3/4A could eradicate HCV with these resistance variants.

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