Prevalence of natural polymorphisms at the HCV NS5A gene associated with resistance to daclatasvir, an NS5A inhibitor

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Background: Daclatasvir (BMS-790052) is an investigational molecule that inhibits the HCV NS5A protein and shows potent antiviral activity apparently across all HCV genotypes. Selection of drug resistance mutations has been reported only for HCV genotype 1, and no information exists for other HCV variants and/or in HIV–HCV-coinfected individuals.

Methods: All interferon-α-naive, HIV–HCV-coinfected patients newly attended at Hospital Carlos III (Madrid, Spain) in 2011 were identified. Changes reported to be associated with daclatasvir resistance in the in vitro replication system for HCV genotype/subtypes 1a/1b (M28T, Q30H/R, L31F/M/V, P32L and Y93C/H/N) were examined.

Results: A total of 78 HIV–HCV-coinfected individuals as well as 635 NS5A sequences deposited at Los Alamos HCV database were analysed. None of the NS5A sequences from HCV-1a or HCV-3 showed changes associated with daclatasvir resistance. By contrast, all NS5A sequences from HCV-4 harboured L31M. The double mutant L31M+Y93H was found in 7% of HCV-1b and 13% of HCV-4. Finally, all NS5A sequences from HCV-1b and HCV-4 harboured changes at codon 28 (M28L) and 30 (L30R), which are of unknown significance. The rate of all these NS5A polymorphisms did not differ significantly when comparing HIV–HCV-coinfected patients and sequences from HCV-monoinfected subject deposited at Los Alamos HCV database.

Conclusions: Primary resistance mutations to daclatasvir, an investigational HCV NS5A inhibitor, are not seen in HCV-1a or in HCV-3 as natural polymorphisms. By contrast, they can be recognized in most HCV-1b and HCV-4 strains, regardless HIV coinfection.

Introduction

The combination of pegylated interferon-α and ribavirin was, until recently, the only therapeutic option for chronic HCV infection, which provided poor rates of cure and frequent side effects [1]. These limitations were further accentuated in HIV–HCV-coinfected patients in whom liver disease evolves more rapidly than in HCV-monoinfected individuals [2]. Thus, the advent of new direct acting antivirals (DAAs) against HCV has been eagerly awaited for the treatment of chronic hepatitis C in HIV-coinfected individuals [3].

DAAs target distinct viral proteins, including the NS3 protease, the NS5B polymerase and the NS5A protein. Two HCV protease inhibitors, telaprevir and boceprevir, have already been marketed and many other compounds are in advance stages of clinical development [3]. Although the antiviral potency of most DAAs is impressive, the rapid emergence of drug resistance in HCV must be considered as a major threat. Moreover, given the large genetic variability in HCV, the efficacy of these drugs can be genotype/subtype-dependent, mainly due to the presence of natural polymorphisms associated with resistance to some of these compounds in distinct viral variants. Indeed, this has already been reported in clinical trials using NS3 protease inhibitors and non-nucleoside analogues blocking the NS5B polymerase, highlighting that HCV-1a might have a lower barrier to resistance than HCV-1b [4,5].

Because the majority of trials are testing new DAAs in patients infected with HCV genotype 1, their efficacy against other HCV genotypes remains largely unknown. This is also the case for the new promising NS5A inhibitors [6], although preliminary data have suggested that they might exert a much broader activity against distinct HCV genotypes. The HCV NS5A
There is an urgent need for information regarding the presence of HIV-associated immunosuppression, with HIV and that HCV variability could be enhanced in chronic hepatitis C who need treatment are coinfected with HIV and daclatasvir interaction site with the NS5A protein [8,9].

These residues likely delineate the resistance profile to daclatasvir has recently been characterized in the in vitro replicon system using HCV-characterized in the replicon system using HCV-1a and HCV-1b [8]. Resistance mutations have been reported at the N terminus of domain 1 at codons 28, 30, 31, 32 and 93. These residues likely delineate the daclatasvir interaction site with the NS5A protein [8,9].

Given that a relatively large proportion of patients with chronic hepatitis C who need treatment are coinfected with HIV and that HCV variability could be enhanced in the presence of HIV-associated immunosuppression, there is an urgent need for information regarding the expected activity of NS5A inhibitors in the coinfected population. The aim of this study was to assess the prevalence of natural polymorphisms at the HCV NS5A protein domain 1 that might be associated with daclatasvir resistance in HIV–HCV-coinfected individuals.

### Methods

All consecutive HIV–HCV-coinfected patients who attended, for the first time, Hospital Carlos III (Madrid, Spain) during the year 2011 were identified. For the purpose of this study, only individuals who had never been exposed to pegylated interferon- α/ribavirin and/or any antiviral drug for treating chronic hepatitis C were chosen. Plasma samples regularly stored at -80°C were recovered from the laboratory refrigerators. Only individuals who had specimens frozen before beginning any anti-HCV therapy were selected.

Plasma HIV RNA was measured using Versant 3.0 bDNA (Siemens, Barcelona, Spain). Serum HCV RNA was quantified using the Abbott real-time HCV quantitative assay (Abbott Laboratories, Madrid, Spain). In HCV viraemic patients, HCV genotype/subtype was determined using LiPA version 2.0, a commercial hybridization assay (Innogenetics, Ghent, Belgium).

The HCV NS5A gene was sequenced using plasma RNA extracted from clinical specimens. HCV RNA was extracted using a commercial QUlamp viral RNA kit (Qiagen, Hamburg, Germany), following the manufacturer’s instructions. The HCV NS5A gene was amplified by reverse transcriptase (RT) nested-PCR using specific primers designed for each HCV genotype/subtype (Table 1). Conditions for the RT-PCR procedures were as follows: 48°C for 45 min, 94°C for 2 min, 45 cycles at 94°C for 35 s, 53–55°C for 30 s and 72°C for 1 min.
and 30 s, and a final extension at 72°C for 10 min. For the nested-PCR step, conditions were as follows: 94°C for 3 min, 45 cycles at 94°C for 35 s, 53–55°C for 35 s, and 72°C for 1 min and 30 s, and a final extension at 72°C for 10 min. PCR amplicons were purified using the High Pure PCR product Purification Kit (Roche, Mannheim, Germany) and directly sequenced in both senses using the ABI PRISM 3100Genetyc Analysyer using the ABI PRISM Rhodamine Terminator reaction kit (Applied Biosystems, Foster City, CA, USA). Finally, HCV sequences were aligned and analysed for drug resistance mutations using the SeqScape version 2.5 software (Applied Biosystems).

Drug resistance mutations at the HCV NS5A gene, such as M28T, Q30H/R, L31F/V, P32L and Y93H/N, all of which have recently been reported to diminish daclatasvir susceptibility in the replication system for HCV-1a and HCV-1b, were examined [6].

**Results**

A total of 78 HIV–HCV-coinfected individuals fit the inclusion criteria and had available plasma stored specimens. Overall, 75% were male with a median age of 45 years. Three quarters of them were on antiretroviral therapy and 80% had undetectable plasma HIV RNA. The median serum HCV RNA was 6.1 log IU/ml (IQR 5.6–6.4). The distribution of HCV genotypes was as follows: 51 HCV-1 (65.5%; being 36 HCV-1a and 15 HCV-1b), 19 HCV-3 (24.3%) and 8 HCV-4 (10.2%).

Table 2 depicts the prevalence of changes associated with daclatasvir resistance in the study population. None of the NS5A sequences from HCV-1a individuals harboured mutations associated with loss of daclatasvir susceptibility. By contrast, one HCV-1b patient harboured the double mutant L31M+Y93H, which has been associated with high-level resistance to daclatasvir in vitro (>4,000 fold change) [8]. All NS5A sequences from HCV-3 harboured an amino acid substitution at codon 30 (Q30A) of unknown significance. Finally, one individual infected with HCV-4 harboured the combination L31M+Y93H. Interestingly, all NS5A sequences from HCV-4 harboured L31M, which has been associated with high-level resistance to daclatasvir (>300 fold change) in HCV-1a replicons. Moreover, all NS5A sequences from HCV-1b and HCV-4 harboured changes at codon 28 (M28L) and 30 (L30R), which are of unknown significance.

The overall prevalence of drug resistance changes at the NS5A gene was additionally investigated in 635 sequences available in the Los Alamos HCV database, which is mainly derived from HCV-monoinfected individuals. They had the following genotype/subtype distribution: 153 HCV-1a, 155 HCV-1b, 282 HCV-3 and 45 HCV-4 (Table 2). Overall, the prevalence of mutations associated with resistance to NS5A inhibitors was similar to that found in our HIV–HCV-coinfected population. None of the NS5A sequences from HCV-1a harboured changes associated with daclatasvir resistance. In HCV-1b isolates, the rate of mutations L31M and Y93H was 7% and 6%, respectively. However, the combination L31M+Y93H, which has been associated with high-level resistance to daclatasvir, tended to be lower in the NS5A sequences from the Los Alamos HCV database (0.6%) compared with our coinfected population (7%). The prevalence of mutations L31M and Y93H in HCV-4 was also comparable (93.3% and 10%, respectively) in the two groups. Finally, changes at codon 28 (M28L) and 30 (L30R) were also recognized in most NS5A sequences from HCV-1b (97.5% and 92.2%, respectively) deposited in the Los Alamos HCV database. Furthermore, Q30A was seen in 90.4% of HCV-3 sequences. Finally, M28L was seen in 82% and L30R in 50% of HCV-4 sequences.

In order to explore the higher genetic barrier demonstrated in genotype 1b compared with 1a, we evaluated the triple nucleoside composition at specific critical codons and the number of nucleotide substitutions.
required to become resistant to daclatasvir (Table 3). Selection of significant resistance mutations required only one nucleotide change in most cases, although two were required in 7% of HCV-1a for L31M and in 18% of HCV-1b for L31F. It is noteworthy that fold changes produced by the same mutations generally compromise, to a greater extent, the susceptibility to daclatasvir in HCV-1a than HCV-1b.

**Discussion**

This study assessed the rate of natural changes at the HCV NS5A domain 1 associated with resistance to the NS5A inhibitor daclatasvir in HIV–HCV-coinfected patients who had never been exposed to pegylated interferon-α/ribavirin and/or any experimental DAA. Overall, the frequency of natural polymorphisms associated with resistance to daclatasvir in the coinfected population was similar to that found in 635 NS5A sequences from HCV-3 harboured an amino acid substitution at codon 30 (Q30A), which is also of unknown significance. Likewise, all NS5A sequences from HCV-4 harboured changes at codons (M28L) and 30 (L30R), which are of unknown significance. P32L and Y93C/H/N [8], was HCV genotype/subtype-dependent. Importantly, this finding was not influenced by the HIV status. Although NS5A sequences belonging to HCV-1a did not harbour any resistance change to daclatasvir, the double mutant L31M+Y93H, which results in high-level resistance to daclatasvir, was seen in 7% of HCV-1b and 13% of HCV-4 strains. In addition, all NS5A sequences from HCV-4 harboured L31M, which has been associated with high-level resistance to daclatasvir in HCV-1a replicons. Moreover, all NS5A sequences from HCV-1b and HCV-4 harboured changes at codons (M28L) and 30 (L30R), which are of unknown significance. The prevalence of mutations associated with resistance to daclatasvir, such as M28T, Q30H/R, L31F/M/V, 

<table>
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<tr>
<th>HCV-1 subtype</th>
<th>Codon</th>
<th>Wild-type amino acid</th>
<th>Resistance mutation*</th>
<th>EC₅₀ fold change*</th>
<th>Number of nucleotide changes</th>
<th>Prevalence*</th>
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<tr>
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*Resistance change is shown in bold. 50% effective concentration (EC₅₀) values derived from in vitro susceptibility testing by Fridell et al. [8]. *Prevalence of each nucleotide codon was examined in 635 NS5A sequences deposited at the Los Alamos HCV database.
NS5A sequences deposited in the Los Alamos HCV database. Only the combination L31M+Y93H (present in 7% of HCV-1b and 13% of HCV-4 coinfected patients) tended to be less common in Los Alamos HCV sequences (0.6%), which is mainly represented by HCV-monoinfected individuals.

There is no information regarding the potential effect of natural polymorphisms at the NS5A gene on the clinical response to daclatasvir across distinct HCV genotypes/subtypes. In Phase II clinical trials, the antiviral activity of this inhibitor was tested in subjects infected with HCV-1. Interestingly, the best responses were seen in HCV-1b patients, with more failures in those infected with HIV-1a, as reported in other studies using other DAAs such as NS3 protease inhibitors and non-nucleoside HCV polymerase inhibitors [4,10]. Although the exact mechanism underlying this observation is not completely understood, a different rate of natural polymorphisms in HCV-1 subtypes may influence the resistance barrier might contribute to explain it. In this regard, it is intriguing that we found natural polymorphisms potentially involved in daclatasvir resistance in most HCV-1b but in none of HCV-1a patients. In the case of HCV protease inhibitors telaprevir and boceprevir, differences in nucleotides at codon 155 of the HCV protease have been claimed to explain the lower resistance barrier in HCV-1a versus HCV-1b [3,4]. We could not find such differences between HCV-1 subtypes examining NS5A codons involved in resistance to daclatasvir, and the number of nucleotide substitutions required to result in amino acid changes at resistance positions was comparable in HCV-1a and HCV-1b. Therefore, the results of our examination of NS5A sequences do not explain the different behaviour of HCV subtypes 1a and 1b with respect to the clinical antiviral activity of daclatasvir.

Among other mechanisms that may contribute to explain the different activity of daclatasvir confronting distinct HCV variants, two merit particular attention. First, in the replication system the loss of susceptibility to daclatasvir associated with selection of resistance mutations at NS5A was more pronounced in HCV-1a (ranging from 233 to 3,350 fold changes) than in HCV-1b (ranging from 3 to 28 fold changes). Thus, in contrast to HCV-1a, more than one resistance mutation might be required to impair significantly daclatasvir activity in most HCV-1b viruses. Secondly, NS5A plays a critical role in regulating viral replication as an essential component of the HCV replication complex through the interaction with other non-structural viral proteins such as NS3, NS4A, NS4B and NS5B. Due to the massive protein–protein interaction in the HCV replication complex, the presence of polymorphisms at NS5A regions other than domain 1 might hypothetically influence the susceptibility of distinct HCV variants to daclatasvir. In this regard, recent data have demonstrated that emergence of resistance to other NS5A inhibitors may involves selection of additional changes at NS3, NS4B and/or NS5B proteins [11].

A limitation of our study refers to the use of bulk sequencing to explore drug resistance mutations in HCV. In this regard, the presence of viral genomes with resistance changes at low proportion might have been missed. To exclude the presence of minority variants, more sensitive tools as allelic-specific PCR or ultra-deep sequencing should have been required. However, at this time it is unclear the clinical and/or therapeutic relevance of the presence of low-level drug-resistant mutant viruses.

In summary, natural polymorphisms at the HCV NS5A at positions that may influence the susceptibility to daclatasvir are rarely observed in HCV-1a and HCV-3, whereas conversely they are common in HCV-1b and HCV-4. Similar findings were recognized in our HIV–HCV-coinfected patients and in NS5A sequences from HCV-monoinfected individuals deposited in the Los Alamos HCV database. This information may provide further insights to understand the different antiviral activity of daclatasvir across distinct HCV genotypes/subtypes and patient populations.

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VS and EP designed the study. ZP and MdMG did the laboratory work. ZP, ES and EP did the data analysis. ZP, EP and VS wrote the manuscript. EV and PB provided the samples, clinical data and critical insights into the manuscript.

Disclosure statement

The authors declare no competing interests.

References