Characterization of Resistance to the Protease Inhibitor Boceprevir in Hepatitis C Virus–Infected Patients

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Boceprevir is a hepatitis C virus (HCV) nonstructural protein (NS) 3/4A protease inhibitor that is currently being evaluated in combination with peginterferon alfa-2b and ribavirin in phase 3 studies. The clinical resistance profile of boceprevir is not characterized in detail so far. The NS3 protease domain of viral RNA was cloned from HCV genotype 1–infected patients (n = 22). A mean number of 47 clones were sequenced before, at the end, and after treatment with 400 mg boceprevir twice or three times daily for 14 days for genotypic, phenotypic, and viral fitness analysis. At the end of treatment, a wild-type NS3 protease sequence was observed with a mean frequency of 85.9%. In the remaining isolates, five previously observed resistance mutations (V36M/A, T54A/S, R155K/T, A156S, V170A) and one mutation (V55A) with unknown resistance to boceprevir were detected either alone or in combination. Phenotypic analysis in the HCV replicon assay showed low (V36G, T54S, R155L; 3.8- to 5.5-fold 50% inhibitory concentration [IC50]), medium (V55A, R155K, V170A, T54A, A156S; 6.8- to 17.7-fold IC50) and high level (A156T; >120-fold IC50) resistance to boceprevir. The overall frequency of resistant mutations and the level of resistance increased with greater declines in mean maximum HCV RNA levels. Two weeks after the end of treatment, the frequency of resistant variants declined and the number of wild-type isolates increased to 95.5%. With the exception of V36 and V170 variants all resistant mutations declined by more than 50%. Mathematical modeling revealed impaired replicative fitness for all single mutations, whereas for combined mutations a relative increase of replication efficiency was suggested. Conclusion: During boceprevir monotherapy, resistance mutations at six positions within the NS3 protease were detected by way of clonal sequence analysis. All mutations are associated with reduced replicative fitness estimated by mathematical modeling and show cross-resistance to telaprevir. (HEPATOLOGY 2009;50:1709-1718.)

According to an estimate by the World Health Organization, approximately 180 million people worldwide are affected by the hepatitis C virus (HCV). Of these, at least 130 million are chronic HCV carriers with a significant risk of developing liver cirrhosis and liver cancer. Chronic hepatitis C is a leading cause of liver transplantation in developed countries.1 HCV is an enveloped, ~9.6 kb, positive-sense, single-stranded RNA virus from the genus Flaviviridae. Viral and host peptidases cleave the large open reading frame, resulting in three structural proteins (core, envelope 1, and envelope 2), a small protein named p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). There is a non-coding region of 324–341 nucleotides at the 5′ end containing the internal ribosome entry site and a 3′ noncoding region of variable length that also plays an important role in the replication process.2 Because of the high replication rate of the virus (1012 virions per day) and the high mutation frequency during replication of the viral RNA, it has been calculated that every possible vari-
nant of the virus is produced every day in an infected individual, leading to the circulation of multiple genetically distinct but closely related virus subpopulations (quasispecies). In addition, recombination of HCV was reported to contribute to the genetic diversity to a small extent as well.\(^5\)

The current standard of treatment for patients with chronic hepatitis C consists of the administration of peginterferon alfa and ribavirin for 24 or 48 weeks.\(^4\) Significant side effects are characteristic for this treatment, and for patients infected with the most prevalent genotype 1, a sustained virologic response only can be achieved in about 50% of the patients.\(^5\)

Thus, better tolerated and more effective treatment options are urgently needed. A promising approach is the development of specifically targeted antiviral therapies for hepatitis C (STAT-C). HCV-specific protease inhibitors target an essential step in HCV replication by blocking the NS3/4A protease-dependent cleavage of the HCV polyprotein. Several peptidomimetic NS3 protease inhibitors have been developed and are currently investigated in clinical trials. Two linear peptidomimetic ketoamides (boceprevir and telaprevir) have entered phase 3 studies\(^6-8\) and several macrocyclic inhibitors (i.e. ITMN-191, BI201335, TMC-435350, and MK7009) are in phase 1/2 development.\(^9-12\) Results from phase 2 studies with boceprevir and telaprevir in naïve patients with genotype 1 infection showed a significant improvement in comparison with the standard of care with sustained virologic response rates up to 75% for triple therapy with boceprevir, peginterferon alfa-2b and ribavirin for 48 weeks. In addition, some studies have suggested that it may be possible to shorten treatment duration for patients who achieve a rapid virologic response.\(^13,14\)

Knowledge of the resistance profile of individual targeted antiviral drugs is critical for predicting which drug combinations are appropriate for use in treatment. Preexisting resistant variants within the HCV virus population in an individual patient can be selected during treatment with a targeted antiviral, leading to treatment failure and development of cross-resistance to related compounds. The HCV replicon has been used as an in vitro system to identify individual resistance mutations for several NS3/4A protease inhibitors.\(^15-18\) However, clonal sequence analysis of the NS3 protease in subjects participating in a telaprevir monotherapy study revealed several previously unknown mutations associated with resistance.\(^19\) In addition to the high sensitivity of clonal analysis for low-level resistant variants, this finding is explained by the fact that some mutations were observed only in subtype 1a isolates, whereas the replicon studies are based on an HCV subtype 1b consensus strain.

For boceprevir, three resistant variants were identified in the replicon system (T54A, A156S, and V170A).\(^17\) In a monotherapy study, mutations at position V170 were detected by population sequencing in a single patient who received boceprevir alone for 2 weeks; however, this method cannot reliably detect mutations at frequencies below 20% to 25%.\(^20\)

In the present study, genotypic resistance analysis using population sequence in baseline samples and clonal sequencing in samples at end-of-treatment and follow-up, which can detect minor variants with a frequency of as low as 5% to 7%, was performed in genotype 1–infected patients treated in a phase 1 study with boceprevir alone for 2 weeks. Phenotypic resistance levels based on replicon IC\(_{50}\) were calculated by curve-fitting of the dose–response curves. In addition, we estimated relative viral fitness based on a multiple variant viral kinetic model to define the relationship between resistance and fitness for variants in the virus population undergoing selection in vivo.

**Patients and Methods**

**Patient Population and Study Design.** Forty-six patients were enrolled into a randomized, double-blind, placebo-controlled, 14-day multiple dose, phase 1b trial and received one of the following doses of boceprevir: 100 mg every 12 hours (12 patients), 200 mg every 12 hours (11 patients), 400 mg every 12 hours (11 patients), 400 mg every 8 hours (12 patients), or placebo. For the present study, patients who received the highest dose (400 mg) every 12 hours (BID) or every 8 hours (TID) were selected because with the lower doses only weak virologic responses have been observed (approximately 0.5 log decline). All patients had a chronic hepatitis C with plasma HCV-RNA levels of \(7 \times 10^4\) IU/mL or greater and negative hepatitis B antigen and antibodies to human immunodeficiency virus 1 and 2. Written informed consent was obtained from each patient in accordance with the 1975 Declaration of Helsinki. For HCV-RNA determination and genotypic analysis presented in this report, serum samples were collected and subsequently stored at \(-80^\circ\text{C}\) from all patients before dosing (baseline), at the end of dosing (day 14), and 14 days after the end of treatment (follow-up). HCV-RNA was measured by extracting total RNA from the sample and using an in-house real-time reverse-transcription polymerase chain reaction (PCR) assay.

**Amplification and Sequencing of the Gene Encoding the HCV NS3 Protease.** Amplification of the complete region encoding the NS3 protease catalytic domain was performed as described by Sarrazin et al.\(^19\) In genotype 1a samples, a 620-bp fragment was amplified by using the following primers: NS3-1a-1s, CCGGGAGA-
TACTGCTCGGAC; NS3-1a-2s, CCGATGGAATGCTCTCCAAGG; NS3-1a-1a, GCTCTGGGGCACTGCTG; and NS3-1a-2a, GAGAGGATGTGCTCCGTGAACAC. A mean number of 47 clones were amplified and sequenced per patient for end of treatment and end of follow-up at J. W. Goethe University Hospital (lower limit of variant detection of 7% with a 95% confidence interval). Population sequencing was performed on baseline samples. The molecular clones and the amplified baseline samples were subjected to sequence PCR according to the manufacturer’s instructions using the M13 forward or M13 reverse primers (BigDye Deoxy Terminators; Applied Biosystems, Foster City, CA). Sequencing was performed by an automat (3100 DNA Sequencer; Applied Biosystems, Foster City, CA).

**Sequence Alignment and Analysis.** Sequences for the N-terminal 543 nucleotides (181 amino acids) of the NS3 protease were aligned and analyzed for mutations using the software Mutational Surveyor (SoftGenetics, State College, PA). Mean frequencies of mutations at end-of-treatment and end-of-follow-up were calculated for each patient compared with the respective baseline consensus sequence. For a summary of all patients with similar viral kinetics (< versus ≥2 log₁₀ maximum decline), mean mutations frequencies were calculated based on the results from single patients. GenBank accession numbers: the GenBank accession numbers for the baseline consensus sequence. For a summary of all patients with similar viral kinetics (< versus ≥2 log₁₀ maximum decline), mean mutations frequencies were calculated based on the results from single patients. GenBank accession numbers: the GenBank accession numbers for the baseline consensus sequence.

**Generation of HCV replicon cells and IC₅₀ determination.** Generation of HCV replicon cells and IC₅₀ determination of antiviral agents in the HCV replicon assay was described above. The parental plasmid expressing His-tagged single chain NS4A-NS3 protease domain, NS4A₂₁-₃₂-GSGS-NS₃₋¹₈₁, as well as the expression and purification protocol was described by Tarem et al. Recombinant proteases were tested using a chromogenic assay as described by Zhang et al. The overall inhibition constant Kᵢ* (where Kᵢ* = VₘₐₓS / (Kₘ(1 + 1/Kᵢ*))) was used to measure inhibitor potency.

**Fitness Analysis.** The multiple kinetic model is described by a differential equation system, which models the behavior of both wild-type and resistant viral variants similar to a multiple variant viral dynamic model to estimate fitness of HCV genotype 1–resistant variants in subjects dosed with telaprevir. The differential equation system has three types of compartments, namely target cells (T), infected cells (I), and virus in plasma (V) where i ∈ {1, K, k}. The dependencies between the different compartments are modeled by a differential equation system:

\[
I_i'(t) = p \sum_{j=1}^{K} m_{ij} f_j (1 - \varepsilon_j) I_j(t) - c V_i(t)
\]

\[
V_i'(t) = \delta I_i(t) - V_i(t)
\]

\[
T(t) = L - \sum_{i=1}^{K} I_i(t)
\]

Here, β is the infection rate, δ the degradation rate of infected cells which was bounded from below by 0.01, p the production rate of wild-type virus, c the viral clearance rate, mᵢⱼ the mutation rate from the jth variant to the ith variant during one life-cycle step, and, finally, εᵢ and fᵢ are the efficacies of the drug for the variants and the relative fitnesses of the variant, respectively. The model uses specific fitness parameters for each variant and treatment efficacies are derived from the individual wild-type efficacy and in vitro replicon IC₅₀ and IC₉₀ values of the variants. Further viral kinetic parameters such as viral clearance, infected-cell loss, and de novo infection rates are assumed to be identical for all variants but can be patient-specific. The model also accounts for a shared and limited HCV replication space by assuming that the total number of infected and uninfected target cells is constant (L). The model was fitted to all HCV patient data (daily HCV-RNA concentrations from day 1 to 14 and at day 28) simultaneously using a maximum likelihood approach iterating between fitting individual parameters on one hand and global variant fitnesses on the other hand. This iteration and the
Table 1. Distribution of Known Resistance Mutations Occurring at End of Therapy (HCV-RNA Decline < 2 log₁₀ IU/mL; HCV RNA decline ≥2 log₁₀ IU/mL)

<table>
<thead>
<tr>
<th>Viral Response Potential</th>
<th>Mutations with Known Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtype 1a (n = 8)</td>
<td></td>
</tr>
<tr>
<td>&lt; 2 log₁₀ decline</td>
<td>V55A 9%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54S 3%, R155K 11%</td>
</tr>
<tr>
<td>&lt; 2 log₁₀ decline</td>
<td>V55A 3%, R155K 3%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>V36A/M 9%, T54S/A 18%, V55A 2%</td>
</tr>
<tr>
<td></td>
<td>R155K 2%, A156S 2%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>V36M 4%, R155K 24%</td>
</tr>
<tr>
<td>Subtype 1b (n = 14)</td>
<td></td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54A 1%, V170L 2%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>V55A 7%, T54A 5%, V170A 5%</td>
</tr>
<tr>
<td>&lt; 2 log₁₀ decline</td>
<td>T54A 4%, V170A/L 1%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54A 17%, V55A 6%, V170A 4%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>V170A 4%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>V55A 5%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54S/A 33%, V55A 7%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54A 21%, A156S 7%</td>
</tr>
<tr>
<td>≥2 log₁₀ decline</td>
<td>T54A 2%</td>
</tr>
</tbody>
</table>

objective function were implemented in MATLAB (MathWorks, Inc., Natick, MA).

In Silico Analysis. Methods used for in silico analysis are described in the Supporting Information.

Statistical Analysis. Continuous variables were compared with Wilcoxon paired sample test and Spearman rank correlation coefficients were calculated. All tests were two-tailed for a significance level of 0.05. Statistical analyses were done using SPSS for Windows, release 16 (SPSS Inc, Chicago, IL).

Results

Virologic Response

The samples analyzed in this study were obtained from 22 nonresponder (to previous standard treatment) patients infected with HCV subtype 1a (n = 8) or 1b (n = 14) who were dosed for 14 days with boceprevir in a randomized, placebo-controlled double-blind, phase 1 study (Table 1). Patients who received 400 mg boceprevir BID or TID had a decline of up to 3 log₁₀ HCV-RNA IU/mL. The patients were divided into two groups according to virologic response: (1) patients with a maximum decline ≥ 2 log₁₀ HCV-RNA IU/mL (n = 15) and (2) patients with a maximum decline < 2 log₁₀ HCV-RNA IU/mL (n = 7). Fig. 1C shows viral kinetics for these two groups, which are also separated for the different subtypes. Samples for sequence analysis were obtained at baseline, at end of treatment (day 14), and at end of follow-up (day 28).

Genotypic Resistance Analysis

No resistant variants were observed by population sequencing in baseline samples. Generally, a relatively high genetic heterogeneity was observed within the HCV virus population at the end of treatment. Based on a mean difference of more than 5% within the HCV virus population when compared with baseline samples, altogether mutations at 31 amino acid positions within the NS3 protease were detected: 7, 8, 14, 18, 33, 36, 46, 48, 49, 54, 55, 61, 71, 72, 80, 86, 87, 91, 117, 119, 122, 127, 131, 146, 147, 153, 155, 156, 170, 174, and 179. A restriction to mutations detectable in more than one patient still resulted in changes at 19 amino acid positions of the NS3 protease. Thus, further analysis was restricted to mutations that were observed in more than one patient and showed a decline in frequency during follow-up (n = 9).

The following mutations were observed at amino acid positions that have been shown to be associated with resistance to other protease inhibitors: V36M/A (three patients), T54S/A (10 patients), R155K/T/P (six patients), A156S (three patients), and V170A/L (five patients); these changes occur either as single mutations or as double mutations (V36M + T54S, V36A + R155K, T54S/A + R155K, and T54S + A156S each in one patient). Amino acid mutations V36M and R155K/T were detected only in subtype 1a isolates, presumably because two nucleotide substitutions are required to create these amino acid changes in subtype 1b isolates. Interestingly, no mutations were observed at position R155 in subtype 1b isolates with the exception of a single clone with R155P in one patient.

In addition, we detected four amino acid sites with novel mutations (V48I, V55A, T72I, and I153V) with so far unknown clinical significance for resistance to a protease inhibitor. Selection of V170A and T72I was restricted to subtype 1b patients. Subtype 1a patients all carried I170 and I72 as wild-type amino acids.

Irrespective of variants which are detectable in a specific HCV subtype only (see above) generally, the frequency of resistant variants detected in subtype 1a and subtype 1b patients was similar.

Distribution of Mutations according to Virologic Response

Patients with a Maximum Decline ≥ 2 log₁₀ HCV-RNA IU/mL. The mean frequency of mutations versus baseline samples at positions V36 (1.4%), T54 (5.0%),
Fig. 1. (A) Frequency of NS3 protease variants in patients with a maximum HCV-RNA decline $\geq 2 \log_{10}$ IU/mL. The pie charts show the frequency of known and unknown mutations at baseline, end of treatment, and end of follow-up. (B) Frequency of NS3 protease variants in patients with a maximum HCV-RNA decline $< 2 \log_{10}$ IU/mL. The pie charts show the frequency of known and so far unknown mutations at baseline, end of treatment, and end of follow-up. (C) Viral kinetics for the different subtypes in the two groups of patients (green, HCV-RNA decline $< 2 \log_{10}$ IU/mL, genotype 1b, n = 3; violet, HCV-RNA decline $< 2 \log_{10}$ IU/mL, genotype 1a, n = 4; blue, HCV-RNA decline $\geq 2 \log_{10}$ IU/mL, genotype 1b, n = 11; red, HCV-RNA decline $\geq 2 \log_{10}$ IU/mL, genotype 1a, n = 4) during therapy (day 1-14) and 14 days thereafter (day 28). Error bars represent the standard deviation.
R155 (6.9%), A156 (0.8%), and V170 (3.9%) was 18% at the end of treatment in patients with a maximum HCV-RNA decline of $\approx 2 \log_{10}$. The frequency of mutations at these positions declined to 6% by the end-of-follow-up ($P = 0.05$). No mutations at position A156 were detected at the end of follow-up. The mean frequency of the potentially new resistance mutations at positions V48, V55, T72, and I153 was 15% at the end of treatment and declined to 10% at the end of follow-up ($P > 0.2$) (Fig. 1A).

**Patients with a Maximum Decline <2 log\textsubscript{10} HCV-RNA IU/mL.** In this group of patients, the mean frequency of known (7% at end of treatment) as well as potentially new resistance mutations (4% at end of treatment) decline to 1% by day 28 ($P > 0.2$ and $P = 0.06$, respectively) (Fig. 1B).

Overall, irrespective of viral kinetics in 7 of 22 and 9 of 22 patients, only wild-type variants without any resistance mutations at positions V36, T54, V55, R155, A156, and V170 were detected at end of treatment and end of follow-up, respectively. For two patients, long-term follow-up serum samples were available, and no resistant variants have been detected by clonal sequencing in either of these patients 2.5 to 4 years after the end of treatment.

**Phenotypic Resistance Analysis**

Replicon IC\textsubscript{50} values from the known and the potentially new mutations are shown in Fig. 2A. As expected, a change at position 156 (A156T) conferred high-level resistance (>120-fold increase in IC\textsubscript{50}) against boceprevir, whereas changes at positions 36, 54, 155, 156, and 170 conferred low to moderate resistance (3.8- to 17.7-fold change in IC\textsubscript{50}). Three of the four newly detected variants (V48I, T72I, and I153V) did not differ from wild-type IC\textsubscript{50}. However, the substitution from valine to alanine at position 55 showed a clear increase in resistance in the replicon model (6.9-fold). Resistance of the V55A mutation to boceprevir was also confirmed using the *in vitro* enzymatic assay for NS3 protease (Table 2).

**Cross-resistance to Telaprevir**

For mutations at several amino acid positions (V36, T54, R155, A156), previously reported resistance at different levels against telaprevir\textsuperscript{19,21} was confirmed in the present study. In addition, cross-resistance to telaprevir was shown for mutations at position V170 and for the newly characterized mutation V55A (Fig. 2B). A direct comparison of the IC\textsubscript{50} values of both compounds is shown in Fig. 3. Rank correlation between the IC\textsubscript{50} values was 0.82 ($P = 0.002$).

**Mathematical Modeling of Relative in Vivo Fitness of Resistant Variants**

Replicative fitness of individual NS3 resistant variants was estimated based on mutational frequencies, phenotypic resistance levels and viral kinetics. The fitting procedure using all patient data converged. In addition to relative fitness levels, estimates also were obtained for infected cell loss rate with a median of 0.11 per day (quartiles: 0.03 and 0.24 per day) and a median treatment efficiency in wild-type of 96.7% (quartiles: 94.4% and 98.6%). The viral kinetic modeling method provides an

| Table 2. Enzyme IC\textsubscript{50} of the V55A Variant Compared with Wild-Type |
|-----------------|-----------------|-----------------|-----------------|
|                 | Wild-Type       | V55A (Mean ± Standard Deviation) |
|                 | K\textsubscript{I} [nM] | K\textsubscript{I} [nM] | V55A/Wild-Type |
| Boceprevir      | 19              | 80 ± 18          | 4.2 ± 0.96     |
| Telaprevir      | 21              | 58 ± 7           | 2.7 ± 0.33     |
estimate of the fitness of each NS3-resistant variant based on that variant’s replacement frequency relative to other NS3 variants (including wild-type) identified within the population.

Overall, an inverse correlation between the resistance potential and the fitness of variants with single mutations can be observed (rank correlation: \(-0.762, P = 0.037\)). Amino acid changes at position 156 conferred the highest level of resistance but had the lowest relative fitness (Fig. 4). As described, relative fitness increased if the variant carried a second resistance mutation (i.e., V36A + R155K).19 No differences were observed for subtypes 1a and 1b. Globally fitted values of estimated relative fitness for all reported mutations are shown in Table 3.

**In silico Analysis**

Results from *in silico* analysis are described in the Supporting Information.

**Discussion**

With the development of direct antiviral drugs, treatment options for HCV-infected individuals will be broadened considerably. Currently, two of the NS3 protease inhibitors are in phase 3 clinical development, boceprevir and telaprevir. Based on the results of the completed phase 2b studies showing a significant increase in sustained virologic response rates,13,14,26 the new standard treatment will likely include the use of a protease inhibitor in combination therapy with peginterferon and ribavirin. However, as with other direct antiviral therapies for other viruses such as human immunodeficiency virus, resistant HCV variants may be selected during treatment with STAT-C compounds.19,27

In the present study, the resistance profile of boceprevir generated during a 14-day monotherapy phase 1b study was assessed using population sequencing for baseline samples and a clonal sequencing approach at the end of treatment and follow-up.

A selection of a high number of variants (n = 25) with a high variability was already reported for telaprevir monotherapy and seems to be typical for HCV.19 An increase of the frequency of a resistant variant without the presence of the antiviral drug seems to be very unlikely, because such a variant should have an equal or even higher replicative fitness than the wild-type variant. For the present study, a restriction to variants with a \(\geq 5\%\) increase, present in more than one patient and with a decline after end of treatment was imposed. Future investigations are required to detect a potential importance of variants detected at lower frequencies.

**Table 3. Values of Relative Fitness**

<table>
<thead>
<tr>
<th>Variant</th>
<th>Relative Fitness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.000</td>
</tr>
<tr>
<td>A156S</td>
<td>0.001</td>
</tr>
<tr>
<td>R155K/P/T</td>
<td>0.500</td>
</tr>
<tr>
<td>T54A</td>
<td>0.143</td>
</tr>
<tr>
<td>T54S</td>
<td>0.182</td>
</tr>
<tr>
<td>V36M/A</td>
<td>0.499</td>
</tr>
<tr>
<td>V55A</td>
<td>0.356</td>
</tr>
<tr>
<td>V170A/L</td>
<td>0.229</td>
</tr>
<tr>
<td>T54S + A156S</td>
<td>0.502</td>
</tr>
<tr>
<td>T54S/A + R155K</td>
<td>0.531</td>
</tr>
<tr>
<td>V36A + R155K</td>
<td>0.877</td>
</tr>
<tr>
<td>V36M + T54S</td>
<td>0.522</td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of the n-fold IC\(_{50}\) change of boceprevir and telaprevir. Blue-filled circles show the n-fold IC\(_{50}\) values for boceprevir and red unfilled circles show the n-fold IC\(_{50}\) values for telaprevir. For wild-type, V48I, T72I, I153V, and the A156T variant one circle with both colors represent the values for both inhibitors. Both values for A156T and telaprevir n-fold IC\(_{50}\) values for R155L and A156S are out of range, so they are positioned at the top of the graph.

Fig. 4. Resistance versus replicative fitness of boceprevir-resistant variants. Relative resistance is expressed as the mean fold change in replicon IC\(_{50}\) values (y axis). Relative fitness is calculated based on a multiple viral kinetic model fit (x axis).
We found mutations at six positions within the NS3 protease to be associated with different levels of resistance to boceprevir. Initially, when using a population-based sequence analysis, only one mutation at position V170 was detected in a single patient in the study. This highlights the ability of clonal sequencing to provide more information about the resistance profile of STAT-C drugs than population sequencing. In addition to boceprevir-resistant mutations previously identified in the replicon system (T54A, A156S, V170A), mutations at several other positions were identified within the NS3 protease based on an increased frequency at the end of treatment followed by a decline thereafter. Phenotypic characterization of all potential resistance mutations identified by clonal sequencing and frequency analysis identified six loci that were clearly associated with resistance to boceprevir (V36, T54, V55, R155, A156, V170). Presence of these variants already at baseline at high frequencies (20% to 25%) was excluded by population-based sequencing in the present study and is highly unlikely at frequencies around 5% based on the results of a previous study with clonal sequencing at baseline.19

The results of this study are consistent with previous in vitro studies15,17,18,28 showing that the cross-resistance profiles of the two linear peptidomimetic ketoamides boceprevir and telaprevir are largely overlapping. Furthermore, previously described differences of variants selected by genotype 1a or 1b HCV subtypes only, were confirmed. However, differences between the frequencies of specific resistance mutations have been observed in the clinical monotherapy studies of boceprevir and telaprevir. These differences in mutation frequency may be related to the different drugs as well as differences in the patient populations and the doses used in the studies. In treatment-naïve and previously treated patients who received telaprevir at doses of 450 mg every 8 hours, 750 mg every 8 hours, or 1,250 mg every 12 hours, approximately 90% of clones sequenced from end of treatment samples contained resistant variants.19 In the present study of interferon nonresponder patients who received boceprevir monotherapy at doses of 400 mg BID or TID, a mean frequency of 85.9% of clones contained wild-type NS3 protease sequence at the end-of-treatment. Overall, the frequency of resistant variants was associated with viral load decline. In patients with a maximum decline of HCV-RNA concentrations of at least 2 log10 steps, resistant variants were observed more frequently than in those with a less pronounced decline. The A156S variant, which has a medium level of resistance but low fitness, was not detected in the patients treated with boceprevir 400 mg TID or BID. The mean viral decline of boceprevir in the present study was lower than that in the phase 1b monotherapy study for different doses of telaprevir.8,20 In subsequent phase 2 studies as well as the currently ongoing phase 3 study, boceprevir is dosed with 800 mg TID in combination with peginterferon and ribavirin. Moreover, the probability of selection of resistant variants followed by a viral breakthrough is significantly reduced by combination therapy of NS3 protease inhibitors with peginterferon and ribavirin.15,14,27 and potentially by the addition of a “lead-in” phase with peginterferon and ribavirin alone. To answer these questions, results of resistance analysis from the phase 2b studies (SPRINT-1, PROVE1/2) have to be awaited.

In addition, several amino acid mutations frequently detected in the patients treated with boceprevir showed some differences in resistance to telaprevir. T54A was detected more frequently in boceprevir treated patients despite a similar resistance profile (less than two-fold difference) in the replicon assay. V170A mutations conferred a higher level of resistance to boceprevir in comparison with telaprevir in the phenotypic assay, and this variant was frequently detected in boceprevir-treated patients but was not described to be a frequent variant in patients who received telaprevir monotherapy.19 In contrast, mutation A156S conferred a higher level of resistance to telaprevir than boceprevir.18 As previously observed for telaprevir, the frequency of resistant variants at the end of treatment generally decreased during follow-up in patients treated with boceprevir.19

In the present study, V55A was identified as a new variant to be associated with resistance in patients treated with boceprevir. Resistance to boceprevir by the V55A variant as well as telaprevir with a relative low IC50 was confirmed in the phenotypic assay. However, V55A was not described to be selected during telaprevir monotherapy or combination therapy with peginterferon in phase 1 trials.19,27 Phenotypic characterization showed slightly higher resistance in replicon assay (6.9-fold versus 3.1-fold) as well as in enzyme assay (4.2-fold versus 2.7-fold) for V55A to boceprevir than to telaprevir. In silico analysis showed that interaction between V55A and S139 or R155 may be responsible for impaired binding of boceprevir. Studies of larger population of patients treated with boceprevir or telaprevir will be required to fully understand the clinical emergence of this novel mutation.

In the present study, only the NS3 protease gene was sequenced. Thus, it is unclear to which extent mutations within the NS4A cofactor and/or at the NS3/4A protease cleavage sites may contribute to confer resistance or to
compensation of impaired NS3/4A function. Mutations outside the NS3 protease are not anticipated, and in a recent study of telaprevir in combination with peginterferon and ribavirin, no mutations at the cleavage sites have been detected.29

Taken together, during boceprevir monotherapy with 400 mg BID and TID resistance mutations at six positions within the NS3 protease (V36M/A, T54A/S, V55A, R155K/T, A156S, V170A) were detected by a highly sensitive, clonal sequencing method. Generally, mutations were observed at relative low frequencies within the HCV virus population and a strong correlation of the presence of resistant variants with a more pronounced decline of HCV-RNA concentrations was observed. All detected variants showed cross-resistance to telaprevir in the phenotypic assay. However, differences for frequency of detection and/or resistance levels between boceprevir and telaprevir were observed, and this was mainly true for the T54A and V170A mutations. Also, the newly described V55A variant seems to be selected in boceprevir-treated patients only. Impaired viral fitness of single resistant variants was estimated in a mathematical model while combined mutations showed a relative increase of replication efficiency. Further detailed analyses are required to determine the potential long-term persistence of the observed variants and to characterize resistant mutations selected during treatment with higher doses of boceprevir in combination with peginterferon and ribavirin.

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