

# Tracking the Evolution of Multiple *In Vitro* Hepatitis C Virus Replicon Variants under Protease Inhibitor Selection Pressure by 454 Deep Sequencing<sup>∇</sup>

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Resistance to hepatitis C virus (HCV) inhibitors targeting viral enzymes has been observed in *in vitro* replicon studies and during clinical trials. The factors determining the emergence of resistance and the changes in the viral quasispecies population under selective pressure are not fully understood. To assess the dynamics of variants emerging *in vitro* under various selective pressures with TMC380765, a potent macrocyclic HCV NS3/4A protease inhibitor, HCV genotype 1b replicon-containing cells were cultured in the presence of a low, high, or stepwise-increasing TMC380765 concentration(s). HCV replicon RNA from representative samples thus obtained was analyzed using (i) population, (ii) clonal, and (iii) 454 deep sequencing technologies. Depending on the concentration of TMC380765, distinct mutational patterns emerged. In particular, culturing with low concentrations resulted in the selection of low-level resistance mutations (F43S and A156G), whereas high concentrations resulted in the selection of high-level resistance mutations (A156V, D168V, and D168A). Clonal and 454 deep sequencing analysis of the replicon RNA allowed the identification of low-frequency preexisting mutations possibly contributing to the mutational pattern that emerged. Stepwise-increasing TMC380765 concentrations resulted in the emergence and disappearance of multiple replicon variants in response to the changing selection pressure. Moreover, two different codons for the wild-type amino acids were observed at certain NS3 positions within one population of replicons, which may contribute to the emerging mutational patterns. Deep sequencing technologies enabled the study of minority variants present in the HCV quasispecies population present at baseline and during antiviral drug pressure, giving new insights into the dynamics of resistance acquisition by HCV.

Chronic hepatitis C virus (HCV) infection can lead to liver fibrosis, cirrhosis, hepatocellular carcinoma, and ultimately liver failure. Approximately 170 million people worldwide are infected with HCV (54a). The current standard of care consists of pegylated alpha interferon (Peg-IFN) plus ribavirin (RBV), providing limited efficacy for genotype 1-infected patients, i.e., a sustained virological response (SVR) in 40 to 50% of the patients. Moreover, Peg-IFN/RBV therapy is associated with significant adverse events (9). Therefore, direct antiviral agents (DAA) (previously also known as “specifically targeted antiviral therapies for hepatitis C” or STAT-C) have been a major focus of drug discovery efforts over the last 2 decades. Several NS3/4A (protease), NS5A, and NS5B (polymerase) inhibitors either alone or in combination with Peg-IFN/RBV have recently shown potent antiviral effects in HCV-infected patients (22, 36). However, viral resistance to these novel agents can occur rapidly when they are dosed as monotherapy (43, 49).

Because of the high mutation rate of the HCV polymerase ( $10^{-3}$  to  $10^{-5}$  misincorporations per nucleotide copied [11]) and the high viral production rates *in vivo* (approximately  $10^{12}$  viruses per patient per day [37]), it can be assumed that HCV exists as a diverse population of nonidentical but closely re-

lated viral genomes, referred to as a quasispecies (10). A viral quasispecies is characterized by a dominant nucleotide sequence, called a master sequence, and a surrounding mutant spectrum, which can harbor minority subpopulations (42). Although in theory all single and double mutants are produced daily in an infected person (6, 40), it is important to note that mutation rates are not equally distributed over the entire genome and that additional factors, such as viral fitness and the replication environment, determine whether a mutation becomes fixed in a viral quasispecies population (12). The diversity of the viral variants present in an infected individual facilitates the adaptation of the quasispecies to external pressure, such as antiviral treatment, improving the survival chances of the population (53). The speed of such adaptation depends mainly on the turnover of the viral nucleic acid acting as a source of new viral genomes. Whereas in HIV the rapid turnover of infected CD4<sup>+</sup> T lymphocytes is responsible for the rapid turnover of nucleic acids, in HCV rapid turnover is explained by the short half-life (~10 h) of HCV RNA strands in the hepatocyte (47). However, if mutation rates exceed a certain limit, called the error threshold, deleterious mutations will accumulate and the viral population will become extinct (4).

Recent reports have demonstrated that mutations known to affect the activities of DAA compounds *in vitro* are present in some treatment-naïve patients as either dominant or minority species (6, 13, 19, 21, 27). With the eradication of variants

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susceptible to the antiviral drugs, resistant viruses initially present as minority species may expand to occupy the freed replicative space, thus becoming the dominant master sequence (1); this may lead to failure of the antiviral regimen. In HIV it has been shown that minority species can play an important role in the accelerated evolution toward resistance to antiretroviral drugs (5). The extent to which preexisting HCV variants may compromise treatment with DAAs, however, is not yet fully understood (3). Depending on the concentration of the antiviral agent, different resistance profiles seem to emerge. In clinical studies, a correlation was noted between the plasma trough levels of the NS3/4A inhibitor telaprevir, the virological response, and the mutations responsible for the drug-resistant phenotype (43). In patients with a low exposure to telaprevir, variants carrying mutations with low resistance to telaprevir *in vitro* were observed, while higher drug levels were associated with variants conferring a greater degree of resistance *in vitro*. Correlations between the inhibitor concentration and the mutation profile were also described in *in vitro* studies (44, 50, 51).

HCV replicon cell culture systems have been widely used to characterize resistance against antiviral inhibitors and to assess the impact of resistance mutations on drug susceptibility and replication fitness *in vitro* (8, 15). Although the information on resistance mutations observed with DAA during clinical trials is still limited, mutations identified *in vitro* appear to be predictive for those mutations that may emerge in patients (17, 20). In addition, analysis of the genetic variability and diversity of a long-term HCV replicon-containing cell culture has shown that mutations accumulate over time at rates comparable to those observed *in vivo*:  $(3.5 \text{ to } 4.8) \times 10^{-3}$  *in vitro* versus  $(1.4 \text{ to } 1.9) \times 10^{-3}$  *in vivo* base substitutions/site/year (16). Hence, HCV replicon systems are considered a useful and relevant surrogate system for analyzing the evolutionary dynamics and variations of HCV in response to selection pressure.

The detailed study of the dynamics of viral variants present in a quasispecies population has long been hampered by the lack of sensitive sequencing methods. The recent development of deep sequencing technologies may facilitate a better understanding of the genetic composition and natural evolution of viral quasispecies in the presence of antiviral drugs (30, 34, 54). Indeed, studies of HIV suggest that these more-sensitive sequencing technologies detect additional minority variants for both treatment-naïve and treatment-experienced patients which could impact the clinical outcome of antiretroviral therapy and may provide important information for treatment planning (2, 23, 41, 46).

TMC380765 (Fig. 1) is a macrocyclic inhibitor of the HCV NS3/4A protease and a potent inhibitor of HCV RNA replication *in vitro*, with median 50% effective concentration ( $EC_{50}$ ) and 90% effective concentration ( $EC_{90}$ ) values of 35 nM and 106 nM, respectively, in the Huh7-Luc replicon using a luciferase readout (25, 39). Other examples of macrocyclic NS3/4A inhibitors include BILN-2061, ITMN-191, MK7009, and TMC435. To assess the effect of its selective pressure on the composition of the replicon population, selection experiments were performed with different concentrations of TMC380765 and sequence changes were determined with population, clonal, and 454 deep sequencing technologies.

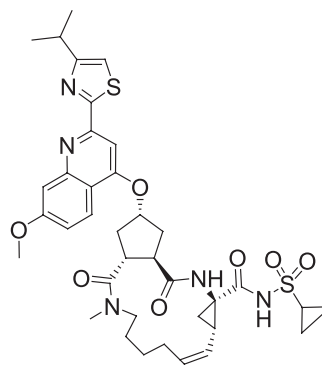


FIG. 1. Structural formulae of TMC380765.

## MATERIALS AND METHODS

**TMC380765.** TMC380765 was synthesized in-house as described previously (9).

**Cell culture and selection with TMC380765.** Huh7-Luc cells (kindly provided by R. Bartenschlager [adapted from reference 26]), containing the genotype-1b (con1b-based) bicistronic subgenomic replicon (clone ET) encoding a firefly luciferase reporter with the cell culture adaptive mutations E1202G, T1280I, and K1846T in the HCV genome, were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 1% L-glutamine, 0.04% gentamicin, and 0.75 mg/ml G418. To select for resistance against TMC380765,  $3 \times 10^5$  replicon-containing cells were seeded in a 10-cm<sup>2</sup> tissue culture dish and cultured for 4 to 6 weeks in the presence of TMC380765 and G418. Replicon-containing cells were split twice per week at a ratio of 1:2 to 1:5, and fresh TMC380765-containing medium was added regardless of whether the cell culture was split. For "low-dose" experiments, 100 nM TMC380765 was used, and "high-dose" experiments used a concentration of 1,000 nM. The concentration of TMC380765 was increased in every passage in the "stepwise-increasing inhibitor" experiments (here referred to as experiments A and B): 0 (pretreatment), 20, 40, 60, 80, 160, 240, 320, 400, 480, 560, 640, 720, 800, 880, and 1,000 nM. As a negative control, replicon-containing Huh7-Luc cells were cultured in the absence of compound. At every passage, approximately 10,000 to 15,000 cells were taken from culture, diluted in 1 ml of cell culture medium, and frozen at  $-80^{\circ}\text{C}$  for RNA extraction.

**RNA extraction and PCR.** Total RNA was extracted from 10,000 to 15,000 cells using an automated EasyMag extraction robot (NucliSens; bioMérieux) and eluted in 25  $\mu\text{l}$  of elution buffer. cDNA for high- and low-dose selection experiments and for experiment A was generated by reverse transcription using an NS3 gene-specific PRO5543R (CCCGATTGCCTTGTGTTT) primer and the Expand reverse transcriptase (RT) kit (Roche). Nested PCR for the NS3/4A gene was performed using the Expand HF system (Roche) and the primer combinations PRO5543R and Prot2519F (GGCTGAAGGATGCCAGAGG) for outer and PRO5502R (CCTGTTCCGATGTAAGGGAGG) and Prot262F (GATAATACCATTGGCGCCTATTACGGCC) for inner PCR at an annealing temperature of  $58^{\circ}\text{C}$ . cDNA for experiment B was generated using the AccuScript high-fidelity 1st-strand cDNA synthesis kit (Stratagene) as described by the manufacturers using the random primers provided with the kit. Two independent sets of primer pairs were designed: primer pair one, containing 1b\_1\_fwd\_SG, (CGTATTCAACAAGGGGCTGA; nucleotides 3541 to 3560 in the encephalomyocarditis virus [ECMV] internal ribosome entry site [IRES] of the HCV replicon) and HCV1b\_3739R (ACCAAGTAAAGG TCCGAGCTG; nucleotides 4009 to 4026 in NS3), and primer pair 2 containing HCV1b\_3648F (AATGTAGACCAGGACCTCGTCTG; nucleotides 3938 to 3959 in NS3) and HCV1b\_4016R (CACCTGGAATGTCTGCGG TAC; nucleotides 4286 to 4306 in NS3). These primer pairs were used to generate two amplicons: amplicon 1b\_1 (488 bp) and 1b\_2 (369 bp), covering the complete protease domain (amino acids 1 to 181) of NS3. NS3 protease amino acid positions 1 to 99 are covered by the first amplicon; amino acid positions 85 to 192 are covered by the second amplicon.

For bidirectional pyrosequencing purposes, A and B sequence adaptors were ligated to forward and reverse primers, respectively, and additional multiplex identifiers (MIDs) (i.e., short barcode sequences) were attached to the adaptor-ligated primers to allow pooling of samples. For PCR, Phusion Hot Start high-fidelity DNA polymerase (Finnzymes) was used to overcome possible primer

dimer formation and ensure accuracy. To maximize the amount of input templates and to minimize variation due to PCR drift, seven replicate RT-PCRs were carried out for each sample and subsequently pooled for sequence analysis (48, 52).

For standard Sanger sequencing of experiment B, the first 181 amino acids (aa) of NS3 were amplified as one amplicon using the forward primer 1b\_1\_fwd\_SG and reverse primer HCV1b\_4016R, resulting in an amplicon of 765 bp.

**Cloning and standard Sanger sequencing.** NS3/4A amplicons derived from cultures of high- and low-dose selection experiments and of experiment A and NS3 amplicons from experiment B were purified (PCR purification kit; Qiagen), bacterially cloned, and sequenced (Agowa Berlin, Germany). For clonal analysis of experiments A and B, 24 and 48 clones per sample were analyzed, respectively. Sequence editing and contig assembly were performed using the SeqScape v2.5 software program (Applied Biosystems).

**454 Deep sequencing (GS-FLX).** 454 deep sequencing analysis of experiment A was performed at the 454 sequencing center (Branford, CT). To this end, 2-kb NS3/4A PCR amplicons were prepared and nebulized to generate smaller fragments for GS-FLX shotgun DNA library preparation followed by sequencing. Sequencing results were analyzed in-house using the EMBOSS software program (<http://emboss.sourceforge.net/>) and custom Perl scripts.

Deep sequencing, library preparation, and analysis of overlapping amplicons of samples of experiment B and of the replicon control experiment were performed using the GS-FLX amplicon approach. Sequencing data were analyzed using the AVA software program.

The average number of sequence reads per position (= coverage) was between ~2,600 and 12,000 (experiment A) or between ~800 and 15,000 (experiment B). Coverage of the second amplicon of the samples from 800 and 880 nM concentrations in experiment B was very low (<100 reads). No replicon RNA copy numbers were determined in experiment A. The average number of HCV replicon RNA copies per sample analyzed in experiment B was determined by quantitative PCR (qPCR), with an average of ~16,500 and minimum of ~2,600 replicon RNA copies.

The NS3 protease region of a control plasmid was processed in parallel. Based on the variation observed on the control plasmid, the limit of detection was defined as a frequency of 0.4% for mutations in single samples (data not shown). Percentages below 0.4% were considered if the same mutations were also present in previous or subsequent samples.

**Transient replicon assay.** The transient replicon assay used to measure the TMC380765  $EC_{50}$ s for wild-type and site-directed-mutants was described previously (24). In brief, Huh7 Lunet cells were transfected with *in vitro*-transcribed replicon RNA from the wild type or mutants. Based on the luciferase luminescence signal of compound-treated and control cells,  $EC_{50}$  (50% effective concentration) values were determined.  $EC_{50}$ s of the mutants were compared to those of the parental wild-type HCV replicon, and fold changes were calculated. Replication capacity was determined in the absence of inhibitors as previously described (28, 35) by measuring luciferase levels 48 h postelectroporation and normalizing to the luciferase levels obtained 4 h postelectroporation.

## RESULTS

**Sanger population sequencing reveals TMC380765 concentration-dependent resistance profiles.** To assess the potential differences in mutation profiles arising in the presence of different TMC380765 concentrations, Huh7-Luc replicon-containing cells were cultured in the presence of G418 and either 100 nM or 1,000 nM or stepwise-increasing TMC380765 concentrations (ranging from 20 to 1,000 nM). Each selection protocol was performed at least twice. Compound concentrations used were well below the levels of toxicity for cell culture (data not shown). At every passage, cell viability was scored microscopically, and cells were harvested, total RNA was extracted, and the protease domain of the NS3 gene was sequenced using standard Sanger population sequencing. For reasons of clarity, we will here limit our analysis to the six amino acid positions (41, 43, 80, 155, 156, and 168) in NS3 known to affect binding and activity of macrocyclic NS3 protease inhibitors (20, 21, 24).

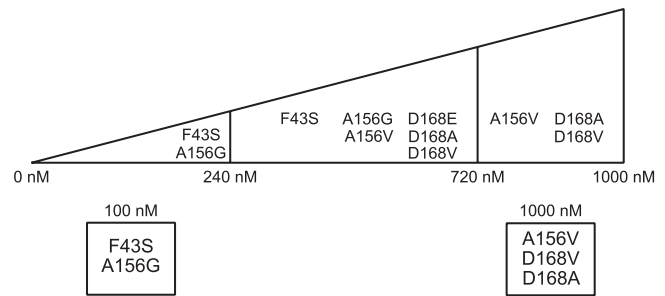


FIG. 2. Summary overview of mutations at NS3 protease positions 43, 156, and 168 detected by population sequencing during selection experiments using 100 nM, 1,000 nM, and stepwise-increasing (0 to 1,000 nM) concentrations of TMC380765. It is important to note that not every individual mutation was observed in each individual experiment and that the majority were detected as mixtures of mutant and wild-type amino acids. Each individual selection protocol was performed at least twice.

Different resistance profiles were observed in the low-concentration (100 nM) and high-concentration (1,000 nM) experiments (Fig. 2). In the low-concentration selection experiments, viability of the replicon-containing cells was not affected. In the high-concentration selection experiments, limited initial cell death was followed by recovery of the cell culture. During low-concentration selection, F43S/F mixtures were very rapidly detected, and these remained present at each time point analyzed until the end of the experiment, whereas in the high-concentration selection experiment, mixtures of A156A/V and D168A/D/V were detected after cell recovery. In addition, at some time points during low-concentration selection, A156A/G and A156A/G/V mixtures were transiently observed. No mutations at residue F43 were detected in the high-concentration experiment, whereas no mutations at residue D168 were present in the experiment with low concentrations. In these experiments, changes at NS3 amino acid position 41, 80, or 155 were not observed.

Population sequence analysis of samples from experiments with stepwise-increasing TMC380765 concentrations suggested three phases in the development of resistance (Fig. 2). Initially F43F/S and/or A156A/G mixtures were observed, whereas at the ends of the experiments, the mutations A156V and A156A/V and D168D/V and D168A/V were present. This is consistent with the mutation profiles observed in the low- and high-concentration selection experiments, respectively. A transition period can be assigned in the middle range of TMC380765 concentrations used, in which, in addition to the A156G and F43S mutations, the D168E/V or D168E/D/V and A156A/G/V mutations were detected, reflecting a mixed population of resistant replicons. No mutation was detected at position 41, 80, or 155. Mutations at other amino acid positions in the NS3 region appeared to reflect background mutations unrelated to TMC380765 pressure, since they were frequently observed in untreated replicon-containing cell cultures as well (24) or were detected only in combination with a mutation at position 156 or 168. In all three experiments with increasing TMC380765 concentrations, no effect on cell growth was observed, indicating relatively stable HCV replication. This observation was confirmed by qPCR analysis of samples from one experiment (data not shown).

**Deep sequencing reveals a complex pattern of mutations.** To better assess the dynamics of changes in the replicon population in experiments with increasing TMC380765 concentrations, 454 deep sequencing was employed on samples of two experiments (here referred to as experiments A and B). 454 deep sequencing widens the window of detection significantly. In contrast to population (bulk) Sanger sequencing, which allows the detection of mutations present with a frequency of at least ~25%, 454 deep sequencing decreases the detection limit to ~0.5%. In addition, it allows quantification of the frequency of multiple replicon variants in parallel. The complete sequence of the NS3/4A gene from replicons obtained in experiment A was determined using the GS-FLX shotgun technology, whereas for replicons obtained in experiment B, only the protease domain of NS3 was sequenced with the GS-FLX amplicon approach. As a control, Huh7-Luc replicon-containing cells were passaged in parallel with experiment B but in the absence of TMC380765 pressure, and replicon RNA was sequenced by the GS-FLX amplicon approach.

Although the absolute mutation frequency levels varied between experiments A and B, the overall trends and patterns of mutations emerging during increasing TMC380765 concentrations were comparable between the two experiments.

In the pretreatment samples (Fig. 3A1 and B1 to A6 and B6), mutations at NS3 amino acid positions 41, 80, and/or 156 were observed at frequencies between 0.3 and 2%. At position 41, the preexisting mutations Q41H and Q41R were observed in both experiments. Interestingly, at position 80, the mutation Q80R was present only in the pretreatment sample from experiment A whereas the Q41K and A156G mutations were present only in the pretreatment sample from experiment B. In addition, at positions 80, 156, and 168, a second codon was observed for the wild-type amino acid variant (indicated with an apostrophe [']); e.g., amino acid D168 is encoded by GAC and the alternative codon GAT. (Fig. 4).

In the control experiment, the preexisting mutations Q41H and A156G were maintained at low frequencies (~1%), as were alternative wild types D168D' and Q80Q'. In addition, at two time points, F43S (~0.5%) was observed. No amino acid changes at position 80, 155, or 168 were detected.

During selection with TMC380765, the most pronounced changes in amino acid frequencies occurred at the NS3 protease positions 156 and 168, where in total three (A156V, A156T, and A156G) and five (D168A, D168E, D168V, D168H, and D168Y) different mutations emerged. In addition, changes were also noted at positions 41, 43, and 80; in each case, one mutation (Q41R, F43S, and Q80R) dominated while some other mutations were present at a low frequency (<1%). At position 155, only mutations R155Q and R155W emerged in response to TMC380765 treatment, but they did not reach frequencies above 1%. Mutations at positions 41, 43, and 80, which emerged early during the selection experiments, dominated the replicon population, with frequencies of up to 25%. A further increase in TMC380765 concentrations resulted in a decrease in the frequency of the latter mutations, with replicon variants with mutations at NS3 protease positions 156 and 168 becoming dominant.

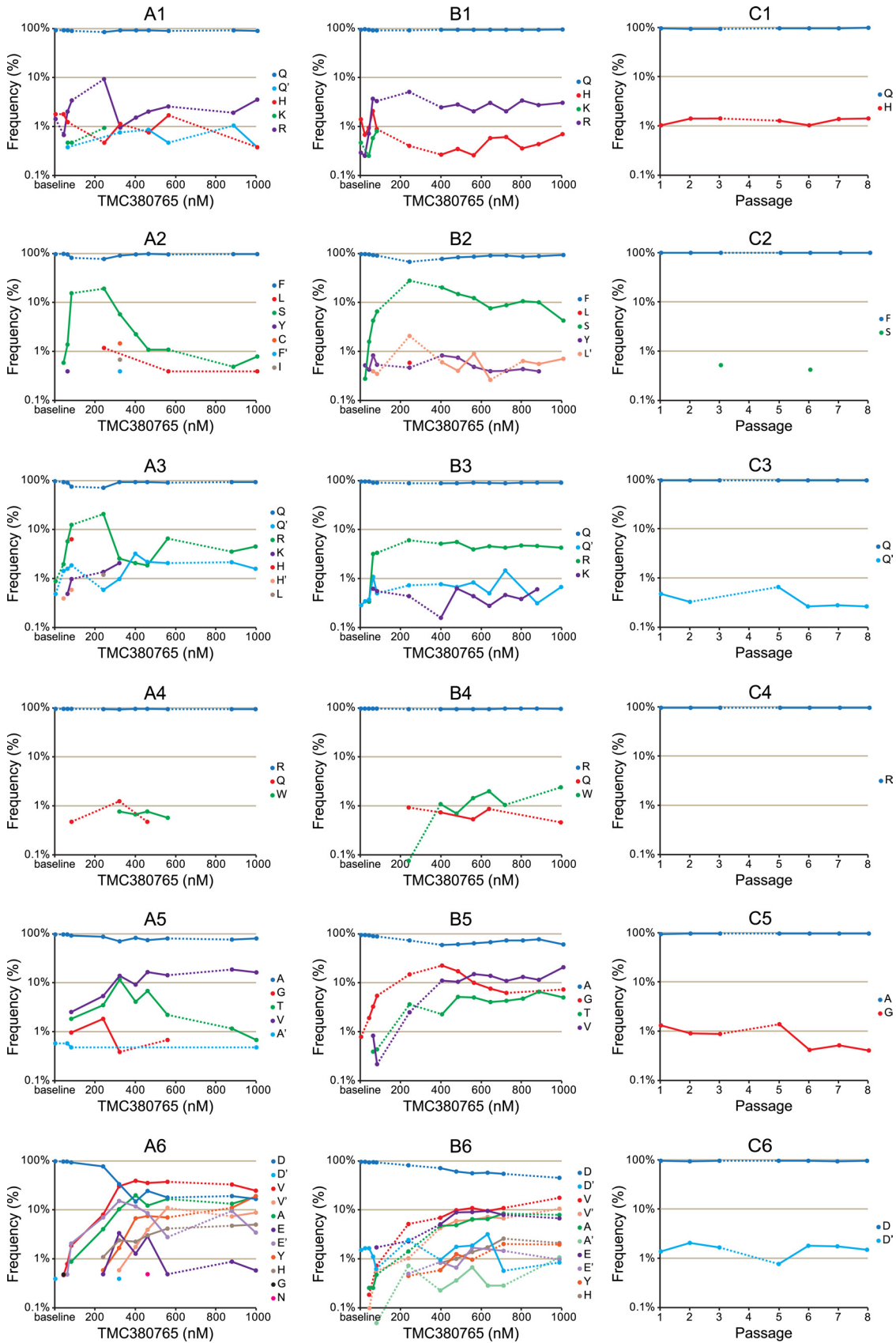
In more detail, the Q41R mutation (Fig. 3A1 and B1) increased from ~1% in the baseline sample up to maxima of 10

and 5% at 240 nM TMC380765 in experiments A and B, respectively; subsequently, the frequency of Q41R decreased and stabilized at 2 to 3%. F43S (Fig. 3A3 and B3) was detected very early during both experiments at a frequency below 1% and increased to 20 to 30% at 240 nM TMC380765 before declining again below 1%. The frequency of the Q80R mutation (Fig. 3A3 and B3) peaked at 20% in experiment A, where it was present at ~1% in the pretreatment sample. In experiment B, Q80R was first detected at 40 nM and only reached a frequency of 5%. At higher drug concentrations, the Q80R mutation stabilized at a frequency of 5% in both experiments. In addition, in one or both experiments, the mutations Q41K, Q41H, F43Y, F43L, F43C, Q80K, Q80H, and Q80L were present as minorities with frequencies around 1%.

Interestingly, in experiment B, the frequency of the A156G mutation increased significantly, from pretreatment levels of 0.8% (Fig. 3C5) to ~25% at 240 nM TMC380765, followed by a decline to 8% at the end of the experiment, whereas this mutation was not detected before treatment and was present only transiently as a minority variant in experiment A. With further-increasing TMC380765 concentrations, the A156V mutation became the dominant mutation at position 156 (Fig. 3A5 and B5), reaching a frequency of 10 to 15% at the end of the selection experiment (Fig. 3A5 and B5). The frequency of the A156T mutation increased to ~10% at a TMC380765 concentration of 320 nM, after which its frequency declined to 1 to 5%.

At position 168, the five amino acid changes detected can be classified into two groups, defined by the concentration range of TMC380765 at which they emerged (Fig. 3A6 and B6). In both experiments, the initial group of mutations was first detected at or below a TMC380765 concentrations of 80 nM, with frequencies of <1%. They consisted of D168V, D168A, and D168E. The mutations D168Y and D168H emerged in both experiments at concentrations at or above 240 nM. Frequencies of the mutations D168A and D168V increased rapidly, to 20 to 30%. D168E displayed a similar rapid increase in frequency (up to 10 to 15%), but in contrast to D168A and D168V (which remained present at these high frequency levels), its prevalence decreased again toward the end of the selection experiment (3 to 7%). In addition, in experiment A, mutation D168Y also reached a high frequency (~20%), whereas in experiment B, it showed only a slight increase in frequency, to 2% (similar to what was observed for D168H in both experiments). Interestingly, the mutations D168V', D168A', and D168E', which are encoded by an alternative codon, were observed at different time points. The codons for these three mutations differ by one nucleotide from the alternative wild-type codon for D168D', whereas there is a two-nucleotide difference from the major wild-type codon. The frequency of the alternative mutation D168V' increased in parallel with that of D168V, although delayed in time, reaching its maximum frequency of ~10% at the end of the selection experiment. In contrast, the alternative mutations D168A' and D168E' remained at average frequencies of around ~1% during the whole experiment.

Whereas our analysis focused on specific amino acid positions, other mutations in the NS3 protease gene that affect susceptibility to a range of different HCV NS3/4A inhibitors have been described (17). These mutations, such as V36A,



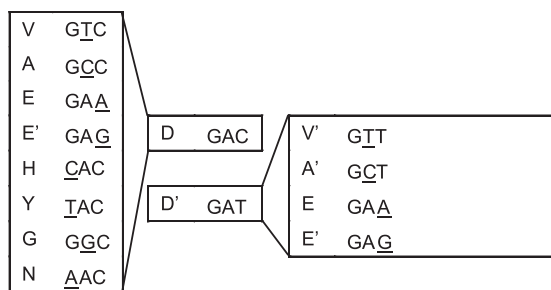


FIG. 4. Codon usage of amino acids detected by 454 deep sequencing during selection experiments at NS3 protease position D168. Two wild-type codons, GAC and GAT, coding for aspartate (D), are shown in the middle box. Boxes on the left and right show mutations detected that are one nucleotide change away from wild-type codons GAC and GAT (nucleotide changes resulting in amino acid changes are underlined).

T54A, V170A, R109K, S122R, and S138T, were detected at low frequencies. None of these mutations appeared to contribute significantly to the HCV replicon mutation profile. In addition, compensatory mutations, such as Q86R, P89L, and G162R (55), known to boost the replication capacity of some resistance mutations, were detected. However, there was no clear linkage with any of the resistance mutations since they were already detected in the pretreatment samples. Moreover, their frequencies did not change markedly with increasing TMC380765 concentrations. It is important to note that changes at other NS3 or NS4A positions were limited and that no consistent pattern was observed, with the exception of changes in the frequency of different codons encoding wild-type amino acids, suggesting that these were linked with mutations at the positions described.

**Mutations confer various degrees of resistance in a transient replicon assay.** To study the impact of the mutations identified in the different selection experiments on the activity of TMC380765, mutant replicons were generated by site-directed mutagenesis and the sensitivity to TMC380765 was assessed in a transient replicon assay (Fig. 5). The mutations observed in the low-concentration selection experiment or at the early or intermediate stages of the experiments with increasing concentrations resulted in reduced susceptibility, ranging from 2-fold for Q80R up to 50-fold for D168E. In contrast, the mutations D168V and A156V, selected by high concentrations of TMC380765, resulted in a greater than 500-fold reduction in susceptibility, whereas D168A conferred a 90-fold change in drug sensitivity.

The replication capacities of the different mutant replicons were determined as a measure of viral fitness using standard procedures (28, 35). The luciferase signal at 48 h posttransfection was normalized to the signal obtained at 4 h to correct for

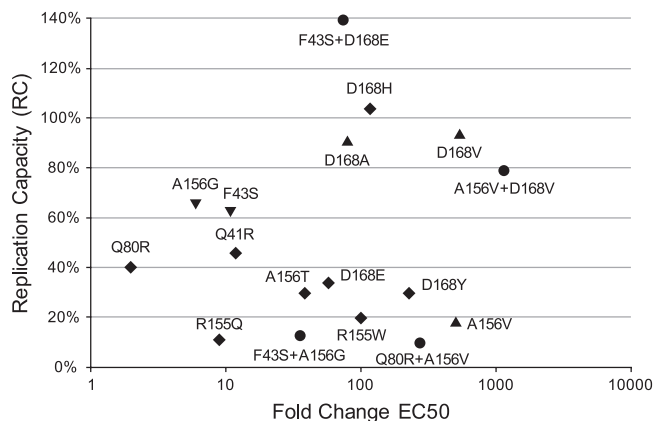


FIG. 5. Change in TMC380765 susceptibility ( $EC_{50}$ ) and replication capacity (RC) values determined using a transient replicon assay with replicons harboring single or double mutations in the NS3 protease domain. Fold change in  $EC_{50}$  =  $EC_{50}$  mutant/ $EC_{50}$  clone ET measured at 48 h.  $RC = (\text{Luc48h\_mut}/\text{Luc48h\_ET})/(\text{Luc4h\_mut}/\text{Luc4h\_ET})$ , where Luc48h\_mut or -ET and Luc4h\_mut and -ET are luciferase levels of the mutant or the ET clone at 48 h and 4 h postelectroporation, respectively. Inverted triangles, diamonds, and upright triangles indicate amino acid changes detected primarily during low, middle-range, and high TMC380765 concentration selection, respectively. Double mutants are indicated by full circles.

differences in transfection efficiencies, and the replication capacities of mutants were calculated relative to that of the wild type. Overall, most mutants showed reduced replication capacity levels compared to the wild type (Fig. 5).

**Replicon sequences carrying more than one mutation.** Sequence analysis of the selection experiments revealed amino acid changes at multiple positions in many samples occurring simultaneously. Neither the population sequencing nor the 454 deep sequencing protocol applied here allows the determination of mutation linkage, that is, that specific changes are on the same replicon genome. In the case of 454 deep sequencing, only mutations closer than 250 bp can be linked due to the limited read length. To overcome this technical limitation, single-genome sequencing using a clonal approach was performed. Starting with the RNA samples, the full NS3 protease domain was amplified by RT-PCR and bacterially cloned (24 or 48 clones were picked per sample for samples of experiment A or B, respectively), followed by DNA sequencing of individual clones. Overall, the individual amino acid frequencies correlated well between clonal and 454 deep sequencing (data not shown). However, clonal sequencing revealed that the seeming persistence of the individual single low-level resistance mutations F43S, A156G, Q41R, and Q80R at frequencies between 5 and 10% at the highest TMC380765 concentrations, as shown by 454 deep sequencing, was due mainly to the occur-

FIG. 3. Codon frequencies at six key amino NS3 acid positions (41, 43, 80, 155, 156, and 168; indicated by 1 to 6, respectively) of selection experiment with stepwise-increasing TMC380765 concentrations (A and B) or in the absence of NS3/4A inhibitor (C), as determined by 454 GS-FLX shotgun (A) or 454 GS-FLX amplicon (B and C) deep sequencing. Frequencies of individual amino acids were calculated as the number of sequence reads per specific codon divided by the total number of reads at that codon position. Dotted lines connect points where no intermittent data points were available. Alternative codon usage is indicated by an apostrophe ('). Based on 454 deep sequencing results of replicon control plasmid (data not shown), the limit of detection was set at 0.4%; therefore, single-codon frequencies below 0.4% were excluded from this analysis. However, percentages below 0.4% were considered significant if mutants were present in previous or subsequent samples.

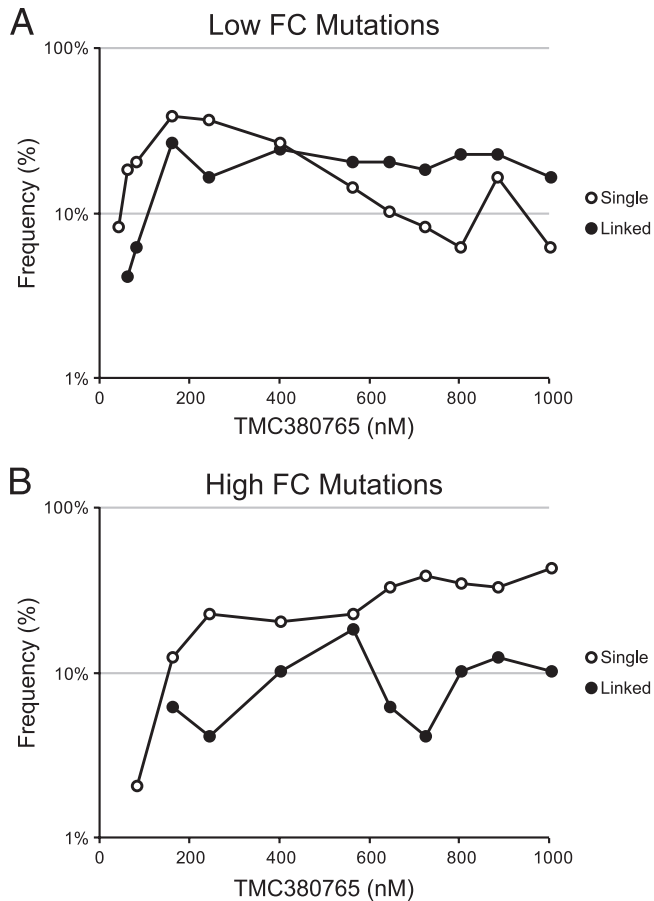


FIG. 6. Combined amino acid frequencies determined by clonal sequencing for mutations Q41R, F43S, Q80R, and A156G (A) (mutations with change in  $EC_{50}$ s < 15-fold) or A156V and D168V (B) (mutations with change in  $EC_{50}$ s > 500-fold) in experiment B. For every group, the frequency of genomes/clones harboring single or multiple mutations is shown.

rence of replicons carrying an additional resistant mutation in addition to these low-level resistant mutations. In contrast, the mutations A156V and D168V, which reduced TMC380765 potency significantly by themselves, were observed mainly as single mutant variants at the end of the selection experiment, although some double/triple mutant variants were detected at lower frequencies (Fig. 6B). These observations were confirmed by clonal analysis of samples from experiment A (24 clones/sample).

Clonal analyses further revealed that with increasing TMC380765 concentrations, the overall wild-type population (sequences carrying no mutation at amino acid positions 41, 43, 80, 155, 156, and/or 168) was totally replaced by mutant variants (Fig. 7A to C). In the pretreatment sample, 44/47 clones (~94%) had wild-type amino acids at the selected positions, whereas 3 clones carried either the F43Y, Q80H, or A156G mutation. Interestingly, the F43Y and Q80H mutations were not detected by 454 deep sequencing, whereas the A156G mutation was observed with a frequency of 0.8% using 454 deep sequencing. At a TMC380765 concentration of 560 nM, wild-type replicon sequences were reduced to 7/44 clones (~15%), while the number of clones with a single (26/44 clones tested; ~60%) or double (11/44 clones tested; ~25%) mutation increased (Fig. 6B). At the highest concentration ranges, no wild-type sequences were detected. Single mutant frequency increased to ~75% (33/45 clones) of the population, whereas the prevalence of variants carrying double mutations remained constant at ~25% (12/45 clones). Interestingly, strains carrying three (R155W, A156V, and D168Y) or even four (F43Y, Q80R, A156G, and D168V) mutations were observed. Overall, a large variety of double mutant variations was detected. The great majority (95%) of all the double mutant genomes detected contained combinations of the low-level resistance mutations Q41R, F43S, Q80R, and/or A156G with either high-level resistance mutations (50%), intermediate-level resistance mutations (such as D168E and/or A156T [15%]), or combinations of two low-level resistance mutations

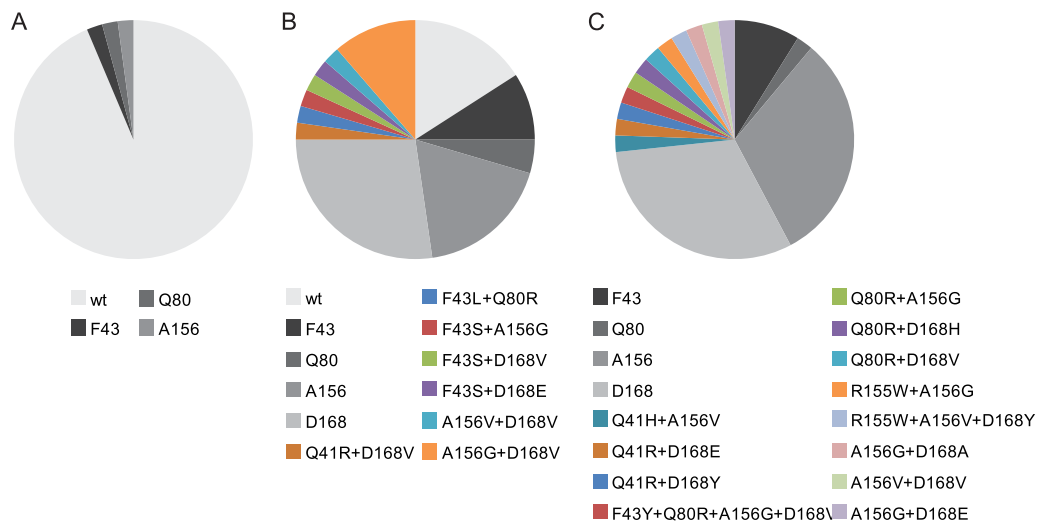


FIG. 7. Amino acid frequencies at a TMC380765 concentration of 20 nM (A), 560 nM (B), or 880 nM (C) detected by clonal sequencing. "wt" indicates wild-type sequences carrying no mutations at any of the six key NS3 protease amino acid positions (41, 43, 80, 155, 156, and 168). Sequences carrying single mutations are marked in gray. Sequences carrying multiple mutations are highlighted in color.

(25%). Although not frequently observed (5% of total) and at only the highest compound concentrations, combinations of two high-level resistance mutations (A156V and D168V) also were present. Analysis of 4 selected double mutations, with combinations of different types of mutations (i.e., combination of low-level with low- or intermediate-level resistance and combination of two high-level resistance mutations) in a transient replicon assay, showed either an increase in the fold change of  $EC_{50}$  values or replication capacity or an increase in both fold change and replication capacity for the double mutants compared to the respective single mutants (Fig. 5).

## DISCUSSION

In this study, the mutational patterns at six key positions of NS3 (41, 43, 80, 155, 156, and 168) emerging in HCV replicon-containing cells that were cultured in the presence of various concentrations of the HCV NS3/4A protease inhibitor TMC380765 were assessed. To this end, we employed population, clonal, and 454 deep sequencing technologies.

Concentration-dependent mutational patterns were observed: low concentrations of TMC380765 selected for mutations conferring a low change in  $EC_{50}$ s, as assessed in a transient replicon assay (such as the mutations F43S or A156G), whereas selection with high concentrations of TMC380765 resulted in the emergence of mutations conferring a high change (such as D168A, D168V, and A156V). In experiments in which the concentration of TMC380765 was increased stepwise, low-level resistance mutations were initially detected by population sequencing, whereas at a higher concentration of TMC380765, these mutant replicons were replaced by replicons carrying mutations conferring a high level of resistance.

A detailed analysis of the selection experiments by clonal and 454 deep sequencing allowed better assessment of the dynamic changes of the replicon population under increasing selective pressure from TMC380765. This technology also allowed the detection of low-frequency mutations not detectable by population sequencing. Overall, many mutations in NS3 previously associated with exposure to NS3/4A protease inhibitors (reviewed in reference 17) were observed during the selection experiments. However, as expected for a macrocyclic NS3 protease inhibitor (7, 24) with a large S2 group and consistent with the results from population sequencing, the most pronounced amino acid changes in selection experiments with TMC380765 were detected at NS3 positions 156 (3 different mutations) and 168 (5 different mutations). As determined by 454 deep sequencing in the experiments with stepwise-increasing TMC380765 concentrations, initial low concentrations selected for a mixed pool of replicons carrying the low-level resistance mutations Q41R, F43S, Q80R, and A156G at frequencies between 10 and 25%, which were suppressed with increasing TMC380765 concentrations and gradually replaced by replicons carrying the high-level resistance mutations A156V, D168A, and D168V. Interestingly, mutations causing small changes in susceptibility were still detected at high TMC380765 concentrations but, as clonal sequencing analysis revealed, primarily in combination with other resistance mutations, probably resulting in phenotypes conferring a higher level of resistance to TMC380765. Phylogenetic analysis of the clonal sequences did not allow assessment of whether these

double mutant variants were the result of the acquisition of additional resistance mutations in addition to the initial or preexisting mutation or whether variants carrying two or more mutations were newly emerging (data not shown). Mutations conferring high-level resistance (such as D168V and A156V) were present predominantly as single unlinked mutations. No or very few changes were observed beyond the six key positions (41, 43, 80, 155, 156, and/or 168) of the NS3 protease or NS3/4A gene during selection.

It has been reported that mutations need to be present at a certain frequency to result in a measurable phenotypic effect (32, 38). In total, 28 different amino acid changes were detected at the six key positions, of which only five changes reached a frequency of approximately 25%. At least half of these 28 amino acid changes did not reach frequencies higher than 10%, of which again half emerged and remained present as a minority, with an average frequency of ~1%. Clonal sequencing, however, revealed that with increasing concentrations of TMC380765, the number of sequences carrying one or more resistance mutations increased, reaching levels close to 100% at the end of the selection experiments. The contribution of all these individual variants to the resistant phenotype of the replicon population is not fully understood and remains a possible subject for further studies.

The level of resistance (measured by the change in  $EC_{50}$ s) conferred by a mutation is a major determinant of viral survival under drug pressure. In addition, replication capacity and, more specifically, replication capacity in the presence of a drug is regarded as a factor critical in predicting the emergence and evolution of mutant viruses during drug treatment (14, 29).

As a third factor, the variants present before treatment influence the emerging mutational patterns (1). The high mutation rate of the HCV polymerase and the detection of multiple mutant replicon variants early during the experiment suggest the presence of many replicon variants at baseline. For example, the A156G mutation, which was detected at baseline in experiment B at 0.8%, reached a maximal frequency of 24% at 400 nM TMC380765, whereas in experiment A, A156G was not detected at baseline and became only transiently detectable during selection, at an average frequency of ~1%. Thus, the higher preexisting frequency of A156G in experiment B seems to confer an advantage in the competition for resources and therefore an initial dominance, consistent with the mechanism suggested by the clonal interference model (33) and in a recently described multivariant, viral dynamic model of genotype 1 HCV (1). In addition, the D168V mutation was not detected in pretreatment samples and did not emerge immediately at low compound concentrations, although it has the most favorable replication capacity and resistance profile *in vitro* of all mutations detected in our experiments. This observation is puzzling and may be related to the absence or very low frequency of this mutation at baseline. Additional studies are needed to better understand the importance of preexisting mutations for the selection and emergence of replicon variants during compound treatment.

For HIV, it has been reported that codons specifying the same amino acid can lead to different pathways of amino acid substitution by single nucleotide changes, a phenomenon called "quasisynonymy" (18). Similarly, for HCV, *in vitro* and *in vivo* exposure to some HCV protease inhibitors favors an



NS3 protease R155K mutation in genotype 1a, whereas this mutation is rarely observed in genotype 1b, probably due to the fact that in genotype 1a only one nucleotide change is needed for this mutation whereas in genotype 1b two nucleotide changes are required (17, 24, 31). Here we have described, to our knowledge for the first time, the presence of two codons encoding wild-type amino acids within one population of replicons and the potential contribution of both codons to the emergence of amino acid variants. Although not proven experimentally, it can be assumed that, as shown in Fig. 4, at position 168 the codons for the resistant amino acids valine (V; GTC) and alanine (A; GCC) have the major wild-type amino acid aspartate (D; GAC) as an ancestor while the alternative resistance mutations valine (V'; GTT) and alanine (A'; GCT) are derived from the minority wild-type aspartate (D'; GAT). The presence of such second wild-type amino acid codons within one population may facilitate alternative pathways to resistance, bringing an extra dimension to the mutational space available for the virus to explore and allowing it to adapt even more rapidly to changing environments (45). Analysis of a large group of genotype 1a and 1b patient NS3 protease nucleotide sequences extracted from the Los Alamos database reveals that 93 and 7%, respectively, were carriers of the major wild-type codon (D; GAC) and the alternative wild type (D'; GAT) at position 168 (data not shown).

In summary, the *in vitro* resistance profile of the macrocyclic HCV NS3/4A inhibitor TMC380765 and the changes in quasispecies composition during selection *in vitro* were assessed using state-of-the-art sensitive sequencing technologies. Depending on the concentration of TMC380765 used during the *in vitro* resistance selection experiments, distinct mutational patterns were selected. This resulted in the emergence and disappearance of multiple replicon variants in response to the increasing selection pressure. In addition, clonal and 454 deep sequencing analysis of the viral sequences in these experiments enabled identification of low-frequency mutations present at baseline (preexisting mutants), which may contribute to the mutational pattern that emerges. The presence of two alternative codons for wild-type amino acids at certain NS3 positions within a single population of replicons is described and is proposed to contribute to the emergence of the drug-resistant geno- and phenotypes. Deep sequencing technologies, in conjunction with clonal sequencing, are a powerful tool for gaining a more profound insight into the dynamics of resistance acquisition in the HCV quasispecies population during antiviral drug pressure.

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