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Comparative Study of the Genetic Barriers and Pathways towards Resistance of Selective Inhibitors of Hepatitis C Virus Replication▽†

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Hepatitis C virus (HCV) inhibitors include direct-acting antivirals (DAAs) such as NS3 serine protease inhibitors, nucleoside and nonnucleoside polymerase inhibitors, and host-targeting antivirals (HTAs) such as cyclophilin inhibitors that have been developed in recent years. Drug-resistant HCV variants have been reported both *in vitro* and in the clinical setting for most classes of drugs. We report a comparative study in which the genetic barrier to drug resistance of a representative selection of these inhibitors is evaluated employing a number of resistance selection protocols. The NS3 protease inhibitors VX-950 and BILN 2061, the nucleoside polymerase inhibitor 2′-*C*-methylcytidine, three nonnucleoside polymerase inhibitors (thiophene carboxylic acid, benzimidazole, and benzothiadiazine), and DEB025 were included. For each drug and passage in the selection process, the phenotype and genotype of the drug-resistant replicon were determined. For a number of molecules (BILN 2061 and nonnucleoside inhibitors), drug-resistant variants were readily selected when wild-type replicon-containing cells were directly cultured in the presence of high concentrations of the inhibitor. Resistance to DEB025 could be selected only following a lengthy stepwise selection procedure. For some DAAs, the signature mutations that emerged under inhibitor pressure differed depending on the selection protocol that was employed. Replication fitness of resistant mutants revealed that the C445F mutation in the RNA-dependent RNA polymerase can restore loss of fitness caused by a number of unfit resistance mutations. These data provide important insights into the various pathways leading to drug resistance and allow a direct comparison of the genetic barriers of various HCV drugs.

Hepatitis C virus (HCV) is a positive single-stranded RNA virus and the only member of the *Hepacivirus* genus within the *Flaviviridae* family. An estimated 170 million people are chronically infected worldwide. Three million to four million people become newly infected each year (57). Chronically infected patients are at increased risk of developing liver cirrhosis and hepatocellular carcinoma. In Western countries, infection with HCV is the most common reason for liver transplantation. The current standard of care for the management of chronic hepatitis C virus infection consists of the combination of pegylated alpha interferon (pegIFN-α) and ribavirin. This therapy is effective in only 50 to 60% of infected patients and is associated with serious side effects (44). Therefore, more tolerable, highly potent inhibitors of HCV replication are urgently needed and are currently also being developed. Antivirals that specifically target viral proteins are referred to as "direct-acting antivirals" (DAAs) for HCV. A number of NS3/NS4A protease inhibitors are currently in clinical development. The first HCV NS3/4A serine protease inhibitor to enter clinical trials was ciluprevir (BILN 2061) (54), but clinical development was halted because of cardiotoxicity. Other protease inhibitors in clinical development include danoprevir (ITMN-191), narlaprevir (SCH 900518), and vaniprevir (MK-7009); telaprevir (VX-950), boceprevir (SCH-503034), and TMC435 progressed into phase III clinical trials. Both nucleoside and nonnucleoside inhibitors of the HCV RNA-dependent RNA polymerase (RdRp) have been identified. Nucleoside analogues mimic natural polymerase substrates and cause chain termination following phosphorylation to their corresponding 5′ triphosphate. Valopicitabine (2′-*C*-methylcytidine [2′-CMC]) was the first nucleoside analogue to enter clinical trials. Development has been discontinued because of modest antiviral efficacy along with significant gastrointestinal side effects (2). RG7128, a prodrug of nucleoside analogue PSI-6130 (β-D-2′-deoxy-2′-fluoro-2′-*C*-methylcytidine), and PSI-7977, a liver-targeted prodrug of the uridine nucleotide analogue PSI-6206 monophosphate, are in phase II clinical trials. A number of structurally unrelated nonnucleoside polymerase inhibitors have been reported; these include, but are not limited to, benzimidazoles, benzothiadiazines, thiophene derivates, benzofuranes, and imidazopyridines (14). Recently, inhibitors of other targets, such as (i) the entry process, (ii) NS4A (74), (iii) NS4B (7, 17), and (iv) NS5A (31), have also been identified (14). Not surprisingly, monotherapy with most DAAs has been associated with the rapid emergence of resistant variants (63).

On the other hand, host factors that are essential for efficient viral replication may also be good antiviral targets. Host-targeting antivirals (HTAs) may have a higher barrier to resistance than (most) DAA inhibitors. A number of cyclophilin-binding molecules such as alisporivir (DEB025), NIM811, and SCY-635 have proven to be potent inhibitors of HCV replication and have shown clinical efficacy (30, 47).

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The rapid emergence of drug-resistant variants of HCV is, as is also the case with HIV, of major concern and results from several factors. These include the poor fidelity and lack of exonucleolytic proofreading capacity of the reverse transcriptase (RT) enzyme in the case of HIV (error rate, 10^{-5} mutations per nucleotide per genomic replication) (4) or the RdRp in the case of HCV (error rate, 10^{-3} to 10^{-5} mutations per nucleotide per genomic replication) (9) and the very high magnitude of replication (HCV, 10^{12} virions/day [48]; HIV, 10^{10} virions/day [51]). As a result, multiple viral variants known as quasispecies (4, 59) are generated. In various clinical studies for both HIV and HCV in which antiretroviral drugs or DAA inhibitors were given as monotherapy, escape mutants were shown to develop very rapidly. For example, the administration of a single dose of the nonnucleoside RT inhibitor (NNRTI) nevirapine to prevent mother-to-child HIV transmission rapidly and routinely selects for NNRTI-resistant mutants (23). Also, for HIV protease inhibitors, more than 80 mutations have been reported (56). When HCV-infected patients were treated with telaprevir for 14 days, viral breakthrough, associated with a number of mutations that confer low-level and high-level resistance to telaprevir, was noted in a significant number of patients (55). Drug-resistant variants also rapidly emerged in clinical trials with other protease inhibitors such as boceprevir (61) and nonnucleoside polymerase inhibitors (49, 69). These drug-resistant HCV variants may be present at frequencies of $<1\%$ in the quasispecies population in treatment-naïve patients (27, 35), which may result in a rapid selection during DAA treatment. These findings are in agreement with those of *in vitro* resistance studies in which HCV is shown to develop (often rapidly) resistance against polymerase and protease inhibitors.

HCV subgenomic replicons have been widely used in the discovery and the development of DAA inhibitors. Drug-resistant HCV replicons have been obtained for most classes of drugs. However, since different resistance selection protocols are used in different studies, it is not possible to directly compare the genetic barrier to antiviral drug resistance of various (classes of) HCV drugs. We here report a comparative study in which the genetic barrier to drug resistance of a selection of reference compounds is evaluated employing a number of resistance selection protocols. The NS3 protease inhibitors (VX-950, BILN 2061), a nucleoside polymerase inhibitor (2′-*C*-methylcytidine), three nonnucleoside polymerase inhibitors (thiophene carboxylic acid, benzimidazole JT-16, and benzothiadiazine A-782759) as well as the cyclophilin-binding molecule DEB025 were used for this purpose.

MATERIALS AND METHODS

Compounds. The following molecules were included in this study: (i) the nucleoside polymerase inhibitor 2′-CMC (the active moiety of NM283) (8), (ii) the nonnucleoside polymerase inhibitors 2-[4-[[4-(acetylamino)-4′-chloro-[1,1′-biphenyl]-2-yl]methoxy]phenyl]-1-cyclohexyl-1*H*-benzimidazole-5-carboxylic acid (JT-16) (21), 3-{isopropyl-[(*trans*-4-methylcyclohexyl)carbonyl]amino}-5-phenylthiophene-2-carboxylic acid (TCA) (33), and 2-[3-(1-cyclobutylamino-4-hydroxy-2-oxo-1,2-dihydro-quinolin-3-yl)-1,1-dioxo-1,4-dihydro-1l6-benzo(1,2,4)thiadiazin-7-yloxy]-acetamide (A-782759) (53), (iv) the protease inhibitors (2*R*,6*S*,12*Z*,13a*S*,14a*R*,16a*S*)-6-[[(cyclopentyloxy)carbonyl]amino]-1,2,3,6,7,8,9,10,11,13a,14,15,16,16a,-tetradecahydro-2-[[7-methoxy-8-methyl-2-[2-[(1-methylethyl)amino]-4-thiazolyl]-4-quinolinyl]oxy]-5,16-dioxocyclopropa[e]pyrrolo[1,2-a][1,4]diazacyclopentadecine-14a(5*H*)-carboxylic acid (BILN 2061) (28), and (1*S*,3a*R*,6a*S*)-2-[2*S*)-2-[[(2*S*)-2-cyclohexyl-2-(pyrazine-2-carbonylamino)acetyl]amino]-3,3-dimethylbutanoyl]-*N*-[(3*S*)-1-(cyclopropylamino)-1,2-dioxohexan-3-yl]-3,3a,4,5,6,6a-hexahydro-1*H*-cyclopenta[c]pyrrole-1-carboxamide (VX-950) (60), and (v) the cyclophilin-binding compound DEB025 (13). All molecules were synthesized in-house by medicinal chemists.

Selection of drug-resistant replicon. Huh-7 cells containing subgenomic HCV replicons I377/NS3-3′/wild type (WT) (Huh 9-13) (39) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 1× minimal essential medium nonessential amino acid solution without L-glutamine (Gibco), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Gibco), and 1 mg/ml G418 (Geneticin-selective antibiotic; Gibco). Huh-Lunet cells, which are derived from a cell clone that was generated by curing Huh-7 replicon cells with a selective drug, were cultured without G418 (19).

Various drug-resistant replicons were selected following culturing of Huh 9-13 replicon-containing cells for 11 passages (5.5 weeks) under constant antiviral pressure (1×, 2×, 5×, 25×, and 125× 50% effective concentration [EC_{50}]). When replicon-containing cells suffered (massive cell death) from compound pressure (because of replicon disappearance), G418 and compound were removed until cells recovered. This particular observation during cell culture was designated "critical phase," and all these events are listed in Tables 1 to 7. Thereafter, cells were recultured in the presence of the antiviral drug and G418 pressure until the predetermined 5.5 culture weeks were completed. Subsequently, Huh 9-13 cells were challenged with gradually increasing doses of antiviral pressure; e.g., cells surviving doses of 5× EC_{50} were subsequently challenged with 25× or 125× EC_{50}. Since certain compounds are, at particular concentrations, toxic for the host cell, drug selection protocols were in this case not carried out with such concentrations. Following selection of drug-resistant cultures, the genotypes and phenotypes of all replicons obtained during the different selection protocols were determined. When the replicon proved ≥4-fold less susceptible to the inhibitor than the wild-type replicon in the case of resistance culture or ≥3-fold less susceptible to the inhibitor than the wild-type replicon in the case of transient transfections, the replicon/mutant was considered resistant. These thresholds were set according to Table SA1 in the supplemental material, in which the variability of the antiviral assays is calculated.

Phenotyping of wild-type or drug-resistant Huh 9-13 replicon cells. Antiviral assays were performed as described before (15). Briefly, cells were seeded at a density of 5×10^3 cells per well in a 96-well cell culture plate in complete DMEM. Following incubation for 24 h at 37°C (5% CO_2), serial dilutions of the test compounds in complete DMEM were added in a total volume of 100 μl and cells were cultured for an additional 3 days. Replicon RNA levels were determined by a reverse transcription-quantitative PCR. Primers used for detection of HCV replicon RNA were 5′-CCG GCT ACC TGC CCA TTC-3′ (forward primer), 5′-CCA GAT CAT CCT GAT CGA CAA G-3′ (reverse primer), and 5′-FAM-ACA TCG CAT CGA GCG AGC ACG TAC-TAMRA-3′ (probe; where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). The EC_{50} was defined as the concentration of compound that reduced HCV RNA replication by 50%. The fold resistance value was calculated as the ratio of the EC_{50} in the resistant replicon to the EC_{50} in the wild-type replicon. Maximum fold resistance values were calculated as the ratio of the highest EC_{50} of the inhibitor tested to the mean EC_{50} in the wild-type replicon.

Cytostatic assays were performed as described before (15). Briefly, cells were seeded at a density of 5×10^3 cells per well in a 96-well cell culture plate in complete DMEM. After 24 h of incubation at 37°C, serial dilutions of the test compounds in complete DMEM were added. After 3 days of incubation at 37°C, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulfate (MTS/PMS) method (Promega). The 50% cytotoxic concentration (CC_{50}) value was defined as the concentration that inhibited the proliferation of exponentially growing cells by 50%.

Population sequencing of resistant replicon RNA. Total RNA of the wild-type or resistant replicon population was extracted from 5×10^4 replicon cells using an RNeasy minikit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. cDNA fragments encompassing selected fragments of the HCV genome were amplified with 0.6 μM HCV-specific primers (see Table SA2 in the supplemental material) using a Qiagen OneStep reverse transcription-PCR kit. The reverse transcription-PCR program was as follows: 30 min at 50°C for reverse transcription and 15 min at 95°C to activate the HotStar *Taq* enzyme, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, 55°C, or 50°C, and 60 s at 72°C. A final elongation step of 10 min at 72°C was performed after cycling. Amplification products were purified using a Wizard SV Gel and PCR cleanup system (Promega Benelux, Leiden, The Netherlands), and nucleotide sequences were determined using the same primers (final concentration, 0.5 μM) used for reverse transcription-PCR and the BigDye Terminator (version 3.1) sequencing system (Applied Biosystems, Nieuwerkerk Ad IJssel, The Netherlands). Muta-

tions that are detected in both wild-type and resistant Huh 9-13 replicon-containing cells were not included in the mutational analysis. Furthermore, no linkage between mutations was implied.

Clonal sequencing of wild-type replicon. HCV RNA was isolated from Huh 9-13 cells using the RNeasy minikit (Qiagen Benelux), according to the manufacturer's instructions. cDNA fragments were synthesized using the Transcriptor high-fidelity cDNA synthesis kit (Roche Diagnostics, Vilvoorde, Belgium). The cDNAs were subjected to amplification by PCR using the 9F/9R primers (also used for population sequencing) and an AccuPrime *Pfx* DNA polymerase kit (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. This polymerase was chosen, as it possesses a proofreading 3′ to 5′ exonuclease activity. The appropriately sized product was than purified by the Wizard SV Gel and PCR cleanup system (Promega) and cloned using a TOPO TA cloning kit for sequencing (Invitrogen). Transformed TOP10 *Escherichia coli* cells were plated on ampicillin-LB agar plates. Colonies were randomly picked, and 96 clones were sent for sequencing using the M13F/M13R primers at Beckman Coulter Genomics (formerly Agencourt Bioscience and Cogenics; Takeley, United Kingdom).

Site-directed mutagenesis. Various published and drug-selected resistance mutations were introduced in pFK I389 Lucibineo EI NS3-3′ET (71), including D168V in NS3 (BILN 2061), S282T in NS5B (2′-CMC), C316Y in NS5B (A-782759), T389A in NS5B (JT-16), M414T in NS5B (A-782759), M423T in NS5B (TCA), C445F in NS5B (A-782759, JT-16, TCA), P495L in NS5B (JT-16), Y452H in NS5B (A-782759), C316Y and C445F in NS5B (A-782759), and C445F and Y452H in NS5B (A-782759).

Initially, the NS5B or NS3 gene sequences were excised from the pFK I389 Lucubineo EI NS3-3′ET construct by SpeI-XhoI or NotI-MluI restriction digestion and subcloned to construct pCRII-HCV5B or pCRII-NS3. Mutations (single or combinations) were introduced into pCRII-HCV5B or pCRII-NS3 (see Table SA3 in the supplemental material). For the double mutants, the C316Y and Y452H mutations were constructed in pCRII-HCV5B containing the C445F mutation. The construction of the T389A mutant will be described elsewhere. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The 50-μl reaction mixture contained 1× reaction buffer, 50 ng plasmid solution, 125 ng forward primer, 125 ng reverse primer, 1 μl deoxynucleoside triphosphate mix, and 2.5 U *PfuTurbo* DNA polymerase. Thermal cycling was performed as follows: denaturation at 95°C 30 s, followed by 12 cycles (for 1 mutation) or 16 cycles (for 2 mutations) of 30 s at 95°C, 1 min at 55°C, and 7 min at 68°C. Following temperature cycling, the reaction mixture was cooled down to 4°C and digested with 10 U DpnI to remove the methylated, nonmutated parental supercoiled double-stranded DNA template. The mutated pCRII-HCV5B or pCRII-NS3 plasmid was then transformed in One Shot TOP10 chemically competent *E. coli* cells (Invitrogen), and positive colonies were subcultured the next day. Plasmid DNA was collected by a Wizard Plus SV miniprep kit (Promega Benelux) and digested with SpeI-XhoI for NS5B or NotI-MluI for NS3 to collect the mutated NS5B/NS3 fragments. The mutated fragments were ligated into digested pFK I389 Lucibineo EI NS3-3′ET and subsequently transformed into One Shot TOP10 chemically competent *E. coli* cells. Plasmid DNA was collected by the Wizard Plus SV miniprep kit. To control for the correct plasmid insert, a restriction digest was performed. In addition, to confirm the presence of the desired mutations, the entire NS5B and NS3 inserts were sequenced. The M423T mutant (a kind gift from W. Zhong, Gilead Sciences) was obtained in a similar way as the other NS3 and NS5B mutants. In short, the genotype 1b PI-Rluc replicon plasmid was created from the pFK I341 PI-Luc/NS3-3′ET construct (38) by replacing the firefly luciferase reporter gene with a *Renilla* luciferase reporter sequence (Promega Benelux). The 1b PI-Rluc construct encoding an M423T mutation in NS5B was produced by replacing the wild-type NS5B BclI-SpeI fragment with an analogous fragment in which the M423T mutant sequence had previously been introduced via site-directed mutagenesis using the QuikChange site-directed mutagenesis kit of Stratagene.

Transient transfection. (i) *In vitro* transcription. Total DNA isolated with the Wizard Plus SV miniprep kit from an overnight *E. coli* culture transformed with mutant HCV replicon was linearized using AseI (New England BioLabs, Germany) and ScaI (Promega Benelux) or only SpeI (Promega Benelux) in the case of M423T. The linearized plasmid was phenol-chloroform extracted, ethanol precipitated, and dissolved in RNase-free water. *In vitro* transcription was performed on 5 μg DNA by a RiboMAX large-scale RNA production system-T7 enzyme mix (Promega Benelux) according to the manufacturer's instructions. After 2 h at 37°C, an additional 5 μl of enzyme mix (T7) was added and the reaction mixture was incubated for an extra 2 h at 37°C. Transcription was ended by adding RQ1 RNase-free DNase (Promega Benelux). RNA was purified and collected by using the RNeasy miniprotocol for RNA cleanup (RNeasy minikit; Qiagen Benelux). The concentration and purity of RNA were spectrophotometrically measured.

(ii) Transfection. Twenty micrograms of *in vitro*-transcribed RNA was mixed with 400 μl of a suspension of 4×10^6 Huh-Lunet cells in a cuvette with a gap width of 0.4 cm (VWR International, Leuven, Belgium). After one pulse at 1,600 V with an ECM 830 Electro Square Porator (BTX Harvard Apparatus), cells were immediately transferred into 20 ml of complete Dulbecco's modified Eagle medium without G418. One hundred-microliter aliquots of the cell suspension were seeded in a 96-well plate (Iwaki, Asahi Techno Glass, Japan) previously filled with serial dilutions of the test compounds in complete Dulbecco's modified Eagle medium without G418. Cells were allowed to proliferate for 4 days at 37°C, after which the luciferase activity was determined using a Steady-Glo luciferase assay system (Promega Benelux); the luciferase signal was measured using a Luminoskan Ascent apparatus (Thermo, Vantaa, Finland). The EC_{50} was defined as the concentration of compound that reduced the luciferase signal by 50% compared to the signal for nontreated transfected cells.

Replication fitness. Transfections were performed in Huh-Lunet cells as described above, with the exception that cells were transfected with 5 μg RNA. To stabilize the input mutant RNA, an additional 5 μg tRNA (Sigma-Aldrich, Bornem, Belgium) was added. Transfected cells were immediately transferred to 24 ml of complete Dulbecco's modified Eagle medium without G418, and a 2-ml aliquot of the cell suspension was added to a 6-well plate (Iwaki). Cells were collected at 4 h (normalization point) and 1, 2, 3, and 4 days after transfection to compare luciferase values with wild-type values, after extraction of the tRNA background signal. Cells transfected with the GND replicon (a replication-deficient subgenomic replicon encoding a GDD-to-GND mutation in NS5B [62]) were used as a negative control reflecting background activity from the residual input RNA.

Three-dimensional structures of HCV inhibitor binding to NS5B. Different HCV NS5B RdRp structures, 1YVF (52), 1OS5 (40), and 2BRK (16), containing A-782759, thiophene carboxylic acid, and JT-16, respectively, were superimposed onto a reference structure, 1GX6 (6), using the Dalilite server (Holmer). Structures were loaded in the Chimera program to create three-dimensional figures. Colors used are blue for the fingers domain (residues 1 to 188 and 228 to 286), yellow for the palm domain (residues 188 to 227 and 287 to 370), and pink for the thumb domain (residues 371 to 563). The different inhibitors are highlighted in green carbons and a transparent surface. Mutations are presented as colored balls.

RESULTS

Huh 9-13 replicon-containing cells were cultured for 11 consecutive passages (5.5 weeks) under continuous antiviral pressure (either 1×, 2×, 5×, 25×, or 125× EC_{50}) with a selection of inhibitors. Massive cell death was documented in those cultures where it appeared as a critical phase. Surviving Huh 9-13 cells were subsequently challenged with gradually increasing concentrations of compound for 11 passages per concentration. At the end of the selection protocol, the genotype (amino acid changes are presented in the tables; codon changes are presented in Table SA4 in the supplemental material) and antiviral phenotype of the replicons (see Tables 1 to 7) thus obtained were determined.

Protease inhibitors (BILN 2061 and VX-950). BILN 2061-resistant variants were readily selected at all steps of the resistance selection procedure, even when wild-type replicon-containing cells were directly cultured in the presence of high drug concentrations (25× or 125× the EC_{50}) (Table 1). Replicons with a reduced susceptibility (~20-fold) to BILN 2061 were obtained following selection at concentrations as low as 2× the EC_{50}. Selecting resistance in the presence of concentrations of 25× and 125× the EC_{50} (either directly or gradually) resulted in pronounced resistance (>50-fold increase in EC_{50}). VX-950-resistant variants were also readily selected in all protocols when wild-type replicon-containing cells were directly cultured in the presence of high drug concentrations (5× the EC_{50}) (Table 2).

BILN 2061 resistance is associated with mutations

TABLE 1. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of BILN 2061[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	EC_{50} ± SD[b] (μM)	NS3 mutation(s) (aa 22-336)[c]	Critical phase[d]
1	2	0.033 ± 0.008	S174S/C	N
2	16	0.24 ± 0.08	**D168D/E/V**, S174S/C	N
5	13	0.19 ± 0.09	**D168D/E/V**, S174S/C	N
25	67	1 ± 0.4	**D168V**, E176E/G	Y
125	200	3 ± 0.5	**D168V**, S174S/C, E176E/G	Y
2-5	17	0.26 ± 0.007	**D168D/E/V**, S174S/C	N
2-25	61	0.92 ± 0.3	**D168V**, S174S/C, E176E/G	N
2-125	133	2 ± 0.2	**D168V**, S174S/C, E176E/G	N
5-25	56	0.84 ± 0.005	**D168D/V**, S174S/C, E176E/G	N
5-125	133	2 ± 0.6	**D168V**, S174S/C, E176E/G	N
25-125	133	2 ± 0.3	**D168V**	N
2-5-25	133	2 ± 0.6	**D168V**, S174S/C	N
2-5-125	133	2 ± 0.6	**D168V**, S174S/C	N
2-25-125	133	2 ± 0.5	**D168V**, S174S/C, S280S/A	N
5-25-125	200	3 ± 0.04	**D168V**, S174S/C, E176E/G, S280S/A	N
2-5-25-125	267	4 ± 0.7	**D168V**, S174S/C	N

[a] EC_{50}, 0.015 ± 0.01 μM; CC_{50}, 22.6 ± 0.1 μM.
[b] Data are mean values for 2 independent determinations of the antiviral phenotype.
[c] Major resistance mutations are indicated in boldface. aa, amino acids.
[d] Y, yes; N, no.

R155Q/K, A156T/V, and D168V/A (41, 68). Mutations at amino acid positions R155 and A156 of the HCV NS3 confer resistance to all NS3 protease inhibitors to date. The main resistance mutation for BILN 2061 identified in the present study is D168V. Whenever this substitution is present in the replicon, at least a 60-fold reduced susceptibility is noted. Mutations S174C and S280A were not reported earlier in the context of BILN 2061 resistance. However, it is unlikely that these mutations contribute to the reduced susceptibility for BILN 2061. E176G was described previously as a cell-culture-adaptive mutation (68).

The dominant resistance mutation reported in the literature for VX-950 *in vitro* is A156S/V/T (36). However, this mutation could be selected only as a multispecies by culturing replicon-containing cells directly in the presence of 5× the EC_{50}. This can be explained by the low concentrations of VX-950 that were used in this study (3 μM), while in previous studies, relatively high concentrations were used (14 and 28 μM) (36). However, substitutions at positions T54 and V170, which were previously reported to be associated with low-level resistance, were observed (63, 73). Like in the BILN 2061-resistant replicons, a previously reported adaptive mutation (E176G) was identified (25). Mutation T254A is most likely an adaptive mutation, as was earlier reported for T254I (25). Mutations at positions T22, T177, I248, and S280 have not been described before. Remarkably, mutation S280A was also observed in some BILN 2061-resistant replicons. These mutations are likely adaptive mutations or may have emerged randomly.

Polymerase inhibitors. (i) Nucleoside analogue (2′-CMC). A concentration of 25× EC_{50} (11 μM) cleared replicons from Huh 9-13 cells (Table 3). No resistance against 2′-CMC could be obtained following 5.5 weeks of culturing under all compound concentrations studied. This is in accordance with previously described observations (3, 29). The mutation A281T/A that was detected in the replicon when Huh 9-13 cells were cultured under the pressure of 1× EC_{50} of 2′-CMC and the T390I/T and A421T/A mutations detected when they were cultured under 2× and then 5× EC_{50} (the 2-5× EC_{50} protocol) of 2′-CMC are most likely adaptive mutations that do not contribute to resistance (Table 3).

(ii) Nonnucleoside analogues. ***(a) Benzothiadiazine (A-782759).*** Under none of the conditions studied was A-782759 able to clear the cells of their replicons. Low-level resistance (4- to 5-fold) against A-782759 was observed when replicon-containing cells were cultured in the presence of 1× EC_{50} (0.075 μM) or 2× EC_{50} (0.15 μM) of the molecule (Table 4).

TABLE 2. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of VX-950[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	EC_{50} ± SD[b] (μM)	NS3 mutations (aa 1-336)[c]	Critical phase[d]
1	1	0.78 ± 0.4	T22T/I, E176E/G	Y
2	3	2.0 ± 0.1	**T54T/S**, E176E/G, I248I/V, T254T/A, S280S/A	Y
5	5	3.6 ± 1.4	**T54T/A**, **A156A/S/T**	Y
2-5	7	5.8 ± 1.0	**T54T/S**, **V170V/A**, E176G, T177T/A, T254A, S280A	Y

[a] EC_{50}, 0.74 ± 0.8 μM; CC_{50}, 24.1 ± 4.4 μM.
[b] Data are mean values for ≥2 independent determinations of the antiviral phenotype.
[c] Major resistance mutations are indicated in boldface. aa, amino acids.
[d] Y, yes; N, no.

TABLE 3. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of 2′-CMC[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	$EC_{50} \pm SD$[b] (μM)	NS5B mutation(s) (aa 246-536)[c]	Critical phase[d]
1	1	0.40 ± 0.04	A281A/T	N
2	1	0.41 ± 0.07	NP	N
5	2	0.99 ± 0.1	NP	Y
25	—	NA	ND	Y
2-5	2	1 ± 0.4	T390T/I, A421A/T	Y
2-25	—	NA	ND	Y
5-25	—	NA	ND	Y
2-5-25	—	NA	ND	Y

[a] EC_{50}, 0.43 ± 0.1 μM; CC_{50}, 26 ± 10 μM.
[b] Data are mean values for ≥2 independent determinations of the antiviral phenotype. —, cell cultures died; NA, not applicable.
[c] Major resistance mutations are indicated in boldface. aa, amino acids; NP, none present; ND, not determined.
[d] Y, yes; N, no.

Mutation S556G was present as a quasispecies in the cultures of replicons cultured in the presence of 2× EC_{50} or higher of the compound. Replicons cultured in the presence of 5× EC_{50} (0.38 μM) or using the 2-5× protocol exerted low-level resistance (11- or 13-fold). The mutation C445C/F was identified in both cases. C445C/F was also identified in the replicons obtained in some of the stepwise-culture protocols. C445R has previously been reported in A-782759-resistant replicons (43). M414T, described as being the dominant resistance mutation for A-782759 (46), was observed in the viral genome selected in the presence of the 2-125×, 5-125×, 2-5-25×, 2-5-125×, and 2-25-125× protocols. Mutation W571P was observed in replicons of the 5×, 25×, 2-25-125×, and 5-25-125× cultures and has not been reported earlier to be a benzothiadiazine resistance mutation. Nonetheless, the mutation appears to contribute to resistance (compare 2× and 25× EC_{50}s in Table 4). Mutation G554D and/or Y555C was identified in the 125×, 2-5×, 2-125×, 2-5-25×, 2-5-125×, 2-25-125×, and 2-5-25-125× cultures and has previously been reported for another benzothiadiazine (i.e., A-837093) (42). Of note, the A73S mutation was selected in replicons of all stepwise-culture protocols. Although this mutation is located in the finger domain outside the benzothiadiazine binding site (Fig. 1A), it may contribute to resistance (compare 2× and 2-25× EC_{50}s). However, a mutation at the nearby residue M71 was previously reported to emerge during resistance selection with a benzothiadiazine derivative; this mutation did not confer resistance to the benzothiadiazine. The C316Y mutation, identified in the replicons that were selected using the 5-125×, 2-5-125×, 5-25-125×, and 2-5-25-125× protocols, has previously been reported for A-837093 (10). Mutations at positions L47, V108, V122, L126, D135, A207, C311, I447, S476, Y452, and T520 have not been described before to contribute to benzothiadiazine resistance. These residues are not located in close proximity to the binding site of A-782759 (Fig. 1A).

(b) TCA. At none of the concentrations used was TCA able to cure cells of their replicons (Table 5). No resistance mutations were observed in the 1× and 2× EC_{50} selection protocols. Cultures from the 5× and 2-5× conditions exhibited low-level resistance. The C445F mutation, located outside the TCA binding pocket (Fig. 1B), was also identified in the genome of replicons that had been selected using 25× EC_{50} and all but one stepwise-culture protocol (2-125×). The low-level resistance observed in cultures from the 5× and 2-5× conditions might be attributed to the M426T mutation, as this amino acid is positioned near the TCA binding site (Fig. 1B). E440E/G and K151K/R most likely present adaptive mutations. L419M, M423T/I/V, and I482L, previously reported to be dominant TCA resistance mutations, were detected in several but not all selection protocols (33). T389T/D likely presents an adaptive mutation (compare 5× and 2-25× EC_{50}s).

TABLE 4. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of A-782759[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	$EC_{50} \pm SD$[b] (μM)	NS5B mutation(s) (aa 1-591)[c]	Critical phase[d]
1	4	0.31 ± 0.05	NP	N
2	5	0.38 ± 0.03	**S556S/G**	N
5	11	0.86 ± 0.6	**C445C/F, S556S/G, W571P**	N
25	120	9 ± 0.7	**S556S/G, W571P**	N
125	293	22 ± 10	**G554G/D, Y555Y/C, S556S/G**	Y
2-5	13	0.99 ± 0.3	**A73A/S, C445C/F, Y555C/R/Y/H, S556S/G**	N
2-25	67	5 ± 1	**A73A/S, S556S/G**	N
2-125	240	18 ± 7	**A73A/S, M414 M/T, G554G/D, S556S/G**	N
5-25	80	6 ± 0.5	**A73A/S**, V122V/L, L126L/V, D135D/G, **C445C/F, S556S/G**	N
5-125	>440	>33	L47L/M, **A73A/S, C316C/Y, M414 M/T, C445C/F**, T520T/I, **S556S/G**	Y
25-125	200	15 ± 1	**A73A/S, C445C/F**, S476S/N, **S556S/G**	N
2-5-25	107	8 ± 0.6	**A73A/S, M414 M/T, G554G/D, Y555Y/C, S556S/G**	N
2-5-125	293	22 ± 2	**A73A/S**, C311C/Y, **C316C/Y, M414 M/T, Y555Y/C, S556S/G**	Y
2-25-125	>440	>33	**A73A/S**, V108V/A, D135D/G, **M414 M/T, C445C/F,** Y452Y/H, **G554G/D, S556S/G, W571P**	N
5-25-125	>440	>33	L47L/M, **A73A/S**, A207A/G, **C316C/Y, C445C/F, S556S/G, W571P**	N
2-5-25-125	>440	>33	**A73A/S, C316C/Y, C445C/F**, I447I/M, **G554G/D, S556S/G**	N

[a] EC_{50}, 0.075 ± 0.01 μM; CC_{50} > 33 μM.
[b] Data are mean values for ≥2 independent determinations of the antiviral phenotype.
[c] Major resistance mutations are indicated in boldface. aa, amino acids; NP, none present.
[d] Y, yes; N, no.

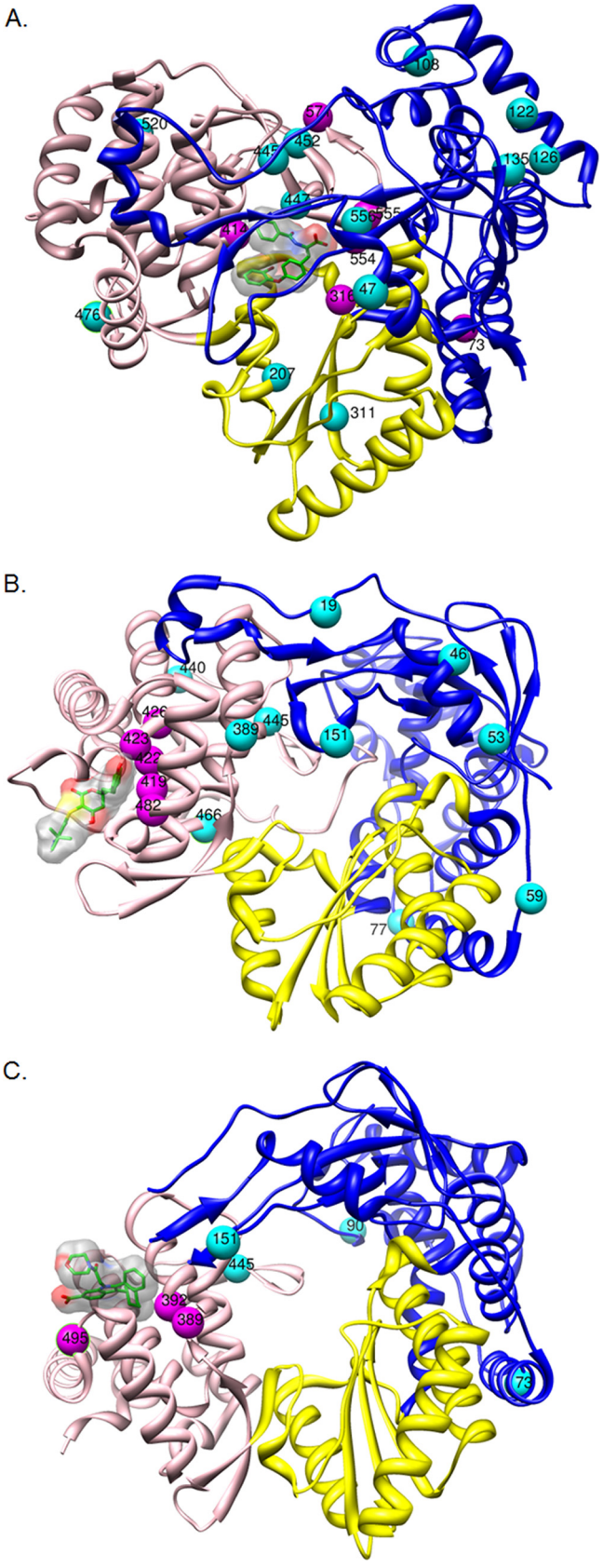

FIG. 1. Three-dimensional structures of NS5B with indication of the amino acid mutations identified following culture in the presence of A-782759 (A), TCA (B), and JT-16 (C). The palm, thumb, and finger domains of HCV NS5B are depicted in yellow, pink, and blue, respectively. The corresponding inhibitor is shown in green. Mutated amino acids conferring resistance are labeled in purple; other mutated amino acids are labeled in magenta.

Although the R422R/K mutation, identified in replicons selected using the 2-125×, 5-125×, and 2-5-125× protocols, is located in the TCA binding pocket (5), this mutation was not earlier reported to be a TCA resistance mutation. Moreover, it was described that R422 is present in the thumb binding pocket of phenylalanine derivatives (72). Mutations at positions T19, S46, T53, V59, T77, and L466 have not previously been described to contribute to TCA resistance. These residues are not located in close proximity to the binding site of TCA (Fig. 1B) and are therefore probably not involved in resistance to TCA.

(c) Benzimidazole (JT-16). JT-16-resistant variants were readily selected in all protocols and were also selected when wild-type replicon-containing cells were directly cultured in the presence of high drug concentrations (5× the EC_{50}) (Table 6). Concentrations of ≥10× EC_{50} proved cytotoxic.

Antiviral resistance to benzimidazole derivatives has been shown to be mainly associated with mutations at positions P495, P496, and V499 in the thumb domain (26, 64). Mutation P495A was detected under the condition 5× EC_{50}. Substitutions at position T389 were not previously reported to be involved in benzimidazole resistance. However, experiments with mutants with the T389A and T389S mutations revealed that these mutations reduced the susceptibility to JT-16 (our unpublished results). L392I, which is located in the proximity of T389 (Fig. 1C), was earlier reported to be responsible for benzimidazole resistance (50). The C445F mutation is a known resistance mutation for benzofuran compounds such as HCV-796 (22) but is probably a compensatory mutation in benzimidazole-resistant replicons. Mutations observed outside the thumb domain (A73S, K90R, K151R) are likely adaptive mutations or may have emerged randomly in that particular population and may be of no biological relevance. These residues are not located in close proximity to the binding site of JT-16 (Fig. 1C).

Cyclophilin inhibitors. Resistance to the cyclophilin-binding compound DEB025 could be selected only following a stepwise selection procedure (Table 7). When wild-type replicon-containing cells were directly cultured in the presence of 5×, 25×, or 125× EC_{50}, the replicon-containing cells were cured of their replicon. Only a stepwise increase of compound concentration resulted in DEB025-resistant replicons (2-5× and 2-5-25× EC_{50} protocols). The highest reduction in antiviral sensitivity was obtained with replicon cultured using protocol 2-5-25× EC_{50}. Mutation D320E in the NS5A protein, reported to be the mutation that contributes the most to resistance (12), was observed in the DEB025-resistant replicons.

Phenotypic analysis of resistant HCV variants. To confirm the impact of the mutations reported above on the actual phenotype, recombinant replicons with a single mutation or two mutations were generated. Most of these had a resistance profile that is in accordance with the profiles described in earlier studies (18, 20, 32, 37, 46, 58) (Table 8). Y452H, not

TABLE 5. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of TCA[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	EC_{50} ± SD[b] (μM)	NS5B mutations (aa 1-591)[c]	Critical phase[d]
1	1	0.21 ± 0.02	NP	N
2	1	0.32 ± 0.03	NP	N
5	2	0.65 ± 0.2	M426T, E440E/G, C445C/F	N
25	17	5 ± 1	T389T/D, **L419L/M, M423 M/T**, C445C/F	N
125	70	21 ± 5	**M423 M/T**, E440E/G	Y
2-5	2	0.57 ± 0.2	K151K/R, M426T, E440E/G, C445C/F	N
2-25	33	10 ± 4	**L419L/M**, **M423 M/T**, C445F	N
2-125	>110	>33	T19T/P, T53T/I, **R422R/K, M423 M/T,** M426T, E440E/G, **I482I/L**	Y
5-25	33	10 ± 2	S46S/G, K151K/R, **M423 M/T/V/A**, C445C/F	N
5-125	77	23 ± 3	S46S/G, **L419L/M, R422R/K, M423 M/T**, E440E/G, C445C/F	N
25-125	100	30 ± 2	V59V/A, T77T/P, **L419L/M, M423T,** M426T, E440E/G, C445C/F	N
2-5-25	13	4 ± 2	K151K/R, **L419L/M, M423 M/T/V/A,** M426T, C445C/F	N
2-5-125	67	20 ± 0.8	**L419L/M**, **R422R/K, M423 M/T**, C445C/F	N
2-25-125	33	10 ± 0.9	**M423T**, C445F, L466L/V	Y
5-25-125	80	24 ± 6	S46S/G, **M423 M/T/V/A**, E440E/G, C445C/F	N
2-5-25-125	80	24 ± 2	T53T/I, **L419L/M, M423 M/T,** M426T, C445C/F	N

[a] EC_{50}, 0.30 ± 0.2 μM; CC_{50} >33 μM.
[b] Data are mean values for ≥2 independent determinations of the antiviral phenotype.
[c] Major resistance mutations are indicated in boldface. aa, amino acids; NP, none present.
[d] Y, yes; N, no.

earlier identified in replicons resistant to benzothiadiazines, resulted in low-level (7-fold) resistance to this molecule. Also, P495L (a benzimidazole resistance mutation) resulted in low-level (8-fold) resistance to the benzothiadiazines.

All recombinant mutations (except for C445F, D168V, and the combination C316Y-C445F) resulted in reduced replication fitness (Fig. 2). Mutations S282T, C316Y, Y452H, and P495L resulted in a >50% reduction in replication fitness.

Preexisting mutations in wild-type replicon. The level of preexisting mutations in wild-type Huh 9-13 replicons was quantified by means of clonal sequencing. A total of 96 colonies were analyzed, and 90 sequences were obtained (see Table SA5 in the supplemental material). In 3 of 89 clones (1/90 sequences had a frameshift deletion), the C445F mutation was identified. Mutation T520I was detected in 3 of 84 clones, and D310G was detected in 1 of 89 clones. All three mutations were observed by the less sensitive population sequencing method when replicons were cultured under specific compound pressure.

TABLE 6. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of JT-16[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	EC_{50} ± SD[b] (μM)	NS5B mutation(s) (aa 1-591)[c]	Critical phase[d]
1	2	2 ± 0.5	C445C/F	N
2	5	6 ± 1	A73A/S, **T389T/A**, C445C/F	Y
5	14	17 ± 0.6	K90K/R, K151K/R, **P495A**	Y
2-5	11	14 ± 3	**T389T/A**, **L392L/I**, C445C/F	N

[a] EC_{50}, 1.2 ± 0.8 μM; CC_{50}, 23 ± 5 μM.
[b] Data are mean values for ≥2 independent determinations of the antiviral phenotype.
[c] Major resistance mutations are indicated in boldface. aa, amino acids.
[d] Y, yes; N, no.

DISCUSSION

Selection of *in vitro* drug-resistant variants of novel HCV inhibitors has been extensively used to identify and characterize mutations associated with drug-resistant phenotypes. A variety of resistance selection protocols have been used in such studies; it is hence not possible to directly compare the genetic barrier to antiviral drug resistance of various (classes of) HCV drugs. In the present study, barriers towards resistance development were compared side by side for HCV DAAs of different classes and a host-targeting antiviral (DEB025) by using different resistance selection protocols. The genetic barrier depends not only on the nature and number of resistance mutations but also on their impact on viral replication, the genetic background of the virus, and the variation in the viral population.

Protease inhibitors are associated with a low genetic barrier to resistance. They have been shown to select resistant HCV variants *in vitro* in less than 10 cell passages in the replicon cell culture system (37, 66, 67). Low- to medium-level (V36, T54, and R155) and high-level (A156) resistance mutations are readily identified in a significant number of patients treated with telaprevir (55). Remarkably, using the selection protocols of this study, the signature high-level resistance mutation A156S/T was observed only as a quasispecies in one of the selection protocols. This can probably be explained by the fact that replicon-containing cells could not be selected in the presence of concentrations above 5× EC_{50}, since the next concentration in this protocol (25× EC_{50}) proved toxic to the host cells. In an independently carried out selection experiment with VX-950 (in which cultures were directly incubated with a high concentration, i.e., 14 μM), VX-950-resistant replicons

TABLE 7. Mutations and antiviral phenotype of replicons obtained following culturing in the presence of DEB025[a] using various selection protocols

Antiviral pressure (fold EC_{50})	Fold increase[b] compared to WT	EC_{50} ± SD[b] (μM)	NS5A mutations (aa 26-447)[c]	Critical phase[d]
1	2	0.094 ± 0.01	NP	N
2	4	0.15 ± 0.05	NP	Y
5	—	NA	ND	Y
25	—	NA	ND	Y
125	—	NA	ND	Y
2-5	10	0.41 ± 0.05	L287L/P, **D320E**, D409D/V	Y
2-25	—	NA	ND	Y
2-125	—	NA	ND	Y
2-5-25	21	0.88 ± 0.007	L287L/P, **D320E**, D409D/V	Y
2-5-125	—	NA	ND	Y
2-5-25-125	—	NA	ND	Y

[a] EC_{50}, 0.041 ± 0.03 μM; $CC_{50,}$ 12 ± 6 μM.
[b] Data are mean values for 2 independent determinations of the antiviral phenotype. —, cell cultures died; NA, not applicable.
[c] The major resistance mutation is indicated in boldface. aa, amino acids; NP, none present; ND, not determined.
[d] Y, yes; N, no.

that carried (only) the resistance mutation A156T/S were selected. Thus, the particular selection protocol used may have an important impact on the selection of drug resistance mutations. In contrast to VX-950, resistance mutations against BILN 2061 (a molecule with a much larger selectivity index than VX-950) were already selected (as quasispecies) following culturing in concentrations of 2-fold the EC_{50}.

Nucleoside HCV polymerase inhibitors have been reported to have a high barrier to resistance (45), and no preexisting mutations to nucleoside inhibitors could be identified in treatment-naïve HCV-infected patients, whereas variants naturally resistant to nonnucleoside inhibitors were observed at a low frequency (27, 35). No resistance-associated mutations were detected following RG7128 monotherapy at various doses for 2 weeks (34). For valopicitabine (NM283; the prodrug of 2′-CMC), an average of 14 to 16 weeks of treatment was needed to select for the S282T mutation (1). In agreement with these findings, we were not able to select for 2′-CMC-resistant variants using the standard protocols employed in the current study. However, we were able to obtain 2′-CMC-resistant replicons (carrying mutation S282T) when a more gradually increasing selection protocol was used (total duration, 3.5 months; data not shown).

Most nonnucleoside RdRp inhibitors have a low genetic barrier to resistance, and depending on the drug class, the target site and the mechanism of action of a variety of resistance mutations are readily selected *in vitro* (10, 33, 64, 65). Employing the different resistance selection protocols described in this study, variants resistant to the various nonnucleoside inhibitors studied were readily selected. Mutations that were reported earlier to be responsible for the respective drug-resistant phenotypes were identified. However, the emergence of signature mutations clearly depended on the drug resistance selection protocol that was used. For instance, when replicons were cultured in the presence of 5× the EC_{50} of the benzimidazole JT-16, mutation P495A emerged, whereas T398A was identified in cultures that were selected using a lower concentration or the stepwise selection protocol. Interestingly, T389A requires a transition, whereas P495A requires a transversion (see Table SA4 in the supplemental material). It was previously demonstrated for HIV that the nucleotide substitution pattern can provide important information on drug resistance (24).

Interestingly, mutation C445F emerged under JT-16 and TCA pressure, although this mutation has not been reported to be associated with resistance against these inhibitors (Table 8).

TABLE 8. Effect of selected mutations on the antiviral phenotype of replicons

Mutation	Fold increase in EC_{50} compared to WT[a]					
	Protease inhibitors		Nonnucleoside RdRp inhibitors			Nucleoside RdRp inhibitor 2′-CMC
	BILN 2061	VX-950	A-782759	TCA	JT-16	
D168V (NS3)	**>265**	0.20 ± 0.16	0.90 ± 0.44	0.24 ± 0.19	0.85 ± 0.31	1.2 ± 0.31
S282T	ND	1.98 ± 1.0	1.4 ± 0.89	1.3 ± 0.40	0.96 ± 1.1	**4 ± 0.78**
C316Y	0.32 ± 0.30	0.21 ± 0.080	**607 ± 177**	0.31 ± 0.35	0.52 ± 0.056	0.83 ± 0.39
316-445	0.74 ± 0.45	1.3 ± 1.3	**>719**	0.27 ± 0.15	0.72 ± 0.42	0.88 ± 039
M414T	1.4 ± 0.70	0.83 ± 0.14	**285 ± 112**	2 ± 1.5	0.84 ± 0.72	0.62 ± 0.33
M423T	ND	1.3 ± 0.31	0.56 ± 0.21	**56 ± 11**	0.88 ± 0.15	ND
C445F	0.93 ± 0.19	0.77 ± 0.66	**3 ± 0.67**	0.17 ± 0.14	1.1 ± 0.21	0.93 ± 0.39
Y452H	0.90 ± 0.60	0.37 ± 0.37	**7 ± 1.4**	1.6 ± 1.3	1.9 ± 1.8	1.6 ± 0.28
445-452	1.2 ± 0.97	0.65 ± 0.69	**4 ± 1.5**	0.35 ± 0.14	1.2 ± 0.47	0.57 ± 0.14
P495L	ND	2 ± 1.1	**8 ± 0.63**	ND	**>30**	0.71 ± 0.33

[a] Resistance was set as ≥3-fold EC_{50} increase compared to WT EC_{50}. Data are mean values for ≥2 independent experiments. Boldface indicates resistance. ND, not determined.

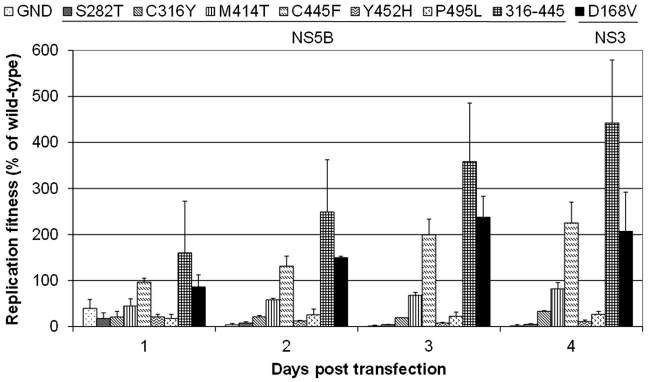

FIG. 2. Replication capacity of replicons carrying different resistance mutations transfected in Huh-Lunet cells. Huh-Lunet cells were transiently transfected with the mutant replicons. RNA replication was measured by means of a luciferase assay for 4 days posttransfection. Data are normalized to the value at 4 h posttransfection to normalize for transfection efficiency. Values shown are expressed as a percentage of the wild-type value at the corresponding time point posttransfection. Data are mean values ± standard deviations for at least three independent experiments.

Clonal sequencing revealed the preexistence at a prevalence of 3.4% of this mutation in the wild-type Huh 9-13 replicon (see Table SA5 in the supplemental material). Moreover, replicons carrying this mutation were markedly more fit than wild-type replicons (Fig. 2). Altogether, these observations suggest that C445F appears under compound pressure to compensate for the loss of replication fitness caused by unfit drug resistance mutations. For example, the replication fitness levels of mutants with the C316Y mutation and both the C316Y and C445F mutations compared to WT (at 96 h) were 33% and 440%, respectively (Fig. 2).

Inhibitors of host factors that are crucial in viral replication may have a higher barrier to resistance than (most) DAA inhibitors. Culturing of cell-containing replicons in the presence of concentrations of 5× EC_{50} or higher of DEB025 resulted in complete clearance of the cells from their replicons (Table 7) and thus did not result in the selection of drug-resistant variants. We observed that resistance against the cyclophilin-binding molecule DEB025 emerged only following a lengthy stepwise selection procedure (2-5-25× EC_{50} protocol). Replicons that were selected to replicate in the presence of 25-fold EC_{50} did not survive when they were cultured in the presence of a higher concentration (125× EC_{50}) of the inhibitor. Remarkably, the reduced susceptibility in the DEB025 resistance selection (maximum 21-fold reduction) was very moderate compared to the reduced susceptibilities to the DAAs in this study. As reported earlier, mutation D320E conferred low-level resistance (3.9-fold) to DEB025 by reducing the need of the HCV replicon for cyclophilin A-dependent isomerization of NS5A (8).

In addition to the determination of the resistant phenotype and genotype, (i) codon analysis was performed (see Table SA5 in the supplemental material), and (ii) a comparison of the critical phases following drug pressure was carried out (Tables 1 to 7). Some molecules readily resulted in a so-called critical phase in the replicon-containing cultures. This means that only a few remaining cells in the culture carry a replicon that possesses the drug resistance mutation(s). Culturing of replicon-containing cells with DEB025 or 2′-CMC resulted in critical phases in (almost) all culture protocols studied. For BILN 2061 and JT-16, the stepwise protocols did not induce massive cell death, whereas dosing the highest concentrations of monotherapy resulted in a critical phase. For A-782759 as well as for thiophene carboxylic acid treatment, critical phases were observed after dosing the highest concentrations of monotherapy and after some stepwise protocols. On the basis of (i) the fold resistance, (ii) the appearance of resistance mutations, (iii) the codon analysis, and (iv) the critical phases, it can therefore be concluded that host-targeting antivirals (e.g., DEB025) and nucleoside polymerase inhibitors have a higher *in vitro* barrier to resistance than other HCV DAA classes. This conclusion is largely in agreement with the findings of earlier reported *in vitro* resistance studies with single molecules and data from clinical studies.

A large body of work on the barrier to resistance of antiretroviral drugs has been gathered. As for HCV nonnucleoside inhibitors, first-generation NNRTIs are associated with a low barrier to resistance, limiting their clinical use. However, second-generation NNRTIs like etravirine and rilpivirine possess an increased resistance barrier, with multiple mutations being necessary to result in resistance (70). Both HCV and HIV protease inhibitors have a low barrier to resistance. The barrier to resistance of nucleos(t)ide reverse transcriptase inhibitors (NRTIs) is more complex for HIV than for HCV. For instance, lamivudine is considered an NRTI with a low barrier to resistance, since only one mutation (M184V) is required to confer high-level resistance to lamivudine. When lamivudine is used as a part of highly active antiretroviral therapy, this mutation is almost always the first to emerge. Other nucleosides, such as zidovudine, require the sequential development of multiple thymidine analogue resistance mutations (TAMs), and resistance to the nucleoside phosphonate tenofovir develops very slowly (11).

In conclusion, in this comparative study we demonstrate that different DAAs may have very different barriers toward resistance. Nucleoside analogues and a cyclophilin-binding molecule(s) have a much higher barrier to resistance than protease and nonnucleoside RdRp inhibitors. A number of factors have to be considered with regard to barriers to resistance: (i) the number of genotypic changes needed for resistance, (ii) the time and difficulty required to select resistant replicons, and (iii) the observed level of resistance of selected replicons. In addition, the development of drug resistance and acquisition of the responsible mutations may depend on the particular selection protocol used. Mutation C445F in the RdRp may compensate for the loss of replication fitness caused by unfit drug resistance mutations. Our observations highlight the importance of in-depth studies on the *in vitro* drug resistance genotypes and phenotypes of inhibitors when (combination) clinical studies are planned.

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