Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients

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Objectives: To characterize the effect of hepatitis C virus (HCV) polymerase intrinsic genetic heterogeneity on the inhibitory activity of nucleoside and non-nucleoside HCV polymerase inhibitors.

Methods: The sensitivity of genotype (GT) 1 HCV NS5B clinical isolates from treatment-naive patients to nucleoside and non-nucleoside polymerase inhibitors was assessed. The genetic diversity at the population level, as well as that of their quasispecies, was correlated with the observed reduced sensitivity to inhibitors.

Results: R1479 and NM107 (nucleoside analogues that have entered Phase 2 clinical trials as prodrugs R1626 and NM283, respectively) were similarly active across the tested clinical isolates. Resistance mutations to nucleoside analogues were not observed in any of the isolates. However, the activity of the non-nucleoside thumb II inhibitor NNI-1, palm I inhibitors NNI-2 and NNI-3, and palm II inhibitor HCV-796 was reduced across different isolates. This reduction in inhibitory activity for non-nucleoside inhibitors (NNIs) was, in most cases, correlated with the existence of known NNI resistance mutations in the NS5B polymerase population of the clinical isolates, as detected by population sequencing. Resistance mutations to NNIs were also observed at a low frequency within the clinical isolates’ viral quasispecies that allowed for their rapid selection upon drug selective pressure.

Conclusions: The higher frequency of known NNI resistance mutations or polymorphisms known to affect their antiviral potency when compared with the lack of detection of resistance mutations to the nucleoside analogues suggests a potential for primary reduced responsiveness as well as faster development of clinically significant resistance.

Keywords: viral resistance, HCV antiviral therapy, nucleoside analogues

Introduction

Hepatitis C virus (HCV), a positive-strand RNA virus, is a member of the genus *Hepacivirus* in the *Flaviviridae* family and is the leading cause of liver disease worldwide. It is estimated that over 170 million individuals are infected with HCV. The current standard of care provides good clinical efficacy in patients infected with genotypes (GTs) 2 and 3, but is less efficacious in the most prevalent GT 1-infected patients, thereby emphasizing the urgent need for more effective specific targeted antiviral therapies for HCV.2,3

The HCV RNA-dependent RNA polymerase (RdRp) is an essential enzyme for viral RNA replication and therefore an attractive therapeutic target. This enzyme has a crystal structure with the shape of an encircled right hand, and in addition to the active site, four allosteric sites have been identified: palm I (palm domain near the active site), palm II (partially overlapping palm I and towards the active site), thumb I (thumb domain near the fingertips) and thumb II (the outer surface of the thumb domain) (Figure 1). There are a number of polymerase inhibitors that have been advanced to Phase 2 clinical trials and have demonstrated clinical efficacy in monotherapy: nucleoside analogues NM283 (1.2 log drop in viral load at 800 mg twice daily)4 and R1626 (3.7 log drop in viral load at 4500 mg twice daily)5 and the non-nucleoside inhibitor (NNI) HCV-796 that binds to the palm II domain of the polymerase (1.4 log drop in viral load at ≥500 mg twice daily at day 4 followed by viral load rebound while on treatment). Other NNIs that inhibit the...
Materials and methods

Clinical isolates

Ninety-two HCV NS5B clinical isolates were obtained from serum samples of untreated GT 1 HCV-infected individuals. Forty-five samples were from the Phase Ib R1626 study and originated from Australia.5 Others were obtained from New Zealand (39) and from a variety of commercial sources (7 from the USA and 1 from Germany).

Plasmid construction

The Con1-adapted transient replicon (rep PI-luc/ET) and cured Huh-7 cells were obtained from Ralf Bartenschlager (Department of Molecular Virology, University of Heidelberg, Germany).18 The transient replicon repPI-luc/ET vector was modified to replace the pBR322 backbone with the pUC18 backbone and to include two restriction sites flanking the start (AsI/SI) and the end (RsrII or SacII) of a truncated NS5B gene. Briefly, the transient replicon includes the poliovirus internal ribosome entry site (IRES), which controls the translation of the firefly luciferase gene. Downstream of the firefly luciferase gene, the IRES from the encephalomyocarditis virus controls the translation of the HCV non-structural genes (NS3, NS4A, NS4B, NS5A and a truncated NS5B). Only vectors containing a full-length NS5B clinical isolate are able to replicate.

The transient replicon containing the GT 1a H77 sequence was adapted from the GT 1b replicon by replacing the non-structural region of the Con1 by the H77 sequence, except the first 75 amino acids of NS3 that remained of Con1 origin. For better replication efficiency, three adaptive mutations were introduced.19 Restriction sites AsI/SI and RsrII were also introduced at the 5’ and 3’ end of the NS5B gene. All constructs were confirmed by double-stranded DNA sequencing.

HCV RNA extraction and NS5B amplification

HCV RNA was extracted from 400 µL of serum from HCV-infected patients using the ZR Whole-Blood Total RNA kit following manufacturer’s instructions (Zymo Research, Orange, CA, USA; catalogue no. R1020). Reverse transcription (RT) was carried out with 5 µL of total RNA and the Taqman RT reagents kit following manufacturer’s instructions (ABI, Foster City, CA, USA; catalogue no. N808-0234) using a 29-mer oligo (da) as primer for RT initiation.

NS5B amplification was carried out using GC-RICH PCR system (Roche Diagnostics, Indianapolis, IN, USA; catalogue no. 12 140 306 001) as follows: 4 µL of cDNA was used in a 50 µL reaction containing 28 µL of water, 5 µL of Buffer #3 (1 × final), 10 µL of Buffer #2 (1 × final), 0.5 µL of forward and reverse primers (0.2 µM final each), 1 µL of dNTPs (0.02 mM final) and 1 µL of enzyme (2 U). PCR reactions were performed as follows: 2 min at 94°C, then 40 cycles of 30 s at 92°C, 1 min at 50°C and 2 min at 72°C, followed by a 5 min extension at 72°C. A nested amplification was carried out in duplicate using the same conditions with primers specific for GT 1a or GT 1b sequences that contained the restriction site sequences for AsI/SI and RsrII (or SacII). After restriction enzyme digestion, GT 1b-amplified products were cloned into the GT 1b shuttle replicon vector, whereas GT 1a-amplified products were cloned into the GT 1a shuttle replicon vector. After overnight ligation and transformation, 96 individual colonies were pooled for each clinical isolate. In vitro-transcribed RNA was then prepared using RiboMAX™ T7 Express (Promega, Madison, WI, USA; catalogue no. R1320).

To determine the error rate (ER) of the PCR amplification procedure, the NS5B region of the H77 DNA plasmid was amplified in two independent experiments following the same protocol as the one used for the amplification of clinical isolates. After TA cloning (TOPO TA cloning vector Invitrogen, Carlsbad, CA, USA; catalogue no. 45-0641), two sets of 29 and 28 clones, respectively, were analysed by sequencing. In each set, 10 nucleotide substitutions were found. The ER was determined using the following formula:

Error Rate (ER) = (Number of Nucleotide Substitutions / Total Number of Nucleotides) × 100%
Effect of HCV genetic diversity on drug inhibitory activity

ER = number of mutation per base pair/number of PCR cycles. For both sets, the ER was calculated at $2.4 \times 10^{-5}/bp/PCR$ cycle.

Sequencing of NS5B polymerases

Sequencing spanning the entire NS5B coding region was performed using primers covering both DNA strands, both populations (direct PCR sequencing) or molecular clones sequencing. Sequencing reactions were performed using ABI 3730 xl DNA Analyzer and chromatograms were analysed using Sequencher and VNTI software.

Compounds

Compounds R1479 (4’azido-cytidine), NM107 (2’-C-Me-C), thiophene-2-carboxylic acid (NNI-1), benzo thiadiazine (NNI-2) and HCV-796 (benzofuran carboxamide) were synthesized at Roche Palo Alto LLC. Thiazine (NNI-3) was synthesized at Array BioPharma. Stocks of 10 mM concentration were prepared in 100% DMSO and stored at $-20{}^\circ C$.

$IC_{50}$ determinations

For $IC_{50}$ determination, 4 million cured Hu7 cells were transfected with 5 μg (or 10 μg) of in vitro-transcribed replicon RNA from GT 1b or GT 1a reference strains or from clinical isolate-derived transient replicons. After electroporation, cells were resuspended in 12 mL of DMEM containing 5% (v/v) fetal bovine serum and plated in 6-well plate at 600 000 cells/well. Compounds (or medium as a control) were added 24 h post-transfection in 3-fold dilutions at a final DMSO concentration of 1% (v/v). Firefly luciferase reporter signal was read 72 h after addition of compounds using the Luciferase Assay system (Promega; catalogue no. E1501). The $IC_{50}$ values were assessed as the compound concentration at which a percentage reduction in the level of firefly luciferase reporter was observed when compared with control samples in the absence of compound. Dose–response curves and $IC_{50}$ values were generated by using the XLfit3 program (ID Business Solutions Ltd, Surrey, UK). The 95% CI was calculated on the basis of 2 × SEM. The variability of the assay was represented as the SD as well as the maximum fold changes relative to the mean $IC_{50}$.

For Con1 and H77 wild-type replicons, the signal-to-noise ratio was 1200- and 2000-fold above background, respectively. Signal-to-noise ratio for the majority of the GT 1b clinical isolates was between 50- and 180-fold and between 50- and 2700-fold for the GT 1a clinical isolates.

Amplification and sequencing of the coding region from replicating transient replicons

Transfected cells were collected 4 days after electroporation and total RNA was extracted using the RNeasy mini kit following manufacturer’s instructions (Qiagen, Valencia, CA, USA; catalogue no. 74104). RT was carried out with 2 μL of total RNA and the Taqman RT reagents kit following manufacturer’s instructions (ABI; catalogue no. N808-0234) using random hexamers as primers for RT initiation. PCR and nested PCR NS5B amplification were carried out in duplicate using the Expand High Fidelity PCR System, following manufacturer’s instructions. The duplicate PCR products were pooled before being purified using the PCR purification kit (Qiagen; catalogue no. 28104), following manufacturer’s instructions. Sequencing was carried out as described earlier.

Selection of mutant variants under drug pressure

For the selection of mutant variants under drug pressure using NS5B clinical isolate-derived transient replicons, 4 million cured Hu7 cells were transfected with 10 μg of in vitro-transcribed replicon RNA from clinical isolate-derived transient replicons. Reference replicons were also transfected as a control. After electroporation, cells were resuspended in 12 mL of DMEM containing 5% (v/v) fetal bovine serum and plated in 6-well plate at 600 000 cells/well. Compounds (or medium as a control) were added 24 h post-transfection at multiples of the $IC_{50}$ of the respective clinical isolate populations with a final DMSO concentration of 1% (v/v) and incubated for 4 days. Sequencing of replicating replicons was performed as described earlier.

For the selection under drug pressure of the NNI-1-resistant mutant M419 in the presence of wild-type replicon Con1 (L419), a 5 μg mixture containing 10% of in vitro-transcribed M419 RNA and 90% of in vitro-transcribed L419 RNA was transfected. As a control, 5 μg of the wild-type replicon Con1 was transfected in parallel. After electroporation, cells were resuspended in 12 mL of DMEM containing 5% (v/v) fetal bovine serum and plated in 6-well plate at 600 000 cells/well. NNI-1 (or medium as a control) was added at 1 ×, 5 × and 10 × $IC_{50}$ with a final DMSO concentration of 1% (v/v) and incubated for 4 days. Amplification and sequencing of replicating transient replicons were performed, as described earlier.

Replication capacity of HCV NS5B clinical isolates

The replication level of either reference strains or clinical isolate-derived transient replicons was determined as the ratio of the firefly luciferase signal at 4 days post-electroporation to the luciferase signal at 4 h post-transfection, to normalize the transfection efficiency. The replication capacity of the clinical isolate-derived replicons was expressed as their normalized replication efficiency when compared with that of the reference strain for each of the GT 1a or GT 1b, set at a value of 1.

Results

The HCV NS5B phenotypic assay mimics the patients’ genetic heterogeneity and enables the determination of drug sensitivity of clinical isolates

The HCV NS5B phenotypic assay enables the determination of the sensitivity of clinical isolates to polymerase inhibitors, as described in the Materials and methods section, by mimicking the intrinsic HCV genetic heterogeneity present in patients (Figure 2).

The assay reproduces the genetic NS5B population present in the patient as shown by the comparison of the NS5B population sequence of the clinical isolate with the NS5B population sequence obtained after 4 days of in vitro replication (post-transfection). The NS5B region from 24 clinical isolates was sequenced either completely (amino acid residues 1–591) or partially (residues 154 and 356), and DNA sequencing data showed no major differences before and after in vitro replication, as exemplified in Figure 3. The reproducibility of the assay was evaluated by repeatedly testing the sensitivity of reference replicons from GT 1b Con1 and GT 1a H77 strains to R1479, NM107, NNI-1 and NNI-2. The mean $IC_{50}$ values, standard deviations, the 95% confidence intervals and the maximum fold changes for four HCV inhibitors were obtained from between 22
and 99 independent determinations (Table 1). Most of the IC50 determinations from independent assays differed by ~2-fold with respect to the mean IC50 (with a maximum of 2.6-fold), similar to other phenotypic assays.

The variability of the phenotypic assay was also tested using NS5B clinical isolate-containing replicons generated after independent HCV RNA extraction from serum and amplification of the NS5B coding region. No significant differences in the replication capacity (data not shown) or in the levels of drug sensitivity to R1479 were observed between the two independently generated NS5B clinical isolate-containing replicons, with IC50 values within 2-fold from each other, similar to what was observed using the Con1 and H77 reference strains (Table 2).

Using this phenotypic assay, a total of 63 clinical isolates from untreated patients (47 GT 1a and 16 GT 1b) were characterized. The replication capacity varied across the different clinical isolates, irrespective of the patients’ viral load (data not shown), with five clinical isolates either not replicating or replicating at low levels that did not allow for an accurate IC50 determination (Figure 4). A total of 58 clinical isolates (92%) demonstrated sufficient replication levels to allow for drug sensitivity determination.

Activity of HCV polymerase inhibitors against genetically diverse GT 1 clinical isolates

The sensitivity of GT 1 clinical isolates from untreated patients to nucleoside analogues R1479 and NM107 as well as to NNIs binding at different allosteric sites of the polymerase, thumb II (NNI-1), palm I (NNI-2 and NNI-3) and palm II (HCV-796) was determined (Figure 5). All clinical isolates were sensitive to R1479 (Figure 5a) and to NM107 (Figure 5b), within 2.5-fold of the mean IC50 (i.e. within the variability of the assay) compared with the reference strains H77 and Con1. The in vitro replication capacity of the clinical isolates did not affect the compound potency.

The majority of the GT 1a isolates (21/23) were sensitive to NNI-1 (Figure 5c), whereas 2 of them (RO-67 and RO-75) demonstrated a reduced sensitivity (>12-fold compared with the reference control). Five of the nine GT 1b clinical isolates showed a decreased sensitivity to NNI-1, which ranged from 3- to 15-fold in IC50 value when compared with the reference control (Figure 5c).

In the case of palm I inhibitor NNI-2, 75% of the tested GT 1a clinical isolates (17/23) showed a decreased sensitivity to the compound that ranged from 3- to 9-fold in IC50 value versus H77 (Figure 5d), whereas one isolate had a greater reduction in sensitivity (17-fold). Six of the 10 GT 1b clinical isolates showed a decreased sensitivity to NNI-2 that ranged from 3.5- to 11-fold when compared with Con1 (Figure 5d).

Sensitivity to palm I inhibitor NNI-3 (Figure 5e) was slightly reduced (3–4-fold), compared with H77 in five of the six GT 1a clinical isolates tested and the remaining isolate showed a reduction in sensitivity of 12-fold. One GT 1b isolate was sensitive to NNI-3, whereas the other tested showed a 12-fold reduced sensitivity when compared with the reference strain Con1.
Sensitivity to HCV-796 was comparable with reference strains H77 and Con1 (Figure 5f) except in two GT 1b isolates that showed a 30-fold reduced sensitivity compared with Con1. The presence of known drug resistance mutations or natural polymorphisms can explain reductions in drug sensitivity.

To investigate the possible correlation between the observed variable sensitivity to some polymerase inhibitors and the natural genetic heterogeneity of HCV, the population sequence of the entire NS5B coding region was obtained for a total of 92 clinical isolates from treatment-naive individuals (72 GT 1a and 20 GT 1b isolates), of which 58 had been phenotypically characterized, as mentioned earlier (Figures 4 and 5). The NS5B DNA sequences were compared with their respective reference strains (H77 for GT 1a and Con1 for GT 1b isolates). No naturally occurring resistance mutations S96T, S96T/N142T or S282T, which confer resistance to nucleoside analogues R147922 and NM107,23 respectively, were observed. In contrast, although 73 of the 92 isolates (79%) showed wild-type sequence at positions related to resistance to NNIs, 19 (21%) contained natural polymorphisms at amino acid residues involved in resistance to inhibitors binding to thumb I, thumb II, palm I or palm II sites of the polymerase. Of this 21%, changes at amino acid residues that correlated with resistance to palm I-binding inhibitors were observed in 8%,7,24 2% contained mutations that conferred resistance to thumb II-binding inhibitors24 and another 2% to HCV-796.16 Amino acid substitution at residue 499 (V to A), related to resistance to thumb I inhibitors,12 was seen in 9% of the isolates and all were of GT 1b origin.

The two GT 1a clinical isolates (RO-67 and RO-75) that demonstrated reduced sensitivity to NNI-1 (Figure 5c) contained the amino acid substitution M423I in the NS5B coding region, known to confer resistance to this compound,24 either as a complete M423I substitution, which correlated with a 35-fold reduced inhibitory activity when compared with the wild-type replicon, or as a mixture of M (wild-type) and I (~30% of the mutant residue), which corresponded to a reduced inhibitory activity of 12-fold when compared with the reference control (Figure 5c and Table 3).

Although the five GT 1b clinical isolates that showed a 3–15-fold decreased sensitivity to NNI-1 had no known NNI-1 resistance mutations, four of them had the amino acid substitution V499A, which is in close proximity to residues in direct contact with the inhibitor and could affect its inhibitory activity. In the case of palm I inhibitor NNI-2, the GT 1a clinical isolate (RO-58) with the lowest sensitivity to the drug (17-fold

**Table 1.** Reproducibility of drug sensitivity determinations

<table>
<thead>
<tr>
<th>Replicon</th>
<th>Statistics</th>
<th>R1479 IC50 (µM)</th>
<th>NM107 IC50 (µM)</th>
<th>NNI-1 IC50 (µM)</th>
<th>NNI-2 IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con 1</td>
<td>mean IC50</td>
<td>1.5</td>
<td>0.9</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.6</td>
<td>0.4</td>
<td>0.1</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>n°</td>
<td>56</td>
<td>22</td>
<td>31</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>95% CI b</td>
<td>0.2</td>
<td>0.2</td>
<td>0.04</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>fold c</td>
<td>2.6</td>
<td>2</td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>H77</td>
<td>mean IC50</td>
<td>1.7</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.67</td>
<td>0.22</td>
<td>0.19</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>n°</td>
<td>99</td>
<td>41</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>95% CI b</td>
<td>0.2</td>
<td>0.06</td>
<td>0.06</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>fold c</td>
<td>2.3</td>
<td>1.8</td>
<td>1.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

°Represents the number of independent experiments.

bThe 95% confidence interval was calculated based on 2 × SEM.

cMaximum fold changes relative to the mean IC50 value.

**Table 2.** Reproducibility of the phenotypic assay using independently generated NS5B clinical isolates containing replicons

<table>
<thead>
<tr>
<th>NS5B origin</th>
<th>R1479 IC50 (µM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO-18</td>
<td>1.30 ± 0.2 (2)</td>
</tr>
<tr>
<td>RO-18</td>
<td>1.80 ± 0.2 (2)</td>
</tr>
<tr>
<td>RO-19</td>
<td>2.31 ± 0.2 (2)</td>
</tr>
<tr>
<td>RO-19</td>
<td>3.15 ± 0.6 (2)</td>
</tr>
<tr>
<td>RO-20</td>
<td>1.94 ± 0.4 (2)</td>
</tr>
<tr>
<td>RO-20</td>
<td>2.52 ± 0.04 (2)</td>
</tr>
<tr>
<td>RO-73</td>
<td>1.21 ± 0.2 (9)</td>
</tr>
<tr>
<td>RO-73</td>
<td>1.32 ± 0.1 (6)</td>
</tr>
<tr>
<td>RO-76</td>
<td>1.17 ± 0.02 (4)</td>
</tr>
<tr>
<td>RO-76</td>
<td>1.15 ± 0.1 (7)</td>
</tr>
<tr>
<td>RO-77</td>
<td>1.28 ± 0.2 (8)</td>
</tr>
<tr>
<td>RO-77</td>
<td>0.86 ± 0.08 (8)</td>
</tr>
</tbody>
</table>

b# denotes independently generated NS5B clinical isolates containing replicons.

Number in parentheses represents the number of independent drug-sensitive phenotypic experiments.
reduction; Figure 5d and Table 3) contained the amino acid substitution H95C, previously shown to confer resistance to this type of compound when the amino acid substitution is an arginine.7,24 Isolate RO-3 showed an ~3-fold decrease of sensitivity to NNI-2 despite not having any known NNI-2 resistance mutations detected by population sequencing (Table 3). The GT 1a clinical isolates with a 3–9-fold decreased sensitivity to NNI-2 had no known NNI-2 resistance mutations (Figure 5d). The highest decrease in sensitivity to NNI-3 (Figure 5e and Table 3) observed in two clinical isolates (GT 1b RO-3 and GT 1a RO-38) was due to amino acid substitution at position 556 (S to G), previously shown to confer resistance to NNI-3.24 Similarly, the 30-fold reduced activity of HCV-796 observed against GT 1b clinical isolates RO-8 and RO-13 (Figure 5f and Table 3) correlated with the presence of an asparagine at amino acid residue 316.

**Genetic diversity of variants within HCV quasispecies**

To investigate the genetic heterogeneity of HCV quasispecies and the existence of minority mutant variants with reduced drug...
sensitivity, the quasispecies of 13 clinical isolates (9 GT 1a and 4 GT 1b) were analysed by DNA sequencing. Three of the selected samples contained mutations at residues 499 and 556 of the NS5B related to viral resistance to thumb I \(^ {12} \) and palm I inhibitors, respectively, \(^ {24} \) whereas the other 10 did not contain any resistance mutation(s) detectable by population sequencing. Amino acid alignments of the quasispecies (comprising approximately 90–100 clones) for each patient sample demonstrated different population structures in each case: within each clinical isolate, clones genetically identical to the isolate’s consensus (major) sequence were detected at frequencies of 5% to 16% in 11 clinical isolates and of 2% to 4% in the other 2 isolates. The rest of the molecular replicon clones (variants) contained between 1 and 12 amino acid differences with respect to their own consensus sequence, the majority of which were unique in the population. This shows that HCV quasispecies contain a high proportion of unique variants that can allow the virus to adapt to changes in the environment.

Among the studied 1110 HCV variants, with a sequencing detection limit of minority variants present at a frequency of 1% in the quasispecies, no amino acid substitutions responsible for resistance to R1479 or NM107 were found. However, the presence of mutations that confer resistance to NNIs binding to the palm and thumb domains of the polymerase was observed within the quasispecies of untreated patients. The most prevalent mutations were those that confer resistance to palm I-binding inhibitors NNI-2 and NNI-3 observed in 11 of 13 isolates (85%) at a frequency ranging from 1% to 5% in each patient (Figure 6). Variants that contained mutations conferring resistance to the palm- and thumb-binding inhibitors co-existed in eight isolates, but were not observed together in the same molecule.

**Drug sensitivity of variants within HCV quasispecies**

We sought to investigate the effect that these minority variants may exert on the drug sensitivity of the clinical isolates. For this purpose, a total of 70 variants from seven clinical isolates were characterized to understand their sensitivity to inhibitors. Clones identical to their respective consensus sequence replicated at a similar or even higher level when compared with the clinical isolate population (data not shown). A qualitative correlation was also found between the number of amino acid substitutions compared with their respective consensus sequence and the variant’s replication capacity: a total of 40 variants from the

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**Table 3.** Amino acid substitutions that confer resistance to thumb II-, palm I- and palm II-binding inhibitors *in vitro* can also confer resistance in the context of clinical isolates

<table>
<thead>
<tr>
<th>NS5B origin</th>
<th>Amino acid substitutions*</th>
<th>Thumb II inh (NNI-1) IC(_{50}) ((\mu)M)</th>
<th>Palm I inh (NNI-2) IC(_{50}) ((\mu)M)</th>
<th>Palm I inh (NNI-3) IC(_{50}) (nM)</th>
<th>Palm II inh (HCV-796) IC(_{50}) (nM)</th>
<th>R1479 IC(_{50}) ((\mu)M)</th>
<th>NM107 IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT 1b Con1</td>
<td></td>
<td>0.2 ± 0.02</td>
<td>0.15 ± 0.01</td>
<td>0.9 ± 0.14</td>
<td>4.3 ± 0.7</td>
<td>1.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>RO-3</td>
<td>V499A, S556G/S(^ {b} )</td>
<td>1.6 ± 0.22</td>
<td>0.4 ± 0.06</td>
<td>10.6 ± 2</td>
<td>ND</td>
<td>1.7 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>RO-8</td>
<td>C316N</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.07</td>
<td>ND</td>
<td>130 ± 14</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>RO-13</td>
<td>C316N</td>
<td>ND</td>
<td>1.3 ± 0.04</td>
<td>ND</td>
<td>140 ± 7</td>
<td>2.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>GT 1a H77</td>
<td></td>
<td>0.5 ± 0.03</td>
<td>0.1 ± 0.01</td>
<td>0.9 ± 0.09</td>
<td>5.6 ± 0.6</td>
<td>1.7 ± 0.1</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>RO-58</td>
<td>H95C</td>
<td>0.9 ± 0.21</td>
<td>1.7 ± 0.34</td>
<td>ND</td>
<td>ND</td>
<td>0.8 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>RO-38</td>
<td>S556G</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.06</td>
<td>11 ± 1.9</td>
<td>ND</td>
<td>1.2 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>RO-67</td>
<td>M423I</td>
<td>17.4 ± 6.9</td>
<td>0.6 ± 0.11</td>
<td>ND</td>
<td>ND</td>
<td>0.6 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>RO-75</td>
<td>M423M/F(^ {c} )</td>
<td>6.1 ± 1.2</td>
<td>0.4 ± 0.06</td>
<td>ND</td>
<td>ND</td>
<td>1.8 ± 0.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

*Only amino acid substitutions related to resistance, compared with the reference strains, are indicated.

**Table 3:** Amino acid substitutions that confer resistance to thumb II-, palm I- and palm II-binding inhibitors *in vitro* can also confer resistance in the context of clinical isolates

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**Figure 6.** Prevalence of NNI resistance mutations among the quasispecies of untreated HCV NS5B clinical isolates. Prevalence of pre-existing NNI resistance mutations in the quasispecies of four GT 1b and nine GT 1a isolates. Amino acid substitutions found at each position are indicated. The number of molecular replicon clones in each clinical isolate containing the specified resistance mutation is indicated as a percentage. For each amino acid residue, the area of the pie chart is proportional to the number of isolates containing the specified resistance mutation in the quasispecies (resistance mutations at positions 95, 316 and 554 were seen in the quasispecies of one isolate each, 411 and 495 in two isolates each, 414 and 423 in three isolates each, 448 in four isolates, 426 and 556 in five each and 451 in six isolates). The code for pie chart represents the NNI binding sites: palm I-related amino acid residues, white; palm II-related amino acid residues, hatching; thumb I-related amino acid residues, light grey; thumb II-related amino acid residues, dark grey.
same seven isolates that contained three or fewer amino acid substitutions compared with their respective consensus sequence were tested and 24 were replication competent (60%). In contrast, 20 clones with four or more amino acid substitutions compared with their consensus sequence were selected from the same seven isolates and all but one were replication incompetent (data not shown).

All replicating variants were sensitive to R1479 and NM107 with a similar IC\textsubscript{50} to their respective originating isolates (data not shown). The sensitivity to NNI-1 and NNI-2 of variants was similar to that of their respective population (data not shown), except in those cases in which known NNI resistance mutations were observed. Those variants exhibited reduced drug sensitivity when compared with their population isolates, as exemplified in Table 4. Isolate RO-65 showed only a moderate decrease in sensitivity to NNI-2. However, minor variants within its quasispecies that contained an NNI-2 resistance mutation at position 414 (methionine to isoleucine) were present at a frequency of 1.3% in the population. The variant containing M414I was isolated, tested and shown to be 15-fold less sensitive to NNI-2 when compared with the RO-65 population (Table 4). Furthermore, this variant showed similar replication capacity to the RO-65 population (data not shown).

Isolate RO-3 contained a mixture at residue 556 (~53% mutant) that correlated with a 12-fold reduced sensitivity of the viral population to NNI-3, compared with the reference strain Con1 (Table 3). To prove that substitution S556G was solely responsible for the observed reduced sensitivity to NNI-3, a single clone containing G556 (variant RO-3-D10) was selected from the isolate’s population and its drug sensitivity assessed, showing reduced sensitivity to NNI-3 (Table 4). Furthermore, when the same variant was used to revert this amino acid substitution to S556, it demonstrated an 80-fold increased sensitivity to NNI-3 against the mutant variant and 50-fold against the isolate population that contained a mixture of mutant and wild-type variants (Table 4). This isolate also contained minor variant M414L (RO-3-D04) present in its quasispecies at a frequency of 5%. The sensitivity of this clinical isolate to NNI-2 was found by the phenotypic assay to be only slightly decreased compared with wild-type (3-fold). However, when a single clone RO-3-D04 (containing NNI-2 resistance mutation M414L) was selected and tested, the sensitivity to NNI-2 was shown to be decreased by >75-fold (Table 4), showing the ability of the assay to detect the effect of minor variants that may be selected upon drug pressure.

**Rapid selection of minor variants upon drug pressure**

Figure 6 shows that mutations that confer resistance to the palm I-binding inhibitors NNI-2 and NNI-3 were observed in 85% of the patients at a frequency ranging from 1% to 5% in each patient. Given their low frequency within the quasispecies, these mutations were only detected by extensive clonal analysis. We sought to investigate the potential effect of drug-resistant variants present at low frequency within the quasispecies upon drug selective pressure. We have shown that isolate RO-3 contained amino acid substitution S556G in ~53% of its variants. Moreover, ~5% of the variants from this isolate contained amino acid substitution M414L, which confers resistance to palm I inhibitors.\(^23\) Given the low frequency of these M414L mutant variants, this isolate’s population showed only a small reduction in sensitivity to NNI-2 (~3-fold; Table 3). Likewise, isolate RO-65 contained variants with reduced sensitivity to NNI-2 (Table 4), albeit at a low frequency in its quasispecies. To understand whether these minority variants would be selected upon drug pressure, clinical isolates RO-3 and RO-65 were transfected and incubated for 4 days in the presence of either no drug or concentrations of NNI-2 at multiples of the IC\textsubscript{50} of the respective clinical isolates’ populations, followed by sequencing of the NS5B region (Table 4 and Figure 7). After incubation with NNI-2 at 10× IC\textsubscript{50}, variants containing 414L were selected from the RO-3 quasispecies over the consensus population (Figure 7a). The replication capacity and sensitivity to NNI-2 of one of the variants containing M414L were tested alone in the transient replicon assay (variant RO-3-D04). Interestingly, this variant showed a very poor replication capacity in the absence of drug when compared with that of the population (20-fold lower). However, in the presence of NNI-2, the replication capacity of the mutant variant increased up to an 8-fold maximum at 3 μM (~5× IC\textsubscript{50}) (data not shown), which could explain the observed rapid selection in the presence of drug observed in Figure 7(a). As shown in Table 4, this variant showed the same sensitivity to NNI-1 as the RO-3 population, but showed reduced sensitivity to NNI-2.

For the RO-65 isolate, a mixture of mutant 414I and wild-type was observed after incubation with 10× and 20× IC\textsubscript{50}

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### Table 4. Palm resistance mutations found at a low frequency in the quasispecies of clinical isolates confer resistance

<table>
<thead>
<tr>
<th>NS5B origin</th>
<th>Amino acid substitutions(^a)</th>
<th>Thumb II inh (NNI-1) IC\textsubscript{50} (μM)</th>
<th>Fold IC\textsubscript{50}</th>
<th>Palm I inh (NNI-2) IC\textsubscript{50} (μM)</th>
<th>Fold IC\textsubscript{50}</th>
<th>Palm I inh (NNI-3) IC\textsubscript{50} (nM)</th>
<th>Fold IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO-3 population</td>
<td>V499A, S556G/S(^b)</td>
<td>1.6 ± 0.22</td>
<td>1</td>
<td>0.4 ± 0.06</td>
<td>1</td>
<td>10.6 ± 2</td>
<td>1</td>
</tr>
<tr>
<td>RO-3-D10</td>
<td>V499A, S556G</td>
<td>2.2 ± 0.02</td>
<td>1.4</td>
<td>0.4 ± 0.04</td>
<td>1</td>
<td>16.2 ± 3.1</td>
<td>1.5</td>
</tr>
<tr>
<td>RO-3-D10-G556S</td>
<td>V499A, G556S</td>
<td>2.4 ± 0.35</td>
<td>1.5</td>
<td>0.4 ± 0.03</td>
<td>1</td>
<td>0.2 ± 0.04</td>
<td>0.02</td>
</tr>
<tr>
<td>RO-3-D04</td>
<td>V499A, M414L</td>
<td>1.4 ± 0.30</td>
<td>1.1</td>
<td>&gt;30</td>
<td>&gt;75</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RO-65 population</td>
<td>ND</td>
<td>0.5 ± 0.12</td>
<td>1</td>
<td>0.5 ± 0.08</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RO-65-B06</td>
<td>M414I</td>
<td>0.3 ± 0.02</td>
<td>0.6</td>
<td>7.4 ± 0.6</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

\(^a\)Only amino acid substitutions related to resistance, compared with the reference strains, are indicated.

\(^b\)S556G/S: G, 53%; S, 47% and M414L: M, 95%; L, 5% (determined by sequencing of the 96 replicon molecular clones present in the population).

\(^c\)RO-65 population does not contain any substitution at known resistance amino acid positions.
Effect of HCV genetic diversity on drug inhibitory activity

Table 5. Decreased NNI-1 sensitivity correlates with the frequency of resistant mutants in replicon population

<table>
<thead>
<tr>
<th>Transfection ratio</th>
<th>M419 (wild-type)</th>
<th>IC50 (µM)</th>
<th>IC50 fold shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>0%</td>
<td>0.2 ± 0.01</td>
<td>1</td>
</tr>
<tr>
<td>90%</td>
<td>10%</td>
<td>0.4 ± 0.05</td>
<td>2</td>
</tr>
<tr>
<td>75%</td>
<td>25%</td>
<td>1.1 ± 0.16</td>
<td>5.5</td>
</tr>
<tr>
<td>65%</td>
<td>35%</td>
<td>3.9 ± 0.54</td>
<td>20</td>
</tr>
<tr>
<td>50%</td>
<td>50%</td>
<td>4.7 ± 0.28</td>
<td>24</td>
</tr>
<tr>
<td>0%</td>
<td>100%</td>
<td>7.9 ± 0.7</td>
<td>40</td>
</tr>
</tbody>
</table>

Discussion

The current standard of care provides good clinical efficacy in patients infected with HCV GTs 2 and 3, but is less efficacious in the most prevalent GT1-infected patients, thereby emphasizing the urgent need for more effective HCV-specific antiviral therapies. This has triggered considerable efforts in drug discovery with a number of HCV inhibitors currently in clinical trials, both NS3/4A protease and NS5B polymerase inhibitors. The high genetic heterogeneity of HCV, due to the error-prone nature of its RdRp, high virus production and turnover represents an opportunity for the virus to evade antiviral treatment, with every possible mutant likely present in a patient’s quasispecies. The potential presence of major or minor HCV variants within a viral population carrying amino acid changes associated with reduced drug sensitivity could affect the sensitivity of untreated patients to HCV inhibitors.

In this study, we describe the development of an HCV NS5B phenotypic assay that allows for the assessment of the sensitivity of NS5B clinical isolates to polymerase inhibitors by mimicking the intrinsic HCV genetic heterogeneity present in infected patients. This approach allows for the quick determination of an isolate’s drug sensitivity and, unlike other previously reported methods, avoids the need to test a large number of individual clones or the selection and testing of individual clones from the population that could potentially have significantly different genetic and phenotypic characteristics from the isolate’s overall population (Table 4). As shown here, the phenotypic assay is able to detect a reduction in drug sensitivity in clinical isolates that contain natural resistance mutations at a frequency as low as 5% in the quasispecies (RO-3; see Table 3) and a significant decrease in sensitivity (12-fold IC50 shift) when mutations present at least in 30% of the quasispecies (Table 3).

The assay described here supports the replication of >90% of genetically diverse GT 1 clinical isolates and allows for drug sensitivity determination, with a calculated variability of 2-fold (and a 2.6-fold maximum variability). Using this technology, the sensitivity of 58 clinical isolates from untreated GT 1 patients to a panel of polymerase inhibitors was determined in order to investigate the effect of HCV NS5B genetic diversity on the inhibitor’s antiviral potency.
The potency of nucleoside analogues R1479 and NM107 was shown to be similar across genetically diverse clinical isolates of GT 1 origin and similar to the GT 1b Con1 and GT 1a H77 reference strains. This correlated with the lack of the in vitro-selected R1479- or NM107-resistance mutations, S96T, S96T/N142T and S282T, respectively, in the population of untreated clinical isolates. Furthermore, none of these resistance mutations was detected among the HCV quasispecies of 13 untreated patients in this study, encompassing 1110 HCV NS5B variants, within the sequencing detection limit of minority variants, which are often detected in the population of untreated clinical isolates. Indeed, using a similar type of assay, variable NNIs (particularly NNI-1 and NNI-2) were found to be more variable across genetically diverse clinical isolates. Indeed, using a similar type of assay, variable activity has been described for compound A-782759, chemically related to NNI-2. Moreover, a number of isolates that exhibited reduced drug sensitivity contained known NNI-1 or NNI-2 resistance mutations (Table 3). Several isolates were slightly less sensitive to these compounds, but did contain any known resistance mutations (or any common mutation that could be related to this phenotype). This could be due to the intrinsic genetic diversity of HCV, by which genetically diverse clinical isolates can show variable sensitivity to inhibitors, as has been previously shown for HIV inhibitors such as enfuvirtide. For HCV, the existence of natural polymorphisms across different GTs has been previously shown to affect sensitivity to drugs, as at amino acid 482 for which leucine is the wild-type consensus in GT 2a and at amino acid 423 for which isoleucine is the wild-type consensus in GT 5, suggesting that the GT 1 isolates with the lowest sensitivity to NNIs compared with wild-type could account for the fact that they were not detected in this extensive study of the NS5B quasispecies.

In contrast, the potency of NNIs (particularly NNI-1 and NNI-2) was found to be more variable across genetically diverse clinical isolates. Indeed, using a similar type of assay, variable activity has been described for compound A-782759, chemically related to NNI-2. Moreover, a number of isolates that exhibited reduced drug sensitivity contained known NNI-1 or NNI-2 resistance mutations (Table 3). Several isolates were slightly less sensitive to these compounds, but did not contain any known resistance mutations (or any common mutation that could be related to this phenotype). This could be due to the intrinsic genetic diversity of HCV, by which genetically diverse clinical isolates can show variable sensitivity to inhibitors, as has been previously shown for HIV inhibitors such as enfuvirtide. For HCV, the existence of natural polymorphisms across different GTs has been previously shown to affect sensitivity to drugs, as at amino acid 482 for which leucine is the wild-type consensus in GT 2a and at amino acid 423 for which isoleucine is the wild-type consensus in GT 5, suggesting that the GT 1 isolates with the lowest sensitivity to NNIs compared with wild-type could account for the fact that they were not detected in this extensive study of the NS5B quasispecies.
In summary, we have described an NS5B phenotypic assay that mimics the patient viral quasispecies and allows for the determination of the sensitivity to NS5B polymerase inhibitors of genetically diverse clinical isolates. The existence of NNI resistance mutations in the virus population correlated with the reduced sensitivity to NNIs observed in some clinical isolates. We also showed that resistance mutations can be present in the viral population of untreated patients at a low frequency in their quasispecies and that these variants can be readily selected upon drug pressure (Figure 7). The use of HCV phenotypic assays such as the one for the characterization of clinical isolates from clinical trials will allow the study of the levels and prevalence of resistant mutants necessary to result in clinically relevant drug resistance that may affect the efficacy of HCV inhibitors. The higher frequency of NNI resistance mutations when compared with the absence of resistance mutations to the nucleoside analogues in the HCV quasispecies of treatment-naive HCV patients suggests a potential for faster development of clinically significant resistance for non-nucleoside therapies.

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Transparency declarations

All authors are employees of Roche Palo Alto, LLC.

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