Replication capacity of minority variants in viral populations can affect the assessment of resistance in HCV chimeric replicon phenotyping assays

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Objectives: Drug-resistant minority viral variants can pre-exist in the viral quasispecies of chronically infected hepatitis C virus (HCV) patients and can emerge gradually upon drug treatment. When heterogeneous clinical samples are tested for drug susceptibility in a chimeric replicon-based phenotyping assay, biphasic dose–response curves may be observed. The effect of drug-resistant minority viral variants on the biphasic phenotype of mixtures was assessed in detail.

Methods: Susceptibility of mutant/wild-type mixtures containing minorities of NS3 mutants with different replication capacities and susceptibilities to protease inhibitors were tested in a transient replicon assay. The contribution of both variants in the mixture to the overall replication level was described with an $E_{\text{max}}$ model.

Results: The 90% and 99% effective concentrations (EC90 and EC99, respectively) provide a more accurate measure of the susceptibility of the population than the determination of EC50 values. Reduced susceptibility at the EC50 level correlated with the replication capacity of the NS3 mutant in the mixture. Using replication-enhanced mutant/wild-type mixtures demonstrated that the relative difference between the replication capacity of the variants present in the mixture results in biphasic dose–response curves. Modelling revealed that in mixtures containing wild-type and resistant variants with low replication capacity, the contributions of the wild-type variants are higher than expected from the replication level of the replicons transfected alone.

Conclusions: Differences in the replication capacity of variants present in HCV replicon-based phenotype assays can lead to biphasic dose–response curves. Using EC90 or EC99 values increases the sensitivity of the assay to minor variants.

Keywords: NS3, replicons, phenotyping, mixture, compensatory mutations

Introduction

With the recent approval of the hepatitis C virus (HCV) NS3/4A protease inhibitors telaprevir and boceprevir, the first approved direct-acting antiviral agents for the treatment of hepatitis C, the treatment paradigm for HCV infections is changing.1–5 In addition, multiple other direct-acting antivirals with different modes of action are in development, potentially providing new therapeutic options for hard-to-treat patient populations as well as for patients who are not eligible for interferon treatment.6,7 However, in hepatitis C, as in other viral diseases, the use of agents that directly target viral proteins essential for the replication of the virus can result in the selection of variant viral populations with reduced susceptibility to these antiviral agents, both in vitro and in vivo.8 To reduce this risk, treatment strategies will require the combination of different antiviral agents to be successful.

For HIV and hepatitis B virus (HBV), resistance testing, i.e. genotypic and phenotypic characterization of a patient’s viral population, has proven to be a useful tool for patient management.9,10 In HCV the future role of resistance testing for treatment management is still unclear. Currently, resistance testing is mainly being used to support drug development. Resistance assays include genotyping (i.e. sequencing of the viral target gene to detect mutations that confer drug resistance) and phenotyping assays (i.e. cell culture-based viral replication assays in the absence or presence of drugs). Both tests have a different ability and sensitivity to measure resistance, and their combined
use has been proposed in HCV treatment management as they may provide complementary clinically relevant information.\textsuperscript{11} When mixtures of susceptible and resistant virus are present, it has been shown in HIV that the resistance profile resulting from genotyping and phenotyping assays and from different laboratories can vary significantly.\textsuperscript{11}

Resistance assays in HCV are not yet standardized and the methodology as well as analysis can differ between different laboratories. To assess phenotypic resistance, biochemical or cellular protease assays and different HCV replicon-based phenotyping assays for assessing drug susceptibilities of NS3, NS5A and NS5B sequences from clinical samples have been described. Most replicon-based phenotyping assays utilize a genotype 1 subgenomic replicon in which the HCV gene of interest derived from patient isolates is inserted. While in some assays the drug susceptibilities of single clonal HCV RNA sequences are assessed,\textsuperscript{12–14} other assays determined the effect on drug susceptibility using a mixture of replicon variants.\textsuperscript{15–23} The latter approach might mirror the effect on drug susceptibility using a mixture of replicon variants.\textsuperscript{24} However, the presence of both wild-type (wt) and pre-existing resistant strains have been found in treatment-naive patients.\textsuperscript{24} The nature of the resistant strain can vary from patient to patient, and the presence of multiple resistant strains in a single patient can make it difficult to interpret and complicate the determination of EC50 and EC90 values.

In this study we characterized an HCV replicon-based phenotypic assay for assessing the drug susceptibility of viral populations containing wt and mutant strains. We analysed the dynamics of biphasic dose–response curves, which are difficult to interpret and complicate the determination of EC50 and EC90 values.

Materials and methods

Compound(s)
The HCV NS3/4A inhibitor TMC380765 was synthesized in house, as described previously.\textsuperscript{25} The NS3/4A protease inhibitors INH-1, BILN-2061 and INH-2 were obtained from Spectrum Info (Kiev, Ukraine), Medivir (Huddinge, Sweden) and Acme Bioscience (Palo Alto, CA, USA), respectively. The HCV non-nucleoside inhibitor thiophene-2 was synthesized in house (compound 16 described by Chan et al.).\textsuperscript{26}

Transient replicon assay

The transient replicon assay was described previously.\textsuperscript{27} In brief, Huh-7-Lunet cells were transfected with in vitro-transcribed replicon RNA of wt, mutants or mutant/wt mixtures. Transfected cells were incubated in the presence of a nine-point dilution range of inhibitor for 48 h and luminescence generated by luciferase activity was measured. Replicon RNA was transcribed in vitro from a plasmid encoding a genotype 1b (con1 based) bicistronic subgenomic replicon (clone ET) with a poliovirus internal ribosome entry site-driven firefly luciferase reporter, and from the cell culture-adaptive mutations E1202G, T1280I and K1846T in the HCV genome (kindly provided by R. Bartenschlager, University of Heidelberg, Germany) (adapted from Lohmann et al.).\textsuperscript{28} Replicons carrying the NS3 mutations F43S, R155K, A156G, A156T, A156V, D168E, D168A and D168V as well as replicons with compensatory mutations R109K and G162R,\textsuperscript{29} and NS3 double mutants G162R-D168V and R109K-D168V were generated by site-directed mutagenesis and used in the transient replicon assay.

\textit{In vitro}-transcribed replicon RNA was transfected either alone or replicon RNA from different replicons was mixed prior to transfection. For each transfection, 10 µg of the total replicon RNA amount was used (e.g. to obtain 90% mutant and 10% wt mixture, 9 µg of mutant and 1 µg of wt RNA were mixed). The mutant/wt ratios tested were 5%/95%, 10%/90%, 25%/75%, 50%/50%, 75%/25% and 90%/10%. Homogeneous (100%) wt and mutant transfections were performed as controls.

Based on the luciferase luminescence signal of inhibitor-treated and control cells, the 50%, 90% and 99% effective concentration values (EC50, EC90 and EC99, respectively) were determined via log-linear interpolation. The EC50 values were log transformed (pEC50 = −logEC50) and the mean pEC50, pEC90 and pEC99, standard deviations (SDs) and 95% CIs of the respective means were calculated. These were then back-transformed to obtain the linear EC50/90/99 values (nM). Fold changes (FCs) in the susceptibility of the single NS3 mutants or NS3 mutant/wt replicon mixtures to the respective protease inhibitors were calculated relative to the control wt replicon (ET). Any activity of a protease inhibitor against a mutant or mutant/wt mixture outside of the mean pEC50/90/99 for wt replicon ±2×SD range was considered as significantly different from wt activity. The 95% CI represents the reproducibility of the drug susceptibility determinations.

The replication capacity of the different mutant replicons in the absence of inhibitors was determined previously,\textsuperscript{27} as previously described.\textsuperscript{28} In brief, luciferase activity 48 h post-electroporation was measured and normalized to the luciferase activity determined 4 h post-electroporation. The replication capacities of the mutants were calculated relative to the replication capacity of the wt replicon.

Results

The dose–response curve of HCV NS3/4A protease inhibitors in NS3 mutant/wt mixtures is biphasic

When assessing the protease inhibitor susceptibility of chimeric replicons containing NS3 protease sequences from clinical isolates, biphasic dose–response curves can be observed when mixtures of mutant and wt sequences are present in a patient’s viral population.\textsuperscript{16,20} For example, a chimeric replicon carrying the NS3 protease region from a clinical isolate, which contains a mixed population of D168 wt and the D168E variant, tested in a replicon-based phenotyping assay, yielded a biphasic dose–response curve for the protease inhibitor BILN-2061 (Figure 1). As
expected, the D168E mutation was shown not to affect susceptibility to the NS5B non-nucleoside inhibitor thiophene-2. Consistently, the dose–response curve of the same sample for this latter HCV polymerase inhibitor is monophasic, showing that the biphasic nature of the BILN-2061 dose–response curve is not an artefact of the assay, but related to the presence of D168E and wt variants.

This observation was studied in more detail by testing the phenotype of mixtures of replicons with variable susceptibilities to HCV NS3/4A protease inhibitors, together with wt replicon. For this purpose, in vitro-transcribed replicon RNA carrying mutations in the NS3 protease was mixed at different ratios with wt replicon RNA prior to transfection into Huh7-Lunet cells. After 48 h incubation in the presence of a dilution range of different protease inhibitors, luciferase activity was measured and dose–response curves calculated.

When the activity of NS3/4A protease inhibitors was assessed in mixtures of wt and mutant replicons carrying NS3 protease-resistant mutations, the resulting dose–response curve displayed two marked sigmoid phases (Figure 2) separated by an intermediate plateau. With increasing concentrations of the NS3 mutant replicon in the mixture, the biphasic dose–response curves converged towards the dose–response curve observed with the 100% mutant replicon. However, the dynamics of convergence differed between the different mutants. For example, with increasing input of the D168V mutant replicon (Figure 2a) the curve shifted gradually from that of the wt to that of the mutant variant. In contrast, the fractional input of A156V replicons (Figure 2b) had to be considerably higher than for D168V before the convergence towards the fully A156V mutant curve became detectable. Interestingly, both A156V and D168V confer similar levels of resistance to BILN-2061, whereas the replication capacity in the absence of inhibitor differs substantially when these two mutants are tested alone [Figure 2 and Table S1, available as Supplementary data at JAC Online]. The intermediate plateau observed in mixtures with highly divergent EC50 values, e.g. those including, respectively, A156V and D168V variants, was largely missing from mixtures that included replicons carrying D168E, a mutation conferring only a limited FC in EC50 values (Figure 2c). However, the curves clearly remain biphasic and, interestingly, the experimental findings with the in vitro D168E/wt replicon mixtures closely mimic the biphasic dose–response curve of the heterogeneous D168D/E population in the chimeric replicon containing the NS3 protease sequence derived from a clinical isolate (Figure 1).

Similar biphasic dose–response curves for the respective mutant/wt mixtures were obtained for the protease inhibitors INH-1, INH-2 and TMC380765 (data not shown). Importantly, the response to NS5B inhibitors, which are equally active against all of the NS3 mutants tested, exhibited, as anticipated, monophasic dose dependence (data not shown).

**Figure 2.** Dose–response curves of in vitro mixtures of RNA replicons carrying NS3 protease mutations (a) D168V, (b) A156V and (c) D168E with wt replicon (ET) to protease inhibitor BILN-2061. Percentages of mutant transfected are indicated on the right of the graph in the key. Each graph represents a representative experiment. FC in EC50 for single D168V, D168E and A156V mutations (100%) and the replication capacity (RC) of the respective mutant are shown above each graph (as determined previously27).
The drug susceptibility of replicon mixtures depends predominantly on the replication capacity of the contributing variants

The level of resistance a mutation confers and its replication fitness are accepted as two key parameters for the survival of mutant variants under drug pressure. In the context of a dose–response measurement, the effect of mutant variants on inhibitor susceptibility is summarized as the FC in EC50, as compared with the EC50 of wt variants. However, if different mutants with different levels of resistance and/or replication capacities are mixed with wt replicons, distinct biphasic dose–response profiles and a range of EC50 values can be observed (Figure 2a–c). To assess the impact of the level of resistance a mutation confers and replication fitness on the determination of EC50/90/99 values, a wide range of mutant/wt mixtures were tested.

First, for each protease inhibitor tested, the mean EC50 for the wt plus 2×SD was calculated (Table S2, available as Supplementary data at JAC Online). If the EC50 value for the mixture was above this threshold, the reduction in susceptibility was considered significant. The same exercise was performed for the EC90 and EC99 values. The lowest percentage of NS3 mutant input (5%, 10%, 25%, 50%, 75%, 90% or 100%) for which a significant reduction in susceptibility was observed in the assay was annotated as the lowest mutant percentage affecting the EC50, EC90 or EC99 (abbreviated as LM%EC50/90/99) (Figure 3a).

The concentration of mutant replicon RNA needed to significantly affect the EC50 values of mixtures (=LM%EC50) is low (25%–50%) for mutants with a relatively high replication capacity, such as D168V and D168A, and high (90%–100%) for mutants with low replication capacity, such as A156V or A156T (Figure 3a). For all mutants and protease inhibitors tested, a correlation between the LM%EC50 and replication capacity was apparent (R² = 0.66, P < 0.0001). Interestingly, this correlation was less observed with the LM%EC90, which was lower than the LM%EC50, while the LM%EC99 was even lower, meaning that 5% of minor mutants for all combinations tested was needed to significantly affect the EC99 values (Figure 3a).

Significant differences in the susceptibilities of some NS3 mutant replicons to the tested HCV NS3/4A inhibitors were observed, e.g. the FC in the EC50 determined for BILN-2061 and INH-1 for mutation A156V was 1344 and 41, and for D168V was 3213 and 80, respectively (Figure 3b). Nevertheless, for each of the tested protease inhibitors a significant correlation between LM%EC50 and the replication capacity was found (Figure 3a). Statistical testing of the hypothesis that all individual correlation curve slopes are equal was, however, rejected using the likelihood ratio test. This indicates that the relation between LM%EC50 and the replication capacity was statistically different between the different compounds; however, the estimates suggest that these are not biologically relevant.

Validation of the role of replication capacity by using replication-enhanced mutant and wt replicons

To corroborate the observed correlation between the replication capacity of resistant minorities and their impact on dose–response curves, a panel of mutant replicons with the replication-enhancing secondary NS3 mutation R109K was engineered and tested.27,32 The introduction of the R109K mutation into a wt ET or D168V replicon backbone significantly increased the replication capacity to 311% for wt ET and 342% for D168V mutant, respectively (data not shown), without changing the EC50 and EC90 of the NS3 protease inhibitors (Figure 4). The introduction of the R109K mutation into either the wt or the D168V mutant replicon in a 25%/75% D168V mutant/wt mixture shifted the intermediate plateau from ~75% inhibition up to ~90% (Figure 5a) or down to ~20% inhibition (Figure 5b), respectively. Remarkably, introducing the R109K mutation into both the D168V and the wt replicons largely rebalanced the replication capacity of the mutant and the competing wt replicon, and lifted the intermediate plateau of the 25%/75% mixture to ~60% inhibition (Figure 5c).

Consistent with the elevated intermediate plateau and thus smaller second phase of inhibition, no FC in the EC50 and EC90 of BILN-2061 was observed when D168V mutants were co-transfected with R109K replication-enhanced wt replicons at a 25%/75% ratio (Figure 4). By comparison, mixtures of standard D168V mutants with wt at the same ratio yielded a 2-fold increase in EC50 and a 600-fold increase in EC90. Substitution of the R109K-D168V replicon in the mixture resulted in a larger second phase of inhibition and raised the FC in EC50 (Figure 4). Finally, simultaneous R109K introduction in both replicons in the 25%/75% mixture resulted in similar FC in EC50 and EC90 as compared with the unenhanced mixture (Figure 4). Similar observations were made with mixtures of G162R-containing wt and D168V replicons (data not shown).

Replication of wt, NS3 mutant and NS3 mutant/wt mixtures in the presence and absence of protease inhibitors

To further elucidate the dynamics of biphasic dose–response curves, the replication levels of the A156V and D168V mutants and wt, transfected alone and as a mixture, in the absence of BILN-2061 and across the BILN-2061 dilution range, were assessed. As a surrogate for replication, the observed luciferase activity levels were used and analysed in an Emax exposure–response model.
Second, the replication levels in the mixtures were assessed using a joined modelling approach, and the replication levels of the mixtures were modelled as a weighted combination of the single wt and mutant replication levels, using the following model:

\[ \mu_{i,(k),\text{mix}} = \pi_{j}(i,j,k) + \pi_{j}(i,j,k) \]

where \( \mu \) thus represents the parameter describing the dose-dependent sigmoidal decline of replication of the single replicon type \( j \) (i.e. wt or mutant) in well \( i \) at increasing inhibitor concentrations \( k \), as modelled by an \( E_{\text{max}} \) dose–response model, and where \( \pi_{j} \) represents the estimated quantification of contribution of each replicon \( j \) to optimally reconstitute the observed dose–response curve of the mixture (\( \mu_{\text{mix}} \)). The model is based on the assumption that the fitness of the replicon variants remains constant over the 48 h incubation period. The model estimates the contributions of wt and mutant replicon \( j \), described as \( \pi_{\text{wt}} \) or \( \pi_{\text{mut}} \), needed to reproduce the dose–response curve observed for the mixed mutant/wt population.

In the 50%/50% A156V/wt or D168V/wt mixture analysed, cells were transfected with equal amounts of both replicons, being for each half the amount used for the individual dose–response, resulting at the start of incubation in a \( \pi_{\text{wt}} = \pi_{\text{mut}} = 0.5 \). If both \( \pi \) values estimated using equation (1) after 48 h still approach the 0.5 value, this suggests an equal contribution and an unaltered fitness of both replicons. If higher, or lower, \( n \) values are estimated at 48 h for a particular replicon variant, this suggests a replication benefit, or disadvantage, respectively, for that variant in the setting of the mixture. For the 50%/50% D168V/wt mixture, \( \pi_{\text{wt}} \) and \( \pi_{\text{D168V}} \) were estimated at a comparatively similar contribution: 0.38 (95% CI 0.28–0.51) and 0.58 (95% CI 0.52–0.64), respectively. This similar contribution of both variants is graphically illustrated by the overlay of the model that assumes equal contribution (Figure 6b, broken line) with the model with the weighted estimates (Figure 6a, continuous lines).

In contrast, in the 50%/50% A156V/wt mixture, \( \pi_{\text{wt}} \) and \( \pi_{\text{A156V}} \) were estimated at a more divergent 1.42 (95% CI 1.34–1.51) and 0.48 (95% CI 0.44–0.53), respectively. This suggests that in the context of a mixture of wt and A156V replicons, the wts have a replication benefit of factor \( \sim 3 \), compared with when transfected alone. The overlay of the model that assumes equal contribution of both wt and A156V variants (Figure 6b, broken line) with the model with estimated weights (Figure 6b, continuous lines) shows that this higher-than-expected contribution of wt in this mixture occurs primarily in the absence of inhibitor and at the lower concentration range (Figure 6b).

**Estimation of mutant and wt EC50s present in mixtures**

Another assumption of the mixture model approach is that the combination of the individual mutant and wt replicons in a mixture does not affect their individual EC50s. Graphically, this assumption is supported by the approximate coincidence of reductions in replication in the overlay of the biphasic response to BILN-2061 curves of the wt/A156V (Figure 7a) and wt/ D168V (Figure 7b) 50%/50% mixtures and the monophasic curves of the constituent replicons.

To actually determine the individual wt and mutant EC50s in the mixtures, baseline and maximum luciferase activity levels
for both sigmoidal phases in the biphasic dose–response curve were estimated and percentage inhibitions for the respective phases were calculated. Subsequently, EC_{50,1} and EC_{50,2} were estimated for the first and second phase (Table 1). The EC_{50} values obtained for wt and both A156V and D168V mutants from mixture experiments were essentially the same as observed when these replicons were tested alone (Table 1).

**Discussion**

Triple therapy of pegylated interferon and ribavirin plus a direct-acting antiviral can significantly increase the sustained virological response (SVR) rate and shorten treatment duration for a majority of patients compared with pegylated interferon/ribavirin alone. However, virological failure can occur with
direct-acting antiviral(s), which is frequently associated with the emergence of resistant viral variants. Different resistant viral variants can emerge at the same time and be present while wt variants are still detectable. When assessed for drug susceptibility in an in vitro chimeric transient replicon assay, such heterogeneous populations often result in biphasic dose–response curves, which complicate the accurate determination of the EC50 values. To mimic these heterogeneous populations, mixtures of NS3 mutants resistant to HCV protease inhibitors with wt replicons were prepared and the antiviral susceptibility was assessed in a transient replicon assay. The dynamics of the dose–response curves obtained with these

![Graph](image-url)

**Figure 6.** Model prediction for 50%/50% mixture of (a) D168V/wt and (b) A156V/wt replicons. Luciferase levels are on the y-axis. BILN-2061 concentration is indicated on the x-axis as log10 µM. Note that the log10 concentration at 10^{-5} represents replication of the respective mixtures in the absence of BILN-2061. Circles represent experimental luciferase values of the mixture. Continuous lines represent model prediction with estimated weights with 95% CI. Broken lines represent the model where contributions \( p_{mut} \) and \( p_{wt} \) were fixed to 0.5 (=equal contribution).

![Graph](image-url)

**Figure 7.** Non-linear regression of the single wt (dark grey continuous line), single mutant (light grey broken line) and mutant/wt mixture (black broken line) luciferase values. Curve fitting of monophasic and biphasic dose–responses was performed using GraphPad Prism 5.0 software (wt, A156V and D168V single transfection: non-linear regression, dose–response—inh (log[inh]) versus response—variable slope; A156V/wt and D168V/wt mixtures: non-linear regression, dose–response—special, biphasic).

<table>
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<th>Single transfection</th>
<th>wt</th>
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<th>A156V</th>
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<td>0.0007</td>
<td>3.04</td>
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<tr>
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<td>3.35</td>
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**Table 1.** EC50s for wt and A156V and D168V alone, and EC50_1 and EC50_2 for mixtures were obtained using GraphPad Prism Software (wt, A156V and D168V single transfection: non-linear regression, dose–response—inh (log[inh]) versus response—variable slope; A156V/wt and D168V/wt mixtures: non-linear regression, dose–response—special, biphasic; in both functions values were weighted 1/y).
mixtures was carefully examined and the factors leading to biphasic dose–response curves were assessed.

Biphasic dose–response curves could be observed for most of the NS3 mutant/wt mixtures tested. However, the proportion of the mutant needed to observe biphasic curves differed substantially between mutants.

Recent reports describing chimeric replicon-based phenotyping assays suggested that reduced replication capacity or a lower level of resistance of the minority mutant variant present in the test sample could result in EC_{50} levels similar to those for wt and thus the contribution of the resistance conferred by the minority variants could be missed.\textsuperscript{15,16,18,20} To assess the role of the replication capacity and the level of resistance in more detail, multiple protease inhibitors and NS3 mutants with a wide range of replication capacities and susceptibilities to these protease inhibitors were compared.

For mutants with low replication capacity, such as A156V, a high concentration of mutant was needed to significantly affect EC_{50} values, while less input of mutant RNA was needed for better replicating mutants, such as D168V. Interestingly, the correlation between the proportions of mutant needed to affect the EC of the protease inhibitor was less pronounced at the EC_{90} or EC_{99} level. At the EC_{90} level, a presence of 5% minority variant (lowest proportion tested) was already sufficient to observe a shift in the EC_{90} values. This suggests that to adequately determine the ECs of heterogeneous populations, the ECs leading to ≥90% inhibition can provide information additional to the EC_{50} value, in line with a recent report.\textsuperscript{20}

Confirmation of the role of the replication capacity in this effect was demonstrated with experiments using the wt and D168V replicons with and without the R109K replication-enhancing secondary mutation. While the EC_{50} values of BILN-2061 were not affected by the R109K mutation, the replication capacity of the wt and the D168V mutant increased ~3-fold.

Following this, different mutant/wt mixtures with and without the R109K mutation were tested. Interestingly, depending on to which subpopulation present in the mixture the R109K mutation was introduced, a dose–response profile closer to that of wt or to that of the more resistant variant was observed. Furthermore, if the R109K mutation was introduced into both the wt and D168V mutants, the dose–response curve was similar to what was observed in the absence of the R109K mutation. This suggests that the difference in the replication capacity between the replicons in the heterogeneous population is driving the different dose–response profiles.

In the replicon phenotyping assay, the dose–response curves describe the relative change in the replicon RNA level at a given inhibitor concentration compared with the replication level in the absence of inhibitor (baseline) and with the maximal inhibition of replication by the inhibitor (control signal). Analysis of the replication levels of the wt, NS3 mutants and mixtures clearly indicated that the first-phase decline in replication at lower drug concentrations results from inhibition of the wt, whereas the intermediate plateau and the second-phase decline in replication observed in the biphasic curves are mainly driven by the fitness and drug susceptibility of the remaining resistant variant. Remarkably, applying the canonical biphasic dose–response function to the response data of the mixtures could retrieve constituent wt and mutant EC_{50}s in the first- and second-phase declines of the biphasic curve, respectively. This suggests that it may be possible to convey information from a phenotyping result about which subpopulations or minorities are present in the mixed sample tested without prior knowledge of the actual (mixed) genotype.

As expected, in the absence of inhibitor, the luciferase level 48 h after transfection of the replication-compromised A156V mutant was significantly lower than that of the D168V mutant. The latter almost reached the levels observed for the wt replicon. Surprisingly, the luciferase levels of A156V/wt 50%/50% mixtures in the absence of inhibitor and at low inhibitor concentrations were consistently higher than the replication levels predicted based on an equal contribution of both mutant and wt replication levels. A model that allows estimation of the contribution of each constituent replicon of a mixture, based on the replication levels of each respective replicon when transfected as a single variant, was developed. Such modeling showed that the contribution of the wt replicon to the mixture dose–response would have to be ~3-fold higher than the actual contribution at the start of incubation. The A156V contribution, in contrast, behaved in line with expectations. These observations suggest a difference in replication rates within the A156V/wt mixture in favour of the wt replicon. In the D168V/wt mixture, on the other hand, the model suggested comparatively similar wt and D168V mutant replication. The higher contribution of wt replicon in the A156V/wt mixture could only be explained by the lesser ability of the A156V mutant to compete with wt for cellular resources. This may provide a larger replicative space for the wt variants to replicate in, resulting in higher luciferase signals, similar to observations made previously.\textsuperscript{15,16} However, to test this hypothesis, experiments with mixtures of replicons with different read-outs would need to be performed.

Importantly, the relatively higher replication levels of the A156V/wt mixture in the absence of inhibitor affect the relative inhibition across the inhibitor dilution range, since a higher luciferase level in the absence of inhibitor will decrease the relative inhibition calculated. Thus, the limited sensitivity of replicon-based phenotyping assays to measure the reduced susceptibility of replicon populations containing poorly replicating variants is due to the lower replication capacity of the poorly replicating variant, and is further aggravated by a higher-than-expected luciferase signal in the absence of inhibitor.

In summary, the difference in the replication levels of the variants present in the replicon population assessed with a transient replicon assay can lead to biphasic dose–response curves when heterogeneous populations, containing resistant variants, are assessed. In addition, testing of different NS3 mutants suggests that the determination of FCs in EC_{90} and EC_{99} values can be used to increase the sensitivity of the phenotyping assay. The replication levels of the variants present in a mixture were assessed and a model was developed to gain insight into the respective contributions of the variants to the overall replication level in a mixture.

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Transparency declarations
T. V., L. V., H. C., G. F. and O. L. are all employees of Janssen Infectious Diseases BVBA. T. J. is an employee of Janssen Pharmaceutica. G. F. and O. L. are shareholders of J&J. All other authors: none to declare.

Supplementary data
Tables S1 to S4 and Figures S1 to S4, as well as supplementary information on Emax modelling, are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


