Research Article
Viral Hepatitis

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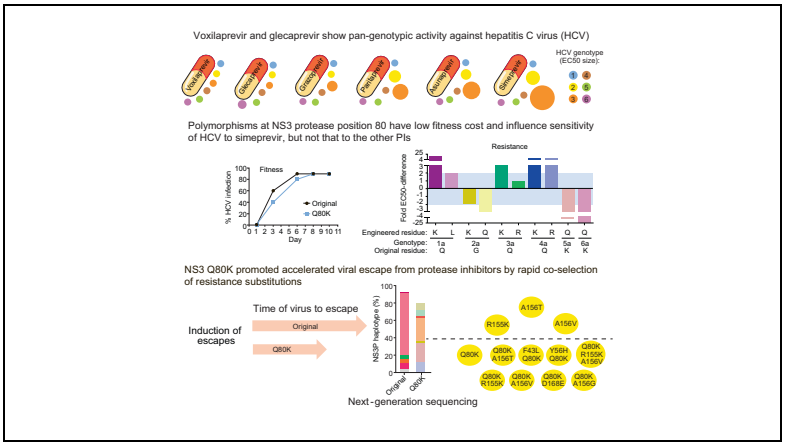
HCV genotype 1-6 NS3 residue 80 substitutions impact protease inhibitor activity and promote viral escape

Graphical abstract



Highlights

- Pan-genotypic activity of voxilaprevir and glecaprevir in cell culture infectious HCV.
- High fitness of engineered HCV genotype 1-6 protease position-80-variants.
- Position-80-variants showed altered sensitivity to simeprevir but not to other PIs.
- Q80K promoted accelerated HCV escape from PIs in long-term treatment.
- Escape was mediated by rapid co-selection of additional resistance substitutions.

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Lay summary

Among all clinically relevant hepatitis C virus protease inhibitors, voxilaprevir and glecaprevir showed the highest and most uniform activity against cell culture infectious hepatitis C virus with genotype 1-6 proteases. Naturally occurring amino acid changes at protease position 80 had low fitness cost and influenced sensitivity to simeprevir, but not to other protease inhibitors in short-term treatment assays. Nevertheless, the pre-existing change Q80K had the potential to promote viral escape from protease inhibitors during long-term treatment by rapid co-selection of additional resistance changes, detected by next generation sequencing.

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HCV genotype 1-6 NS3 residue 80 substitutions impact protease inhibitor activity and promote viral escape

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Background & Aims: Protease inhibitors (PIs) are of central importance in the treatment of patients with chronic hepatitis C virus (HCV) infection. HCV NS3 protease (NS3P) position 80 displays polymorphisms associated with resistance to the PI simeprevir for HCV genotype 1a. We investigated the effects of position-80-substitutions on fitness and PI-resistance for HCV genotypes 1-6, and analyzed evolutionary mechanisms underlying viral escape mediated by pre-existing Q80K.

Methods: The fitness of infectious NS3P recombinants of HCV genotypes 1-6, with engineered position-80-substitutions, was studied by comparison of viral spread kinetics in Huh-7.5 cells in culture. Median effective concentration (EC50) and fold resistance for PIs simeprevir, asunaprevir, paritaprevir, grazoprevir, glecaprevir and voxilaprevir were determined in short-term treatment assays. Viral escape was studied by long-term treatment of genotype 1a recombinants with simeprevir, grazoprevir, glecaprevir and voxilaprevir and of genotype 3a recombinants with glecaprevir and voxilaprevir, next generation sequencing, NS3P substitution linkage and haplotype analysis.

Results: Among tested PIs, only glecaprevir and voxilaprevir showed pan-genotypic activity against the original genotype 1-6 culture viruses. Variants with position-80-substitutions were all viable, but fitness depended on the specific substitution and the HCV isolate. Q80K conferred resistance to simeprevir across genotypes but had only minor effects on the activity of the remaining PIs. For genotype 1a, pre-existing Q80K mediated accelerated escape from simeprevir, grazoprevir and to a lesser extent glecaprevir, but not voxilaprevir. For genotype 3a, Q80K mediated accelerated escape from glecaprevir and voxilaprevir. Escape was mediated by rapid and genotype-, PI- and PI-concentration-dependent co-selection of clinically relevant resistance associated substitutions.

Conclusions: Position-80-substitutions had relatively low fitness cost and the potential to promote HCV escape from clinically relevant PIs *in vitro*, despite having a minor impact on results in classical short-term resistance assays.

Lay summary: Among all clinically relevant hepatitis C virus protease inhibitors, voxilaprevir and glecaprevir showed the highest and most uniform activity against cell culture infectious hepatitis C virus with genotype 1-6 proteases. Naturally occurring amino acid changes at protease position 80 had low fitness cost and influenced sensitivity to simeprevir, but not to other protease inhibitors in short-term treatment assays. Nevertheless, the pre-existing change Q80K had the potential to promote viral escape from protease inhibitors during long-term treatment by rapid co-selection of additional resistance changes, detected by next generation sequencing.

Introduction

Regimens based on direct-acting antivirals (DAAs) have revolutionized treatment of patients with chronic infection with hepatitis C virus (HCV), which globally has been estimated to cause 70–150 million chronic infections and at least 400,000 deaths annually.[1,2] Approved DAAs target the HCV protease (NS3P), NS5A and NS5B.[3,4] Protease inhibitors (PIs), available since 2011, constitute an important component of DAA-based combination therapies.[3–5] While the initially developed PIs telaprevir and boceprevir have been discontinued, simeprevir might be used for treatment of patients infected with genotype 1, and grazoprevir or paritaprevir for genotypes 1 and 4.[6,7] Asunaprevir, approved in Asia and the Middle East, is used only for subtype 1b.[3] The novel PIs glecaprevir and voxilaprevir are recommended for genotypes 1-6.[6,7]

A subset of DAA-treated patients experience treatment failure, which is associated with selection of resistance-associated substitutions (RASs) that are either naturally occurring and pre-existing at baseline or rapidly acquired during treatment.[3,4] Several clinical studies have provided evidence that baseline RASs in DAA targets can compromise DAA treatment efficacy.[4,5]

Keywords: Q80K; Direct-acting antiviral; Resistance; Resistance associated substitutions; Linkage analysis; Simeprevir; Grazoprevir; Paritaprevir; Asunaprevir; Glecaprevir; Voxilaprevir, Hepatitis C virus.
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The prevalence of pre-existing RASs depends on their effect on viral fitness, influencing persistence and spread in human populations. Of described naturally occurring NS3P RASs, Q80K shows the highest prevalence: for genotype 1a-infected patients, 19%, 48% and 9% are carrying Q80K in Europe, North America, and South America, respectively.[3,8] Strong regional deviations in Q80K prevalence have been observed, with European prevalence rates ranging from 5% in Norway to 75% in Poland.[8] Q80K is rarely observed for genotype 1b and for other genotypes limited data are available.[4] For genotypes 1a and 1b, Q80K and the less prevalent Q80R caused resistance to simeprevir *in vitro*,[9–11] and for 1a, baseline Q80K resulted in decreased efficacy of simeprevir-based treatment regimens.[4,5] For other genotypes, data on the effect of Q80K on PI efficacy are not available.[4] While PI RASs can confer extensive cross-resistance among different PIs, little is known about the impact of Q80K on the efficacy of PIs other than simeprevir.[4] Finally, molecular mechanisms underlying treatment failure mediated by baseline RASs have not been studied.

We aimed to characterize the effect of NS3P position-80-substitutions on viral fitness and resistance to the approved PIs simeprevir, asunaprevir, paritaprevir, grazoprevir, glecaprevir, and voxilaprevir for HCV genotypes 1-6 using infectious cell culture systems. In addition, for selected genotypes we studied the impact of pre-existing Q80K on viral escape from simeprevir, grazoprevir, glecaprevir and voxilaprevir. Using next generation sequencing (NGS) and haplotype linkage analysis, we investigated resistance evolution underlying accelerated viral escape mediated by pre-existing Q80K.

Material and methods

HCV genotype 1-6 viruses

Cell culture adapted recombinants with NS3P of genotypes(isolates) 1a(H77),[12] 1a(TN),[13] 1a(HCV1),[12] 1b(DH1),[14] 2a(J6),[15] 2a(JFH1),[16] 3a(S52),[17] 5a(SA13)[17] and 6a(HK6a)[18] were used; the previously reported 4a(ED43) recombinant[17] contained 7 novel culture adaptive substitutions (Fig. S1). Mutations in these recombinants were introduced by QuikChange Site-Directed Mutagenesis Kit (Agilent). HCV sequences of final DNA preparations were confirmed by Sanger sequencing (Macrogen).

Evaluation of viral fitness

Huh-7.5 cells were cultured in DMEM (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Sigma) and penicillin 100 U/mL/streptomycin 100 µg/ml (Sigma) at 37 °C and 5% CO_2 and split every 2–3 days. For transfection, 3.5×10^5 Huh-7.5 cells/well plated the previous day on 6-well plates were transfected with RNA transcripts of HCV recombinants (supplementary information). Transfection cultures were split every 2–3 days and the percentage of HCV-infected cells was monitored by HCV-antigen immunostaining (supplementary information). For position-80-variants, spread kinetics monitored by immunostaining were compared to those of the respective original viruses, transfected in the same experiment. For variants with additional RASs, at different timepoints following transfection, HCV supernatant infectivity titers were determined (supplementary information). Supernatants from the peak of infection (≥80% HCV antigens positive cells) achieved in transfection or 1st viral passage experiments were used for inoculation of naïve Huh-7.5 cells. First or 2nd passage virus stocks were generated by collection of supernatants from these viral passage cultures at the peak of infection, and their NS3P sequence was analyzed by Sanger sequencing (see below).

Short-term treatment assay for assesment of PI sensitivity

The PIs simeprevir, asunaprevir, paritaprevir, grazoprevir and glecaprevir (Acme Biosciences), as well as voxilaprevir (Gilead Sciences), were dissolved in DMSO. In established concentration-response experiments,[18] 6×10^3 Huh-7.5 cells/well, plated the previous day on poly-D-lysine-coated 96-well plates (Nunc), were infected with NS3P sequence confirmed 1st or 2nd passage virus stocks. Infected cells were treated with dilution series of the PIs 24 h later; each concentration was tested in triplicates. Immunostaining was carried out and single HCV-infected cells were counted automatically 72 h post-infection (supplementary information). Counts from treated wells were related to means of counts from infected, non-treated wells. Following transformation of x-values, sigmoidal concentration–response curves were fitted and EC50 values were calculated using GraphPad Prism 6.[19] Applied PI concentrations were non-cytotoxic, tested here for glecaprevir and voxilaprevir, and previously for the other PIs.[17–19]

Long-term PI treatment assay

For induction of viral escape, Huh-7.5 cells were plated at 3.5×10^5 cells/well on 6-well plates and infected the following day with NS3P sequence confirmed virus stocks of 1a(HCV1) or 3a(S52) with or without Q80K. On day 1 post-infection, infected cells were trypsinized and transferred to T25 culture flasks, where they were maintained throughout the experiment. Every 2–3 days cells were split and plated for immunostaining, which was evaluated by fluorescence microscopy for determination of the percentage of HCV-infected cells. For simeprevir- and grazoprevir-experiments, infected with the original virus and Q80K-variant at the same multiplicity of infection, treatment was initiated on day 2 and 4 post-infection, respectively. Glecaprevir and voxilaprevir treatments were initiated on day 8 post-infection, when both viruses had spread to all culture cells. PI treatment was applied when cells were split, every 2–3 days, using the specified fold-EC50 for 1a(HCV1) or 3a(S52). Non-treated control cultures were included for comparison.

HCV sequence analysis

For Sanger sequencing, HCV RNA was extracted from cell culture supernatants, followed by RT-nested PCR spanning NS3P-NS4A using genotype-specific primers (supplementary information). From these amplicons, NS3P was sequenced (Macrogen). Sequence analysis was done with Sequencher (GeneCodes).

For NGS, we amplified the entire coding genome sequence (supplementary information). NGS was carried out in-house by Illumina Miseq using the v2 500 cycle kit. Minor single-nucleotide polymorphism (SNP) variants were identified as described.[20] Linkage of NS3P substitutions was done by selecting coding SNPs >0.5% and running LinkGE on all positions for each sample.[21] Haplotypes constituting >2% of the viral population were plotted in GraphPad Prism 6. When different nucleotide changes resulted in the same amino acid (aa) change, the variants were grouped as the same haplotype.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

Activity of clinically relevant PIs against HCV genotypes 1-6

We investigated the activity of 6 clinically relevant PIs against 10 cell culture infectious recombinants with NS3P of HCV

A

Genotype	1a	1a	1a	1b	2a	2a	3a	4a	5a	6a
Isolate	H77	TN	HCV1	DH1	J6	JFH1	S52	ED43	SA13	HK6a
PI	EC50 (nM)									
Simeprevir	9	31	3	1	216	74	2,397	5	84	110
Asunaprevir	25	18	15	3	157	76	1,861	27	37	73
Paritaprevir	8	4	3	3	112	52	290	2	10	24
Grazoprevir	3	1	1	1	6	2	28	1	1	5
Glecaprevir	5	1	2	2	4	7	4	2	1	10
Voxilaprevir	6	1	1	2	3	6	2	1	2	5

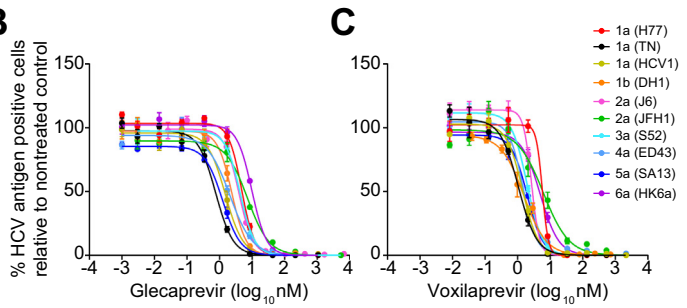


Fig. 1. Activity of PIs against original HCV genotype 1 to 6 recombinants. (A) EC50s of specified PIs for the original recombinant viruses with NS3P of the given genotypes and isolates were determined in short-term treatment assays (Materials and Methods). Representative EC50 values from 1-3 independent experiments are given. (B,C) Concentration-response profiles used for EC50 determination are shown for novel PIs (B) glecaprevir and (C) voxilaprevir. Data points are means of triplicates ± SEM; for curve-fitting, see Materials and Methods. One representative dataset of at least 2 independent experiments is shown. HCV, hepatitis C virus; PIs, protease inhibitors.

A

B

Genotype	1a	1a	1a	1b	2a	2a	3a	4a	5a	6a
Isolate	H77	TN	HCV1	DH1	J6	JFH1	S52	ED43	SA13	HK6a
Position 80 aa										
Q		+15			+13	+15			+11	
K				+2	+33	+10	+2	+2		
R		+7		+2			+5	+2		
L		+10								
G										
original aa	comparable spread				delayed spread					

Fig. 2. NS3P position-80-substitutions had differential effects on fitness of HCV genotype 1-6 recombinants. (A) Alignment of NS3P amino acid sequences of HCV recombinants of indicated isolates–genotypes included in this study (Fig. S1). Green boxes: Cell culture adaptive substitutions V14L (HK6a-6a), K26R (H77-1a), and P70L (J6-2a). Red box: Position 80. (B) Fitness of engineered position-80-variants, specified by genotype, isolate and position-80 amino acid residue, was evaluated by comparison of viral spread kinetics (Materials and Methods). Green, original virus; blue and red, engineered variants, showing comparable *vs.* delayed spread. Numbers in red boxes indicate the difference in spread of infection to the majority of the culture cells (days) observed for engineered variant in comparison to the original virus. HCV, hepatitis C virus.

genotypes 1-6 using short-term concentration-response assays (Fig. S1). In line with previous findings, simeprevir, asunaprevir, and paritaprevir showed the highest activity against genotypes 1 and 4 and the lowest activity against genotype 3. For grazoprevir we found higher potency and less genotype-specific EC50 differences, however, relatively low activity against genotype 3 (Fig. 1A).[13–15,17–19,22] Here, we investigated for the first time the activity of the new PIs glecaprevir and voxilaprevir, which showed true pan-genotypic activity with similar activity against all tested genotype 1-6 cell culture viruses (Fig. 1).

Substitutions at NS3P position 80 had differential effects on viral fitness and PI-resistance for HCV genotypes 1-6

At NS3P position 80, 1a(H77), 1a(HCV1), 1b(DH1), 3a(S52) and 4a(ED43) originally had Q, 1a(TN), 5a(SA13) and 6a(HK6a) had K, while 2a(J6) and 2a(JFH1) had G (Fig. 2A). We engineered

20 position-80-variants to investigate the impact of K and Q on each recombinant, and of R or L on selected recombinants (Fig. 2B). RNA transcripts of engineered variants were transfected into Huh-7.5 cells, and viral spread kinetics were evaluated by HCV-immunostaining. Variants of 1a(H77), 1a(HCV1), 1b(DH1), 3a(S52), 4a(ED43) and 6a(HK6a) showed comparable or slightly impaired spread kinetics compared to the respective original viruses (Fig. 2B). In contrast, for variants of 1a(TN), 2a(J6), 2a(JFH1) and 5a(SA13) viral spread was significantly delayed compared to that of the original viruses. However, all variants proved viable and maintained the engineered substitutions after 1st and/or 2nd viral passage in naïve cells. NS3P sequencing revealed additional substitutions in NS3P for the following variants: K122T and S160C in 2a(J6)G80K; T174S in 2a(J6)G80Q; N72N/H in 4a(ED43)Q80K, and D112d/A in 5a(SA13)K80Q.

Position-80-substitutions had a differential impact on PI sensitivity, determined by treatment of passage virus stocks in short-term treatment assays (Fig. 3). The most pronounced effect was observed for simeprevir: Q80K conferred 3- to 21-fold resistance to all tested recombinants (Fig. 3). For viruses that originally did not have Q at position 80, K80Q caused an 8- to 24-fold increase in sensitivity, while G80Q/K had no-to-little impact on sensitivity. A change to R at position 80 conferred no- to 13-fold resistance. Finally, a change to L conferred no- to 5-fold increased sensitivity.

For the remaining PIs the engineered position-80-substitutions had no-to-little impact on sensitivity (Fig. 3). Threefold resistance was observed for asunaprevir against 1a(HCV1)Q80K and 1b(DH1)Q80R, as well as for paritaprevir, grazoprevir and glecaprevir against 3a(S52)Q80K. Finally, 2a(J6)G80Q showed 3-fold resistance to glecaprevir and voxilaprevir.

Q80K promoted accelerated escape of 1a(HCV1) by concentration-dependent co-selection of RASs under treatment with simeprevir and grazoprevir

In the short-term assay we observed relatively small differences in EC50 for position-80-variants treated with PIs other than simeprevir (Fig. 3). We hypothesized that such differences, however, could result in more pronounced differences in a long-term treatment assay, better reflecting a clinical setting. Therefore, we subjected 1a(HCV1)Q80K and original 1a(HCV1) viruses to long-term treatment with the same concentrations of grazoprevir, widely used in the clinic for treatment of genotype 1, applying 1- to 128-fold EC50, relating to the EC50 determined for 1a(HCV1) (Fig. 1). For comparison, we also carried out treatment with 2- to 128-fold EC50 of simeprevir. Interestingly, under treatment with both PIs and at all concentrations,

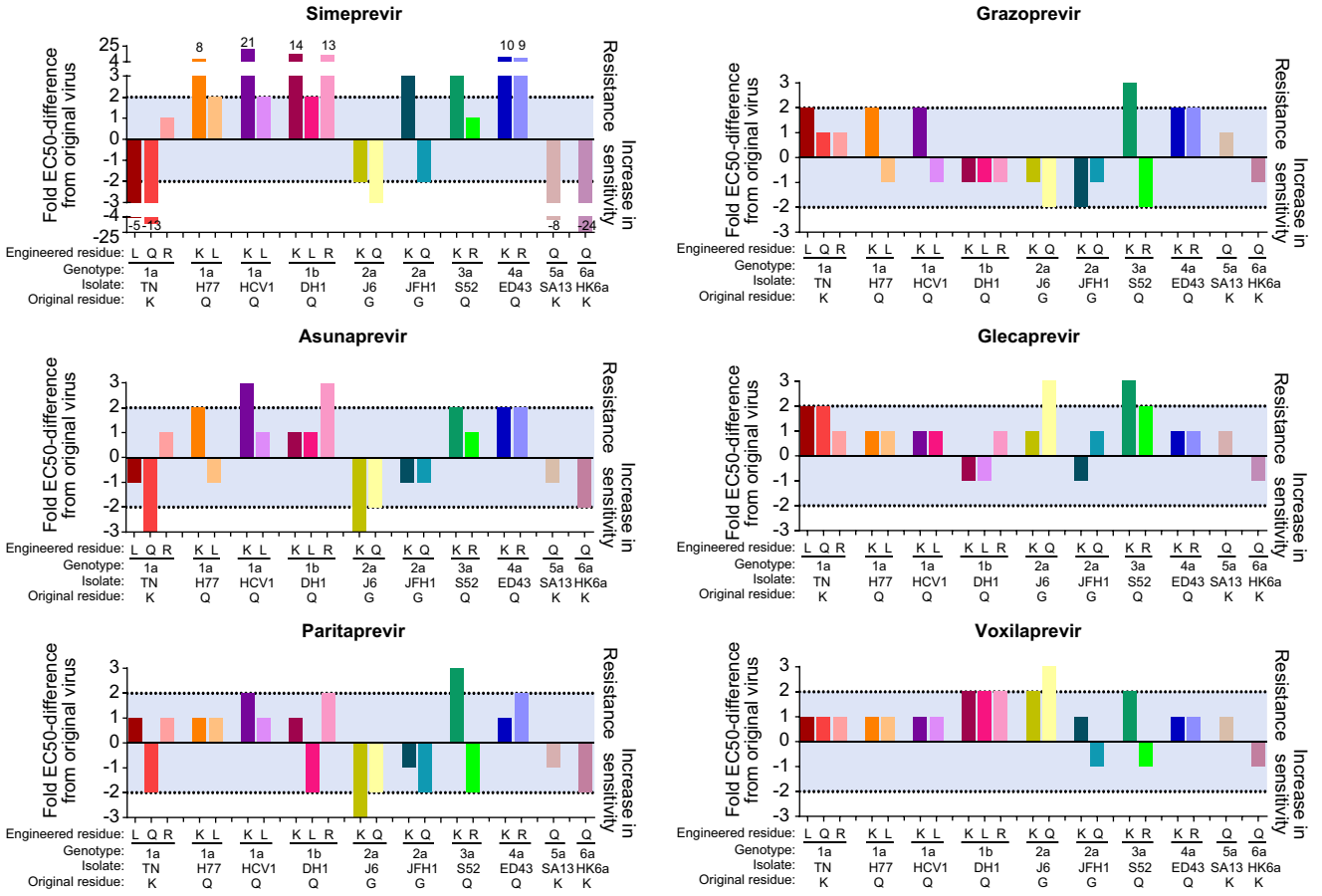


Fig. 3. NS3P position-80-substitutions had differential effects on PI sensitivity for HCV genotypes 1-6. Short-term treatment assays with the indicated PIs employed sequence confirmed virus stocks of the indicated variants, specified by the engineered and the original amino acid at position 80, genotype, and isolate (lower x-axis). EC50s were calculated (Materials and Methods); fold EC50 differences (y-axis) were calculated as [EC50 of position-80-variant/EC50 of original virus included in the same assay], and are color coded according to the respective position-80-variant. Numbers are rounded off. For simeprevir, numbers above bars indicate fold EC50 differences ≤−5 and ≥5. HCV, hepatitis C virus; PIs, protease inhibitors.

Q80K promoted viral escape with all 1a(HCV1)Q80K cultures showing accelerated spread kinetics compared to the original 1a(HCV1) cultures (Fig. 4A,B); viral suppression was observed for simeprevir at 128-fold EC50 for 1a(HCV1), but not 1a(HCV1)Q80K.

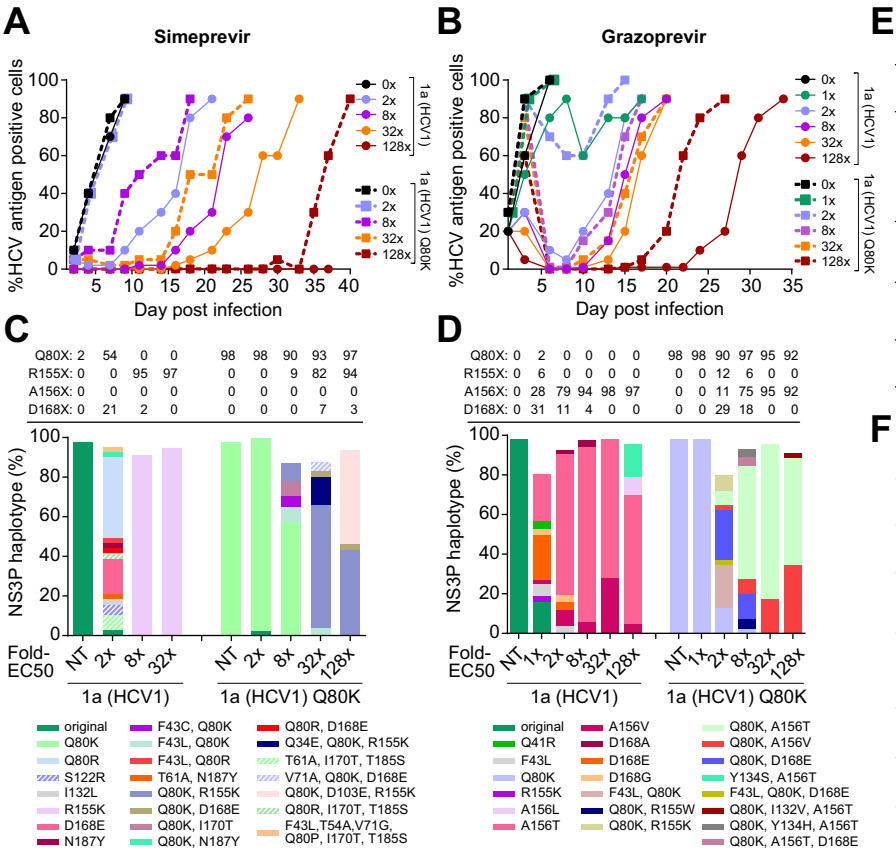
To elucidate molecular mechanisms causing viral escape, viruses that spread under PI treatment were subjected to NGS and NS3P linkage analysis. For both PIs, similar patterns regarding the acquisition of substitutions were observed (Fig. 4C-F). At low concentrations, 2-fold EC50 for simeprevir and 1-fold EC50 for grazoprevir, pre-existing Q80K was sufficient to mediate viral escape, resulting in a homogeneous virus population with Q80K as the single dominant NS3P substitution. In contrast, under these concentrations, for the original 1a(HCV1) a heterogeneous virus population was selected harboring various NS3P RASs.

At intermediate PI concentrations, 8-fold EC50 for simeprevir and 2-fold EC50 for grazoprevir, escape of the original 1a(HCV1) was mainly mediated by acquisition of single high-level resistance RASs, predominantly R155K under simeprevir and A156T under grazoprevir. In contrast, these substitutions were only selected in a minority of 1a(HCV1)Q80K viruses, where escape was mediated by Q80K alone or by co-selection of different RASs in addition to Q80K. Thus, following escape from grazoprevir, 1a(HCV1)F43L + Q80K and 1a(HCV1)Q80K + D168E predominated.

At higher PI concentrations, >8-fold EC50 for simeprevir and >2-fold EC50 for grazoprevir, for the original 1a(HCV1) escape was mainly mediated by the single high-level resistance substitutions R155K and A156T, respectively, as observed under intermediate concentrations. However, for 1a(HCV1)Q80K, in contrast to our findings at intermediate concentrations, R155K and A156T were also co-selected with pre-existing Q80K.

Pre-existing Q80K aided selection of escape variants with fitness or resistance advantages

To investigate the effect of pre-existing Q80K on the characteristics of resulting escape variants, we engineered the 1a(HCV1)Q80K escape variant 1a(HCV1)Q80K + R155K, selected under 32- and 128-fold EC50 of simeprevir. This variant showed higher fitness than the 1a(HCV1) escape variant 1a(HCV1)R155K, also selected under 32-fold EC50 of simeprevir, as determined by comparison of HCV infectivity titers in transfection experiments (Fig. 5A). In addition, compared to 1a(HCV1)R155K, 1a(HCV1)Q80K + R155K showed increased simeprevir resistance (Fig. 5B). The 1a(HCV1)Q80K escape variants 1a(HCV1)Q80K + F43L and 1a(HCV1)Q80K + D168E,



E

NT	Original	None
	Q80K	Q80K
2x	Original	Q80R; S122R; I132L; D168E; N187Y; F43L+Q80R; T61A+N187Y; Q80R+D168E; Q80K+N187Y; T61A+I170T+T185S; Q80R+I170T+T185S; F43L+T54A+V71G+Q80P+I170T+T185S
	Q80K	Q80K
8x	Original	R155K
	Q80K	Q80K; F43C+Q80K; F43L+Q80K; Q80K+R155K; Q80K+I170T
32x	Original	R155K
	Q80K	F43L+Q80K; Q80K+R155K; Q80K+D168E; Q34E+Q80K+R155K; V71A+Q80K+D168E
128x	Original	n.a.
	Q80K	Q80K+R155K; Q80K+D168E; Q80K+D103E+R155K

F

NT	Original	None
	Q80K	Q80K
1x	Original	Q41R; F43L; R155K; A156T; A156V; D168E; D168G
	Q80K	Q80K
2x	Original	F43L; A156T; A156V; D168A; D168E; D168G
	Q80K	Q80K; F43L+Q80K; Q80K+R155K; Q80K+A156T; Q80K+A156V; Q80K+D168E; F43L+Q80K+D168E
8x	Original	A156V; A156T; D168A
	Q80K	Q80K; Q80K+R155W; Q80K+A156T; Q80K+A156V; Q80K+D168E; Q80K+Y134H+A156T; Q80K+A156T+D168E
32x	Original	A156V; A156T
	Q80K	Q80K+A156T; Q80K+A156V
128x	Original	A156L; A156T; A156V; Y134S+ A156T
	Q80K	Q80K+A156T; Q80K+A156V; Q80K+I132V+A156T

Fig. 4. For 1a(HCV1), pre-existing Q80K promoted escape from simeprevir and grazoprevir by concentration-dependent RASs co-selection. 1a(HCV1) and 1a(HCV1)Q80K escape from (A) simeprevir and (B) grazoprevir in long-term treatments with the indicated concentrations of PIs (fold EC50) (Materials and Methods). (C,D) NGS-based NS3P SNP linkage analysis revealed NS3P haplotype distributions in (C) simeprevir or (D) grazoprevir escape variants. Haplotypes >2% of the viral population, included in bars. Percentages of sequences with high-level RASs, shown above bars considering haplotypes >0.5%. (E,F) Summary of RASs in (E) simeprevir or (F) grazoprevir escape variants, including all RASs in (C,D). NT, non-treated. n.a., non-available due to viral supression. HCV, hepatitis C virus; NGS, next generation sequencing; RAS, resistance-associated substitution; SNP, single nucleotide polymorphism.

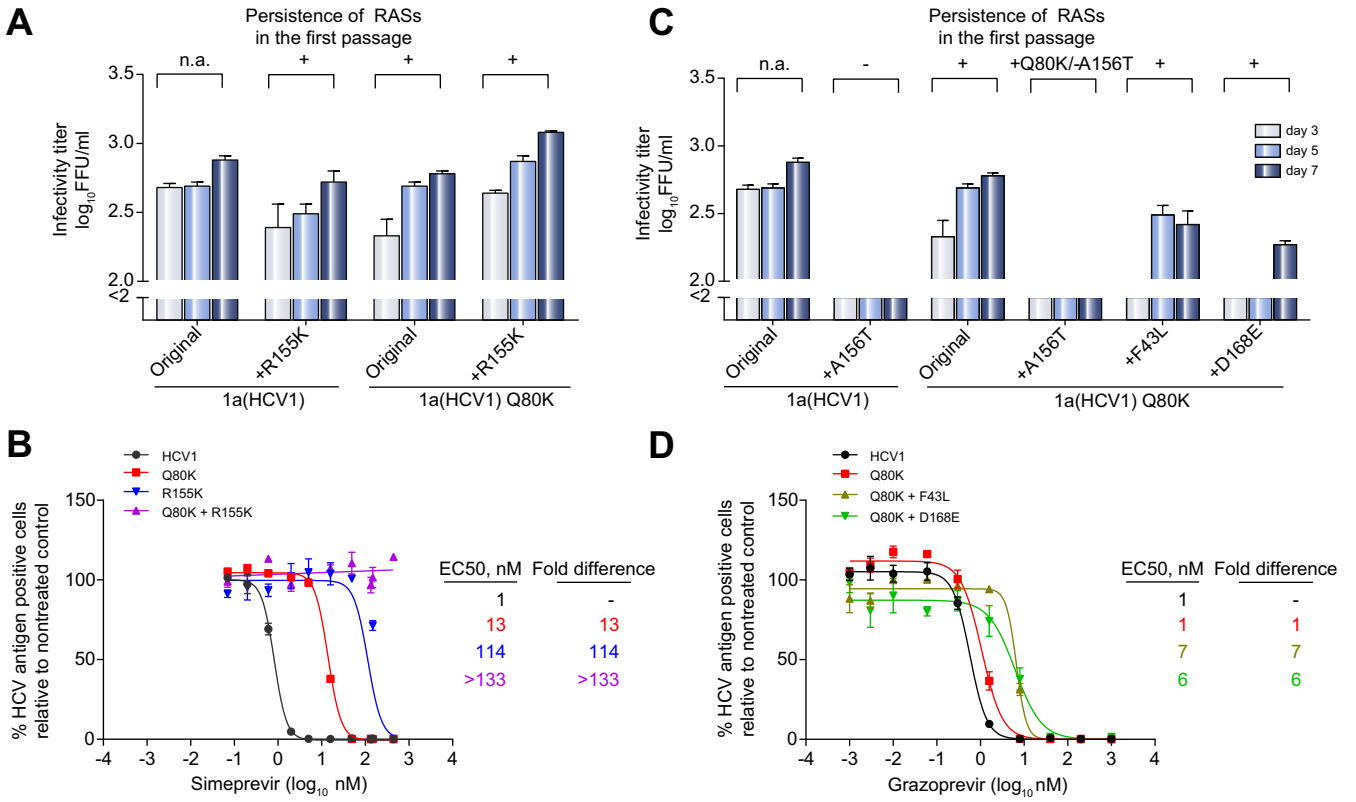


Fig. 5. Fitness and resistance of engineered simeprevir and grazoprevir 1a(HCV1) escape variants. (A,C) Fitness of indicated (A) simeprevir and (C) grazoprevir escape variants, evaluated by HCV infectivity titrations (Materials and Methods). Data points are means of triplicates ± SEM. +, persistence; −, reversion of RASs in 1st passage variants. (B,D) Concentration-response profiles of variants for (B) simeprevir or (D) grazoprevir in short-term treatment assays. Data points are means of triplicates ± SEM; for curve-fitting, see Materials and Methods. Derived EC50s and fold EC50 differences [EC50 of variant/EC50 of original 1a(HCV1) included in the same assay] are given. n.a., non-applicable. HCV, hepatitis C virus.

selected under 2- and 8-fold EC50 of grazoprevir, had higher fitness than 1a(HCV1)A156T, selected under the same concentration from 1a(HCV1) (Fig. 5C) and showed resistance to grazoprevir (Fig. 5D). The RAS A156T reverted in both 1a (HCV1)Q80K + A156T and 1a(HCV1)A156T, selected under grazoprevir from 1a(HCV1)Q80K and 1a(HCV1), respectively. Low fitness of position-156-variants and reversion to A156 is in line with previous *in vitro* findings.[9,19,22–24]

Under treatment with glecaprevir and voxilaprevir, Q80K had limited effect on escape of 1a(HCV1), but promoted accelerated escape of 3a(S52)

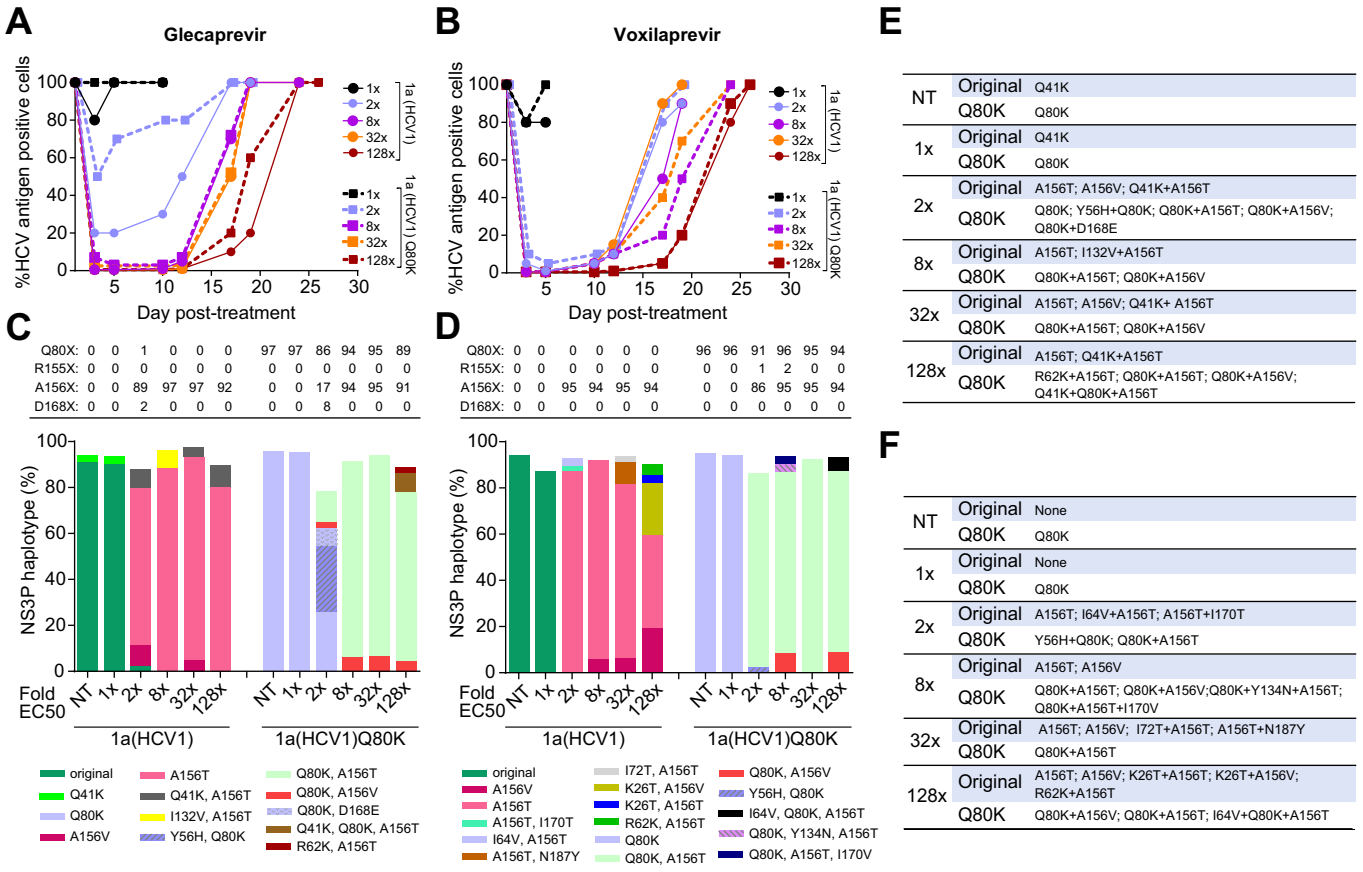
While escape occurred in all genotype 1a cultures, Q80K promoted accelerated escape only under 2- and 128-fold of glecaprevir (Fig. 6A); Q80K did not promote accelerated escape under treatment with any concentration of voxilaprevir (Fig. 6B). NGS and NS3P linkage analysis revealed that under treatment with both PIs at most concentrations A156T dominated for 1a(HCV1) and Q80K + A156T dominated for 1a(HCV1)Q80K escape variants (Fig. 6C-F). Only under 2-fold EC50 of glecaprevir a different pattern was observed. Here, 1a(HCV1)Q80K escape variants comprised various populations harboring Q80K in combination with substitutions at positions different from 156, including Y56H and D168E; in contrast, A156T dominated in 1a(HCV1) escape variants. Under 1-fold EC50 of both PIs both genotype 1a viruses mostly spread without selection of substitutions.

In contrast to our findings for genotype 1a, for genotype 3a pre-existing Q80K promoted accelerated escape under treatment with all concentrations of glecaprevir and voxilaprevir (Fig. 7A,B). At high PI concentrations (>8-fold EC50 for glecaprevir and >2 fold EC50 for voxilaprevir), A156V dominated for 3a(S52) and Q80K + A156V with or without R155K dominated for 3a(S52)Q80K escape variants (Fig. 7C-F). Intermediate concentrations (8-fold EC50) of glecaprevir led to selection of A156T/V in 3a(S52) and to co-selection of several different substitutions in 3a(S52)Q80K, including A156G. Under treatment with 1- and 2-fold EC50 of both PIs, genotype 3a viruses mostly spread without selection of substitutions.

Discussion

Among all clinically relevant PIs only glecaprevir and voxilaprevir showed pan-genotypic activity. HCV genotype 1-6 position-80-variants showed relatively high fitness. In classical short-term resistance assays, Q80K conferred simeprevir resistance across genotypes, but no-to-little resistance to other PIs. However, Q80K had the potential to promote accelerated viral escape from other PIs in long-term treatments. Carrying pre-existing Q80K, genotype 3a appeared to be more prone to escape from glecaprevir and voxilaprevir than genotype 1a. Thus, we describe here for the first time for HCV genotypes 1-6 the impact of position-80-substitutions on fitness and resistance to all 6 clinically relevant PIs and reveal that pre-existing position-80-substitutions facilitate accelerated escape from PIs.

In vitro testing is necessary to study DAA activity and putative RASs.[25] Compared to enzymatic assays and replicons, HCV



E

NT	Original	Q41K
	Q80K	Q80K
1x	Original	Q41K
	Q80K	Q80K
2x	Original	A156T; A156V; Q41K+A156T
	Q80K	Q80K; Y56H+Q80K; Q80K+A156T; Q80K+A156V; Q80K+D168E
8x	Original	A156T; I132V+A156T
	Q80K	Q80K+A156T; Q80K+A156V
32x	Original	A156T; A156V; Q41K+ A156T
	Q80K	Q80K+A156T; Q80K+A156V
128x	Original	A156T; Q41K+A156T
	Q80K	R62K+A156T; Q80K+A156T; Q80K+A156V; Q41K+Q80K+A156T

F

NT	Original	None
	Q80K	Q80K
1x	Original	None
	Q80K	Q80K
2x	Original	A156T; I64V+A156T; A156T+I170T
	Q80K	Y56H+Q80K; Q80K+A156T
8x	Original	A156T; A156V
	Q80K	Q80K+A156T; Q80K+A156V;Q80K+Y134N+A156T; Q80K+A156T+I170V
32x	Original	A156T; A156V; I72T+A156T; A156T+N187Y
	Q80K	Q80K+A156T
128x	Original	A156T; A156V; K26T+A156T; K26T+A156V; R62K+A156T
	Q80K	Q80K+A156V; Q80K+A156T; I64V+Q80K+A156T

Fig. 6. For 1a(HCV1), pre-existing Q80K had limited effect on escape from glecaprevir and voxilaprevir. (A,B) Escape and (C-F) NGS-based NS3P SNP linkage analysis for 1a(HCV1) and 1a(HCV1)Q80K for (A,C,E) glecaprevir and (B,D,F) voxilaprevir. For details, compare Fig. 4 legend. NT, non-treated. HCV, hepatitis C virus; NGS, next generation sequencing; SNP, single nucleotide polymorphism.

infectious culture systems reflect the full viral life cycle and previous results in these systems reflected clinical data.[13–15,17–19,22–24] However, cell culture adaptive substitutions, especially those found in NS3P of the 1a(H77),[12] 2a(J6)[15] and 6a(HK6a)[18] recombinants, might influence study results.

Amino acid residues at position 80 in the used HCV recombinants were representative for the respective genotypes in treatment-naïve HCV-infected individuals. Thus, in genotype 1a infected patients position 80 displayed mainly Q and K, but also G, L, N and R.[10,26] Similar observations were made by analysis of sequences deposited in databases:[27,28] among 307 genotype 1a sequences Q and K were predominant at position 80, while G, L, N and R were found in a minor percentage. For genotype 1b, among 327 sequences, Q was predominant, while K and L were observed to a minor extent. For genotype 3a, Q was found in all of 170 sequences. For genotypes 2a, 4a, 5a and 6a the number of available sequences was limited: for 2a, all 19 sequences had G, for 4a all 17 had Q, for 5a both had K and for 6a 14/16 had K, 1/16 had Q and 1/16 had L. For genotype 5a, 10/11 additional sequences had K, while 1/11 had Q.[29,30] For genotype 6a, 4 additional sequences had K.[30]

The relatively high fitness of engineered position-80 variants is in line with the high prevalence of position-80-polymorphisms *in vivo*, and with position-80-polymorphisms having little-to-no impact on genotype 1 replication capacity *in vitro*.[9,23] Interestingly, Q80K acted as a fitness compensating substitution for R155K. Thus, position-80 RASs can be expected to persist in patients carrying these RASs also without PI pressure. Except for 6a(HK6a), Q80 decreased fitness when introduced in recombinants, originally harboring different aa residues.

Our findings on PI activity against original genotype 1-6 viruses in short-term treatment assays are in line with previous *in vitro* activity and clinical efficacy studies.[5,14,17–19,22,31–36] Sensitivity of the original viruses to simeprevir depended on the residue at position 80. Except for 3a(S52), Q80 resulted in relatively high and K/G80 in relatively low sensitivity. Natural resistance of genotype 3a isolates to simeprevir and other PIs is probably explained by the presence of other NS3P polymorphisms such as Q168 or V170.[5,18,31] The impact of position-80-substitutions on the effect of simeprevir depended on the simeprevir sensitivity of the original recombinants and thus also on the original aa at position 80. Thus, Q80K conferred a higher level of simeprevir resistance than G80K. Also, except for genotype 3a, Q80R but not K80R conferred simeprevir resistance. While this is the first study using genotype 1-6 infectious cell culture systems and a broad panel of all clinically relevant PIs for *in vitro* evaluation of position-80-polymorphisms, our results agree with results previously obtained in genotype 1a and 1b replicon studies.[9,10,37]

Small differences in EC50 might be of clinical relevance.[10] Also in this study, minor differences in EC50 in short-term

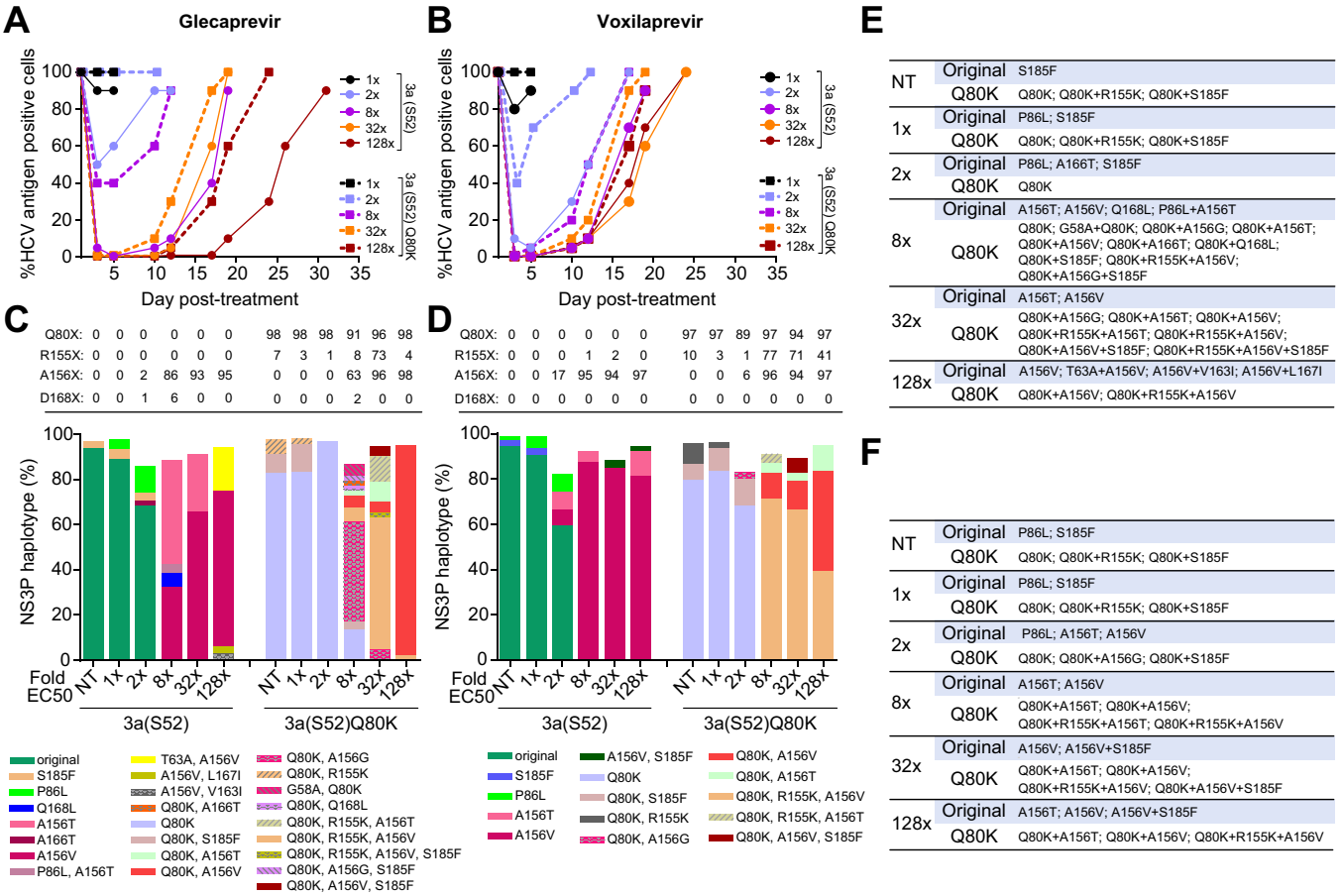


Fig. 7. For 3a(S52), pre-existing Q80K promoted escape from glecaprevir and voxilaprevir. (A,B) Escape and (C-F) NGS-based NS3P SNP linkage analysis for 3a(S52) and 3a(S52)Q80K for (A,C,E) glecaprevir and (B,D,F) voxilaprevir. For details, compare Fig. 4 legend. NT, non-treated. HCV, hepatitis C virus; NGS, next generation sequencing; SNP, single nucleotide polymorphism.

assays translated into more pronounced effects during long-term treatment for grazoprevir, glecaprevir and voxilaprevir. The fact that Q80K had a greater potential to promote escape from novel pan-genotypic PIs for genotype 3a than for genotype 1a might add to the reasons why genotype 3a is emerging as difficult to treat with DAAs.[5] However, prevalence of Q80K in genotype 3a is rare and current treatment regimens use PIs in combination with NS5A and/or NS5B inhibitors, which is likely to minimize the effect of pre-existing Q80K and other pre-existing RASs on overall treatment efficacies.[38] Nevertheless, in the clinical setting, there is preliminary evidence that Q80K might have a negative effect not only on the efficacy of certain simeprevir- but also of certain asunaprevir- paritaprevir-, grazoprevir- and voxilaprvir-based treatment regimens in genotype 1 infected patients.[4,5,39,40] The impact of pre-existing Q80K could vary for the same PI given in combination with different antivirals. Thus, Q80K reduced treatment efficacy of simeprevir/peginterferon/ribavirin more than that of simeprevir/sofosbuvir in genotype 1 infected patients.[4,5] Given the high cost of DAAs and thus the relevance of choosing the most efficient treatment regimen, the clinical effects of pre-existing Q80K on DAA combinations containing different PIs remain to be investigated in detail.

NGS substitution linkage analysis and haplotype reconstruction was previously only used for short genome segments and not in the context of antiviral resistance.[21] We optimized this approach to cover a complete viral protein and major DAA target, enabling us to investigate resistance associated NS3P haplotypes in unprecedented detail. Mostly for simeprevir and grazoprevir, and to a lesser extent for glecaprevir, increasing PI concentrations created an evolutionary bottleneck, driving the selection of highly resistant viruses. While low PI concentrations selected for various NS3P haplotypes with different substitutions occurring individually or in various combinations, high PI concentrations selected for few NS3P haplotypes dominated by high-level resistance RASs such as R155K and A156T.[5] In contrast, for voxilaprevir A156T/V dominated in all escape cultures, possibly because of its increased activity against several low-level resistance RASs.[34] RASs selected in original viruses were comparable to those selected in replicons and in patients under PIs used in this study.[3–5,11,33,35,36,39–46] In addition, selection of RASs R155K, D168E and I170T under simeprevir and of position-168 RASs under grazoprevir in genotype 1a infected patients with baseline Q80K highlights the clinical relevance of our results.[10,40,47,48] Our data underline the central importance of NS3P position 156 for resistance to the clinically highly important PIs grazoprevir, glecaprevir and voxilaprevir.[3–5]

The capacity to escape high PI concentrations was not directly linked to resistance exerted by Q80K. Rather, Q80K facilitated rapid co-selection of additional RASs, leading to faster escape and to escape variants with higher fitness and/or resistance compared to escape variants selected in experiments with

the original viruses. We hypothesize that upon treatment initiation, Q80K variants might have an evolutionary advantage due to increased growth capacity under treatment, allowing faster selection of variants carrying additional RASs and/or fitness compensating substitutions.[49]

Studies of the highly polymorphic NS3P position 80 in HCV genotypes 1-6 are of relevance given that the indication for PI-based treatment regimens has recently been expanded to include all major HCV genotypes. Increased knowledge on the prevalence and effect of baseline polymorphisms in DAA targets on different DAAs could help clinicians make optimal treatment choices and thus avoid the development of a resistance-bearing patient cohort.

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Conflict of interest

Voxilaprevir was provided by Gilead Sciences.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design (L.V.P., S.B.J., U.F., Q.T., J.B., J.M.G.); acquisition of data (L.V.P., S.B.J., U.F., M.S.P., Q.T., L.G., S.R., D. H., S.B.N.S., K.S.), analysis of data (L.V.P., S.B.J., U.F., M.S.P., Q.T., J.M.G.); drafting of the manuscript (L.V.P., S.B.J., U.F., J.B., J.M. G.); study supervision (J.B., J.M.G.).

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Supplementary data

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References

Author names in bold designate shared co-first authorship

[1] Polaris Observatory HCV Collaborators. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. Lancet Gastroenterol Hepatol 2017;2:161–176.

[2] Bukh J. The history of hepatitis C virus (HCV): Basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control. J Hepatol 2016;65:S2–S21.

[3] Pawlotsky JM. Hepatitis C Virus resistance to direct-acting antiviral drugs in interferon-free regimens. Gastroenterology 2016;151:70–86.

[4] Sarrazin C. The importance of resistance to direct antiviral drugs in HCV infection in clinical practice. J Hepatol 2016;64:486–504.

[5] Sorbo MC, Cento V, Di Maio VC, Howe AYM, Garcia F, Perno CF, et al. Hepatitis C virus drug resistance associated substitutions and their clinical relevance: Update 2018. Drug Resist Updat 2018;37:17–39.

[6] Pawlotsky JM, Negro F, Aghemo A, Berenguer M, Dalgard O, Dusheiko G, et al. EASL recommendations on treatment of hepatitis C 2018. J Hepatol 2018;69:461–511.

[7] AASLD-IDSA. HCV Guidance: Recommendations for testing, managing, and treating hepatitis C. https://www hcvguidelines org/ Accessed July 9, 2018.

[8] Sarrazin C, Lathouwers E, Peeters M, Daems B, Buelens A, Witek J, et al. Prevalence of the hepatitis C virus NS3 polymorphism Q80K in genotype 1 patients in the European region. Antiviral Res 2015;116:10–16.

[9] Lenz O, Verbinnen T, Lin TI, Vijgen L, Cummings MD, Lindberg J, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother 2010;54:1878–1887.

[10] Lenz O, Verbinnen T, Fevery B, Tambuyzer L, Vijgen L, Peeters M, et al. Virology analyses of HCV isolates from genotype 1-infected patients treated with simeprevir plus peginterferon/ribavirin in phase IIb/III studies. J Hepatol 2015;62:1008–1014.

[11] Verbinnen T, Fevery B, Vijgen L, Jacobs T, De Meyer S, Lenz O. In vitro activity of simeprevir against hepatitis C virus genotype 1 clinical isolates and its correlation with NS3 sequence and site-directed mutants. Antimicrob Agents Chemother 2015;59:7548–7557.

[12] **Li YP**, **Ramirez S**, Mikkelsen LS, Bukh J. Efficient infectious cell culture systems of the hepatitis C virus (HCV) prototype strains HCV-1 and H77. J Virol 2015;89:811–823.

[13] Li YP, Ramirez S, Jensen SB, Purcell RH, Gottwein JM, Bukh J. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc Natl Acad Sci U S A 2012;109:19757–19762.

[14] Pham LV, Ramirez S, Carlsen THR, Li YP, Gottwein JM, Bukh J. Efficient hepatitis C virus genotype 1b core-NS5A recombinants permit efficacy testing of protease and NS5A inhibitors. Antimicrob Agents Chemother 2017;61:e00037–17.

[15] Li YP, Ramirez S, Gottwein JM, Scheel TK, Mikkelsen L, Purcell RH, et al. Robust full-length hepatitis C virus genotype 2a and 2b infectious cultures using mutations identified by a systematic approach applicable to patient strains. Proc Natl Acad Sci U S A 2012;109:E1101–E1110.

[16] Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–626.

[17] Li YP, Ramirez S, Humes D, Jensen SB, Gottwein JM, Bukh J. Differential sensitivity of 5'UTR-NS5A recombinants of hepatitis C virus genotypes 1-6 to protease and NS5A inhibitors. Gastroenterology 2014;146:812–821.

[18] **Gottwein JM**, **Scheel TK**, Jensen TB, Ghanem L, Bukh J. Differential efficacy of protease inhibitors against HCV genotypes 2a, 3a, 5a, and 6a NS3/4A protease recombinant viruses. Gastroenterology 2011;141:1067–1079.

[19] **Jensen SB**, **Serre SB**, Humes DG, Ramirez S, Li YP, Bukh J, et al. Substitutions at NS3 residue 155, 156, or 168 of hepatitis C virus genotypes 2 to 6 induce complex patterns of protease inhibitor resistance. Antimicrob Agents Chemother 2015;59:7426–7436.

[20] Fahnoe U, Pedersen AG, Drager C, Orton RJ, Blome S, Hoper D, et al. Creation of functional viruses from non-functional cDNA clones obtained from an RNA virus population by the use of ancestral reconstruction. PLoS ONE 2015;10 e0140912.

[21] **Wilker PR**, **Dinis JM**, Starrett G, Imai M, Hatta M, Nelson CW, et al. Selection on haemagglutinin imposes a bottleneck during mammalian transmission of reassortant H5N1 influenza viruses. Nat Commun 2013;4:2636.

[22] Serre SB, Jensen SB, Ghanem L, Humes DG, Ramirez S, Li YP, et al. Hepatitis C virus genotype 1 to 6 protease inhibitor escape variants. in vitro selection, fitness, and resistance patterns in the context of the infectious viral life cycle. Antimicrob Agents Chemother 2016;60:3563–3578.

[23] Shimakami T, Welsch C, Yamane D, McGivern DR, Yi M, Zeuzem S, et al. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. Gastroenterology 2011;140:667–675.

[24] Imhof I, Simmonds P. Genotype differences in susceptibility and resistance development of hepatitis C virus to protease inhibitors telaprevir (VX-950) and danoprevir (ITMN-191). Hepatology 2011;53:1090–1099.
[25] Ramirez S, Bukh J. Current status and future development of infectious cell-culture models for the major genotypes of hepatitis C virus: essential tools in testing of antivirals and emerging vaccine strategies. Antiviral Res 2018;158:264–287.
[26] Cuypers L, Libin P, Schrooten Y, Theys K, Di Maio VC, Cento V, et al. Exploring resistance pathways for first-generation NS3/4A protease inhibitors boceprevir and telaprevir using Bayesian network learning. Infect Genet Evol 2017;53:15–23.
[27] Combet C, Garnier N, Charavay C, Grando D, Crisan D, Lopez J, et al. euHCVdb: the European hepatitis C virus database. Nucleic Acids Res 2007;35:D363–D366.
[28] Kuiken C, Hraber P, Thurmond J, Yusim K. The hepatitis C sequence database in Los Alamos. Nucleic Acids Res 2008;36:D512–D516.
[29] Wose Kinge CN, Espiritu C, Prabdial-Sing N, Sithebe NP, Saeed M, Rice CM. Hepatitis C virus genotype 5a subgenomic replicons for evaluation of direct-acting antiviral agents. Antimicrob Agents Chemother 2014;58:5386–5394.
[30] Ku KS, Chodavarapu RK, Martin R, Miller MD, Mo H, Svarovskaia ES. Sequencing analysis of NS3/4A, NS5A, and NS5B genes from patients infected with hepatitis C virus genotypes 5 and 6. J Clin Microbiol 2016;54:1835–1841.
[31] Lenz O, Vijgen L, Berke JM, Cummings MD, Fevery B, Peeters M, et al. Virologic response and characterisation of HCV genotype 2–6 in patients receiving TMC435 monotherapy (study TMC435-C202). J Hepatol 2013;58:445–451.
[32] Pilot-Matias T, Tripathi R, Cohen D, Gaultier I, Dekhtyar T, Lu L, et al. In vitro and in vivo antiviral activity and resistance profile of the hepatitis C virus NS3/4A protease inhibitor ABT-450. Antimicrob Agents Chemother 2015;59:988–997.
[33] Summa V, Ludmerer SW, McCauley JA, Fandozzi C, Burlein C, Claudio G, et al. MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. Antimicrob Agents Chemother 2012;56:4161–4167.
[34] Taylor JG, Appleby T, Barauskas O, Chen X, Dvory-Sobol H, Gong R, et al. Preclinical profile of the pan-genotypic HCV NS3/4A protease inhibitor GS-9857. J Hepatol 2015;62:S681.
[35] Ng TI, Tripathi R, Reisch T, Lu L, Middleton T, Hopkins TA, et al. In vitro antiviral activity and resistance profile of the next-generation hepatitis C Virus NS3/4A protease inhibitor glecaprevir. Antimicrob Agents Chemother 2018;62:e01620–17.
[36] Rodriguez-Torres M, Glass S, Hill J, Freilich B, Hassman D, Di Bisceglie AM, et al. GS-9857 in patients with chronic hepatitis C virus genotype 1-4 infection: a randomized, double-blind, dose-ranging phase 1 study. J Viral Hepat 2016;23:614–622.
[37] McPhee F, Friborg J, Levine S, Chen C, Falk P, Yu F, et al. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. Antimicrob Agents Chemother 2012;56:3670–3681.
[38] Sarrazin C, Cooper CL, Manns MP, Reddy KR, Kowdley KV, Roberts SK, et al. No impact of resistance associated substitutions on the efficacy of sofosbuvir, velpatasvir, and voxilaprevir for 12 weeks in HCV DAA-experienced patients. J Hepatol 2018;69:1221–1230.
[39] Jacobson IM, Lawitz E, Kwo PY, Hezode C, Peng CY, Howe AYM, et al. Safety and efficacy of elbasvir/grazoprevir in patients with hepatitis C virus infection and compensated cirrhosis: an integrated analysis. Gastroenterology 2017;152:1372–1382.
[40] Zamor PJ, Vierling J, Ghalib R, Luketic V, Ravendhran N, Balart L, et al. Elbasvir/grazoprevir in black adults with hepatitis C virus infection: a pooled analysis of phase 2/3 clinical trials. Am J Gastroenterol 2018;113:863–871.
[41] Dietz J, Susser S, Vermehren J, Peiffer KH, Grammatikos G, Berger A, et al. Patterns of resistance-associated substitutions in patients with chronic HCV infection following treatment with direct-acting antivirals. Gastroenterology 2018;154:976–988.
[42] Krishnan P, Schnell G, Tripathi R, Beyer J, Reisch T, Dekhtyar T, et al. Integrated resistance analysis of CERTAIN-1 and CERTAIN-2 studies in hepatitis C virus-infected patients receiving glecaprevir and pibrentasvir in Japan. Antimicrob Agents Chemother 2018;62(2).
[43] Poordad F, Felizarta F, Asatryan A, Sulkowski MS, Reindollar RW, Landis CS, et al. Glecaprevir and pibrentasvir for 12 weeks for hepatitis C virus genotype 1 infection and prior direct-acting antiviral treatment. Hepatology 2017;66:389–397.
[44] Lawitz E, Reau N, Hinestrosa F, Rabinovitz M, Schiff E, Sheikh A, et al. Efficacy of sofosbuvir, velpatasvir, and GS-9857 in patients with genotype 1 hepatitis C virus infection in an open-label, phase 2 trial. Gastroenterology 2016;151:893–901.
[45] Lawitz E, Yang JC, Stamm LM, Taylor JG, Cheng G, Brainard DM, et al. Characterization of HCV resistance from a 3-day monotherapy study of voxilaprevir, a novel pangenotypic NS3/4A protease inhibitor. Antivir Ther 2018;23(4):325–334.
[46] **Zeuzem S**, **Foster GR**, Wang S, Asatryan A, Gane E, Feld JJ, et al. Glecaprevir-pibrentasvir for 8 or 12 weeks in HCV genotype 1 or 3 infection. N Engl J Med 2018;378:354–369.
[47] Lawitz E, Sulkowski MS, Ghalib R, Rodriguez-Torres M, Younossi ZM, Corregidor A, et al. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. Lancet 2014;384:1756–1765.
[48] Reesink HW, Fanning GC, Farha KA, Weegink C, Van Vliet A, Van 't Klooster G, et al. Rapid HCV-RNA decline with once daily TMC435: a phase I study in healthy volunteers and hepatitis C patients. Gastroenterology 2010;138:913–921.
[49] Pham LV, Ramirez S, Gottwein JM, Fahnøe U, Li YP, Pedersen J, et al. HCV genotype 6a escape from and resistance to velpatasvir, pibrentasvir, and sofosbuvir in robust infectious cell culture models. Gastroenterology 2018;154:2194–2208.