Supplementary On-Line Information

Protease Inhibitor-resistant Hepatitis C Mutants with Reduced Fitness due to Impaired Production of Infectious Virus

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Supplementary Methods

Cells and reagents. Huh7, FT3-7 and Huh-7.5 (Huh7 derivatives) cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% fetal calf serum, penicillin, streptomycin, L-glutamine and non-essential amino acids. Stock solutions of ciluprevir (BILN2061), boceprevir (SCH 503024), danoprevir (ITMN-191), and vaniprevir (MK7009) were prepared in DMSO. All final dilutions contained 0.5 % DMSO.

Plasmids. pH77S.2 is a modification of pH77S[1] that contains an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al., in preparation). To insert the Gaussia luciferase (GLuc)-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A (FMDV2A) protein-coding sequence, MluI, EcoRV, and SpeI restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the MluI and EcoRV sites of the modified plasmid after PCR amplification using the primers: 5'- ATA ATA TTA CGC GT*A TGG GAG TCA AAG TTC TGT TTG CC*-3' (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to MluI underlined) and 5'-ATA AAT AGAT ATC *GTC ACC ACC GGC CCC CTT GAT CTT*-3'(C-terminal GLuc italicized and EcoRV underlined). A DNA fragment encoding the 17 amino acids of the FMDV2A protein was generated by annealing the following complementary oligonucleotides: 5'- ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3' and 5'-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT -3' (underlined sequences indicate EcoRV and SpeI). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A (Fig. 1A). pH77S/GLuc2A/AAG is a replication-defective pH77S/GLuc2A mutant. It was generated by inserting an AfeI/AscI restriction fragment containing the GLuc-2A sequence between the corresponding sites of pH77S/AAG, in which the GDD motif of the polymerase, NS5B, is replaced with AAG[1] .

Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S[1]. Recent work in our laboratory has shown that it is not essential for production of infectious virus (Yi et al., in preparation), and, as we demonstrate below, may contribute to weak resistance against some PIs. We removed this mutation from the pH77S.2 and pH77S.2/GLuc2A constructs, replacing Gln_{41} with the wild-type Arg by site-directed mutagenesis of a PCR fragment spanning the AfeI and BsrGI sites. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were re-designated pH77S.3 and pH77S.3/GLuc2A, respectively.

Candidate PI-resistance mutations within the N-terminal domain of the NS3 protein, including V36A, V36G, V36L, V36M, F43S, and T54A, were created by site-directed mutagenesis of the AfeI and BsrGI fragment, which was then inserted back into the pH77S.3 and pH77S.3/GLuc2A constructs. To create mutations downstream in the NS3 sequence, including Q80R, R109K, S138T, R155K, R155T, R155G, R155Q, A156S, A156T, A156V, D168A, D168E, D168H, D168G, D168V, D168N, I170A, and I170T, an intermediate vector was constructed. The BsrGI and NsiI fragment of pH77S.2 was inserted into pGEM-T Easy (Promega) after creating new BsrGI and NsiI sites in the vector. Mutations generated by site-directed mutagenesis were inserted between the BsrGI and BglII sites of this intermediate vector, pGEM-T-BsrGI/NsiI, and subsequently cloned back into pH77S.3 and pH77S.3/GLuc2A. The sequences of all manipulated DNA segments were confirmed by DNA sequencing.

RNA transcription and transfection. RNA was synthesized with T7 MEGAScript reagents (Ambion) after linearizing plasmids with XbaI. Following treatment with RNase-free DNase to remove template DNA, RNA was purified using the RNAeasy Mini Kit (Qiagen). RNA transfection was carried out with the Trans-IT mRNA transfection kit (Mirus) according to the manufacturer's suggested protocol. Transfection protocols were optimized for determination of antiviral susceptibility, RNA replication capacity, and infectious virus yield. Briefly, for cell-based antiviral activity assays, 150 ng RNA was transfected into $4x10^4$ cells seeded into wells of 48-well plates. To test the replication capacity of GLuc-containing constructs, 250 ng RNA was transfected into $8x10^4$ cells in 24-well plates. To test the capacity of the RNA to produce infectious virus, 1.25 µg RNA was transfected into $6x10^5$ cells in 6-well plates.

Luciferase activity assay. Following RNA transfection, cell culture supernatant fluids were collected and fresh medium added at 24h intervals. Secreted GLuc activity was measured in 25-µl aliquots of the supernatant fluids using the GLuc Assay Kit (New England Biolabs) according to the manufacturer's suggested protocol. The luminescent signal was measured on a Synergy 2 (Bio-Tek) Multi-Mode Microplate Reader.

Virus yield determination. Cells were split at a 1:1 ratio 24h after RNA transfection, and the medium replaced with fresh media containing 10 % HEPES every 24h thereafter. Cell culture supernatant fluids were collected at 72h, 96h and 120h after transfection for virus titration. To determine intracellular infectious virus titers, cell pellets harvested after trypsinization were resuspended in complete medium, washed twice with PBS, and lysed by four cycles of freezing and thawing. Lysates were clarified by centrifugation at 4,000 rpm for 5 min prior to inoculation onto naïve cells. Infectious virus was quantified using a fluorescent focus virus titration assay described previously[1]. Briefly, cells were seeded in 48-well plates at a density of $1x10^5$ cells/well 24h prior to inoculation with 50-100 µl of virus-containing medium, clarified freeze-thaw cell lysates, or gradient fractions. Cells were maintained at 37 °C in a 5 % CO_2 environment and fed with 300 µl of medium at 24h later. Following 48 h of additional incubation, cells were fixed in methanol-acetone (1:1) at room temperature for 9 min and stained

with monoclonal antibody C7-50 (Affinity BioReagents, Golden, CO) to the HCV core protein (1:300). After extensive washing, the cells were stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) antibody. Clusters of infected cells staining for core antigen were considered to constitute a single infectious focus-forming unit (FFU); virus titers are reported as FFU/ml. GLuc activity was also measured in supernatant fluids collected at 24 hr intervals beginning 48h after infection.

Virus neutralization. A 100 µl aliquot of supernatant fluids from H77S.2/GLuc2A or H77S.3/GLuc2A transfected cells was mixed with an equal volume of AP33 antibody kindly provided by A. Patel (Medical Research Council Virology Unit, Glasgow, U.K.) or mouse monoclonal IgG antibody (BD Pharmingen) at a concentration of 5 µg/ml and incubated at 37 °C for 1 hour prior to inoculation of the virus/antibody mixture onto cells seeded 24h previously into a 24 well plate. After incubation in a 5% CO_2 environment at 37°C for 24 h, the virus/antibody mixture was removed and 500 µl of fresh medium was added. GLuc activity secreted into the media by the infected cells was monitored as described above.

Antiviral activity assays. Wild-type and mutant viral RNAs were transfected as described above. The medium was replaced with fresh medium containing serial dilutions of the antiviral compounds at 24h, and at 24h intervals thereafter, and secreted GLuc activity determined 72 h post-transfection, as described above. The concentration of each compound required to reduce the amount of secreted GLuc activity by 50% (antiviral EC_{50}) was determined using a 3-parameter Hill equation (SigmaPlot 10.0).

Northern blot analysis. Total RNA, isolated from RNA-transfected cells with the RNeasy Mini Kit (Qiagen) was analyzed using materials provided with the NorthernMax® kit (Applied Biosystems). Briefly, 5 µg of RNA was resolved on a 0.9% denaturing formaldehyde agarose gel, transferred to a BrightStar-Plus nylon membrane (Applied Biosystems) by downward capillary transfer, and hybridized overnight at 68°C with [^{32}P]-labeled antisense riboprobes specific for the HCV 5' nontranslated RNA (5'UTR, 340 nts) (JFH1 virus sequence, which also detects H77 RNA) and actin (included as a loading control). Following extensive washing, the membranes were subjected to phosphor imaging on a Personal Molecular Imager (PMI, Bio-Rad) and band densities were quantified using Quantity One software (Bio-Rad).

Equilibrium ultracentrifugation. Filtered supernatant fluids collected from transfected cell cultures (no FCS) were concentrated 60-fold using an Amicon Ultra-15 Centrifugal Filter Unit (100-kDa exclusion) (Millipore), then layered on top of a preformed continuous 10–40% iodixanol (OptiPrep, Sigma-Aldrich) gradient in Hanks' balanced salt solution (HBSS; Invitrogen). Gradients were centrifuged in a SureSpin 630 Swinging Bucket Rotor (Thermo Scientific) at 30,000 rpm for 24 h at 4°C, and fractions (500 µl each) were collected from the top of the tube. The density of each fraction was measured with a refractometer (Atago). RNA was isolated from each fraction using a QIAamp Viral RNA kit (Qiagen) and subjected to quantitative TaqMan RT-PCR for HCV RNA. Infectious virus titer of each fraction was determined by a fluorescent focus titration assay as described above.

qRT-PCR for HCV RNA. Quantitative TaqMan RT-PCR analysis was carried out using primer pairs and a probe targeting a conserved 221-base sequence within the 5' nontranslated RNA segment of the genome: HCV84FP, GCCATGGCGTTAGTATGAGTGT; HCV 303RP, CACCCTATCAGGCAGTACCACAA (for JFH-1, CGCCCTATCAGGCAGTACCACAA); and HCV146BHQ, FAM-TCTGCGGAACCGGTGAGTACACC-DBH1. TaqMan assays used

reagents provided with iScript One-Step RT-PCR Kit for Probes (Bio-Rad Laboratories) and the Bio-Rad CFX96 Real-Time PCR Detection System. Reactions were incubated at 50°C for 15 min and 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Supplementary Results

Replication competence of H77S.2/GLuc2A RNA. pH77S is a modified infectious molecular clone of the genotype 1a H77c virus; genome-length RNA transcribed from it replicates efficiently and produces infectious virus when transfected into permissive Huh7 cells[1]. The HCV sequence in this plasmid contains five cell-culture adaptive mutations. pH77S.2 is a second-generation derivative of this clone that contains an additional cell culture-adaptive mutation in E2 (see Supplementary Fig. S1A). Virus produced by cells transfected with synthetic RNA derived from pH77S.2 is infectious in Huh7 cells and also in the chimpanzee (Yi et al., in preparation). To facilitate monitoring replication of H77S.2 RNA in transfected cells, we created an in-frame insertion of the Gaussia luciferase (GLuc) sequence, fused at its C terminus to the FMDV 2A autoprotease, between the p7 and NS2 sequences of pH77S.2, as described in the Supplementary Methods above (see also Fig. S1A). GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase[2]. In addition, a signal sequence directs its secretion into cell culture media, allowing real-time, dynamic measurements of GLuc expression without the need for cell lysis. A similar strategy was reported recently for monitoring replication of the chimeric J6/JFH1 virus[3]. For use as negative controls, we prepared parallel constructs in which the GDD motif in NS5B was altered to AAG, thus ablating the ability of the RNA to replicate.

We transfected these RNAs into Huh-7.5 cells and determined the GLuc activity secreted in the media. GLuc activity increased as early as 6h after transfection of either H77S.2/GLuc2A or its related AAG mutant, and continued to rise comparably for each until 24h. After 24h, GLuc activity secreted by the AAG-transfected cells declined while that expressed by H77S.2/GLuc2A-transfected cells began to increase exponentially (Fig. S1B, left panel). These data indicate that GLuc secreted into the media during the first 24h following transfection is derived almost entirely from translation of the input RNA, and thus reflects the transfection efficiency and translational activity of the RNA. Subsequent increases in GLuc activity are due to viral RNA amplification. To quantify this, we collected supernatant fluids at 24h intervals after transfection, calculating the fold-increase in GLuc activity over that present in the 24h sample (Fig. S1B, right panel). While the insertion of the GLuc sequence between p7 and NS2 significantly degraded the replication competence of H77S.2 RNA (see below), the GLuc activity secreted by transfected cells was more than sufficient to provide a robust yet simple system for monitoring viral RNA replication.

An important attribute of this system is that, under the conditions used in these experiments (restricted time frame and relatively low yields of extracellular virus as described below), spread of GLuc-expressing virus within the cell culture does not contribute significantly to the quantity of GLuc detected in extracellular fluids. This was confirmed by demonstrating the absence of any difference in GLuc expression when AP33, a potent murine monoclonal neutralizing antibody[4, 5], was added to the culture fluids following RNA transfection (data not shown). We also observed no increase in the percentage of cells expressing HCV core protein

between 48 and 96 hrs after transfection of H77S.3 RNA (see below) when this was measured directly by flow cytometry (data not shown). These preliminary studies confirmed that the expression of GLuc from these constructs reflects RNA replication in the transfected cells, and has no appreciable contribution from extracellular spread of infectious virus in the cell culture.

One of the cell culture-adaptive mutations in H77S.2, Q41R, is located in the NS3 protease domain[1]. Since this mutation causes resistance to danoprevir and boceprevir in genotype 1b HCV (Table 1), we removed it from H77S.2/GLuc2A, restoring the wild-type residue at this position, Gln_{41} (designated H77S.3/GLuc2A). GLuc expression was unchanged (Fig. 2B), indicating that Q41R is not essential for the replication of H77S.2 RNA. We thus similarly restored the wild-type residue in H77S.2 (designated H77S.3). To further characterize RNA replication, we analyzed RNA by northern blotting 96h and 120h after transfection (Fig. 1C). This revealed comparable abundances of H77S.3 and H77S.2 RNA, confirming that removal of the Q41R mutation had not impaired the efficiency of viral RNA amplification. The abundance of H77S.3/GLuc2A was also similar to that of H77S.2/GLuc2A, but both were only about 40% that of the respective parental genome (Fig. S1C), indicating that insertion of the GLuc2A sequence imposes a cost on replication fitness. Nonetheless, the secreted GLuc activity correlated well with the HCV RNA copy number determined by real-time RT-PCR assay for both H77S.2/GLuc2A and H77S.3/GLuc2A (R^2=0.993, data not shown). These results validate the measurement of GLuc activity as a surrogate indicator of viral RNA replication.

We also assessed the ability of H77S.3 and H77S.3/GLuc2A RNA to produce infectious virus. Media collected 72h and 96h after transfection of H77S.3 RNA contained approximately $4.0x10^3$ and $2.3x10^3$ FFU/ml cell culture-infectious HCV, respectively (Fig. S2A). This is 20- to 40 fold more virus than produced by H77S RNA[1]. The insertion of the GLuc2A sequence in H77S resulted in a 10-fold reduction in the virus yield, with H77S.3/GLuc2A transfected cells producing about $3.0x10^2$ FFU/ml at 72h and 96h post-transfection. We also assessed the infectivity of medium from H77S.3/GLuc2A-transfected cells by measuring GLuc activity. We collected media 96h after transfection of cells with H77S.3/GLuc2A RNA, and inoculated it onto naïve Huh-7.5 cells. This resulted in a 7-fold increase in GLuc activity in the media between 48h and 120h after infection. This was sharply reduced by neutralization of the inoculum with a murine monoclonal antibody to E2, AP33 (5 μg/ml)[6], but not an isotype-control antibody (Fig. S2B). These data confirm that H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA. This is not surprising, in that the GLuc2A insertion was placed between p7 and NS2 (Fig. S1A), both of which play roles in the assembly and release of infectious virus.

Supplementary Table 1. Mutations in the NS3 protease domain reported to confer resistance against selected PIs.

NS3 Residue*	VX-950 Telaprevir	SCH503034 Boceprevir	SCH446211	SCH567312	ITMN191| Danoprevir	TMC435	BILN2061 Ciluprevir	MK7009 Vaniprevir
V36	A,G,L,M†	A,L,M						
Q41		R		R	R	R		
F43		C,S		S	S	I,S,V		
T54	A,S	A		A,S				
V55		A						
Q80						H,K,R	R	
R109			K			K		
S138					T			
R155	G,I,K,L,M,S,T	K,M,Q,T,				K	Q	E,G,K,M,N,Q,S,T
A156	I,S,T,V	S,T,V	T,V	D,T,V	S,T,V	G,T,V	T,V	T,V
D168					A,E,G,H,N,V	A,E,H,I,N,T,V,Y	A,V	E,G,V,Y
V170‡		A,T		A				
S489					R			

Footnotes to Table 1

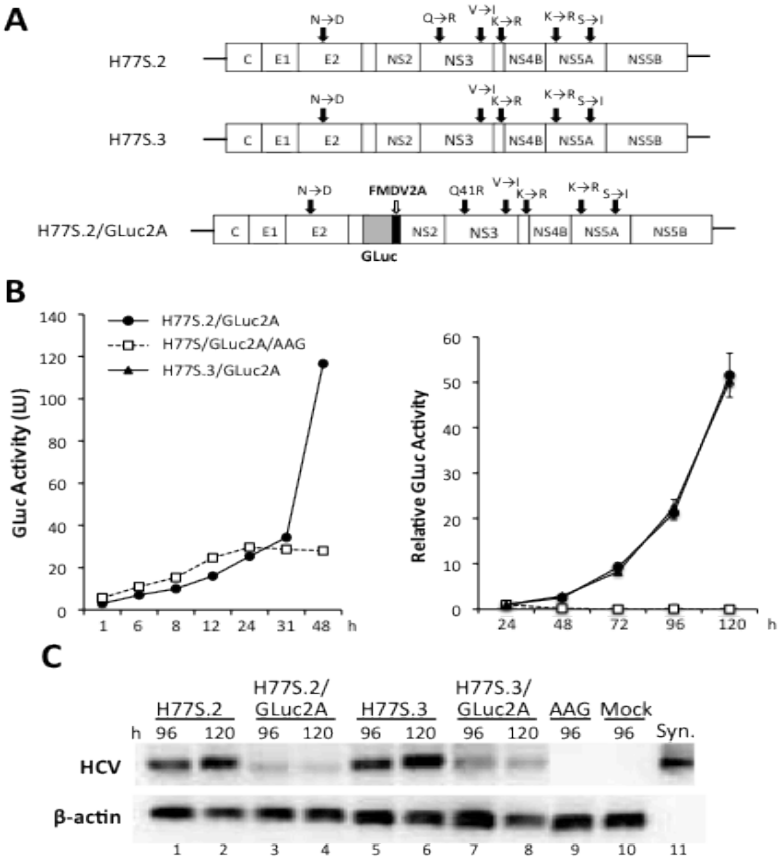
*Numbered from the first residue of NS3.

†Mutations identified in clinical studies are underlined.

‡In H77, residue 170 is not Val but Ile.

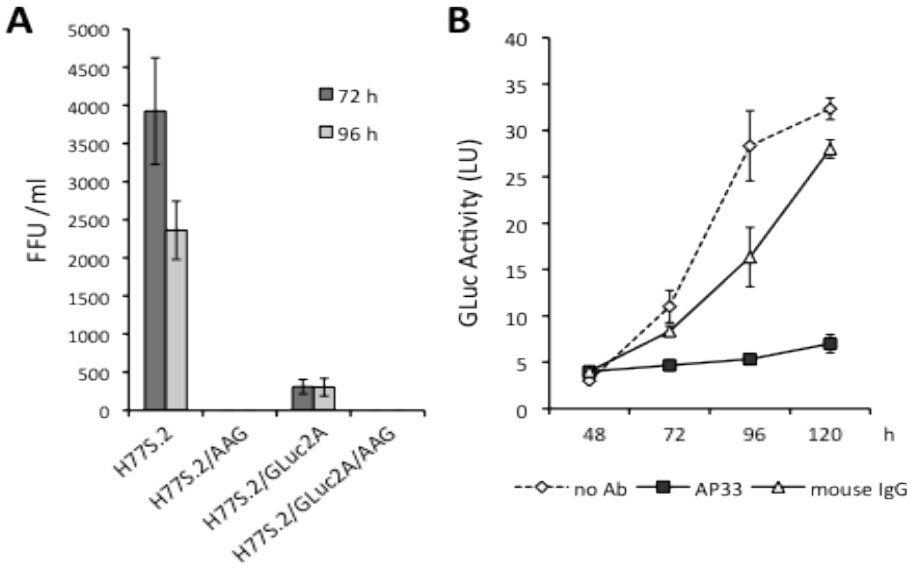
Sources: telaprevir[8, 14, 27]; boceprevir[9, 16, 19, 28]; SCH446211[15]; SCH567312[16]; danoprevir, Seiwert, S. et al. 2006, *International Workshop on Hepatitis C: Resistance and New Compounds*; TMC435[5, 29]; ciluprevir[14, 27, 30]; MK7009, Barnard, R. et al. 2009, *4th International Workshop on Hepatitis C: Resistance and New Compounds*

Supplementary Figure 1



Supplementary Figure 1. Genotype 1a H77S constructs expressing GLuc. (A) Schematic representation of the H77S.2 and H77S.3 genomes. The H77S.2 polyprotein contains six cell-culture adaptive mutations from the wild-type H77c virus sequence: N476.D, Q1067R (NS3 Q41R), V1655I, K1691R, K2040R, and S2204I (numbered according to H77c sequence, GenBank accession No. AF011751). H77S.3 is identical except for the removal of the Q41R mutation (thus H77S.2 is H77S.3/R41Q). The Gluc2A cassette was inserted into the H77S.2 and H77S.3 genomes between the p7 and NS2 sequences (H77S.2/GLuc2A shown only). GLuc, Gaussia luciferase; FMDV, foot-and-mouth disease virus. **(B)** GLuc activity expressed in RNA-transfected cells. H77S/GLuc2A/AAG is a replication-lethal H77S mutant containing the GLuc2A insertion in which the GDD motif of NS5B was altered to AAG[1]. (left panel) The H77S.2/GLuc2A and H77S/GLuc2A/AAG RNAs were transfected as described in Methods, and GLuc activities determined 4, 6, 8, 12, 24, 31, and 48h later. Results are shown as absolute light units and are the mean of results from triplicate samples. (right panel) The indicated RNAs were transfected as described. Medium was collected and replaced at 24h intervals, and GLuc activity determined over five successive days. Results shown are normalized to GLuc activity at 8 hrs and are the mean ± S.D. of triplicate assays. **(C)** Northern blot analysis of HCV RNA. Lanes 1-8, RNA samples extracted from Huh-7.5 cells 96h and 120h after transfection of the indicated RNA; lane 9, RNA extracted 96h after transfection of H77S/GLuc2A/AAG; lane 10, mock transfection, lane 11, in vitro transcribed H77S.3/GLuc2A RNA. β-actin RNA was included as a loading control.

Supplementary Figure 2



Supplementary Figure 2. Infectious virus production by H77S.3/GLuc2A. (A) Supernatant culture fluids were collected 72h and 96h after transfection of the indicated RNA, and immediately inoculated onto naive cells for determination of virus titer using an immunofluorescence assay to detect foci of infected cells. Results shown represent the mean ± S.D. from 3 independent experiments. **(B)** Culture medium was collected 96h after transfection of H77S.3/GLuc2A RNA, then incubated with the indicated antibodies at 37 °C for 1h prior to inoculation onto naïve cells. GLuc activity was determined at 48, 72, 96, and 120h after infection. Results shown represent the mean ± S.D. from 3 independent experiments.

Supplementary References

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