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Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034

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Abstract

HCV NS3 protease variants resistant to the protease inhibitor SCH 503034 were selected. Three mutations, T54A, V170A and A156S mutations conferred low to moderate levels of resistance (<20-fold). Longer exposure (>10 passages) or selection with higher levels of compound led to the selection of a more resistant variant, A156T (>100-fold). [Lin, C., Lin, K., Luong, Y.P., Rao, B.G., Wei, Y.Y., Brennan, D.L., Fulghum, J.R., Hsiao, H.M., Ma, S., Maxwell, J.P., Cottrell, K.M., Perni, R.B., Gates, C.A., Kwong, A.D., 2004. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. J. Biol. Chem. 279(17), 17508–17514; Lu, L., Pilot-Matias, T.J., Stewart, K.D., Randolph, J.T., Pithawalla, R., He, W., Huang, P.P., Klein, L.L., Mo, H., Molla, A., 2004. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro. Antimicrob. Agents Chemother. 48(6), 2260–2266.] Combination with IFN-α drastically reduced the number of emergent colonies. Resistant colonies showed no change in sensitivity to IFN-α.

Although the A156T mutation conferred the highest level of resistance to SCH 503034, it significantly reduced the colony formation efficiency (CFE) of the mutant replicon RNA, and rendered replicon cells less fit than those bearing wild-type replicons. Replicon cells bearing mutation A156S were less fit than wild-type in co-culture growth competition assays but showed no impact on CFE. The V170A mutation, on the other hand, did not affect replicon fitness in either assay, which was consistent with its emergence as the dominant mutant after 12 months of continuous selection. The reduced fitness of the most resistant variant suggests that it may be rare in naïve patients and that development of high-level resistance may be slow. Combination therapy with IFN-α should also greatly reduce the potential emergence of resistance.

Keywords: Hepatitis C virus; Protease; Inhibitor; Resistance; Antiviral

1. Introduction

One of the major issues in development of antiviral therapy is the emergence of drug-resistant variants. In HIV patients treated with protease inhibitors, resistant viruses have been isolated and shown to correlate with relapse of viral replication (Eastman et al., 1998; Romano et al., 2002). Viral resistance can also be developed by selecting the virus with protease inhibitors in vitro, and in many cases the mutations that confer resistance in vitro are the same as those observed clinically (Carrillo et al., 1998; Condra et al., 1995; Hirsch et al., 1998; Markowitz et al., 1995; Molla et al., 1996; Tisdale et al., 1995). These results suggest that resistance studies in vitro could provide insight into what to expect in future clinical trials.

Like most RNA viruses, HCV has a high mutation rate and exists as a complex population of genetically distinct, but closely related, variants commonly referred to as a quasispecies (Cabot et al., 2001; Farci et al., 2002; Martell et al., 1992). Pre-existing minor viral species, resistant to the selecting drug, would gain a growth advantage over the existing wild-type viral population and rapidly become the dominant genotype (Harrigan et al., 2001; Hirsch et al., 1998; Lech et al., 1996; Paolucci et al., 2001).

In addition to reduced susceptibility to the selecting drug, another important factor affecting the outgrowth of the resistant population is the fitness of the mutant viruses. The replicative fitness of resistant viruses is also a critical parameter of viral resistance and has prognostic value with regard to achieving sustained virological response in the clinic. For example, the relative fitness of resistant HIV variants in response to treatment with various protease inhibitors can help guide which of the protease inhibitors should be used in combination therapy

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(Martinez-Picado et al., 2000). Understanding viral fitness is also essential to the predication of prevalence and transmission of resistant viruses in the population especially when the treatment is widely prescribed (Blower et al., 2001; Brenner et al., 2002).

Recently, several groups have reported selection of HCV resistant mutants against two protease inhibitors using the replicon system. Two major resistance loci were reported: mutations at 168 were found to confer resistance to BILN 2061 (a modified macrocyclic peptide, Fig. 1) and its analogues; whereas mutations at 156 impacted both BILN 2061 and VX-950 (a peptidic ketoamide, Fig. 1) (Lin et al., 2004, 2005; Lu et al., 2004; Trozzi et al., 2003). In this report, the selection and characterization of resistance mutations against a novel peptidic ketoamide inhibitor, SCH 503034 (Fig. 1) (Malcolm et al., in press), currently undergoing clinical evaluation, is described. In addition, the impact of the three major resistance mutations (V170A, A156S and A156T) on HCV replicon fitness was studied and the implications for future clinical utility are discussed.

2. Materials and methods

2.1. Cell culture and selection with SCH 503034

Human hepatoma cell line Huh-7 (Nakabayashi et al., 1982) was grown in Dulbecco's minimal essential medium (DMEM) supplemented with 2 mM glutamine, non-essential amino acids (NEAA), 10 mM HEPES, 0.075% sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin, and 10% fetal bovine serum (all cell culture reagents are from BioWhittaker). Cell lines containing replicons were cultured in 0.5 mg/ml of G418 (Cellgro) except for clone 16, for which 1 mg/ml G418 was used. The establishment of HCV replicon cell lines has been previously described (Blight et al., 2000; Lohmann et al., 1999). The replicon sequence used in the study was the same as in (Malcolm et al., in press) where the adaptive mutation S1179I identified by Blight et al. was introduced to increase the colony formation efficiency (Blight et al., 2000).

To select replicon cells resistant to SCH 503034, subconfluent monolayers of clone 16 replicon cells and parental line Huh-7 were cultured with 2.5, 0.5, 0.1, 0.02, or 0 μM SCH 503034. All cells were passed at 1:10 ratio upon reaching 95% confluence.

2.2. Sequence analysis

To identify mutations which conferred resistance to SCH 503034, total cellular RNA was isolated from pooled colonies and amplified by RT-PCR. The primers used to amplify the NS3 coding sequence were: 5′ primer NS3-1642f, GTCAAATGGCTCTCCTCAAGCGTA; 3′ primer NS3-3815r, AAGATGATCCTGCCCACAATGACC. The RT-PCR reactions were carried out following manufacturer's instructions (Titan One Tube RT-PCR, Boehringer Mannheim). Briefly, 0.5–1 μg of RNA was reverse transcribed at 50 °C for 30 min, followed by 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 7 min. The RT-PCR products were purified using QIAquick PCR purification kit (Qiagen) and sequenced using CEQ 2000 Cycle Sequencing kit (Beckman Coulter). Alternatively, the RT-PCR products were cloned into TOPO TA vector (Invitrogen) and plasmid DNA from 6 to 12 bacterial colonies was sequenced. To sequence the NS3/4A and 4A/4B cleavage junctions of NS3 protease, total cellular RNA was RT-PCR amplified using 5′ primer NS3-1642f, GTCAAATGGCTCTCCTCAAGCGTA; and 3′ primer 4B-4048r, ACATATGCTTCGCCCAGAAGGCTTCG. To sequence the 4B/5A and 5A/5B cleavage junctions, total cellular RNA was RT-PCR amplified using 5′ primer F/NS4B, CAGTGGATCAACGAGGACTGCTCC; 3′ primer R/NS5B, GTTGCTCAGTGCATTGATGGGCAG. The sequences were aligned using Lasergene software (DNASTAR).

2.3. Site-directed mutagenesis

To generate mutant proteases carrying a single resistance mutation, the nucleotide changes were introduced using the Quik-change mutagenesis kit (Stratagene). The parental plasmid expressing His-tagged single chain NS3 protease domain, $NS4A_{21–32}$-GSGS-$NS3_{3–181}$ (plasmid p24), was described by Taremi et al. (1998). The Quik-change primers are listed below. For V170A (T to C) mutation, NS3bk-3902f, GAAGGCGGTGGACTTTGCGCCCGTAGAGTCCATGG and NS3bk-3936r, CCATGGACTCTACGGGCGCAAAGTCCACCGCCTTC were used. For A156S (G to T) mutation, NS3BK3861-GTf, GTGGGCATCTTCCGGTCTGCCGTATGCACCC and NS3BK3891GTr, GGGTGCATACGGCAGACCGGAAGATGCCCAC were used. For A156T (G to A) mutation, NS3BK-3861GAf, GTGGGCATCTTCCGGACTGCCGTATGCACCC and NS3BK3891GAr, GGGTGCATACGGCAGTCCGGAAG-

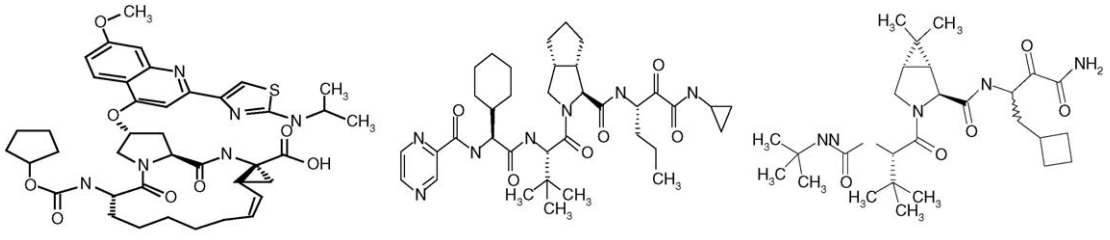

Fig. 1. Compound structures.

ATGCCCAC were used. The mutated nucleotides are underlined.

2.4. Expression and purification of recombinant mutant proteases

The expression and purification protocol was described in detail by Taremi et al. (1998). Briefly, plasmid DNAs encoding mutant proteases were transformed into JM109 cells. Single colonies were used to initiate bacteria culture in 25 μg/ml Kanamycin at 37 °C. When the cell density reached OD_{600} ~1.5, the culture was induced with 0.4 mM IPTG and grown at 23 °C for 4 h. The cell pellet was resuspended in buffer A (25 mM HEPES, pH 7.3, 300 mM NaCl, 0.1% β-octylglucoside, 10% glycerol, 2 mM β-mercaptoethonal or 0.2 mM DTT), and cells were lysed by passage through a microfluidizer (Microfluids Corp). The lysed supernatants were incubated with Ni-NTA beads (Qiagen) for 2 h at 4 °C and then loaded onto columns. The Ni-columns were washed with buffer A supplemented with 20 mM imidazole and 1 M NaCl. The bound His-tagged protease was eluted with buffer A supplemented with 250 mM imidazole. The eluted fractions were pooled and dialyzed at 4 °C for 18 h against 50 mM HEPES, 300 mM NaCl, 5 mM DTT, 0.1% β-octylglucoside and 10% glycerol. The purified proteases were analyzed on 4–12% Novex NuPAGE gel (Invitrogen) and aliquoted for storage at −80 °C.

2.5. Protease activity assay

Recombinant proteases were tested using a chromogenic assay as described by Zhang et al. (1999). The assays were performed at 30 °C in 96-well microtiter plate. Hundred microlitres of protease was added to 100 μl of assay buffer (25 mM MOPS, pH 6.5, 20% glycerol, 0.3 M NaCl, 0.05% lauryl maltoside, 5 μM EDTA, 5 μM DTT) containing chromogenic substrate Ac-DTEDVVP(Nva)-O-PAP based on the NS5A carboxyl terminus coupled to *p*-nitrophenol. The reactions were monitored at an interval of 30 s for 1 h for change in absorbance at 370 nm using a Spectromax Plus microtiter plate reader (Molecular Devices). To determine enzyme concentration to be used in the assay, proteases were tested (1.6–100 nM) to achieve ~12% substrate depletion over the course of the assay. To evaluate kinetic parameters of recombinant proteases, a range of substrate concentrations (0.293–150 μM) was used. Initial velocities were determined using linear regression and kinetic constants were obtained by fitting the data to the Michaelis–Menton equation using MacCurveFit (Kevin Raner Software). Turnover rates were then calculated using the nominal enzyme concentration (2–9 nM). To assess the potency of protease inhibitors, the inhibition constants were determined at fixed concentrations of enzyme (2–9 nM) and substrate (40 μM). The data were fitted to the two step slow-binding inhibition model: $P = v_s t + (v_0 - v_s)(1 - e^{-kt})/k$ of Morrison and Walsh (1988) using SAS (SAS Institute Inc.). The overall inhibition constant K_i^* ($v_s = V_{max} S/(K_m(1 + I/K_i^*))$)was used to measure inhibitor potency.

2.6. Construction of mutant replicon RNA bearing resistance mutations

The replicon sequence used in the study was the same as in Lohmann et al. (1999) except that the adaptive mutation S1179I identified by Blight et al. (2000) was introduced to increase the colony formation efficiency. The resistance mutations were introduced into the S1179I replicon using QuikChange mutagenesis kit (Stratagene). The QuikChange template was plasmid pUC18 1–5 which contained the 5′ half of the subgenomic replicon sequence (T7 promoter to EcoRI site in NS5A). The QuikChange primers were listed below. For V170A (T to C) mutation, NS3Bart2322TCf, GAAGGCGGTGGACTTTGCACCCGTCGAGTCTATGG, and NS3Bart2356TCr, CCATAGACTCGACGGGTGCAAAGTCCACCGCCTTC were used. For A156S (G to T) mutation, NS3Bart2281GTf, GTGGGCATCTTTCGGTCTGCCGTGTGCACCC, and NS3Bart2310GTr, GGGTGCACACGGCAGACCGAAAGATGCCCAC were used. For A156T (G to A) mutation, NS3Bart2281GAf, GTGGGCATCTTTCGGACTGCCGTGTGCACCC, and NS3Bart2310GAr, GGGTGCACACGGCAGTCCGAAAGATGCCCAC were used. The mutated nucleotides are underlined. The BsrGI-EcoRI fragment which contained the coding sequence for NS3 to the N-terminal of NS5A was sequenced to confirm the engineered mutations and used to replace the same region in pUC18 Bart which contained the complete subgenomic replicon sequence.

2.7. Colony formation efficiency (CFE) of mutant replicon RNA and establishment of mutant replicon cell lines

Five micrograms of each replicon RNA was transfected into 5×10^6 Huh-7 cells in 400 μl of PBS at room temperature. Electroporation conditions were 950 μF and 250 V in 0.4 cm cuvette using a Gene pulser system (Bio-Rad). Transfected cells were seeded in 6 or 10 cm dishes and dosed with various concentrations of SCH 503034. Cells were selected with 500 μg/ml G418 for 2–3 weeks until cell colonies were established. One set of the dishes were stained with crystal violet (from Sigma, 0.48 mg/ml in 3% formaldehyde, 30% ethanol, 0.16 mg/ml NaCl) and the numbers of colonies were recorded. The colony formation efficiency was designated as number of colonies established/μg of input RNA. Cells from the duplicate set were pooled and expanded for further analysis.

2.8. Ex vivo dose response studies

To measure dose response to SCH 503034, replicon cells were seeded at 4000 cell/well in 96-well collagen I-coated Biocoat plates (Becton Dickinson). Twenty-four hours post-seeding, protease inhibitors were added to replicon cells. The final concentration of DMSO was 0.5%, fetal bovine serum was 5%, and G418 was 500 μg/ml. Media and inhibitors were refreshed daily for 3 days, at which point the cells were washed with PBS and lysed in 1× cell lysis buffer (Ambion cat #8721). The replicon RNA level was measured using real time PCR (Taqman assay). The amplicon was located in 5B.

The PCR primers were: 5B.2F, ATGGACAGGCGCCCTGA; 5B.2R, TTGATGGGCAGCTTGGTTTC; the probe sequence was FAM-labeled CACGCCATGCGCTGCGG. GAPDH RNA was used as endogenous control and was amplified in the same reaction as NS5B (multiplex PCR) using primers and VIC-labeled probe recommended by the manufacture (PE Applied Biosystem). The real-time RT-PCR reactions were run on ABI PRISM 7900HT Sequence Detection System using the following program: 48 °C for 30 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The ΔCT values ($CT_{5B} - CT_{GAPDH}$) were plotted against drug concentration and fitted to the sigmoid dose response model using SAS (SAS Institute Inc.) or Graphpad PRISM software (Graphpad Software Inc.). IC_{50} was the drug dose necessary to achieve ΔCT = 1 over the projected baseline. IC_{90} was the drug dose necessary to achieve ΔCT = 3.2 over the baseline. Alternatively, to quantitate the absolute amount replicon RNA, a standard curve was established by including serially diluted T7 transcripts of replicon RNA in the Taqman assay. All Taqman reagents were from PE Applied Biosystem.

2.9. Growth competition between mutant and wild-type replicon cell lines

A total of 2×10^5 cells were mixed at ratios of 0/100, 20/80, 50/50, 100/0 of wild-type and resistant replicon cells. Cells were grown in the absence of SCH 503034 and passed at 1:10 when reaching confluence. Cell samples were harvested at various time points as indicated. Total cellular RNA was isolated using RNeasy kit (Qiagen). The percentage of resistant mutants in the mixed culture was measured by pyrosequencing of the mutation sites (Lahser et al., 2003). For mutation at V170, the primers used to generate the amplicon for pyrosequencing analysis were forward PCR primer, CAT CTT TCG GGC TGC; reverse PCR primer, 5′-Biotin-GAC GAG TTG TCC GTG AAG AC. The sequencing primer was AGG CGG TGG ACT TTG. For mutation at A156, the PCR primers were forward PCR primer, TCTTCGGGCGGTCCAC; reverse PCR primer, 5′-Biotin-CTGGAATGTCTGCGGTACGG. The sequencing primer was TGGGCATCTTTCGG. The RT-PCR reactions were carried out following manufacturer's instructions (Titan One Tube RT-PCR, Boehringer Mannheim). Briefly, 0.5–1 μg of RNA was reverse transcribed at 50 °C for 30 min, followed by 94 °C for 2 min, 50 cycles of 94 °C for 15 s, 58 °C for 30 s, 72 °C for 15 s, and final extension at 72 °C for 5 min. The RT-PCR products were processed and assayed using the PSQ 96 System (Pyrosequencing AB, Sweden).

2.10. Estimation of fitness of mutant replicon cells

The fitness of mutant replicon cells was estimated based on the method described by Maree et al. (2000). The ratio of mutant allele over wild-type allele (M_t/W_t) was plotted against time and fitted using the equation:

$$\frac{M_t}{W_t} = \left(\frac{M_0}{W_0}\right) e^{-kt}$$

In which M_0/W_0 is the ratio of the two alleles at time zero, and $k = -rs$ where r is the replication rate constant of wild-type replicon cells (per day); s is the fitness difference (selection coefficient) between mutant and wild-type. The r for wild-type replicon is estimated to be 1 per day based on the observation that the level of replicon RNA seemed to have reached equilibrium with the replicon cell doubling time of 24 h (data not shown); the mutant is therefore expected to replicate with a rate constant of $r_m = 1 + s$ (per day). To determine whether the differences in allele ratio and relative fitness were significant, 95% confidence intervals (95% CI) were calculated for both M_t/W_t and s.

2.11. Western blot analysis

Cell lysates were run on 4–12% Novex NuPAGE gel (Invitrogen) and transferred to PVDF membrane (Invitrogen). The NS3 antibody was a rabbit polyclonal developed in-house and was used at 1:5000. The NS5A antibody (1126) was a monoclonal antibody provided by Dr. Johnson Lau and was used at 1:5000. The NS5B antibody (20A12C7) was a monoclonal antibody developed in-house and was used at 1:5000. The NS4A monoclonal antibody was provided by Dr. Johnson Lau, and was used at 1:5000. The Western blot analysis was carried out using the ECF Western blot kit (Amersham Life Science). The blots were scanned using a Phosphoimager and visualized using ImageQuant software (Molecular Dynamics).

3. Results

3.1. Generation of HCV protease mutants resistant to SCH 503034

Replicon cells were cultured under various concentrations of SCH 503034 with antibiotic selection (G418). Surviving cells were able to maintain a minimal level of replication in the presence of the compound and were evaluated for resistance to SCH 503034. Treatment with up to 10 μM SCH 503034 had no effect on the growth of naïve Huh7 cells. When clone 16 replicon cells were treated with SCH 503034, no effect on cell growth was observed with low concentrations of compound (0.25, 0.5, and $1.2 \times IC_{90}$). Selection of replicon cells was only observed at 2.5 μM ($6 \times IC_{90}$ for clone 16 cells).

3.2. Phenotypic analysis of replicon cells selected with SCH 503034

The sensitivity of replicon cells that had survived SCH 503034 treatment was determined by dose response studies following replicon RNA level directly by real time PCR. Pooled clone 16 replicon cells that had survived 2.5 μM SCH 503034 showed a 12-fold increase in IC_{90} values (Fig. 2). At relatively late passages ($p > 10$), an additional 4-fold increase in IC_{90} was obtained (Fig. 2). The increases in IC_{50} values were similar to those in IC_{90} values (Fig. 2).

In order to investigate the effect of treatment dose on resistance, 10 μM ($25 \times IC_{90}$) SCH 503034 was used to select clone

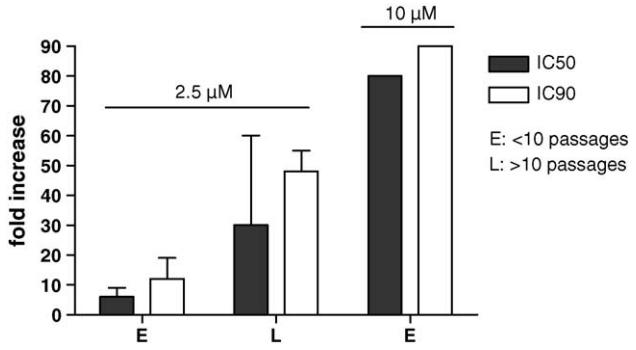

Fig. 2. Increase in replicon sensitivity to SCH 503034. Replicon cells were selected with 2.5 μM or 10 μM SCH 503034. Cells which survived the selection were pooled at early passages ($p < 10$) or late passages ($p > 10$) and assayed for sensitivity to the inhibitor using real time RT-PCR. The fold increases in IC50 and IC90 values are plotted. Error bars represent standard deviation of five independent experiments with the exception of the 10 μM group ($n = 1$).

16 replicon cells as described above. High level of compound resulted in a more significant increase in resistance even at early passage (90-fold increase in IC_{90}, Fig. 2).

In addition to pooled cells, individual replicon cell colonies were also isolated and analyzed for sensitivity to the compound. A 10–14-fold increase in IC_{90} was observed with three colonies selected with 2.5 μM of SCH 503034, and a 55–150-fold increase in IC_{90} was observed with three colonies selected with 10 μM of the compound.

3.3. Identification of mutations associated with resistance to SCH 503034

Resistant cells treated with 2.5 μM of SCH 503034 were pooled, RT-PCR amplified and cloned, and DNA plasmids from individual bacterial colonies were sequenced. Three major resistance loci were identified: T54, A156 and V170, with frequencies ranging from 12 to 31% (Table 1). Two known adaptive mutations, Q86R (Blight et al., 2000) and E176G (Krieger et al., 2001) were also found in some of the resistant cells, consistent with the notion that they might confer certain growth advantages during the outgrowth of cell colonies. No mutations were found in any of the four NS3 cleavage junctions.

Given the observation that resistance to SCH 503034 increased with treatment time, cells that had been passaged under drug for an extended period of time (>p10) were also analyzed by sequencing. Mutation A156T had become dominant in the population (75%), Mutations T54A and A156S remained largely unchanged (~20%). In contrast, V170A was no longer detected (Table 1).

As previously discussed, replicon cells selected with high doses of SCH 503034 exhibited high levels of resistance even at low passage. Sequencing analysis of cells that had survived 10 μM SCH 503034 revealed that mutation A156T was present in 100% of the samples sequenced, whereas A156S and V170A were not detected. The prevalence of the adaptive mutation E176G also increased in parallel (Table 1).

In addition to pooled cells, individual replicon colonies that had survived SCH 503034 selection were also isolated and subjected to sequencing analysis. Similar to results obtained with pooled cells, treatment with 2.5 μM of SCH 503034 gave rise to mutation V170A and A156S (data not shown), whereas higher doses of the inhibitor selected mutation A156T as well as a new mutation at the same position, A156V. Both mutations were associated with adaptive mutations Q86R and E176G (Table 1).

Taken together, the data suggest that mutations T54A, V170A and A156S appear early during low dose of compound selection and confer low levels of resistance. Mutation A156T/V becomes the dominant variant when replicon cells are grown under compound for extended period of time or selected with high doses of the compound, and confers a higher level of resistance. Two known adaptive mutations, Q86R and E176G are consistently observed in association with mutation A156T/V, suggesting that these are compensatory mutations that may help to improve replicon fitness.

Table 1
Frequency of mutations identified in the NS3 protease domain

Mutation	2.5 μM		10 μM
	Early ($p < 10$) (%)	Late ($p > 10$) (%)	Early ($p < 10$) (%)
T54A	31	17[a]	0
A156S	31	17[a]	0
A156T	12	75	100
V170A	25	0	0
Q86R	50	25	56
E176G	44	17	95
A156V, E176G[b]	NA	NA	83
A156T/V, Q86R, E176G[b]	NA	NA	17

NA: not applicable.
[a] A double mutation T54A + A156S was identified in one of the clones.
[b] Mutations identified in resistant replicon colonies.

3.4. Effect of SCH 503034 dose and combination with IFN-α on the emergence of resistant mutants

The frequency of emergence of resistant mutants in relation to SCH 503034 dosage was investigated. The number of colonies that emerged during selection of each compound concentration was used to calculate the frequency of resistant mutants (No. of colonies/No. of input cells). At 6 × IC_{90} the frequency of resistant cells was ~0.14%. A 2-fold increase in drug dose (6 × IC_{90} to 12 × IC_{90}) reduced the number of surviving colonies by an additional 7-fold (~0.02%). A further doubling of drug dose had only a marginal effect (~0.013%) (Fig. 3).

Since IFN-α has been widely used to treat HCV patients (Battaglia and Hagmeyer, 2000; Boyer and Marcellin, 2000; Hoofnagle, 1999), the effect of IFN-α on the emergence of resistant mutants was investigated (Fig. 3). Combination of 6 × IC_{90} of SCH 503034 and 1 × IC_{90} of IFN-α drastically reduced the number of resistant colonies (30-fold) compared with protease inhibitor alone. As shown in Table 2, replicon cells resistant to SCH 503034 were fully responsive to IFN-α. No change in IFN-α sensitivity was observed in either the early passage (≤p10) or

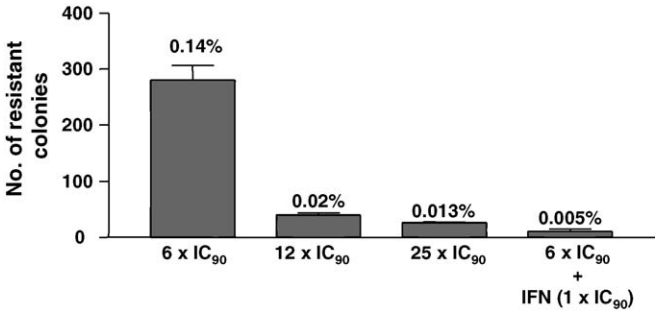

Fig. 3. Effect of SCH 503034 dose and IFN on the emergence of resistant colonies. The IC90 of SCH 503034 was 400 nM, the IC90 of IFN-α was 1 U/ml. Error bars represent standard deviations of 3–9 independent experiments.

Table 2
Dose response of resistant replicon cells to IFN-α

Cells	SCH 503034	$IC_{50} \pm$ S.D. (U/ml)	$IC_{90} \pm$ S.D. (U/ml)
Untreated	0	0.06 ± 0.08	0.8 ± 0.5
Early passage ($p < 10$)	2.5 μM	0.04 ± 0.02	0.6 ± 0.2
Late passage ($p > 10$)	2.5 μM	0.06 ± 0.06	0.7 ± 0.4

late passage (≥p16) cells when compared with parental replicon cells.

3.5. Impact of resistance mutations on protease activity and inhibitor binding

To investigate the impact of representative mutations (T54A, V170A, A156S and A156T) on proteolytic activity, each substitution was introduced into the single chain form of the genotype 1b NS3 protease (in which the essential core region of the NS4A cofactor was fused to the N-termius of the protease domain) (Taremi et al., 1998). Mutation A156S and V170A had little effect on the catalytic efficiency (k_{cat}/K_m) of the protease against a chromogenic peptide substrate (Zhang et al., 1999). Both mutations T54A and A156T decreased the catalytic efficiency by ~5-fold (Table 3).

In contrast to the minimum to modest effect on protease activity, these mutations significantly reduced SCH 503034 binding. Mutation A156T had the biggest impact on inhibitor binding (250-fold increase in K_i^*), whereas T54A, A156S and V170A showed a relatively modest increase in K_i^* (4–19-fold) (Table 3). Taken together these analyses suggested that the mechanism of resistance was mostly due to reduction in enzyme affinity for the inhibitor.

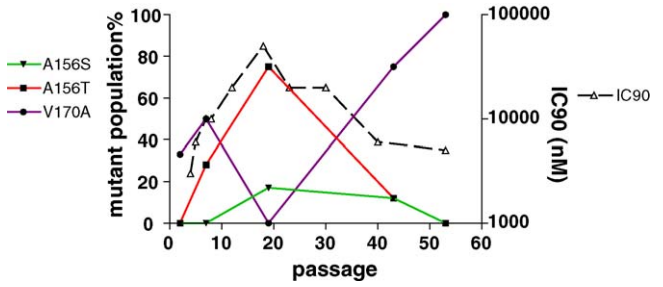

Fig. 4. Relationship between susceptibility to SCH 503034 and prevalence of individual resistance mutations over a 12-month period. Pooled replicon cells which had survived selection with 2.5 μM SCH 503034 were cultured in the presence of the compound for 12 months (53 passages). The percentage of each resistance mutation in the pool was determined by sequencing cloned RT-PCR products of the protease domain. The inhibition of replicon RNA by SCH 503034 was measured by a 10-point dose titration using real-time quantitative PCR (Taqman).

3.6. Long-term study of pooled replicon cells carrying multiple resistance mutations

To study the effect of long-term treatment on resistance mutations, resistant replicon pools were cultured under 2.5 μM SCH 503034 for 12 months (53 passages). Sensitivity to SCH 503034 and prevalence of resistance mutations were monitored periodically. As shown in Fig. 4, The IC90 values of the pool initially increased with time, reached a peak level around passage 20 and then gradually declined. Mutation A156S remained a minor component throughout the course of the experiment (<20%). In contrast, the frequency of mutations A156T and V170A oscillated considerably. The prevalence of mutation A156T in the population correlated with the increase of IC90 values, confirming the previous observation that A156T conferred high levels of resistance to SCH 503034. The prevalence of V170A, on the other hand, was inversely correlated with IC90 values. Mutation V170A was dominant at earlier time points, then decreased below detection level, but eventually became dominant again (to ~100% of the population).

3.7. Impact of resistance mutations on replicon fitness

In order to study the impact of individual mutations on replicon fitness, replicon RNAs carrying single resistance mutations were constructed and used to establish mutant replicon cell lines. As expected, mutations T54A, V170A and A156S caused moderate increases in IC_{90} values (6–15-fold), whereas

Table 3
Impact of resistance mutations on protease activity and inhibitor potency

Mutations	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	Increase in K_i^* (fold)	Increase in IC90 (fold)
WT	9 ± 1	25 ± 5	46000 ± 10000		
T54A	4.5 ± 1	2.5 ± 0.5	9000 ± 3000	4 ($n = 1$)	6 ($n = 1$)
A156S	10 ± 2	19 ± 1	32000 ± 6000	19 ± 8	8 ($n = 1$)
A156T	24 ± 4	17 ± 5	12000 ± 4000	290 ± 30	80 ($n = 1$)
V170A	5.8 ± 0.1	13 ± 1	37000 ± 3000	12 ± 7	16 ($n = 1$)

Standard deviations were calculated from two to five independent experiments unless otherwise indicated.

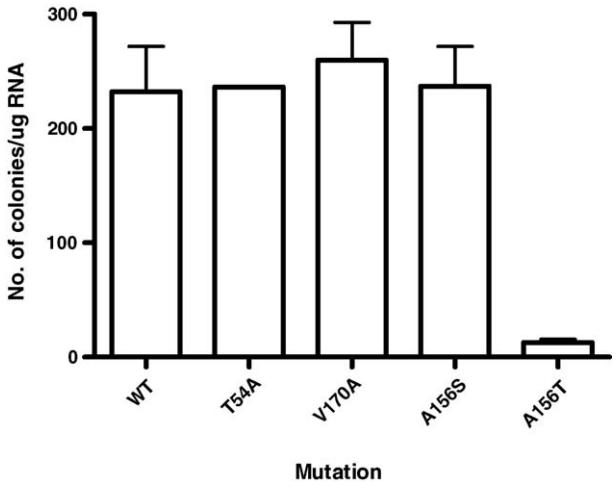

Fig. 5. Colony formation efficiency of replicon RNAs bearing resistance mutations. Replicon RNAs were transfected into Huh7 cells followed by selection with 500 mg/ml G418 for 2–3 weeks until cell colonies were established. The number of replicon colonies/μg input RNA was plotted. Error bars depict the standard error of three independent studies.

the A156T mutation conferred the highest level of resistance (80-fold) (Table 3).

To ascertain replicon fitness, the colony formation efficiency of replicon RNA bearing resistance mutations and direct growth competition studies were performed. As shown in Fig. 5, mutations T54A, V170A and A156S had no adverse effect on colony formation efficiency compared with wild-type RNA, whereas mutation A156T significantly reduced the number of colonies being generated (<5% of wild-type).

In the growth competition experiment, cells containing wild-type and resistant replicons were mixed at various ratios and grown in the absence of SCH 503034. The frequency of mutant cells in the population was determined by monitoring the presence of the mutant allele by pyrosequencing (Lahser et al., 2003). The term relative fitness (1 + s) of a variant represents its relative growth rate over the competing virus. The parameter *s* is defined as the coefficient of selection and can be calculated from the rate constant of the change in the ratio of the two variants (Maree et al., 2000). As shown in Fig. 6A, the ratio of replicon cells bearing V170A remained unchanged when co-cultured with wild-type cells. A slow but measurable decrease in relative abundance was observed with cells bearing A156S at the 50/50 and the 80/20 mixing ratios (Fig. 6B). In contrast, cells bearing the A156T mutation were quickly overgrown by wild-type cells at both mixing ratios (Fig. 6C), suggesting a significant growth disadvantage conferred by A156T. The average selection coefficient of A156S and A156T replicon cells was calculated to be −0.08 and −0.6, respectively (Table 4), therefore the relative fitness (r_m/r) of the two mutant cell lines was 90 and 40% of the wild-type cell lines, respectively. The selection coefficients were similar at both mixing ratios (Table 4), as would be expected for an intrinsic growth property of a cell line.

Table 4
Estimation of selection coefficient (*s*) of mutant replicon cells

Mut/WT	V170A (95% CI)[a]	A156S (95% CI)[b]	A156T (95% CI)[b]
50/50	∼0	−0.10 (−0.19 to −0.001)	−0.8 (−1.4 to −0.2)
80/20	∼0	−0.05 (−0.15 to 0.04)	−0.4 (−0.7 to −0.15)
Average	∼0	−0.08 (−0.12 to −0.03)	−0.6 (−1.0 to −0.2)

[a] No significant change in fitness was observed as shown in Fig. 6A.
[b] Calculated from the decay constant (*k*) from Fig. 6B and C (see Section 2 for details).

There was generally good agreement between the targeted cell mixing ratio (50/50 or 80/20) and the actual initial ratio of replicon RNAs estimated from data shown in Fig. 6. However, it was noted that when A156T containing cells were mixed with wild-type, the initial RNA ratios were lower than expected (i.e. the A156T mutant RNA copy numbers per cell were lower than wild-type). To confirm this, total cellular RNA was isolated and replicon RNA was quantitated by Taqman analysis. V170A and A156S containing cells had similar levels of replicon RNA as did that of wild-type replicon bearing cells, whereas A156T replicon cells contained only about one-third as much. Based on these results, the predicted ratios of A156T/wild-type alleles, when mixed at nominally 50/50 and 80/20 (based on cell count) should be 0.3 and 1.3, respectively—consistent with the data obtained by pyrosequencing (Fig. 6C).

It should be noted that although cells bearing the A156T replicon were less fit than wild-type replicon cells, the mutant cells were relatively stable in the absence of inhibitor or competition from wild-type cells. When the three mutant replicon cell lines were cultured alone without SCH 503034, no spontaneous reversion to wild-type sequence was observed.

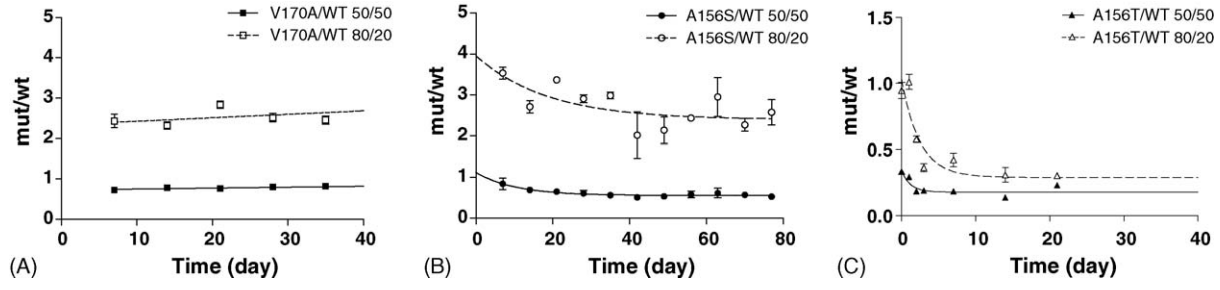

Fig. 6. Relative fitness of mutant replicons in competition with wild-type replicon cells. Mutant replicon cells bearing resistance mutations were mixed with wild-type replicon cells at various ratios and grown in the absence of G418 for up to 11 weeks. The percentage of mutants in the mixed culture was determined by pyrosequencing at the mutation sites ($n = 2$–7). The ratio of V170A versus wild-type replicon cells remained unchanged and was fit by linear regression (4A). The cell population bearing A156S or A156T allele decreased with time, and the data were fit to an exponential decay model (4B and 4C).

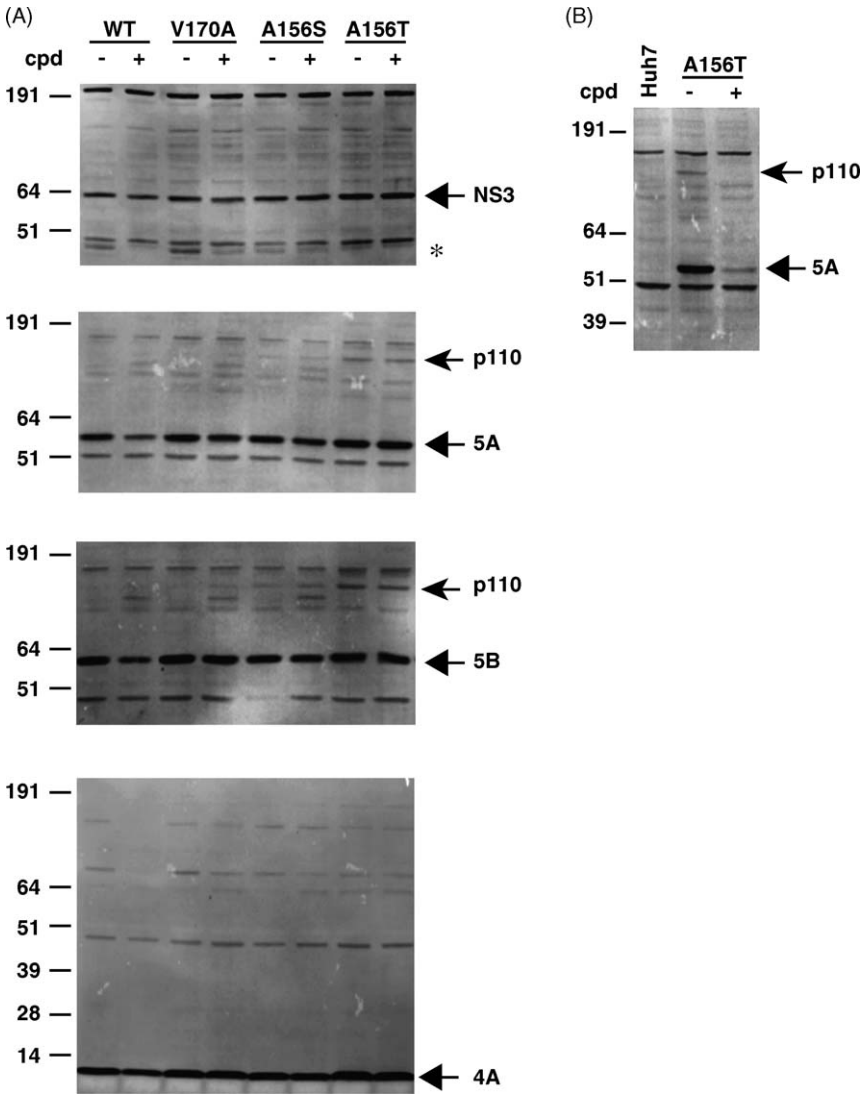

Fig. 7. Polyprotein processing in replicon cells bearing resistance mutations. (A) Replicon cells were treated with or without 2.5 μM SCH 503034. Cells lysates were analyzed by Western blot for NS3, NS4A, NS5A, and NS5B. The processing intermediate (p110) was indicated. The band indicated by the asterisk is likely the internal cleavage product of NS3. (B) Replicon cells bearing A156T mutation were treated with and without 20 μM SCH 446211 and analyzed by Western blot for NS5A. The proteins were quantitated using the ImageQuant software (Molecular Dynamics).

3.8. Polyprotein processing in mutant replicon cell lines

To further investigate the mechanism of reduced fitness in A156T mutant replicon cells, the processing of HCV non-structural proteins in those cells was analyzed by Western blot analysis (Fig. 7A). Parental wild-type replicon cells expressed abundant amounts of HCV proteins (NS3, NS5A, NS5B and NS4A). A polypeptide around 40–45 kDa was detected by NS3 antibody, possibly the internal cleavage product of NS3 (Yang et al., 2000). Treatment with 2.5 μM SCH 503034 drastically decreased the HCV protein level in wild-type cells. In contrast, SCH 503034 had little effect on HCV protein level in the three mutant replicon cell lines, consistent with their resistance phenotype. Interestingly, a 110 kDa (p110) processing intermediate (accounted for ∼7% of the mature proteins) was detected in A156T mutant cells using antibodies against NS5A and NS5B, but not NS3 or 4A (Fig. 6A). Treatment with a high dose (20 μM) of another potent protease inhibitor SCH 446211 (aka SCH 6 (Foy et al., 2003; Yi et al., 2005)) reduced the level of 5A as well as p110 (Fig. 7B), providing further evidence that p110 was a processing intermediate. P110 was likely the uncleaved 5A-5B product based on its size and antibody reactivity. Together with the previous results that mutation A156T decreased peptide substrate binding to NS3 protease in vitro, these data suggest that the reduced fitness of the A156T mutant replicon was at least partly due to decreased polyprotein processing.

4. Discussion

Development of drug resistance is considered a major cause for failure of antiviral therapy. In this study, resistance mutations in the HCV protease domain were generated by culturing replicon cells in the presence of SCH 503034. Similar to what has been reported on HIV resistance mutations, a step-wise increase

in resistance was observed which was associated with emergence of specific mutations. Mutations T54A, V170A and A156S arose after 2–3 passages under drug treatment, and conferred low to moderate levels of resistance. Prolonged treatment of cells with SCH 503034 led to a further increase in resistance and the concomitant increase in prevalence of mutation A156T. Mutations at position 156 have been previously shown to confer resistance to two other protease inhibitors (BILN-2061 and VX-950) (Lin et al., 2004, 2005; Lu et al., 2004). So far no mutations in the polyprotein cleavage sites have been observed with SCH 503034 selection, unlike what has been seen with some of the HIV protease inhibitors (Carrillo et al., 1998; Zhang et al., 1997).

Each of the resistance mutations identified only required a single nucleotide change. The baseline prevalence of these resistance mutations in HCV natural isolates is not clear. Based on analysis of available sequences from Genbank, A156 is conserved in 250 HCV isolates analyzed whereas V170 is more variable. One isolate of genotype 1b (accession number D84265, Tokita et al., 1998) has Ala at position 170 (Ping Qiu, personal communication), therefore the baseline frequency for V170A in clinical isolates could approach 0.4%. In this study 25% of the resistance mutations selected at $6 \times IC_{90}$ of SCH 503034 was V170A. The frequency of resistant colonies (presumably reflecting combined frequency of V170A in the naïve population) was ~0.04%, consistent with the aforementioned estimate of prevalence from natural isolates. Among the factors that were found to influence the rate of emergence were the dose of SCH 503034 and the co-administration of IFN-α. Since more complete suppression of the viral replicon resulted in fewer resistant colonies emerging, it is worth considering designing the therapy to maximally inhibit viral replication early on during treatment.

Mechanistically, the decrease in susceptibility of mutant proteases to SCH 503034 was largely due to reduction in inhibitor binding. Mutations V170A and A156S had no apparent effect on the catalytic efficiency of the protease as measured by a peptide-based assay, but caused a 10- and 20-fold increase in the inhibition constants (K_i^*). Mutation A156T decreased peptide substrate binding by 4-fold, but its impact on inhibitor binding was much more significant (250-fold increase in K_i^*).

Mutations at two other positions were also identified: Q86R and E176G, which seemed to preferentially associate with A156T/V. Both Q86R and E176G had been previously identified as two of the adaptive mutations found in cell-cultured adapted replicons (Blight et al., 2000; Krieger et al., 2001). Furthermore, mutation E176G has been observed in natural isolates at the frequency of about 2% based on the alignment of over 200 HCV sequences (P. Qiu, personal communication). Compensatory mutations are very common in highly resistant HIV strains, and they have been shown to offer growth advantage even in the absence of drug selection (Borman et al., 1996).

Of the mutations conferring resistance to SCH 503034, the two mutations A156S and V170A both gave rise to moderate levels of resistance to the inhibitor, but their impact on replicon fitness was somewhat different. V170A had no observable effect on replicon fitness, whereas the A156S mutant replicon was slightly less fit as measured by direct growth competition with wild-type replicon cells. Mutation A156T, although conferring the highest level of resistance, significantly reduced replicon fitness in both assays. Consistent with these findings, mutation A156T also caused a decrease in protease activity in the peptide-based assay and an accumulation of a polyprotein processing intermediate in replicon cells. Similar phenomena have been reported for HIV protease inhibitors. Certain mutations which confer high levels of resistance also show decrease in enzyme activity and viral fitness (Croteau et al., 1997; Maguire et al., 2002; Mammano et al., 1998). However, the association of A156T with two known adaptive mutations (Q86R and E176G) suggests that compensatory mutations can arise that help improve the fitness of mutant replicons. Recent studies have shown that Q86R indeed partially restores the fitness of A156T replicon (Yi et al., 2005).

To study the effect of long-term selection on resistance mutations, pooled HCV replicon cells composed of a mixed population bearing various resistance mutations (V170A, A156S and A156T) were passaged for 12 months (53 passages) under selection with SCH 503034. The overall resistance of the population and prevalence of individual mutations changed over time. The initial increase of IC_{90} values correlated with the prevalence of mutation A156T. However, mutation A156T gradually disappeared from the population and was replaced by mutation V170A with a concomitant decrease in IC_{90} values for the pool. The exchange can be explained by the relative fitness of these two mutations under the selection conditions. Mutation A156T, although conferring higher levels of resistance (80-fold), had such reduced fitness, that it was eventually outgrown by replicon cells carrying the V170A mutation. The moderate increase in resistance conferred by V170A (16-fold) was apparently sufficient for the cells to survive the selection regimen used in the experiment ($6 \times IC_{90}$), and its superior fitness allowed this variant to dominate the population. Mutation A156S, having no advantage in either fitness or resistance, remained only as a minor component in the population. These data suggest that the replicative fitness of resistant viruses will play a major role in viral dynamics during treatment.

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