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Characterization of resistance mutations against HCV ketoamide protease inhibitors

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Abstract

An issue of clinical importance in the development of new antivirals for HCV is emergence of resistance. Several resistance loci to ketoamide inhibitors of the NS3/4A protease have been identified (residues V36, T54, R155, A156, and V170) by replicon and clinical studies. Using SCH 567312, a more potent protease inhibitor derived from SCH 503034 (boceprevir) series, we identified two new positions (Q41 and F43) that confer resistance to the ketoamide class. The catalytic efficiency of protease enzymes was not affected by most resistance mutations, whereas replicon fitness varied with specific mutations. SCH 503034 and another ketoamide inhibitor, VX-950 (telaprevir), showed moderate losses of activity against most resistance mutations ($\leq$10-fold); the highest resistance level was conferred by mutations at A156 locus. Although SCH 503034 and VX-950 bind similarly to the active site, differences in resistance level were observed with specific mutations. Changes at V36 and R155 had more severe impact on VX-950, whereas mutations at Q41, F43 and V170 conferred higher resistance to SCH 503034. Structural analysis of resistance mutations on inhibitor binding is discussed.

Keywords: Resistance mutation; Boceprevir; SCH 503034; HCV replicon; Protease

1. Introduction

Development of resistance to antiviral medications is an important factor that may limit the effectiveness of therapy for hepatitis C virus (HCV) infections. RNA viruses, such as HCV, have high mutation rates and typically exist as a complex population of genetically distinct but closely related viral variants, commonly referred to as quasispecies (Martell et al., 1992; Farci et al., 2002; Cabot et al., 2001). The high turnover rate of HCV virions *in vivo* (Neumann et al., 1998) is predicted to result in even greater complexity of quasispecies than HIV in chronically infected patients. Upon treatment, the pool of viral variants (10^{12} virions per day) may allow rapid selection and outgrowth of viruses with reduced susceptibility to an antiviral drug. Indeed, natural strains carrying known resistance mutations against HCV protease inhibitors have been detected in HIV/HCV coinfected patients receiving no HCV inhibitors (Morsica et al., 2006).

The HCV NS3 protease has been an important target of drug discovery efforts to develop inhibitors of HCV replication. The HCV genome is translated as a single polyprotein precursor which is processed by cellular and viral proteases into four structural proteins (C, E1, E2 and p7) followed by six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). The N-terminal third of NS3 is a serine protease responsible for the *cis*-processing of the HCV polyprotein at the NS3–NS4A junction. NS3 assembles as a noncovalent complex with NS4A to form the mature protease responsible for the further processing of the non-structural proteins at the NS4A–NS4B, NS4B–NS5A and NS5A–NS5B junctions (for review see Reed and Rice, 2000). The NS3 protease is essential for viral replication (Kolykhalov et al., 2000) and represents an important target for antiviral therapy. Clinical efficacy of NS3 protease inhibitors has been demonstrated with three compounds

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Table 1
Resistance loci against peptidomimetic HCV NS3 protease inhibitors

Locus	Systems used to identify mutations			Reference
	SCH 503034[a]	VX-950	BILN 2061	
V36	Not detected	Replicon/phase I	Not detected	Sheaffer et al. (2004); Sarrazin et al., 2007b
Q41	Replicon	Replicon	Not detected	Sheaffer et al. (2004); this study
F43	Replicon	Not detected	Not detected	This study
T54	Replicon/phase I	Replicon/phase I	Not detected	Sheaffer et al. (2004); Sarrazin et al., 2007b; Tong et al. (2006a); Zeuzem et al. (2005b)
R155	Not detected	Phase I	Replicon	Sarrazin et al., 2007b; Lu et al. (2004)
A156	Replicon	Replicon/phase I	Replicon	Sheaffer et al. (2004); Sarrazin et al., 2007b; Tong et al. (2006a); Lin et al. (2004); Lu et al. (2004)
V170A	Replicon/phase I	Not detected	Not detected	Tong et al. (2006a); Zeuzem et al. (2005a)
D168V	Not detected	Not detected	Replicon	Sheaffer et al. (2004); Lin et al. (2004); Lu et al. (2004)

[a] Identified with SCH 503034 or its analog SCH 567312.

targeting the enzyme active site: SCH 503034 (boceprevir) and VX-950 (telaprevir), both ketoamide derivatives (Zeuzem et al., 2005a; Sarrazin et al., 2007a,b); and BILN 2061, a macrocyclic tripeptide (Lamarre et al., 2003). Mutations conferring resistance to these small molecule inhibitors of the NS3 protease have been identified in the protease domain by selection of replicon cells in the presence of the compounds; many of these mutations have also been detected in patients during clinical trials of VX-950 and SCH 503034 (Table 1) (Sheaffer et al., 2004; Tong et al., 2006a; Zeuzem et al., 2005a,b; Lin et al., 2004; Lu et al., 2004; Sarrazin et al., 2007b). In addition to single mutations, second site mutations have been detected which were shown to confer

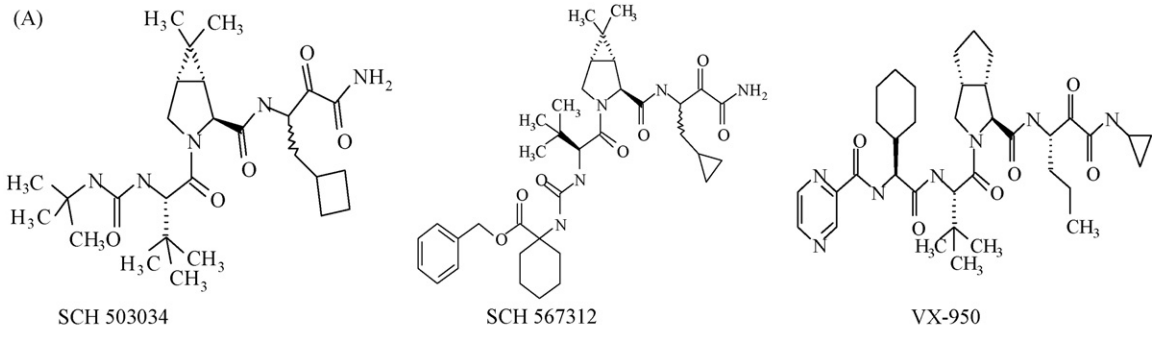

Fig. 1. (A) Structures of HCV NS3 protease inhibitors used in the study. (B) Inhibitor binding to the protease active site. Compound structures are presented as stick model. Magenta, SCH 503034; cyan: SCH 567312; copper, VX-950. The catalytic triad (Asp^{81}, His^{57}, and Ser^{139}) is also shown using CPK model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

higher level of resistance or to increase the fitness of mutant viruses (Yi et al., 2006; Sarrazin et al., 2007b).

Resistance mutations may alter inhibitor–virus interactions in several ways. In addition to reduced susceptibility to inhibitors, mutations may change the fitness of the mutant virus. In the case of the HCV NS3 protease inhibitors, characterization of effects of mutations on enzyme activity and on replication efficiency is an important aspect of interpreting results of clinical trials and assessing long-term efficacy. Furthermore, as shown with HIV, the prevalence and transmission of resistant viruses in the population when therapy is widely prescribed may be affected by the fitness of mutant viruses (Brenner et al., 2002).

Further modification of SCH 503034 has lead to the synthesis of a new compound, SCH 567312 (Bogen et al., 2007), with improved antiviral activity in the replicon system compared with SCH 503034 (Malcom et al., 2006) and VX-950 (Perni et al., 2006) (Fig. 1). The resistance mutations associated with this new compound and further analysis of resistance mutations that affect the ketoamide protease inhibitors SCH 503034, SCH 567312, and VX-950 will be presented in this report.

2. Materials and methods

2.1. Cell culture and selection with protease inhibitors

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 HyClone. Cell lines containing replicons were cultured in 0.5–1 mg/ml G418. The 1b replicon sequence used in this study contains the adaptive mutation S1179I identified by Blight et al. (2000). The establishment of HCV replicon cell lines has been previously described (Blight et al., 2000; Lohmann et al., 1999; Malcom et al., 2006).

To select replicon cells resistant to SCH 567312, 2×10^5 replicon cells and parental Huh-7 cells were seeded in 6-cm tissue culture plates and cultured with 0.25 μM (∼5× IC_{90}), 0.5 μM (∼10× IC_{90}) or 1.5 μM (∼30× IC_{90}) of SCH 567312 and 0.5 mg/ml G418. All cells were passaged at a 1:10 ratio upon reaching 95–100% confluence. Surviving replicon colonies were counted and expanded for further analysis.

2.2. Sequencing analysis

To identify mutations that confer resistance to compounds, 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 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 a QIAquick PCR purification kit (Qiagen) and sequenced using a CEQ 2000 Cycle Sequencing kit (Beckman Coulter). Alternatively, the RT-PCR products were cloned into TOPO TA vector (Invitrogen) and plasmid DNA from 7 to 24 bacterial colonies was sequenced. The sequences were aligned using Lasergene software (DNASTAR).

2.3. Construction and purification of recombinant mutant proteases

To generate mutant proteases carrying a single resistance mutation, the nucleotide changes were introduced using the QuikChange mutagenesis kit (Stratagene). The parental plasmid expressing His-tagged single chain NS4A–NS3 protease domain, $NS4A_{21-32}$–GSGS–$NS3_{3-181}$, was described by Taremi et al. (1998).

The expression and purification protocol was described in detail by Taremi et al. (1998). Briefly, plasmid DNAs encoding mutant proteases were transformed in JM109 cells. When the cell density reached $OD_{600} \sim 1.5$, 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-mercaptoethanol or 0.2 mM DTT), and cells were lysed by passage through a microfluidizer (Microfluids Corp.). The lysed supernatants were incubated with Ni-NTAbeads (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.

2.4. 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 plates. One hundred microliters of protease were 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 assess the potency of protease inhibitors, the inhibition constants were determined at fixed concentrations of enzyme (which would achieve approximately 12% substrate depletion) 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 $K_i^*(v_s = V_{max}S/(K_m(1 + 1/K_i^*)))$ was used to measure inhibitor potency.

2.5. Construction of mutant replicon plasmids

The replicon construction has been described previously (Tong et al., 2006a). The QuikChange (Stratagene) template was plasmid pUC18 1-5, which contained the 5′-half of the subgenomic replicon sequence (T7 promoter to EcoRI site in NS5A). The PmeI-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.

2.6. 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 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 study compounds. Cells were selected with 500 μg/ml G418 for 2–3 weeks until cell colonies were established. One set of the dishes was 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.

2.7. Replicon assay

To measure anti-replicon activity of compounds, replicon cells were seeded at 4000 cells/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%. 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 probed as recommended by the manufacturer (PE Applied Biosystem). The real-time RT-PCR reactions were run on the AIB PRISMS 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.

2.8. X-ray structural analysis

The crystal structure of SCH 503034 complexed with the NS3/4A protease was solved in house (PDB code 2OBO). The coordinates of SCH 567312 was modeled based on those of SCH 503034. VX-950 was modeled based on SCH 503034 and of another ketoamide inhibitor which has the same N-terminal cap (PDB code 1RGQ).

3. Results

3.1. In vitro activity of SCH 567312

SCH 567312 is a potent inhibitor of NS3 protease, and structural modeling showed it to bind to the enzyme active site in a similar fashion as two previously published ketoamide inhibitors, SCH 503034 and VX-950 (Fig. 1B). The inhibition constant (K_i^*) of genotype 1b protease was 60 ± 9 nM as measured by a continuous spectrophotometric assay (Zhang et al., 1999). In the replicon system, the drug concentrations required to suppress viral RNA level by 50% (IC_{50}) and 90% (IC_{90}) were 11 ± 7 nM and 40 ± 18 nM in a 3-day replicon (1b) assay.

3.2. Selection of resistance mutants against SCH 567312

Replicon cells were cultured in the presence of the antibiotic G418 and various concentrations of SCH 567312. The number of surviving replicon colonies was counted and the frequency of resistant colonies was found to decrease as the dose of SCH 567312 was increased, from >0.25% at a dose of 5× IC_{90}, to 0.25% at 10× IC_{90}, to 0.05% at 30× IC_{90}.

3.3. Identification of mutations associated with resistance to SCH 567312

Replicon RNA was purified from pooled resistant replicon cells, amplified by RT-PCR and cloned; DNA plasmids from individual bacterial colonies were sequenced for the NS3 protease region. Mutation patterns were found to change with dose of SCH 567312 and also with the number of passages during incubation with SCH 567312 (Table 2). With lower concentration (5× IC_{90}) of SCH 567312, during early passages (<10), the population was a mixture of wild type sequences and several mutant species, Q41R, T54A/S and A156T/V/D. At later passages (>10), the wild-type population had almost disappeared, and two additional mutant species (F43S and V170A) were detected. Out of the five resistance loci, T54, A156 and V170 have been shown with SCH 503034 (Tong et al., 2006a), whereas Q41R and F43S are novel mutations.

With higher concentration of SCH 567312 (30× IC_{90}), wild-type replicon RNA was more efficiently inhibited and was not detected at the end of the selection process. The mutation pattern was distinct from that with lower concentration of SCH 567312. Changes at A156 locus were the dominant species and two double mutations emerged. The Q86R component of one of these double mutations, A156T/V/D + Q86R, is a known adaptive mutation (Blight et al., 2000) and has been shown to increase

Table 2
Frequency and effect of resistance mutations identified with SCH 567312

Mutation	Frequency[a]				Enzyme resistance[b] (K_i^*-fold/WT)
	Selected with 5× IC_{90} (0.25 μM)		Selected with 30× IC_{90} (1.5 μM)		
	Passage <10	Passage >10	Passage <10	Passage >10	
None (wild-type)	43%	4%	0	0	1
Q41R	28%	21%	0	0	10
F43S	0	21%	0	0	4 (F43C)
T54A/S	14%	12%	0	0	4 (T54A)
A156T/V/D	14%	8%	92%	67%	940 (A156T)
V170A	0	33%	0	0	10
A156T/V/D + Q86R	0	0	8%	25%	860 (A156T + Q86R)
A156T/V/D + F43S	0	0	0	8%	Not done
Number of clones tested	7	24	12	12	

[a] Number of colonies with mutation per number of total colonies sequenced.
[b] For resistance loci with multiple mutations, one representative mutant was tested as indicated in parenthesis.

replication efficiency of a replicon carrying the A156T mutation (Yi et al., 2006); whereas the other, A156T/V/D + F43S, consists of two resistance mutations.

The resistance phenotype of protease mutations identified with SCH 567312 was confirmed in the enzymatic assay (Table 2 and see also Section 3.4). The two newly identified mutations at Q41 and F43 conferred moderate level of resistance (10- and 4-fold, respectively). The highest level of resistance was conferred by A156T. The fold increase in resistance by A156T was similar to that by the double mutant A156T + Q86R (Table 2), and Q86R by itself did not confer resistance to SCH 567312 (data not shown), confirming that Q86R is an adaptive mutation which does not contribute to resistance of SCH 567312.

As highlighted in Fig. 2, the various resistance loci against ketoamide class of protease inhibitors can be grouped into two clusters, one on each side of the catalytic Ser^{139}. The P′-side cluster consists of V36, Q41, F43 and T54; the P-side cluster consists of R155, A156, and V170.

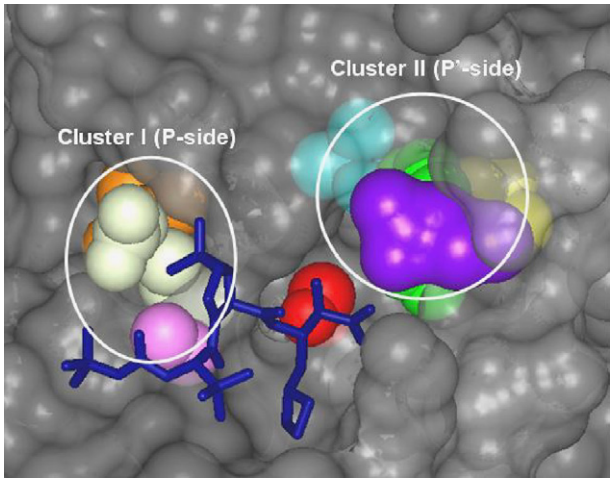

Fig. 2. Structural view of resistance loci in NS3 protease. The structure of SCH 503034 is presented as a stick model. Side chains of key residues are shown using CPK models on the Connolly surface of the NS3 protease. Colors of residues are as follows: red, active site Ser^{139}; orange, Val^{170}; white, Arg^{155}; pink, Ala^{156}; purple, Gln^{41}; cyan, Thr^{54}; green, Phe^{43}; yellow, Val^{36}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Enzyme kinetics of mutant proteases

To investigate whether the mutations in the NS3 protease selected by SCH 567312 affected proteolytic activity, each substitution was inserted into the single-chain form of the 1b NS3 protease (Taremi et al., 1998). The F43S mutant protein was found to be unstable in bacterial culture, and a different substitution (F43C) was constructed. In addition, several most prevalent resistance mutations detected in clinical trials (Sarrazin et al., 2007b) were included in the study. Two double mutants carrying mutations from each of the two clusters were also constructed to evaluate potential interactions between different resistance loci. Among the SCH 567312 selected mutations, the kinetic parameters of mutant proteases T54A, V170A, A156T and A156T + Q86R have been previously described (Tong et al., 2006a; Yi et al., 2006). In general, resistance mutations did not affect catalytic activity of the enzyme (<3-fold) as measured with a P-side substrate (Table 3).

3.5. Colony formation efficiency of mutant replicon cell lines

In order to assess the effect of mutations on viral fitness, replicon RNAs carrying representative resistance mutations were transfected into Huh-7 cells to determine colony formation efficiency. F43S, T54A, and V170A mutations produced similar growth characteristics to wild-type and the Q41R mutation exhibited 10-fold higher colony formation efficiency than wild-type replicon cells. In contrast, V36M and R155K mutations were associated with fivefold lower colony formation efficiency and a double mutation of V36M + R155K was associated with a 17-fold lower colony formation efficiency compared with wild-type cells (Fig. 3). As shown previously, the A156T mutation reduced the number of replicon colonies by ∼20-fold, whereas the adaptive mutation Q86R partially rescued the double mutant A156T + Q86R (Yi et al., 2006).

Table 3
Enzyme kinetic parameters of mutant NS3 proteases

Enzyme mutation	Mean (S.E.) K_m (μM)	Mean (S.E.) k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1}$)
None (wild-type)	3.9 (0.4)	24 (1)	100,000
V36A	4 (1)	12 (1)	50,000
V36M	7.6 (0.5)	146 (4)	320,000
Q41R	1.2 (0.4)	10 (3)	140,000
F43C	2.6 (0.6)	2.6 (0.2)	17,000
T54S	9 (1)	108 (3)	200,000
R155K	6 (1)	13.3 (0.5)	37,000
R155Q	6.7 (0.4)	8.2 (0.2)	20,000
V36M + R155K	12 (1)	67 (2)	93,000
T54S + R155K	15 (1)	53 (2)	59,000

S.E., standard error.

3.6. Comparative protease resistance study of two clinical candidates

SCH 503034 and VX-950 are two ketoamide protease inhibitors currently under clinical trial, and resistant viruses have been observed in patients undergoing treatment. The capacity of mutations in the NS3 protease to confer enzymatic (Table 4A) and replicon (Table 4B) resistance to these two compounds was assessed. Both assays showed that most of the mutations in NS3 protease moderately affected the activity of SCH 503034 and VX-950, with ≤10-fold increase in K_i^* or replicon IC_{50} values. For both compounds, the highest level of resistance was conferred by the A156T mutation. The double mutants carrying mutations from each of the two clusters appear to confer the level of resistance that is multiplicative of individual mutations, suggesting that the impact of these changes on binding free energy is additive.

To evaluate whether there are differences in inhibition of mutant proteases by SCH 503034 and VX-950, the relative resistance (log[fold resistance against SCH 503034/fold resistance against VX-950]) for each mutation was calculated from data presented in Table 4. The estimated 95% confidence interval of the assays (calculated from multi-day averages) is 1.5–2-fold, we therefore set 1.5-fold (i.e. a relative resistance of 0.2) as the threshold for qualifying differences in resistance. Mutations at some positions (T54 and A156) affected SCH 503034 and VX-950 to the same extent (relative resistance ~0), as measured by both enzymatic (Fig. 4A) and replicon assays (Fig. 4B). SCH 503034 was more affected by Q41R, F43C/S and V170A (relative resistance ≥ 0.2), whereas VX-950 lost more activity against mutations at V36 and R155 (relative resistance ≤ −0.2). The P-side mutation in the two double mutants is R155K. Consistent with the results from single mutants, SCH 503034 was only moderately affected by the changes (~10-fold), whereas VX-950 lost significant activity (30–70-fold) on the two double mutants.

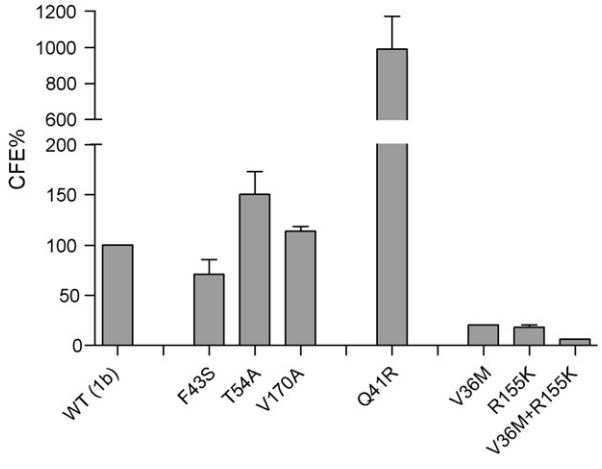

Fig. 3. CFE of mutant replicon cells bearing resistance mutations compared with wild-type replicon cells. Replicon RNAs were transfected into Huh-7 cells followed by selection with 0.5 mg/ml G418 for 2–3 weeks until cell colonies were established. The percent of CFE normalized to wild-type RNA was plotted.

Table 4
Fold of resistance conferred by mutations in NS3 protease against SCH 503034 and VX-950 in (A) enzyme assay and (B) replicon assay

Mutation	SCH 503034	VX-950
(A) Enzyme resistance (K_i^*-fold over wild-type enzyme)[a]		
V36M	2	3
V36A	2	5
Q41R	2	1
F43C	7	2
T54A	6	10
R155K	3	10
R155Q	3	7
A156S	24	33
A156T	300	400
V170A	7	3
V36M + R155K	14	71
T54S + R155K	12	34
(B) Replicon resistance (IC_{50}-fold over wild-type replicon cells)[b]		
V36M	3	10
Q41R	3	1
F43S	5	2
T54A	6	5
R155K	4	10
A156S	16	15
A156T	85	>80
V170A	12	4
V36M + R155K	10	70

[a] Average K_i^* on WT enzyme is 20 nM for SCH 503034 and 30 nM for VX-950.
[b] Average IC_{50} on WT replicon is 200 nM for both SCH 503034 and VX-950.

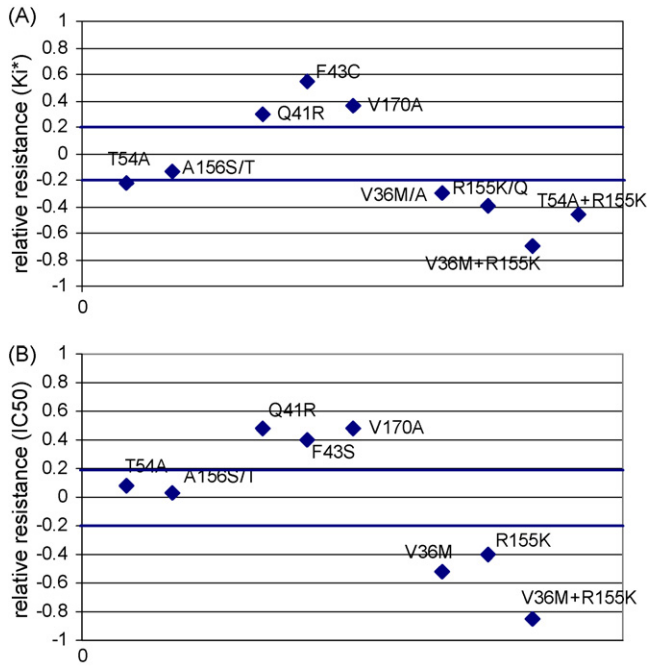

Fig. 4. Comparison of resistance mutations on inhibition by SCH 503034 and VX-950 using (A) enzyme assay (K_i^*) and (B) replicon assay (IC_{50}). Relative resistance of a specific mutation is defined as log[fold resistance against SCH 503034/fold resistance against VX-950].

4. Discussion

We have identified two novel resistance loci (Q41 and F43) using SCH 567312, a more potent derivative of the HCV protease inhibitor SCH 503034, and further characterized resistance mutations associated with ketoamide inhibitors such as SCH 503034 and VX-950. The known mutations that confer resistance to these compounds can be grouped into 2 clusters, one on each side of the catalytic Ser^{139} (P- and P′-side). Although resistance mutations reduced inhibitor binding, mutant proteases cleaved substrate efficiently *in vitro*, suggesting that the peptide substrate can accommodate the changes in the mutant active site probably due to its greater flexibility. The effect of resistance mutations on replicon fitness was dependent on specific mutations; some mutations had no effect, others reduced replicon fitness, and one mutation (Q41R) was found to increase colony formation efficiency by 10-fold. The observation that certain active site resistance mutations can change replicon fitness without affecting protease activity *in vitro* may indicate that enzymatic cleavage of the natural substrate (polyprotein) may have different requirements from the assay substrate and/or NS3 protease activity may be modulated by other viral proteins. The rank order of replicon fitness of resistant mutants (A156T < R155K∼V36M < T54A) as measured by colony formation efficiency is similar to the results (A156T < R155K∼V36M∼T54A) obtained with transient replication assay (Zhou et al., 2007a,b). Moreover, the lower fitness of these resistant viruses was confirmed by recent clinical results (A156T < R155K < T54A < V36M) (Sarrazin et al., 2007b). The exception is mutant V36M, which seems to exhibit better *in vivo* fitness either as a single mutant or in the context of double mutants (V36M + R155K). Clinical data on additional mutants would be useful in assessing the relevance of replicon fitness studies.

As observed with SCH 503034 (Tong et al., 2006a), the frequency of emergence of resistant mutants was reduced by higher dose of SCH 567312, suggesting that high inhibitor exposure coverage in the clinic may be one way to suppress resistance. At higher dose of SCH 567312, the increased selection pressure favored outgrowth of mutants with changes at A156, which have been shown to confer high level of resistance but suffer significant loss in replicon fitness. The compensatory mutation (Q86R) which has been shown previously to improve A156T fitness (Yi et al., 2006) was also detected during high dose SCH 567312 selection, and the frequency of A156T + Q86R double mutant increased with continuous compound treatment. Thus the emergence and prevalence of specific resistance mutations seems to evolve with drug selection pressure and treatment time.

Double mutations consisting of mutations from each of two clusters have been found in replicon studies and clinical trials, and are shown here and by Zhou et al. (2007b) to confer higher level of resistance than that of individual mutations. The impact of these double changes on binding free energy appears to be additive, suggesting that inhibitors bind to the P and P′-side independently. The replicon fitness of double mutants, however, is significantly lower than replicons bearing either of the two single mutations, as shown by this report (V36M + R155K) and by Zhou et al. (2007b) (V36M + A156T). Combined with the higher genetic barrier (i.e. two mutational events), the emergence of double mutants in the clinic probably requires high drug selection pressure and/or long-term treatment.

The two ketoamide inhibitors currently in clinical trials, SCH 503034 and VX-950, have been shown to form similar hydrogen bonding networks with residues at the active site, and exhibit similar resistance profiles against mutations A156T and D168V/Q (Guo et al., 2006). Our results showed that changes at T54 also had similar impact on the two inhibitors. On the other hand, certain mutations appear to differentially affect SCH 503034 and VX-950. Of particular interest are mutations R155K and V170A. R155K is a major resistance mutation identified in clinical trials of VX-950 and confers higher resistance to VX-950 than SCH 503034. The X-ray structure of the mutant protease R155K has recently been published (Zhou et al., 2007a,b). In the wild type protease structure, the Arg^{155} side-chain makes several van der Waals contacts with the bicyclic P2 group of VX-950. In the R155K mutant protease, the side chain of Lys^{155} adopts a conformation extending away from the P2 group, and makes only one or two direct contacts with the inhibitor. The P2 group of SCH 503034 (isopropyl-proline) is smaller than that of VX-950 (cyclopental-proline), and is likely to interact less with the side chain of Arg^{155} in the wild type enzyme, thus is predicted to be less affected by any mutations at position 155. Another clinically relevant mutation is V170A, which has been detected in phase I trials of SCH 503034 (Zeuzem et al., 2005a), but so far has not been reported with VX-950. Our results showed that SCH 503034 lost more activity on the V170A mutant than VX-950. Structural modeling predicted that the side chain of Val^{170} is in van der Waals contact

with the side chain of Arg^{155} (Fig. 2). Mutation of Val to the smaller residue Ala may allow more movement of Arg^{155} side chain therefore loosening the contacts with the small P2 group of SCH 503034. In the case of VX-950, the effect of the minor movement of the side chain of Arg^{155} in the V170A mutant may be less dramatic due to the larger size and more flexibility of its P2, thereby retaining more interaction between Arg^{155} and the inhibitor.

In summary, single mutations at most resistance loci confer only low to moderate level of resistance (≤10-fold) to SCH 503034 and VX-950. Modeling suggest that their impact on active site conformation is minimal (data not shown), reflecting the natural conservation of active site (Tong et al., 2006b). In the clinical setting, achieving high trough coverage may be critical to the suppression of such mutant viruses with low level of resistance. The level of resistance conferred by A156T and the double mutants is higher, and inhibition of these mutants may require drug concentration beyond what is achievable with current ketoamide inhibitors which have limited plasma exposure and short half-life in animals (Kempf et al., 2007). It remains to be determined how the lower fitness of these mutant viruses may affect viral dynamics and disease manifestation. As has been successfully demonstrated with HIV therapy, combination treatment (e.g. protease inhibitors with other anti-HCV agents such as interferon/ribavirin and polymerase inhibitors) may enhance efficacy and suppress emergence of resistance.

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