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A novel HCV NS3 protease mutation selected by combination treatment of the protease inhibitor boceprevir and NS5B polymerase inhibitors

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ABSTRACT

Boceprevir (SCH 503034) is an orally active novel inhibitor of the hepatitis C virus (HCV) NS3 protease currently in clinical development for the treatment of hepatitis C. In this *in vitro* study, we demonstrate that combination of boceprevir with a nucleoside analog or a non-nucleoside HCV NS5B polymerase inhibitor was superior to treatment by single agents in inhibiting viral RNA replication in replicon cells. In the presence of boceprevir (at $5\times EC_{90}$), the addition of 2′-C-methyl-adenosine or an indole-*N*-acetamide targeting the polymerase finger-loop site (at $1\times EC_{90}$) significantly reduced the emergence of resistant replicon colonies. A higher dose ($5\times EC_{90}$) of either of the polymerase inhibitors in combination with boceprevir suppressed replicon resistance further to below detectable levels. Sequencing analysis of replicon cells selected by the combination treatment revealed known resistance mutations to the two polymerase inhibitors but no previously reported resistance mutations to boceprevir. Interestingly, a novel mutation (M175L) in the protease domain was identified. The dually resistant replicon cells were monitored for over 30 passages and sensitivity to polymerase inhibitors was found to decrease over time in a manner that correlated with the increasing prevalence of specific resistance mutations. Importantly, these cells remained sensitive to interferon-α and different classes of polymerase inhibitors. These findings support the rationale for clinical evaluation of combination treatment of HCV protease and polymerase inhibitors.

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Despite recent advances, chronic hepatitis C remains a challenging infection to eradicate. Approximately 50% of patients infected with the most prevalent form of the virus (genotype 1) fail to respond to treatment with the current standard of care of pegylated interferon-α in combination with ribavirin (Manns et al., 2001; Fried et al., 2002). Thus, there is great unmet medical need for the development of new HCV antivirals to improve sustained virological response (SVR). The HCV genome is translated as a single polyprotein precursor which is processed by cellular and viral proteases into structural proteins (C, E1, E2) followed by nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The N-terminal region of NS3 encodes a serine protease responsible for the processing of the HCV polyprotein at the NS3–4A, NS4A–4B, NS4B–5A and NS5A–5B junctions (for review see (Reed and Rice, 2000)). The replication of viral RNA is catalyzed by the NS5B RNA-dependent RNA polymerase, which exhibits the classic "right-hand" shape as other polymerases, with palm, thumb and finger-loop subdomains (Lesburg et al., 1999; Bressanelli et al., 1999). Both the HCV NS3 protease and the NS5B polymerase are essential for HCV replication and have been targets of intense drug discovery efforts.

Boceprevir (SCH 503034) is a ketoamide inhibitor targeting the active site of the NS3 protease (Malcolm et al., 2006) and has demonstrated antiviral activity as monotherapy and in combination with pegylated interferon-α (Sarrazin et al., 2007; Zeuzem et al., 2005a). Mutations conferring resistance to boceprevir have been identified in the NS3 protease domain by selection of replicon cells in the presence of protease inhibitors (Tong et al., 2008; Tong et al., 2006); resistance mutations have also been detected in patients during clinical trials (Zeuzem et al., 2005a,b). The major resistance loci for boceprevir are V36, Q41, F43, T54, R155, A156 and V170, all located near the inhibitor binding site. Against the NS5B polymerase, several classes of inhibitors targeting the active site (nucleoside analogs) and allosteric sites (non-nucleoside inhibitors) have been described (for review see (Carroll and Olsen, 2006; Beaulieu, 2007)). The 2′-C-methyl modified nucleoside analogs (such as 2′-C-methyl-adenosine, -guanosine and -cytidine) have been shown to be chain-terminating inhibitors of NS5B-catalyzed RNA replication. The S282T mutation located

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within the active site confers resistance to this series of compounds (Migliaccio et al., 2003). Different classes of non-nucleoside allosteric inhibitors have exhibited distinct resistance profiles. The major resistance loci are C316 and M414 for palm site binders, M423 and L419 for thumb site inhibitors, and P495 for finger-loop inhibitors (for review see (Beaulieu, 2007)).

Although clinical proof-of-concept has been achieved by HCV protease and polymerase inhibitors such as boceprevir (Zeuzem et al., 2005a), a nucleoside analog (NM283) (Godofsky et al., 2004), and a non-nucleoside polymerase inhibitor (HCV-796) (Villano et al., 2006), the emergence of viral resistance is likely to limit the use of these direct antivirals as monotherapy. Clinical studies combining a small molecule inhibitor with interferon have shown improved antiviral activity and suppression of on-therapy viral load rebound (Sarrazin et al., 2007; Kneteman et al., 2009; Villano et al., 2007). To improve response rates in HCV-infected individuals, especially in interferon non-responders, the combination of two or more small molecule inhibitors with different mechanisms and/or non-overlapping resistance profiles may be required. In this report, we used the replicon system to study the combination of a protease inhibitor boceprevir with two classes of polymerase inhibitors (a nucleoside analog and a finger-loop allosteric inhibitor), and demonstrated that combination treatment enhanced the inhibition of replicon RNA and suppressed the emergence of resistant replicon colonies. Sequencing analysis of dually resistant replicons revealed known resistance mutations in the NS5B polymerase and a novel resistance mutation in the NS3 protease domain, suggesting that novel resistance pattern may arise following combination treatment.

2. Materials and methods

2.1. Replicon assay

Replicon cells (genotype 1b) carrying the adaptive mutations S1179I in NS5A (Blight et al., 2000) were seeded in 96-well plates and dosed with compounds in a 10-point, 1:2 serial dilution for three days. The reduction in replicon RNA was measured using real-time RT-PCR (Taqman) assay (Malcolm et al., 2006).

2.2. Combination treatment

Replicon cells were treated for 3 days with boceprevir which was serially diluted at 1:2.5 for a 5-point titration. At each concentration of boceprevir, a polymerase inhibitor was titrated in at 1:2.5 serial dilutions. The replicon RNA level was measured using real-time PCR (Taqman assay), with GAPDH RNA used as an endogenous control. Relative replicon RNA inhibition (ddCT) is calculated as below:

$$\mathrm{dCT} = \mathrm{NS5BCT} - \mathrm{gapdhCT}, \qquad \mathrm{ddCT} = \mathrm{dCT} - \mathrm{dCT\ of\ no\ cpd}$$

2.3. Selection and long-term treatment of resistant replicon cells

To evaluate replicon resistance to combination treatment, 2×10^5 replicon cells were seeded in 6-cm tissue culture plates and cultured with boceprevir and polymerase inhibitors as indicated in the presence of 0.5 mg/ml G418. All cells were passaged at a 1:10 ratio upon reaching 95% confluence. Surviving replicon colonies were counted and frequency of resistant colonies was calculated as percent of input cells. For long-term treatment, replicon cells were cultured in the presence of G418 and inhibitors at the indicated dose for over 30 passages. To measure replicon fitness, 5 μg of replicon RNA was transfected into 5×10^6 Huh-7 cells. The colony formation efficiency (CFE) was designated as the number of colonies established per μg of input mutant RNA normalized against that of wild type RNA. Cells from a duplicate set were pooled and expanded for further analysis.

2.4. Amplification and sequencing of replicon RNA from resistant cells

To identify mutations that confer resistance to compounds, total cellular RNA was isolated from pooled colonies using RNeasy mini kit (Qiagen) and amplified by RT-PCR. The primers used to amplify the NS3 coding sequence were: 5′ forward primer NS3-1642f, GTCAAATGGCTCTCCTCAAGCGTA; 3′ reverse primer NS3-3815r, AAGATGATCCTGCCCACAATGACC. The primers used for the NS5B gene were: 5′ forward primer SR5720, CGGTTGTCCTGTCAGAATC; 3′ reverse primer 5BRRT-1, GGAGTGTTTAGCTCCCCGTT. 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 about 20 bacterial colonies was sequenced. The sequences were aligned using Lasergene software (DNASTAR).

2.5. Generation and activity determination of recombinant mutant proteases

To generate mutant proteases carrying a single resistance mutation, nucleotide changes were introduced using the QuikChange mutagenesis kit (Stratagene). The parental plasmid expressing His-tagged single chain NS4A–NS3 protease domain, $\mathrm{NS4A_{21-32}}$–GSGS–$\mathrm{NS3_{3-181}}$, as well as the expression and purification protocol have been previously described (Taremi et al., 1998). Recombinant proteases were tested using a chromogenic assay as described by Zhang et al. (1999). 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 (achieveing approximately 12% substrate depletion) and substrate (40 μM). The data were fitted to a 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.

3. Results

3.1. Combination treatment with HCV protease and polymerase inhibitors

Boceprevir (SCH 503034) is a mechanism-based ketoamide inhibitor of HCV NS3 protease (Malcolm et al., 2006); 2′-C-methyl-adenosine is a chain-terminating nucleoside inhibitor of NS5B polymerase (Migliaccio et al., 2003); the indole-*N*-acetamide compound is a non-nucleoside inhibitor targeting the finger-loop site of NS5B (Harper et al., 2005). Compound structure are shown in Fig. 1. While boceprevir alone efficiently inhibited replicon RNA, addition of 2′-C-methyl-adenosine (Fig. 2A) or indole-*N*-acetamide (Fig. 2B)

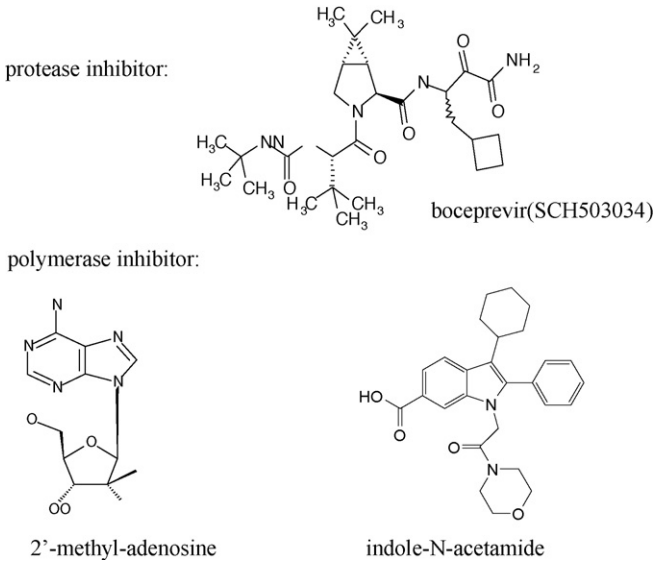

Fig. 1. Compounds used in the study. Boceprevir (SCH 503034) is a mechanism-based inhibitor of NS3 protease (Malcolm et al., 2006); 2′-C-methyl-adenosine is a nucleoside analog inhibitor of NS5B polymerase (Migliaccio et al., 2003); and indole-*N*-acetamide (Harper et al., 2005) is a non-nucleoside inhibitor targeting finger-loop site of NS5B.

Table 1
Frequency of resistant replicons (% of input cells) selected by combination treatment.

	Polymerase inhibitor				
	None	Indole-*N*-acetamide		2′-Methyl-adenosine	
		1×EC90	5×EC90	1×EC90	5×EC90
Boceprevir					
None	Monolayer	>0.3	0.01	>0.3	0.001
5×EC90	0.2	0.01	<LOD	0.07	<LOD

LOD: 0.001%.

to boceprevir enhanced replicon inhibition in a dose-dependent manner.

Replicon cells resistant to boceprevir were generated by selection with 5×EC90 of the compound for 3–4 weeks. Resistant colonies emerged with a frequency of approximately 0.2%, similar to previous results (Tong et al., 2006). Selection with 5×EC90 of either indole-*N*-acetamide or 2′-C-methyl-adenosine alone resulted in resistant colonies with a frequency of 0.01% and 0.001%, respectively (Table 1). The result that fewer resistant colonies were selected with 2′-C-methyl-adenosine is consistent with a previous study in which nucleoside analogs appear to present higher barrier to resistance in replicon cells (McCown et al., 2008). In combination with 5×EC90 boceprevir, the frequency of resistant replicons was significantly reduced compared to treatment with 1×EC90 of either 2′-C-methyl-adenosine or indole-*N*-acetamide alone. Higher doses of polymerase inhibitors (5×EC90) in the presence of boceprevir suppressed resistant colonies to below detectable level (<0.001%) (Table 1).

3.2. Phenotypic and genotypic characterization of replicon cells during long-term combination treatment

We have previously shown that long-term treatment of replicon cells with boceprevir resulted in selection of resistance mutations in the protease domain (Tong et al., 2006). To study how resistant mutants might evolve under long-term combination treatment of boceprevir and polymerase inhibitors, replicon cells were grown in the presence of boceprevir (5×EC90) and the finger-loop polymerase inhibitor (1.5×EC90 of indole-*N*-acetamide) for about 30 passages. At various time points, replicon cells were tested for sensitivity to the two inhibitors and replicon RNA was sequenced for resistance mutations in the NS3 protease and NS5B polymerase regions by population and clonal sequencing (~20 clones per sample). As shown in Fig. 3A, an approximate 10-fold resistance to boceprevir was observed throughout the course of the compound treatment. Sequencing analysis revealed three predominant mutations in the protease domain: a known adaptive mutation (E176G) (Krieger et al., 2001) as well as two genetically linked changes in the protease domain, V113I and M175L, neither of which has been previously described. The genetic linkage of V113I and M175L mutations was confirmed by clonal sequencing. Another mutation, M179V, was detected only transiently at low levels. It is noteworthy that none of the known boceprevir resistance mutations were identified. In contrast to the relative constant level of resistance to boceprevir, resistance to indole-*N*-acetamide increased with treatment time, from 9-fold at passage 2 to >30-fold after passage 20. Sequencing analysis identified P495S, a mutation known to confer resistance to finger-loop inhibitors (Kukolj et al., 2005; Tomei et al., 2003). At passage 2, about 50% of replicon cells contained the P495S mutation, the remaining possessing the wild type sequence. At later passages, P495L became the predominant mutation (80% and 100% of the population at passages 20 and 27, respectively). The evolution of replicon cells from a mixture of wild type and mutant sequences to 100% mutant at the P495 locus corresponded to the observed increase in resistance phenotype during treatment.

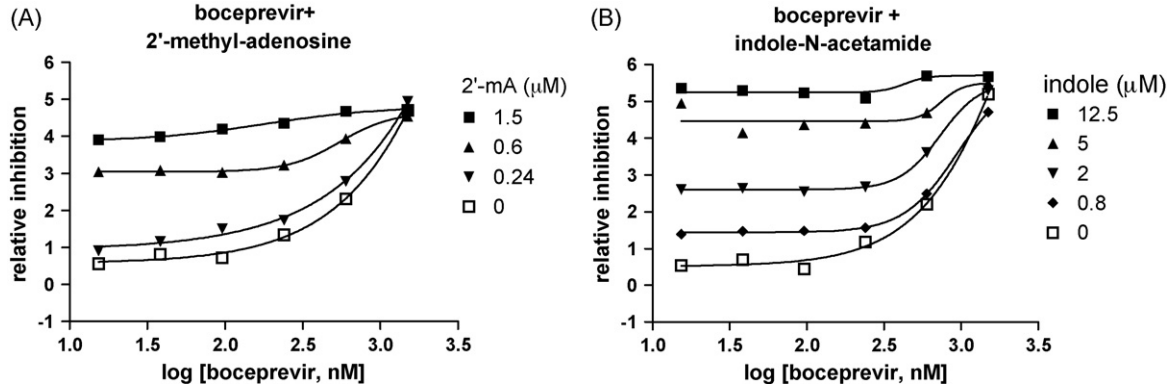

Fig. 2. Inhibition of replicon RNA by boceprevir is enhanced by inhibitors against the NS5B polymerase. (A) Combination with the nucleoside analog, 2′-methyl-adenosine (2′-mA). (B) Combination with the non-nucleoside inhibitor, indole-*N*-acetamide (indole). Relative inhibition (see Section 2) is plotted against the log concentration of boceprevir.

It has been shown that the NS5B S282T mutation, which confers resistance to 2′-C-methyl nucleoside analogs, significantly reduces replicon fitness and it can be difficult to obtain replicon colonies even at relatively low doses of the inhibitor (Migliaccio et al., 2003; McCown et al., 2008). In order to facilitate the selection of replicon cells resistant to both boceprevir and 2′-C-methyl-adenosine, the concentration of the nucleoside analog was increased in a step-wise manner, from 0.25×EC90 to 3.5×EC90 over the course of 38 passages, while the concentration of boceprevir was kept constant at 5×EC90. As shown in Fig. 3B, low level exposure to 2′-C-methyl-adenosine (0.25×EC90) did not give rise to replicon cells resistant to the compound (with less than 2-fold change in EC90). One change in the NS5B region (K209R) was detected by sequencing analysis. Replicon cells resistant to 2′-C-methyl-adenosine were generated when its concentration was increased to 1.5×EC90 and higher. Concomitantly, the S282T mutation appeared in these cells, whereas the K209R mutation was no longer detected; we did not observe any sequences in which both K209R and S282T were present, thus it is unlikely that K209R facilitated the emergence of S282T. The replicon resistance to the protease inhibitor boceprevir remained constant (~10-fold) throughout the course of the experiment. However, the mutation pattern in the protease domain was found to change with the dosing regimen of 2′-C-methyl-adenosine. At low dose of 2′-C-methyl-adenosine (0.25×EC90), the predominant protease mutation was A156S, a well characterized resistance mutation to boceprevir (Tong et al., 2006). Interestingly, when the dose of 2′-C-methyl-adenosine increased to 1.5×EC90 and higher and as the NS5B S282T mutation appeared, the protease mutation A156S was no longer detected; instead, four genetically linked novel changes in the protease domain were identified (R24W/K68R/N77S/M175L). The genetic linkage of these four mutations was confirmed by clonal sequencing. As with the combination treatment of boceprevir and indole-*N*-acetamide, there was also a transient appearance of changes at M179 in early passages.

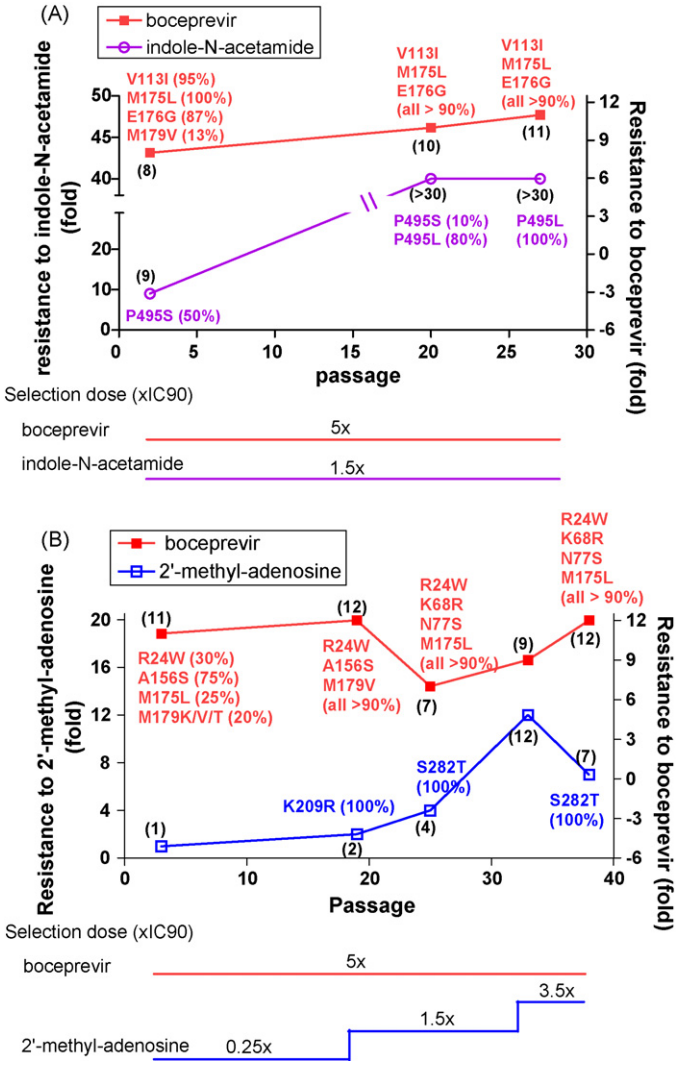

Fig. 3. Selection and identification of resistance mutations in NS3 protease and NS5B polymerase during long-term combination treatment. Replicon cells were grown for 30–40 passages in the presence of boceprevir and a polymerase inhibitor at doses as indicated. At various passages, replicon cells were tested for sensitivity to boceprevir and polymerase inhibitors; the fold increase in resistance is shown in parentheses. The protease and polymerase regions were sequenced by population and clonal sequencing (~20 clones per sample); treatment-associated mutations and their frequencies are reported in parentheses. (A) Selection with combination of boceprevir and indole-*N*-acetamide. (B) Selection with combination of boceprevir and 2′-C-methyl-adenosine.

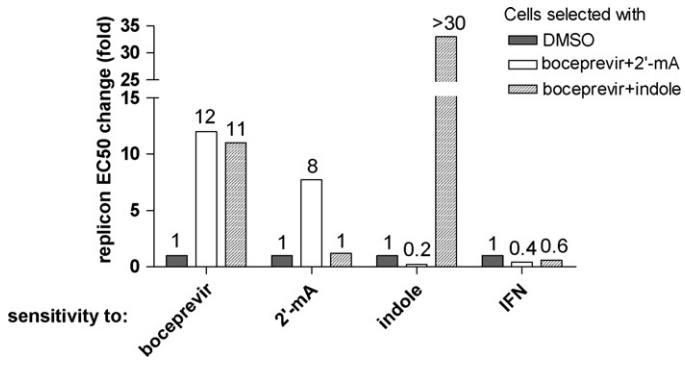

Fig. 4. Dually selected cells remain sensitive to different classes of inhibitors. After dual selection with boceprevir and 2′-C-methyl-adenosine, or boceprevir and indole-*N*-acetamide, replicon cells were expanded and dosed with boceprevir, 2′-C-methyl-adenosine, indole-*N*-acetamide, or interferon-α (IFN) and susceptibility to each agent was measured. The fold change of replicon EC50 (normalized against DMSO treated cells) is shown.

Replicon cells under combination treatment of boceprevir with either the indole-*N*-acetamide compound or 2′-C-methyl-adenosine were tested for cross-resistance to different classes of polymerase inhibitors and sensitivity to interferon-α. As shown in Fig. 4, replicon cells selected by boceprevir and indole-*N*-acetamide compound remained sensitive to 2′-C-methyl-adenosine; likewise, replicon cells selected by boceprevir and 2′-C-methyl-adenosine remained sensitive to the indole-*N*-acetamide compound. All cells were sensitive to interferon-α.

3.3. Characterization of novel mutations in the protease domain

The novel changes in the protease domain identified during combination treatment are well conserved (>90%) among genotype 1 isolates available from Genbank (Table 2A). To characterize these changes, each mutation was introduced into a 1b single chain NS3/4A protease construct. As shown in Table 2A, all mutant enzymes exhibited similar kinetic parameters (K_m and k_{cat}) to the wild type protein. Resistance to boceprevir was measured by fold change of inhibition constant (K_i^*) of mutants over that of the wild type. Four out of the five changes (R24W, K68R, N77S, V113I) did not confer resistance to boceprevir as single mutations. M175L, a novel mutation selected in both combination experiments, conferred ~3-fold resistance to boceprevir. Since V113I and M175L mutations were found to be genetically linked, as was the case for R24W, K68R, N77S and M175L, the double and quadruple protease mutants were constructed. The presence of these other mutations together with M175L did not increase boceprevir resistance compared to M175L alone (Table 2A).

Table 2
Characterization of protease mutations identified during combination treatment in enzyme (A) and replicon (B) assays.

(A)

	Polymorphism[a]	K_m (μM)	k_{cat} (min^{-1})	Resistance (K_i* fold/WT)
WT		3.9	24	
R24W	R: 100%	3.6	49	1
K68R	K: 95.7%, R: 1.1% Q: 2.1%, S: 1.1%	4	35	1.5
N77S	N: 99.5%, S: 0.5%	3	36	1
V113I	V: 99.5%, I: 0.5%	3.9	25	1.5
M175L	M: 93.6%, L: 6.4%	4.3	31	3.5
V113I + M175L	NA	4	34	2
R24W + K68R + N77S + M175L	NA	4.6	42	3

(B)

	Replicon EC50, μM (fold change over WT)		
Cell lines	Boceprevir[b]	Indole-*N*-acetamide[c]	2′-Methyl-adenosine[c]
WT	0.43	0.38	0.53
M175L	0.87 (2)	0.46 (1)	0.53 (1)
M175L + P495L	1.1 (2.5)	>20 (>50)	0.55 (1)
M175L + S282T	0.87 (2)	0.41 (1)	6.1 (12)
P495L	0.49 (1)	>20 (>50)	0.6 (1)
S282T	0.46 (1)	0.28 (0.7)	6.8 (13)

NA: not applicable.
[a] From ~190 genotype 1 isolates available from Genbank.
[b] From 3 to 6 experiments.
[c] From 1 to 2 experiments.

To confirm the resistance phenotype observed with purified enzyme, the novel protease mutation M175L was cloned into 1b replicon as a single mutation and in conjunction with polymerase resistance mutations P495L or S282T observed in the combination experiment. Consistent with *in vitro* results, M175L conferred ~2-fold resistance to boceprevir as a single mutation as well as in the presence of the NS5B mutations P495L or S282T (Table 2B). As expected, P495L and S282T conferred resistance to indole-*N*-acetamide and 2′-C-methyl-adenosine respectively. The presence of M175L in the protease domain did not change the level of resistance to polymerase inhibitors conferred by the two NS5B mutations.

To determine the replicon fitness of mutants carrying protease and polymerase mutations, the colony formation efficiency (CFE) of each construct was measured (Table 3). Replicon carrying the M175L mutation replicated slightly better than the wild type (130% of wild type). The replicon fitness was significantly reduced in replicons carrying the single mutation of NS5B P495L (1% of wild type) (Kukolj et al., 2005; Tomei et al., 2003) or S282T (5% of wild type) (Migliaccio et al., 2003; McCown et al., 2008; Ludmerer et al., 2005) as previously reported. The CFE of double mutants M175L/P495L (2% of wild type) and M175L/S282T (5% of wild type) were better or similar to that of the corresponding NS5B single mutant. As described earlier, under dual selections of boceprevir and 2′-C-methyl-adenosine (at 1.5×EC90 or higher), the A156S protease mutation was replaced by M175L as resistance mutation to the nucleoside developed at S282T in NS5B, thus yielding the double mutant M175L/S282T but not A156S/S282T. To test the hypothesis that the double mutant A156S/S282T might not have emerged due to its poor fitness, a mutant replicon carrying A156S/S282T was constructed and used to establish replicon colonies. The CFE of the double mutant A156S/S282T was found to be 2% of wild type, lower than that of M175L/S282T (Table 3).

Table 3
Colony formation efficiency (CFE) of replicons carrying protease and polymerase mutations.

Cell lines	CFE% of WT (±SD)[a]
WT	100
M175L	130 ± 1
M175L + P495L	2 ± 1
M175L + S282T	5 ± 2
P495L	1 ± 0.1
S282T	5 (n = 1)
A156S + S282T	2 ± 1

[a] SD: standard deviation from 2 to 4 experiments unless indicated otherwise.

4. Discussion

A number of small molecule inhibitors of HCV from different pharmacological classes are currently undergoing clinical development, exemplified by NS3 protease inhibitors, nucleoside and non-nucleoside NS5B polymerase inhibitors. Due to the high mutation rate and turnover rate of HCV (Neumann et al., 1998), the development of drug resistance to monotherapy is a major issue for small molecule anti-HCV agents. Several clinical studies have shown that combination of a small molecule inhibitor with the current standard of care (pegylated interferon-α and ribavirin) improved antiviral activity, and a recent report of a clinical trial of combination of a protease and a polymerase inhibitor in genotype 1 patients offered encouraging results (Gane et al., 2009). However, the impact on emergence of resistance by such small molecule inhibitor combinations remains to be fully explored.

In this study, we demonstrated that combination of an NS3 protease inhibitor (boceprevir) with a nucleoside analog or a non-nucleoside NS5B polymerase inhibitor enhances inhibition of HCV replicon RNA and suppresses the emergence of resistant replicon cells. To characterize resistance under combination treatment, replicon cells resistant to both protease and polymerase inhibitors were generated. The dually resistant cells remain sensitive to interferon-α or to polymerase inhibitors targeting different sites. Sequencing analysis identified known resistance mutations against polymerase inhibitors in the NS5B gene (S282T in the active site and P495S/L in the finger-loop site) from dually resistant cells. However, none of the previously described NS3 protease resistance mutations were detected in conjunction with NS5B mutations; instead, a novel mutation M175L in the protease domain was consistently selected under combination treatment of boceprevir with

either 2′-C-methyl-adenosine or indole-*N*-acetamide. Our previous work has shown that selection with boceprevir alone at a comparable dose (~5×EC90) gave rise to a panel of protease resistance mutations (T54A, A156S/T, V170A) (Tong et al., 2006). Interestingly, an unexpected profile was observed with A156S, a boceprevir resistance mutation which has been shown to have minimal effect on replicon fitness in the CFE assay (Tong et al., 2006). When replicon cells were selected with a moderate dose of boceprevir (5×EC90) and a low dose of 2′-C-methyl-adenosine (0.25×EC90), the surviving cells were resistant only to boceprevir. In these replicon cells, A156S was the predominant mutation in the protease domain, whereas S282T in NS5B was not detected. As the selection pressure of 2′-C-methyl-adenosine increased (to >1×EC90), replicon cells became resistant to the nucleoside inhibitor and the S282T mutation emerged in the population. Phenotypically, replicon cells remained resistant to boceprevir, which was kept at a constant dose throughout the experiment; genetically, however, the mutation A156S was replaced by M175L, suggesting that A156S may be incompatible with the S282T mutation in NS5B. It is possible that simultaneous mutations in the protease active site and certain polymerase sites cannot be tolerated, and protease mutations at less critical positions are favored. Consistent with the hypothesis, the double mutant A156S/S282T was found to exhibit very poor replicon fitness (2% of wild type); whereas the double mutant carrying the novel protease mutation (M175L) and S282T showed modestly improved colony formation efficiency (5% of wild type), which may account for its outgrowth under dual selection conditions. M175 is located at the C-terminal end of the protease domain distal from the active site. It confers a few fold of resistance and moderately improves replicon fitness compared to wild type, both of which may contribute to its preferential selection under combination treatment conditions.

It is noteworthy that in a recent study by Flint et al. (2009) of replicon cells treated with combination of boceprevir and HCV-796 (a palm site polymerase inhibitor), most of the dually resistant replicon clones had the major resistance mutation against HCV-796 (C316Y) without any known protease resistance mutations; only one out of 15 clones sequenced possessed mutations in both the protease (V170A) and polymerase domains (C316Y). Similar to our results with boceprevir and indole-*N*-acetamide, the protease adaptive mutation E176G was selected, which may contribute to the resistance phenotype by increasing general replication efficiency, but it remained to be determined in that study whether any novel protease mutations may also account for the observed phenotypic resistance to boceprevir. Furthermore, no mutations in NS4A, NS4B or NS5A, or in the NS3 cleavage sites were detected under their dual selection conditions (Flint et al., 2009). Taken together, the findings of boceprevir in combination with three different classes of polymerase inhibitors (a nucleoside analog, a palm site and a finger-loop inhibitor) suggest that the prevalence and complexity of resistance mutations to a protease inhibitor can be influenced at least *in vitro* by co-administration of a polymerase inhibitor, and novel mutations in the protease domain may arise as a result of the combination treatment. More *in vitro* combination studies will be required to further evaluate the interactions between resistance mutations against different classes of small molecules antivirals. Our study supports the rationale for clinical evaluation of combination of HCV protease and polymerase inhibitors and suggests that combination therapy may result in changes in the resistance profile from that of individual antiviral agents.

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