

The Hepatitis C Virus (HCV) NS4B RNA Binding Inhibitor Clemizole Is Highly Synergistic with HCV Protease Inhibitors

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Background. We recently identified a compound, clemizole hydrochloride, that inhibits NS4B's RNA binding and hepatitis C virus (HCV) replication. Although significant, clemizole's antiviral effect is moderate (50% effective concentration of 8 μ M against an HCV genotype 2a clone). We hypothesized that the combination of clemizole with other anti-HCV agents can increase the antiviral effect over that achieved with each drug alone and could also decrease the emergence of viral resistance.

Methods. Luciferase reporter-linked HCV replication assays were used to study the antiviral effects of drug combinations that included clemizole. Data were analyzed using Loewe additivity and Bliss independence models for synergy, and resistance studies were performed using HCV colony formation assays.

Results. Clemizole's antiviral effect was highly synergistic with the HCV protease inhibitors SCH503034 and VX950, without toxicity. In contrast, combinations of clemizole with either interferon, ribavirin, or the nucleoside (NM283) and nonnucleoside (HCV796) HCV polymerase inhibitors were additive. Furthermore, combination of clemizole with SCH503034 decreased the frequency of drug-resistant mutants, compared with treatment with either drug alone. Finally, no cross-resistance to clemizole of SCH503034-resistant mutants (or vice versa) was observed.

Conclusions. Clemizole can yield high-level synergy with the protease inhibitor class. Inclusion of clemizole in future anti-HCV cocktails can represent an attractive paradigm for increasing current virologic response rates.

Over 150 million individuals are infected with hepatitis C virus (HCV) worldwide. Current standard of care (SOC), which consists of interferon-ribavirin combi-

nations, is inadequate for many of the patients [1]. The 9.6-kb positive single-stranded RNA HCV genome encodes a polyprotein that is cleaved into structural and nonstructural (NS) proteins [2]. The NS3/4A protease and the NS5B RNA-dependent RNA polymerase have been the main focus of anti-HCV-specific drug discovery efforts thus far. Indeed, several inhibitory compounds against these targets suppress viral replication in HCV-infected patients [3]. Monotherapy, however, was associated with the emergence of viral resistance leading to treatment failure [3]. Furthermore, drug-resistant mutations conferred resistance to additional inhibitors from the same class, excluding this class from these patients' armamentarium [3]. Although combining these emerging drugs with SOC regimens improves response rates [3], resistance and inadequate efficacy in nonresponders to SOC remain important problems. Thus, there is an urgent need for drugs directed at novel HCV targets. Such drugs could be used in combination with SOC regimens or, hopefully, in future interferon-sparing cocktails.

Received 6 August 2009; accepted 20 December 2009; electronically published 20 May 2010.

Potential conflicts of interest: J.S.G. is a consultant to, receives research funding from, or has an equity interest in: Romark Laboratories, Roche, Genentech, Merck, and Eiger BioPharmaceuticals. S.E. and H.D.S. have an equity interest in Eiger BioPharmaceuticals. E.G.: no conflicts.

Presented in part: Western Regional Meeting, January 2009, Carmel, California, and Infectious Diseases Society of America (IDSA) Annual Meeting [abstract 1242], October 2009, Philadelphia, Pennsylvania.

Financial support: National Institutes of Health (NIH) K08-AI079406-01 (to S.E.), a Stanford Digestive Disease Center Pilot/Feasibility Award (to S.E.), NIH P30 DK56339, NIH R01 DK066793 and R01AI087917, Burroughs Wellcome Fund Clinical Scientist Award in Translational Research (to J.S.G.), and the Stanford SPARK program.

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The Journal of Infectious Diseases 2010;202(1):65–74

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0022-1899/2010/20201-0008\$15.00

DOI: 10.1093/653080

The NS4B protein is a key player in HCV replication. It induces the formation of a novel intracellular membrane structure [4], which represents the site of HCV replication, and is required to assemble the other NS proteins within these membrane-associated replication complexes [5]. Disrupting NS4B function thus represents an attractive new anti-HCV strategy.

We have recently shown that an arginine-rich-like motif within NS4B mediates binding to the 3'-terminus of the negative HCV strand and HCV RNA replication [6]. Eighteen pharmacological inhibitors of this activity were identified in a high-throughput screen using a novel microfluidics platform [6]. A lead compound, clemizole, was also found to inhibit HCV RNA replication [6]. This H1-histamine receptor antagonist has been used in humans (albeit for a different indication) and was well tolerated [7]. Although significant, clemizole's antiviral effect is moderate, with a 50% effective concentration (EC_{50}) of 8 μ M against HCV genotype 2a in the human hepatoma-derived cell line Huh7.5. Like other anti-HCV agents, genotype 1 clemizole-resistant mutants can be selected in vitro [6].

We hypothesized that combining clemizole with other anti-HCV agents could increase the antiviral effect achieved with 1 active drug alone and decrease emergence of viral resistance. Here, we report that clemizole's antiviral effect is highly synergistic with the emerging anti-HCV-specific drugs SCH503034 and VX950, which target the HCV protease, without host cell toxicity. These results have exciting implications for novel strategies for combating HCV infections.

METHODS

Compounds. Clemizole and ribavirin were purchased from Sigma, and Interferon- α -B2 was purchased from PBL Biomedical. SCH503034, VX950, NM283, and HCV796 were a gift from Leslie Holsinger (Virobay). Ten millimole per liter stocks were prepared and stored at -20°C . Clemizole and ribavirin were solubilized in H_2O . SCH503034, VX950, NM283, and HCV796 were solubilized in dimethyl sulfoxide (DMSO). Interferon was solubilized according to the manufacturer's instructions.

Plasmids. FL-J6/JFH-5'C19Rluc2AUbi that consists of a full-length genotype 2a HCV genome and expresses *Renilla* luciferase was a gift from Dr. C. M. Rice [8]. Bart-79I has been described elsewhere [9]. Bart-79I-luc was made by replacing the neomycin-phospho-transferase gene by a *Firefly* luciferase gene in the Bart-79I plasmid.

Cell cultures and electroporation. Huh-7.5 cells were electroporated with in vitro transcribed FL-J6/JFH-5'C19Rluc2AUbi or Bart79I-luc RNAs, as described elsewhere [6]. Cells were pooled and seeded in 96-well plates ($2-3 \times 10^4$ cells/well). Medium was replaced at 24 h and daily after. Cells were grown in 4 replicates in the presence of serial dilutions of the inhibitory compounds. Untreated cells with or without corresponding

concentrations of DMSO were used as negative controls for DMSO and water-soluble compounds, respectively. After 72 h, cells were subjected to alamarBlue-based viability assays and luciferase assays.

Viability assays. Cells were incubated for 2 h at 37°C in the presence of either 10% alamarBlue reagent (TREK Diagnostic Systems) or CellTiter-Blue reagent (Promega). Fluorescence was detected using FLEXstationII 384 (Molecular Devices). Depending on the inhibitory compound's solvent, water or DMSO, signal was normalized relative to untreated samples or samples grown in the presence of DMSO, respectively.

Luciferase assays. Viral RNA replication was determined using *Renilla* (for genotype 2a replicons) or *Firefly* (for genotype 1b replicons) luciferase assays (Promega). Cells were washed with phosphate-buffered saline and shaken in lysis buffer. After 15 min incubation at -80°C and thawing, luciferase assay buffer containing the assay substrate was injected, and luciferase activity was measured using a Berthold LB96V luminometer. Signal was normalized as described above. Experiments were repeated 3 times, each time with 4 replicates.

Focus formation assay. To perform the focus formation assay, 2×10^4 Huh7.5 cells were infected in triplicates with cell culture-grown HCV titered at 1.2×10^4 50% tissue culture infective dose per mL, as described elsewhere [10]. Two hours after infection, cells were washed and treated daily with various concentrations of clemizole and SCH503034, either alone or in combination. After 72 h, samples were subjected to viability assays, followed by fixation in 4% formaldehyde and permeabilization with saponin. HCV core protein was detected with primary anti-core monoclonal and secondary goat anti-mouse Alexa-594-conjugated antibodies. Foci were counted under an inverted microscope.

Colony formation assays. Huh7 cells electroporated with genotype 1b subgenomic HCV replicon (Bart-79I) [11] were treated in duplicates with various concentrations of clemizole and SCH503034, either alone or in combination. G418 was included to provide selective pressure on HCV replicon cells, such that cells bearing wild-type replicons that are susceptible to the antiviral drugs are expected to die, whereas cells bearing resistant replicons are expected to grow and form colonies after 3 weeks. Plates were stained with crystal violet, and the frequency of resistance was determined (colonies per input cells).

Selection of resistant mutants. Established HCV replicon-harboring cells [11] were passaged in the presence of neomycin and an increasing concentration of either clemizole (1–16 μ M) or SCH503034 (0.25–2.5 μ M) in 5 replicates. Colonies that grew in the presence of the compounds were pooled and passaged 15–20 times, and the replicating HCV RNA was subjected to sequence analysis [9]. Whole-cell RNA electroporations were performed as described elsewhere [6].

Analysis of combination data. Combination data were an-

Table 1. Inhibitory Activity and Cytotoxicity of the Drugs Used in This Study

DrugCompound	EC ₅₀ ^a			CC ₅₀ ^b		
	2a Genotype	1b Genotype	Measured by others (1b genotype)	2a Genotype	1b Genotype	Measured by others (1b genotype)
Clemizole	8 μM	23 ± 7 μM	NA	35 ± 0.5 μM	40 ± 5 μM	NA
SCH-503034	0.8 μM	0.21 ± 0.034 μM	0.21–0.57 μM	>100 μM	>100 μM	>100 μM
VX-950	0.3 μM	ND	0.35–0.56 μM	ND	ND	26.7–82 μM
Interferon ^c	2.8 ± 0.4 U/mL	ND	1.8 U/mL ^d	ND	ND	>10,000 U/mL
Ribavirin	14 ± 3 μM	ND	15 μM ^d	>100 μM	ND	NA
NM-283	1.16 ± 0.2 μM	ND	1.23 ± 0.52 μM	ND	ND	>100 μM
HCV-796	0.068 ± 0.008 μM	ND	0.017 ± 0.005 μM ^e	ND	ND	>100 μM

NOTE. Data are mean value ± standard deviation. CC₅₀, 50% cytotoxic concentration; EC₅₀, 50% effective concentration; HCV, hepatitis C virus.

^a Antiviral effect was determined using HCV J6-JFH (genotype 2a) and/or Bart791 (genotype 1b) luciferase reporter-linked replication assays after 3 days of treatment. EC₅₀ values were compared with EC₅₀ values previously measured by others using conventional assays using genotype 1b replicons.

^b Cell viability was determined by an alamarBlue-based assay in parallel with the EC₅₀ determinations.

^c Note the low EC₅₀ values for the polymerase inhibitor HCV796.

^d Interferon-α has important interactions with the adaptive and innate immune responses, however it also has potent antiviral activity. The latter is not believed to be mediated by a direct action on the virus or replication complex but, rather, appears to act by inducing interferon-stimulated genes, which establish a non-virus-specific antiviral state within the cell [38].

^e Measured in 2a genotype.

alyzed using the Loewe additivity and Bliss independence drug interaction models [12, 13]. CalcuSyn™ (Biosoft) was used to quantify differences between observed effects and predicted ones. Drugs were mixed at fixed molar ratios that matched their equipotent concentrations, which were maintained during serial dilutions [12–14]. An isobologram was plotted [12, 13] that demonstrated lines of theoretical additivity and experimental EC₅₀, EC₇₀, and EC₉₀ values for the combination. Synergy, antagonism, and additivity are indicated by values plotted to the left of the corresponding lines of additivity, to their right, or on these lines, respectively. Combination indices (CIs) at the EC₅₀, EC₇₀, and EC₉₀ levels were also determined [15]. Indices <1 indicate synergy, indices equal to 1 indicate additivity, and indices >1 indicate antagonism.

The MacSynergy II program (kindly provided by M. N. Prichard) was used to analyze data according to the Bliss independence model [16, 17]. The combination's effect is determined by subtracting the experimental values from theoretical additive values [16]. A 3-dimensional differential surface plot demonstrates synergy as peaks above a theoretical additive plane and antagonism as depressions below it [16]. Matrix data sets in 4 replicates were assessed at the 95% confidence level for each experiment [13, 16, 17]. Synergy (volume under the curve) and log volume were calculated. As suggested by Prichard et al [17], such data sets should be interpreted as follows: volumes of synergy or antagonism at values of <25 μM²% are insignificant, 25–50 μM²% are minor but significant, 50–100 μM²% are moderate and probably important in vivo, and >100 μM²% are strong and likely to be important in vivo.

Statistical analysis. EC₅₀ and 50% cytotoxicity concentration (CC₅₀) values were measured by fitting data to a 3-param-

eter logistic curve using the formula $Y = a + (b-a)/(1 + 10^{(x-c)})$ (where *a*, *b*, and *c* represent minimum binding, maximum binding, and log EC₅₀ or log CC₅₀, respectively) (BioDataFit; Chang Bioscience).

RESULTS

Clemizole's antiviral effect is highly synergistic with SCH503034, an HCV protease inhibitor (PI).

We hypothesized that clemizole's antiviral effect may be synergistic with emerging anti-HCV specific agents that target other viral proteins. We first studied the antiviral activity of clemizole in combination with a PI currently studied in phase 2 trials, SCH503034 (Boceprevir) [18]. Following electroporation with J6/JFH (genotype 2a) HCV RNA genome harboring a luciferase reporter gene [8], Huh7.5 cells were grown in the presence of various concentrations of the individual compounds and their combinations. Luciferase assays and alamarBlue-based viability assays were performed at 72 h. Treatment with either compound alone resulted in a concentration-dependent inhibition of HCV replication. The mean EC₅₀ of clemizole alone was 8 μM (*P* < .05), as previously reported [6], with a CC₅₀ of 35 ± 0.5 μM (*P* < .05) (measured by alamarBlue-based assays and CellTiter-Blue assays). Although EC₅₀ values between ~0.2 μM and 0.574 μM have been reported for SCH503034 [18, 19] and have been similarly determined by us for genotype 1b, the mean EC₅₀ of the PI, using 2a genotype luciferase reporter gene assay, was 0.8 μM with a CC₅₀ >100 μM (*P* < .05) (Table 1).

The SCH503034-clemizole combination resulted in a greater inhibition than either compound alone at all tested concentrations (Figure 1A). For example, although SCH503034 alone

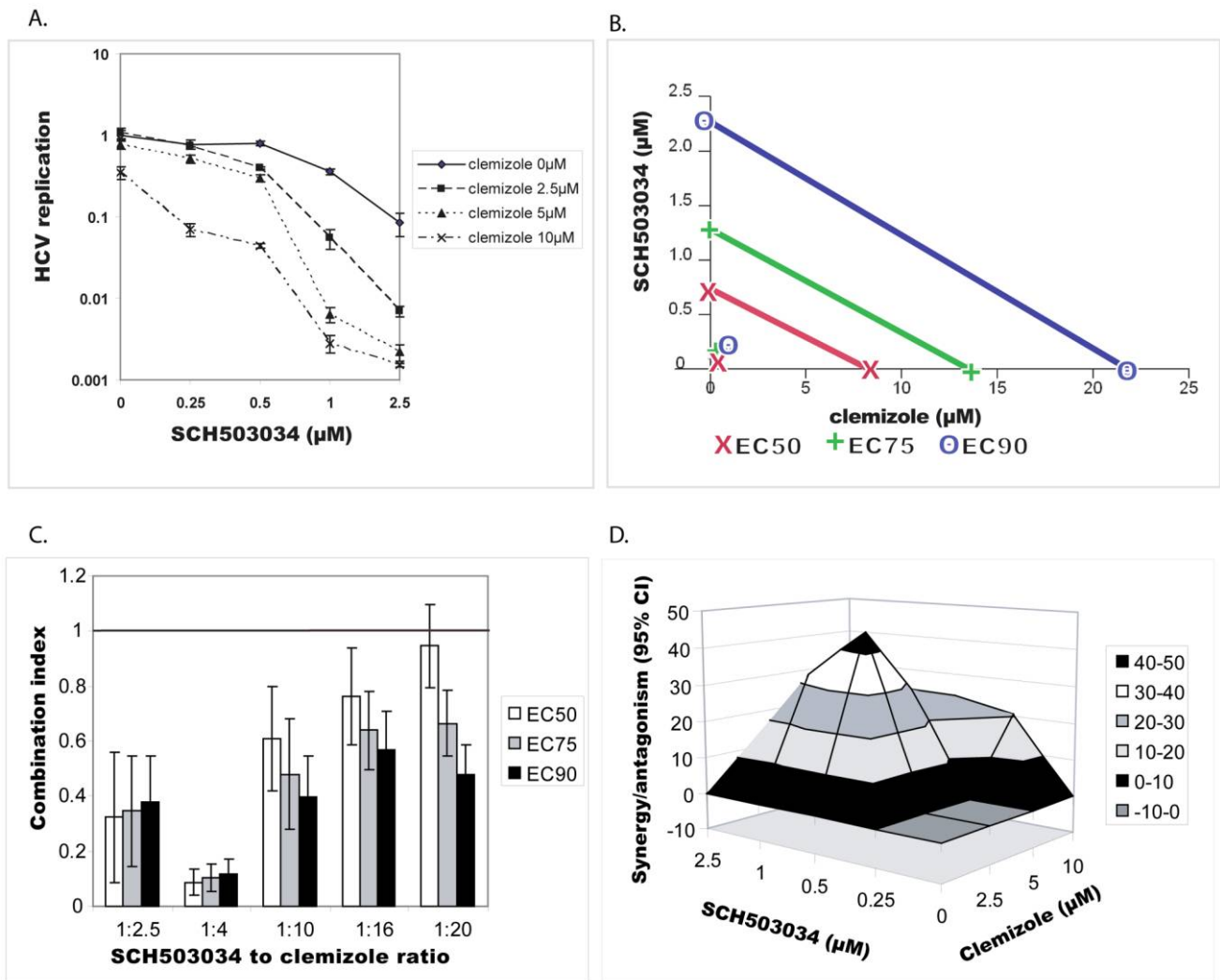


Figure 1. Clemizole's antiviral effect is highly synergistic with SCH503034, a hepatitis C virus (HCV) protease inhibitor. Huh7.5 cells electroporated with a full-length J6/JFH HCV RNA genome harboring a luciferase reporter gene were grown in the presence of increasing concentrations of clemizole and SCH503034 either individually or in various combinations. Luciferase reporter-linked replication assays were performed at 72 h in parallel with alamarBlue-based viability assays. The experiment was performed 3 times each with 4 replicates. Four types of analyses are presented. *A*, Dose response curves of SCH503034 in the absence or presence of increasing concentrations of clemizole. Error bars represent standard deviation. *B*, Isobolograms (generated using CalcuSynTM). The lines denote the expected additive 50% effective concentration (EC_{50}), EC_{75} , and EC_{90} values for the drug combination as calculated from the monotherapies. The experimental EC_{50} , EC_{75} , and EC_{90} values for the combination are marked by x, +, and o, respectively. Note that these values plot to the far left of the corresponding lines, indicating synergy. *C*, Combination indices of SCH503034-clemizole combinations at the EC_{50} , EC_{75} , and EC_{90} values measured at various drug ratios. A combination index (CI) ≥ 1.0 indicates antagonism, and a CI < 1.0 indicates synergism. Error bars represent standard deviation. *D*, Differential surface plot at the 95% confidence level (CI) (generated using MacSynergyII). The 3-dimensional plot represents the differences between the actual experimental effects and the theoretical additive effects at various concentrations of the 2 compounds. Only statistically significant (95% CI) differences between the 2 compounds were considered at any given concentration. Peaks above the theoretical additive plane indicate synergy, whereas depressions below it indicate antagonism. The colors indicate the level of synergy or antagonism.

at a concentration of 2.5 μM decreased viral replication by ~ 1 log, when combined with 5 μM of clemizole, viral replication was inhibited by ~ 3 logs. Furthermore, no significant cytotoxicity was measured with either compound alone or with the above combinations (data not shown). These results suggest

that even relatively low concentrations of clemizole have a dramatic effect on viral replication when added to SCH503034.

To formally characterize the SCH503034-clemizole combination's interaction, a data subset in which the drugs were mixed at a fixed molar ratio that matched their equipotent

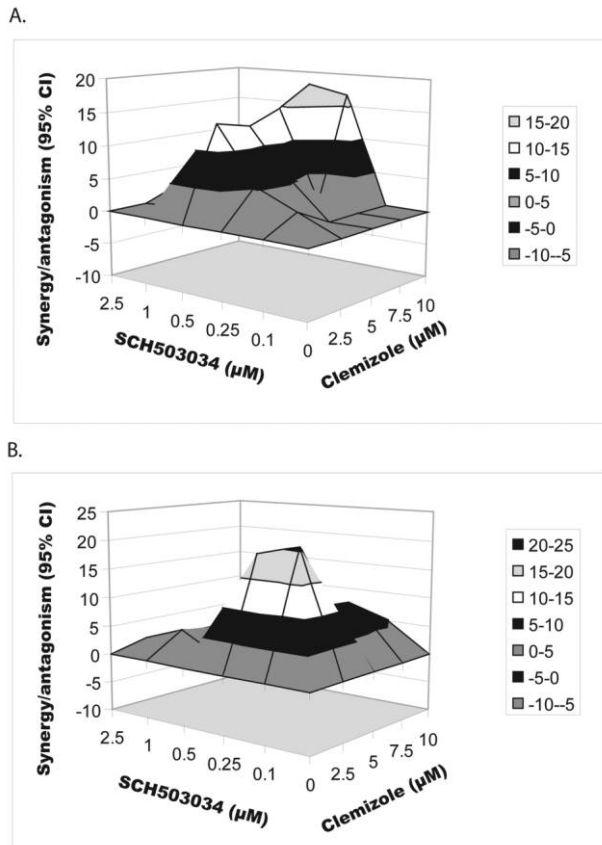


Figure 2. The clemizole-SCH503034 combination is synergistic across genotypes and in hepatitis C virus (HCV)-infected cells. The antiviral effect of the clemizole-SCH503034 combination was determined using (A) genotype 1b luciferase reporter-linked replication assays and (B) focus formation assays in cells infected with cell culture-grown HCV. Assays were performed following 72 h of treatment. Differential surface plot at the 95% confidence level are shown (generated using MacSynergyII). Peaks above the theoretical additive plane indicate synergy, whereas depressions below it indicate antagonism. The colors indicate the level of synergy or antagonism.

concentrations (SCH503034 to clemizole ratio of 1:10), was analyzed using CalcuSyn™. As shown in the resulting isobolgram (Figure 1B), the calculated EC_{50} , EC_{75} , and EC_{90} values for the SCH503034-clemizole combinations plotted far to the left of the corresponding lines of additivity, which suggested that the tested combinations are indeed synergistic [12, 13]. At an equipotent ratio of 1:10, the CIs at the EC_{50} , EC_{70} , and EC_{90} were 0.61, 0.479, and 0.397, respectively (Figure 1C) [15]. Being below 0.9, these indices confirm that the interaction is synergistic. These CIs are similar in magnitude to the most potent synergistic interaction measured by others between HCV PIs and polymerase inhibitors [20]. Although the interaction was found to be synergistic at any tested ratio, lowest CIs were measured at a SCH503034 to clemizole ratio of 1:4 (Figure 1C).

To confirm the nature of this interaction, to adjust for the various concentration ratios, and to better quantify the degree of the observed synergy, we further analyzed the data by means of a mathematical model, MacSynergy [16, 17]. Four-by-3 matrix data sets in 4 replicates were assessed at the 95% confidence level for each of the 3 experiments performed. The clemizole-SCH503034 combination had antiviral effects that were significantly more potent than the theoretical additive effects, which supported that this combination was indeed synergistic (Figure 1D). No evidence of antiviral antagonism was seen with any of the tested doses. The calculated synergy and log volume were $210 \mu M^2\%$ and 19, respectively. According to the criteria suggested by Prichard et al [17] (see Methods), the clemizole-SCH503034 combination is considered strong and will likely be important in vivo. Importantly, because there was no cellular toxicity with either drug alone at the studied concentrations and no increase in cytotoxicity when used in combinations, the measured synergy is indeed specific and does not reflect synergistic toxicity.

The synergy of clemizole-SCH503034 combination is not genotype specific. To determine whether the observed synergy of the clemizole-SCH503034 combination is genotype specific, the experiments outlined above were repeated with use of a subgenomic genotype 1b Bart79I HCV replicon harboring a luciferase reporter gene [8]. In contrast to its effect in genotype 2a, very mild concentration-dependent inhibition of HCV replication was measured after 72 h of treatment with clemizole alone, with an average EC_{50} of $24 \pm 6 \mu M$ ($P = .01$; $R^2 = 0.85$) and a mean CC_{50} of $40 \pm 5 \mu M$ ($P < .05$) (Table 1). It is

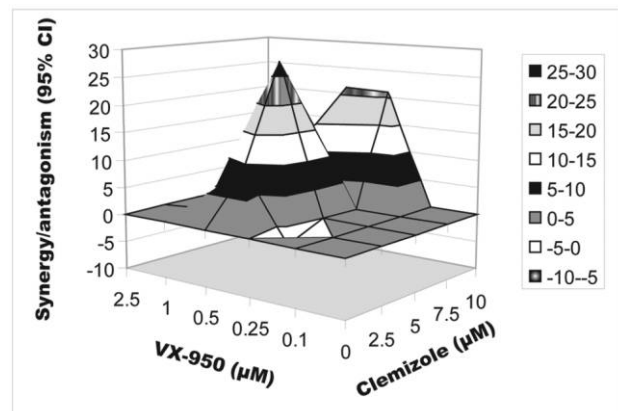


Figure 3. The synergistic effect of NS4B RNA binding inhibitors and protease inhibitors combinations appears to be generalizable. MacSynergy analysis of the antiviral effect of clemizole in combination with VX950 by luciferase reporter-linked replication assays after 72 h of treatment. A differential surface plot at the 95% confidence interval (CI) is shown. Peaks above the theoretical additive plane indicate synergy, whereas depressions below it indicate antagonism. The colors indicate the level of synergy or antagonism.

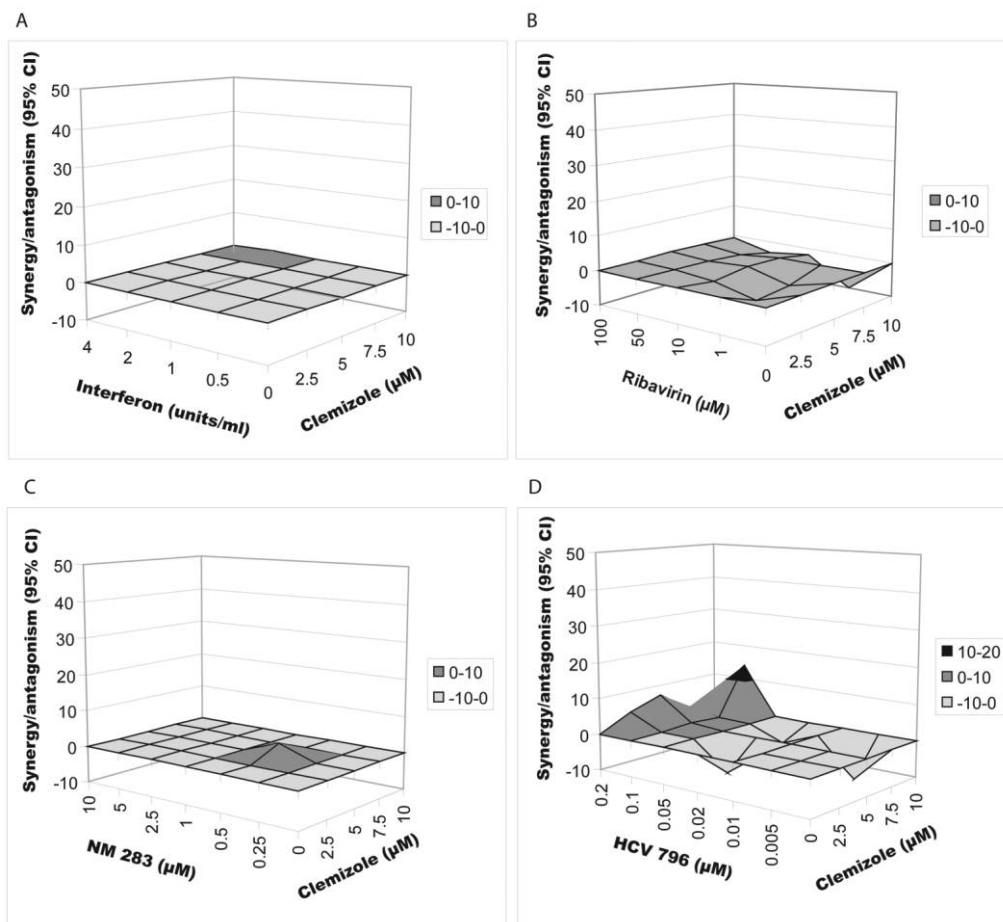
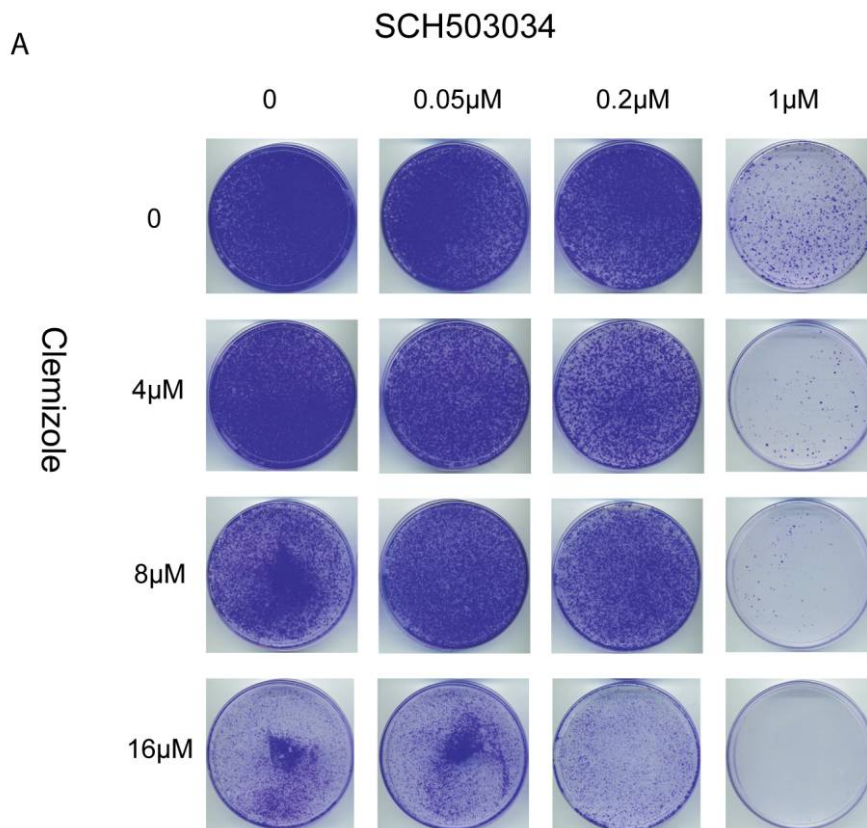


Figure 4. Combinations of clemizole with either interferon, ribavirin, a nucleoside analog, or a nonnucleoside analog polymerase inhibitors are not synergistic but additive. MacSynergy analysis of the antiviral effect of clemizole in combination with interferon alpha (*A*), ribavirin (*B*), NM283 (*C*), and HCV796 (*D*), as measured by luciferase reporter-linked replication assays after 72 h of treatment. Differential surface plots at the 95% confidence interval (CI) are shown. Peaks above the theoretical additive plane indicate synergy, whereas depressions below it indicate antagonism. The colors indicate the level of synergy or antagonism.

possible that the lower sensitivity of this assay, compared with the 2a luciferase reporter gene assay (resulting from a lower level of genotype 1b replication, compared with that of the genotype 2a clone) accounts for the difference in clemizole's EC_{50} values between genotypes. Alternatively, the difference might result from differential antiviral activity of clemizole against the 2 genotypes. Selection of clemizole-resistant mutants in 1b genotype replicon cells [6] suggests that clemizole does have an antiviral effect against genotype 1b. We thus favor the former possibility. Nevertheless, even low concentrations of clemizole surprisingly had a significant effect on genotype 1b viral replication when added to escalating concentrations of SCH503034, with a synergy volume of $100.04 \mu M^2\%$ (MacSynergy) (Figure 2A). Importantly, no cellular toxicity was measured at the concentrations used. These results suggest that the highly synergistic antiviral effect of combined clemizole-SCH503034 treatment is not genotype-specific. Because infec-

tion with genotype 1 HCV is the most common form of HCV infection in the United States [21] and tends to be the least responsive to current SOC regimens [22], the synergistic antiviral effect of the clemizole-SCH503034 combination is important.

Clemizole-SCH503034 combination is synergistic in HCV-infected cells. To determine whether the clemizole-SCH503034 combination is synergistic in inhibiting direct viral replication (versus indirect assessments using luciferase reporter genes), we studied its antiviral effect by focus formation assays using cell culture-grown HCV [10]. While the mean foci number in untreated wells was 46, lower numbers were counted with each drug alone in a dose-dependent manner. When combined, the 2 drugs resulted in substantially more-potent antiviral effects than either compound alone. Importantly, neither drug alone nor the combinations showed cytotoxicity at the concentrations tested (data not shown). The synergy volume was $113 \mu M^2\%$ (Mac-



B

		SCH503034			
		0 μ M	0.05 μ M	0.2 μ M	1 μ M
Clemizole	0 μ M	0.24 \pm 0.02	0.136 \pm 0.06	0.15 \pm 0.004	0.04 \pm 0.03
	4 μ M	0.2 \pm 0.01	0.1 \pm 0.005	0.08 \pm 0.002	0.007 \pm 0.004
	8 μ M	0.14 \pm 0.003	0.1 \pm 0.005	0.07 \pm 0.001	0.005 \pm 0.001
	16 μ M	0.08 \pm 0.001	0.07 \pm 0.002	0.03 \pm 0.0008	0.00008 \pm 0.0001

Figure 5. Combinations of clemizole with SCH503034 significantly reduce the frequency of phenotypic resistance. Huh7 cells electroporated de novo with a genotype 1b subgenomic hepatitis C virus (HCV) replicon were treated in duplicate with various concentrations of clemizole and SCH503034, either alone or in combinations, in the presence of G418 selection. After 3 weeks of treatment, plates were stained with crystal violet. *A*, Representative plates. *B*, Colonies were counted using Image-J (National Institutes of Health), and the number of colonies was used to calculate the frequency of resistance (colonies per input cells).

Synergy) (Figure 2*B*). These results suggest that the highly synergistic antiviral effect of the clemizole-SCH503034 combination is also achieved in the context of viral infection.

The synergistic effect of NS4B RNA binding inhibitors and PIs combinations appears generalizable. We hypothesized that the observed synergistic antiviral effect is also achieved when combining other NS4B RNA binding inhibitors with different HCV NS3 PIs. The antiviral effect of clemizole in combination with VX950 (Telaprevir), another PI [23], was thus determined. Genotype 2a luciferase reporter-linked assays and viability assays were performed as described above. The EC₅₀

of VX950 alone was measured at ~300 nM, similarly to prior reports [23, 24] (Table 1). In most concentrations tested, the combined drugs resulted in substantially more potent antiviral effects than did the corresponding single agents (Figure 3) with a synergy volume 97.51 μ M²% (MacSynergy). An insignificant antagonistic effect appeared in a single combination mixture with an antagonism volume of -2.83 μ M²%. Importantly, neither drug alone nor the combinations showed cytotoxicity at the concentrations tested (data not shown). Furthermore, we have recently embarked on a clemizole derivatization program and identified a variety of such derivative molecules that have

potency similar to or greater than that of clemizole (unpublished data). When combined with SCH503034, 1 tested clemizole derivative demonstrated significant synergistic effects that were similar to those of the parental compound (data not shown). Taken together, these results suggest that the synergistic antiviral effect of the clemizole-SCH503034 combination may be generalizable and may reflect a broad synergism potential between the PI and NS4B RNA-binding inhibitor classes of drugs. Because SCH503034 and VX950 are both ketoamide PIs, however, it remains to be determined whether combinations of the macrocyclic PIs, such as ITMN191 and BILN2061, with NS4B RNA binding inhibitors are similarly synergistic.

Combinations of clemizole with either interferon, ribavirin, a nucleoside analog, or nonnucleoside analog polymerase inhibitors are not synergistic but additive. We then studied the antiviral activity of clemizole in combination with either interferon, ribavirin, or 2 polymerase inhibitors [25, 26]: NM283 (valopicitabine), which is a nucleoside analog, or HCV796, which is a nonnucleoside analog. Genotype 2a luciferase reporter-linked assays were performed as described above. EC_{50} values of the individual compounds are shown in Table 1 [27]. The combination of clemizole with any of these compounds resulted in antiviral effects that were not significantly different from the theoretical additive effects (Figure 4) (MacSynergy). Synergy volumes of $0.4 \mu M^2\%$ and $3.57 \mu M^2\%$ and an antagonism volume of $-15 \mu M^2\%$ were measured for the combinations of clemizole with interferon, NM283, and ribavirin, respectively, indicating additivity. The synergy volume for the clemizole-HCV796 combination was $31.35 \mu M^2\%$ with an antagonistic volume of $-33.26 \mu M^2\%$, which suggest that, overall, this combination is largely additive as well. Two-dimensional analysis using CalcuSyn yielded similar results (data not shown).

Clemizole-SCH503034 combinations significantly reduce the frequency of phenotypic resistance. To test the hypothesis that clemizole-SCH503034 combinations can decrease the emergence of phenotypic viral resistance, we performed HCV colony formation assays in the presence of clemizole and/or SCH503034. There was an inverse correlation between the number of colonies and the compounds' concentration (Figure 5). The addition of clemizole even at a low concentration to SCH503034 significantly decreased the frequency of drug-resistant colonies, compared with SCH503034 alone. Other combinations of anti-HCV treatment were similarly shown by others to have such an effect on frequency of resistance [28, 29].

To confirm that the emerged colonies indeed harbored mutations associated with resistance to the respective inhibitors, HCV RNA replicating in cells from pools of drug-resistant colonies was isolated and subjected to sequence analysis. As expected, replicons selected under SCH503034 pressure harbored mutations within the NS3 coding region, such as the

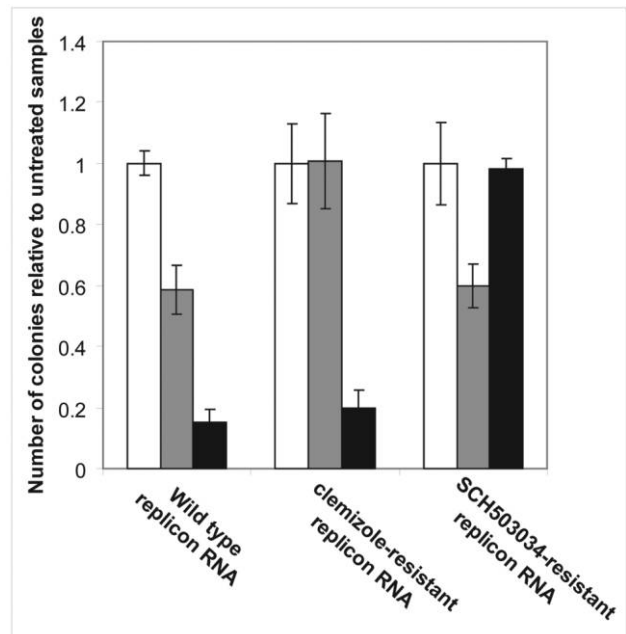


Figure 6. There is no cross-resistance between clemizole and SCH503034. Hepatitis C virus (HCV) replication in Huh7.5 cells electroporated with 50 μg of whole-cell RNA extracted from cells harboring either wild-type, the W55R NS4B mutant clone, or A156T NS3 mutant clone followed by growth in the absence (white bars) or presence of 8 μM of clemizole (gray bars) or 2.5- μM SCH503034 (black bars). Results represent relative numbers of colonies obtained compared with each corresponding untreated control.

A156T/V mutations previously shown to confer resistance to SCH503034 [28]. Similarly, previously described clemizole-resistant mutations were again selected within the NS4B coding region and the 3'-terminus of the negative viral genome in replicons extracted from cells treated with clemizole [6]. The effect of clemizole on the frequency of HCV resistance provides further rationale for its use in combination therapy with NS3 PIs.

There is no cross-resistance between clemizole and SCH503034. To confirm that there is no cross-resistance among these 2 classes of inhibitors, either SCH503034 or clemizole-resistant mutants were selected in HCV replicon-harboring cells, and the HCV RNA was subjected to sequence analysis. None of the 5 independent SCH503034-treated pooled clones harbored replicons with mutations that mapped to the NS4B or the 3'-negative terminus. Similarly, no replicons that harbored mutations in NS3's coding region were identified in 5 pooled clones treated with clemizole.

Lastly, Huh7.5 cells transfected with whole-cell RNA extracted from a clemizole-resistant clone harboring the W55R mutation [6] were unaffected by 8 μM of clemizole but remained sensitive to 2.5 μM of SCH503034 (Figure 6). Reciprocally, decreased susceptibility to SCH503034 but not to clem-

izole was demonstrated in Huh7.5 cells transfected with whole-cell RNA extracted from a SCH503034-resistant clone harboring the A156T mutation (Figure 6). Cells transfected with whole-cell RNA extracted from wild-type replicon cells were susceptible to both drugs.

DISCUSSION

As with HIV infection, effective pharmacologic control of HCV infection will likely best be achieved by a cocktail of drugs against independent virus-specific targets. Our results demonstrate that the antiviral effect of the recently discovered NS4B RNA binding inhibitor clemizole [6] is highly synergistic with HCV PIs and additive with interferon, ribavirin, or HCV polymerase inhibitors. Importantly, combining clemizole with PIs does not increase host cell toxicity. Moreover, the clemizole-SCH503034 combination decreases emergence of drug resistance without conferring cross-resistance.

Two major models, Loewe Additivity and Bliss Independence theory, are used for analyzing interactions between drugs in combinations. Although results are usually concordant, discordant results may be obtained when analyzing data in these 2 models [30, 31]. Both models defined the antiviral effect of the clemizole-SCH503034 combination as synergistic, excluding potential bias and validating the results. Furthermore, the magnitude of the clemizole-SCH503034 combination's synergy was characterized as strong [17], further emphasizing its potential relevance in vivo. Clinical trials are needed to determine the maximally tolerated dose of clemizole (based on preclinical animal data, a no observed adverse events level is estimated to be 100 mg/kg/day [32], and the dosage used for antihistamine therapy in humans was typically only ~2 mg/kg/day), and pharmacokinetics in patients with HCV infection to estimate what are the achievable serum and liver concentrations.

In addition to synergistically increasing the antiviral effect, the SCH503034-clemizole combination decreases emergence of drug resistance. This is likely a result of the distinct mechanisms of action of these 2 drugs and the resulting different drug resistance profiles. The increased antiviral effect likely also contributes. Moreover, each of these drugs can select for drug-resistant mutants that have decreased fitness [6, 28]. Importantly, there was no evidence of cross-resistance between the 2 drugs. Last, the described synergistic antiviral effects may also permit a reduction in the dose or dosing frequency of individual agents, thereby minimizing potential toxicity and adverse effects. Taken together, these major advantages further emphasize clemizole's potential as an important component of future combination regimens.

Synergy between antimicrobial drugs often implies a mechanistic interaction between the 2 targets [33, 34]. NS4B and NS3 have been shown to bind each other biochemically [35]. Furthermore, there is genetic evidence for their interaction [36].

We thus hypothesize that the interaction between these proteins, perhaps involving conformational changes, is the mechanism for the observed synergistic interaction between NS4B RNA-binding inhibitors and PIs. Alternatively, it is possible that inhibiting both of these proteins affects 2 steps in a common pathway that is critical for viral replication, thus resulting in synergy by a sequential blocking mechanism [37].

In summary, these results suggest that combination of even a moderate NS4B RNA-binding inhibitor with PIs represents an attractive paradigm for increasing virologic response rates. Although we hypothesize that more-potent inhibitors than clemizole can be obtained, because clemizole has already been extensively used in humans (albeit for a different indication), it may find immediate use as a critical component of next-generation anti-HCV strategies.

Acknowledgments

We thank Dr. Robert Prichard, for kindly providing us the MacSynergy software and for helpful and insightful comments; Leslie Holsinger (Virobay), for kindly providing us some of the compounds used in this work; the Stanford University SPARK Program; and Clinical and Translational Science Award.

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