Simultaneously Targeting the NS3 Protease And Helicase Activities For More Effective Hepatitis C Virus Therapy

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Abstract

This study examines the specificity and mechanism of action of a recently reported hepatitis C virus (HCV) non-structural protein 3 (NS3) helicase-protease inhibitor (HPI), and the interaction of HPI with the NS3 protease inhibitors telaprevir, boceprevir, danoprevir, and grazoprevir. HPI most effectively reduced cellular levels of subgenomic genotype 4a replicons, followed by genotypes 3a and 1b replicons. HPI had no effect on HCV genotype 2a or dengue virus replicon levels. Resistance evolved more slowly to HPI than telaprevir, and HPI inhibited telaprevir-resistant replicons. Molecular modeling and analysis of the ability of HPI to inhibit peptide hydrolysis catalyzed by a variety of wildtype and mutant NS3 proteins suggested that HPI forms a bridge between the NS3 RNA-binding cleft and an allosteric site previously shown to bind other protease inhibitors. In most combinations, the antiviral effect of HPI was additive with telaprevir, boceprevir, minor synergy was observed with danoprevir and modest synergy was observed with grazoprevir.

Keywords

Direct-acting antivirals; ATPase; motor protein; peptidase

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Introduction

The hepatitis C virus (HCV) causes a common liver disease that if left untreated causes cirrhosis, hepatocellular carcinoma, and liver failure. HCV is a positive-sense RNA virus with a single long open reading frame encoding a ~3,000 amino acid long polyprotein, which is cleaved by host and viral proteases into structural (core, E1, and E2) and nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B).[1] HCV is a blood-borne pathogen that replicates quickly after entering hepatocytes. Like most RNA viruses, HCV evolves rapidly, and these various hepatitis C viruses now comprise a diverse species with 7 genotypes (1–7), and several subtypes (e.g. 1a, 1b), whose sequences differ by typically 20–25%.[2]

Finding treatments for HCV has been challenging because the virus is challenging to study in the lab. HCV was not isolated until 1988,[3] and HCV could not be cultivated in the laboratory until 2005.[4] Until recently, HCV infection was treated with pegylated human interferon (pegIFN) and ribavirin, a regimen with limited efficacy and poor tolerability. PegIFN/ribavirin therapy eliminates detectable HCV in some patients, but side effects are worse than most early stages of hepatitis caused by HCV. Direct acting antivirals (DAAs) for HCV, in contrast, inhibit one of the HCV encoded proteins or enzymes needed for replication.[5] DAA targets include the NS3 protein, which is an RNA helicase and a protease activated by NS4A, the NS5A RNA binding protein, and the NS5B RNA-dependent RNA polymerase. Most DAAs for HCV inhibit the NS3/NS4A protease, the NS5A protein, or the NS5B polymerase. The USA Food & Drug Administration recently approved the NS3-NS4A protease inhibitors telaprevir, boceprevir, simeprevir, and paritaprevir, the NS5B inhibitors sofosbuvir and dasabuvir, and the NS5A inhibitors ledipasvir and ombitasvir. All-oral DAA combination therapies combining these and other DAAs are effective even in the absence of PegIFN/ribavirin.[6] However, costs of new DAA therapy still limit patient access, and new HCV drugs might still be valuable for HCV eradication.

We have been studying the helicase portion of NS3 as another possible drug target.[7, 8] Recently, we designed a series of NS3 helicase inhibitors from a component of the yellow dye primuline,[9] one of which is also a NS3 protease inhibitor and an effective antiviral against HCV subgenomic replicons in cell culture.[10] Similar concentrations of this new helicase-protease inhibitor (HPI, PubChem CID #50930749, Fig. 1) inhibit both the NS3 helicase and protease functions *in vitro*, but not the ability of NS3 to cleave ATP, the fuel for helicase movement.[10]

The goals of this study were to better understand how HPI inhibits both the NS3 helicase and protease, and how HPI interacts with other protease inhibitors used to treat HCV infections. One possible explanation for the ability of HPI to inhibit both the NS3 protease and helicase would be if HPI non-specifically bound both NS3 domains. A lack of specificity might also explain the observed HPI antiviral affect if HPI also inhibits cellular proteins. We therefore first examined HPI specificity using the same Huh7.5 cell line stably transfected with HCV subgenomic replicons made from various HCV genotypes, and another cell line harboring subgenomic dengue virus replicons. We then attempted to select

for HCV resistant to HPI, and we examined the sensitivity of telaprevir-resistant HCV isolates to HPI.

Since the resulting data suggested that HPI specifically inhibits certain HCV genotypes in a manner different from peptidomimetic protease inhibitors, we then used molecular modeling to understand how HPI could inhibit both the NS3 helicase and protease. Prior studies with HPI analogues suggested that HPI binds in the NS3 RNA binding cleft,[11] and in this pose, the fluorinated end of HPI protrudes from the helicase domain to contact a recently reported allosteric protease inhibitor-binding site.[12] To test this binding model, we examined the ability of HPI to inhibit various recombinant NS3-NS4A complexes, proteins harboring amino acid substitutions in the putative HPI-binding cleft, and proteins with mutations in the region where peptidomimetic protease inhibitors bind.[13] Results support the notion that HPI inhibits NS3-catalyzed peptide cleavage by binding an allosteric site, suggesting that HPI might be used to make HCV more sensitive to protease inhibitors that bind the NS3 protease active site. For example, HPI might lock NS3 in a conformation where NS3 would be more likely to bind other protease inhibitors. To test this idea, we examined subgenomic replicon sensitivity to various concentrations of HPI in combination with various concentrations of telaprevir, boceprevir, danoprevir[14] or grazoprevir[15] (Fig. 1). Synergy was observed between HPI and the macrocyclic inhibitors, but not the linear peptidomimetic inhibitors, supporting the notion that HPI might be a useful addition to antiviral therapies utilizing this new generation of HCV drugs.

Results

In an earlier report, Ndjomou et al. (2012)[10] studied the effects of a series of benzothiazole compounds derived from the yellow dye primuline on the various functions of the HCV NS3 protein. Many of these compounds, like NIH molecular probe ML283,[16] specifically inhibit NS3-catalyzed RNA and DNA unwinding, but they do not potently inhibit the ability of NS3 to cleave ATP and peptides. Primuline derivatives do not block helicase activity by inhibiting NS3-catalyzed ATP hydrolysis.[11] Instead, they inhibit unwinding by causing the protein to release its nucleic acid substrate before unwinding is complete.[17] Some primuline derivatives inhibit the ability of NS3 to unwind DNA & RNA and also the ability of NS3 to cleave peptides. Ndjomou et al. (2012) showed that one such dual-acting inhibitor, called "HPI" here (Fig. 1A), acts as a DAA by disrupting HCV replicase complexes in cells. Effects of HPI on NS3 protease were established using standard depsipeptide cleavage assays,[18] and effects on the NS3 helicase were investigated using a molecular beacon-based helicase assay (MBHA).[19] An MBHA monitors the ability of a helicase to displace a molecular beacon from a bound oligonucleotide upon ATP addition to cause an observable fluorescence decrease (Fig. 1B). HPI inhibits full-length NS3 in an MBHA (Fig. 1B), but the NS3 protease inhibitors telaprevir, boceprevir, danoprevir, and grazoprevir do not inhibit NS3 in an MBHA (Fig. 1C).

HPI specifically targets only some HCV genotypes

Previously, the antiviral potential of HPI was tested using a genotype 1b (con1 strain) subgenomic HCV replicon in which *Renilla* luciferase was fused to the neomycin

transferase used for cell selection (HCVsg 1b(con1)-*R*luc).[10] To examine the effect of HPI on other HCV isolates, using the same *R*luc reporter, a similar replicon was constructed using the genotype 2a JFH1 isolate.[20, 21] HPI showed little activity against the HCVsg 2a(JHF1) replicon (Fig. 2A). HPI did not affect the viability of cells containing either HCVsg 1b(con1) or HCVsg 2a(JFH1) at concentrations up to 100 μM, as was observed previously (data not shown).[10] HPI was also tested in a *Renilla* lucifer-ase-tagged subgenomic dengue virus replicon,[22] and no antiviral activity, and no effect on cell viability were observed (Fig. 2A). To test HPI on a wider variety of HCV genotypes, genotype 3a and 4a hepatitis C virus replicons[23] were also used to examine the antiviral activity of HPI. About half the concentration of HPI was needed to lower RNA levels of both the genotype 3a and 4a replicons by 50% than was needed to lower the concentration of the genotype 1b replicon to the same extent (Fig. 2B). When colony-formation assays were used to compare the effect of HPI on HCV genotype 1b and 2a replicons, some antiviral activity was noted against genotype 2a (Fig. 2C).

To understand why HCV genotype 2a seems to be less sensitive to HPI than HCV genotypes 1b, 3a, and 4a, we aligned the replicon sequences (Fig. S1, supporting information) and examined the location of amino acids present in genotype 2a but not the other HCV genotypes (Fig. 2D). Forty-one amino acids in genotype 2a NS3 are not conserved in the other three genotypes, and these are evenly distributed throughout each NS3 domain. While any of these substitutions could explain the resistance of genotype 2a to HPI, three unique genotype 2a residues are within 5 Å of the site in which HPI can bind NS3 in a computer-generated model (see below). For example, Ala482 replaces a proline in the other genotypes. In the model, Pro482 appears to contact the fluorinated end of HPI. Two conserved threonines near HPI in the model are likewise not present in genotype 2a. Thr295 contacts the other end of HPI, and Thr435 contacts the center of HPI in the model (Fig. 2D).

HPI has higher barrier to resistance than the protease inhibitor telaprevir

To better understand how HPI might interact with NS3, we next attempted to select for HCV alleles encoding HPI resistance. Even after continued incubation of numerous replicon-bearing cell lines with HPI, no noteworthy resistance to HPI could be detected. For example, when HCVsg 1b(con1) Huh7.5 cells were incubated with telaprevir for 3 weeks, the cells became resistant to telaprevir (Fig. 3A). In contrast, when the same cells were incubated twice as long with HPI, the sensitivity of the cell line to HPI did not change more than 2-fold (Fig. 3B), and no mutations could be detected in the NS3 region. Cells that become resistant to telaprevir upon incubation retained sensitivity to HPI, and cells that were incubated with HPI retained sensitivity to telaprevir (data not shown).

We next examined if HPI was able to reduce cellular replicon levels if the replicons contained the telaprevir-resistant mutations R155K[24] and V36A.[25] In control experiments, 4.2 times more telaprevir was required to inhibit replication of HCVsg 1b(con1) replicons harboring a R155K by 50% than was needed to inhibit wild type HCVsg 1b(con1), and 24 times more telaprevir was needed to inhibit HCVsg 1b(con1) replicons harboring the R155K and V36A mutations (Fig. 4A). In contrast, HPI was equally active on HCVsg 1b(con1) replicons and telaprevir-resistant HCVsg 1b(con1) replicons (Fig. 4B).

A molecular model predicting how HPI inhibits both the NS3 helicase and protease functions

To examine how HPI might modulate both the helicase and protease functions of NS3, we used molecular modeling to examine possible interactions of HPI with the known RNA-binding cleft of the full-length NS3 protein using PDB file 1CU1[26] and UCSF Dock 6.[27] The modeling suggested that HPI could bind to full-length NS3 such that the fluorines decorating the terminal phenyl stack within 5 Å of His 57 in the catalytic triad of the NS3 protease active site, while the other end of the molecule stacks in the helicase RNA binding cleft (Fig. 5A).

To test the validity of the modeled complex, we examined the ability of HPI to inhibit the protein crystallized in the 1CU1 complex, which is a "single-chain" recombinant protein where the activating region of NS4A is covalently tethered to the NS3 N-terminus (called here scNS4A-NS3).[12, 26, 28, 29] In addition to the "wildtype" protein, we also examined the sensitivity of various HCV genotype 1a scNS4A-NS3 proteins harboring amino acid substitutions near the model-predicted HPI-binding site (S483A, M485A, V524A), and near the binding site of peptidomimetic protease inhibitors (F438A, Q526A, H528A).[30, 31]

Aydin et al. previously characterized the protease, helicase and ATPase functions of each of the mutant scNS4A-NS3 proteins used here.[13] All of the proteins retain the ability to hydrolyze ATP, cleave peptides, and unwind duplex nucleic acids at rates comparable to the wildtype proteins.

We next sought to understand how HPI interacts with the NS3 helicase and protease active sites. Since both functions are difficult to monitor under steady-state conditions, we instead monitored HPI inhibitory effects under pseudo-first order conditions, where substrate concentrations were far lower than substrate K_m's observed in either the helicase assay[32] or protease assay.[18] Under these conditions, the concentration of HPI needed to reduce initial reaction rate by 50% would approximate a K_i if HPI acts as either a competitive or non-competitive inhibitor. The same concentration of HPI was needed to inhibit the ability of all the proteins tested here to unwind DNA by 50% in MBHAs (data not shown). However, K_i values describing the ability of HPI to inhibit peptide cleavage catalyzed by the various recombinant NS3 proteins were notably different (Fig. 5B) assuming the enzyme variants all had K_m values in the same range as those previously published for wildtype scNS4A-NS3 (51 ± 4 μM)[33] or NS3 in the presence of pep4A (18 ± 1 μM).[33]

The M485A substitution had the largest effect on the ability of HPI to inhibit NS3-catalyzed peptide cleavage. Specifically, replacement of Met485 with a smaller Ala, enhanced the ability of HPI to inhibit NS3-catalyzed peptide cleavage by about 5-fold (Fig. 5B). In our model, Met485 appears to block HPI access to the protease active site. HPI does not appear to bind like most peptidomimetic inhibitors because neither the Q526A nor H528A substitutions influenced the ability of HPI to inhibit the NS3 protease. Both Q526A and H528A were previously shown to affect the affinity of NS3 for a variety of different NS3 peptidomimetic protease inhibitors.[35]

Since NS4A and NS3 are not covalently tethered in the cell, we also examined the sensitivity of a recombinant full-length NS3 in the presence and absence of its NS4A cofactor to HPI. Interestingly, when NS3 was not fused to NS4A, considerably more HPI was needed to inhibit NS3-catalyzed peptide cleavage, both in the presence and absence of NS4A (Fig. 5B).

To understand if the putative HPI-binding site overlaps with other known protease inhibitor binding sites, the model was aligned with the crystal structures of various protease inhibitors bound to NS3 protease, such as 3M5L in which danoprevir is bound, and 3SUD in which grazoprevir is bound to the NS3 protease.[31] The modeling suggested that NS3 could accommodate both HPI and grazoprevir (Fig. 5C), and as discussed below, these data support a model in which HPI facilitates grazoprevir binding by trapping NS3 in a compact conformation more likely to interact with the protease inhibitor.

Synergy between HPI and a macrocyclic NS3 protease inhibitor

To study the effect of HPI on HCV replicons in combination with other protease inhibitors, the effect of each agent alone on a stable subgenomic HCV replicon cell line was first examined (Table 1). Each agent was applied to Huh 7.5 cells with stable HCVsg 1b(con1)-Rluc.[36] After 72 hours, Rluc activity in cell extracts was measured.[10] In control reactions, cell viability was monitored with various concentrations of each protease inhibitor, and the effect of each compound on RLuc activity present in cell extracts was assessed. The concentration of each compound needed to reduce cell viability by 50% (CC_{50}) always greatly exceeded the concentration needed to reduce replicon content by 50% (EC_{50}) (Table I).

The resulting EC_{50} values (Table 1) were used to design combination experiments where various concentrations of each of two compounds were applied in 7-point 2-fold dilution series with concentrations above the observed EC_{50} values and concentrations below the EC_{50} values obtained with each compound alone. Reactions were performed in triplicate, normalized, and compared to inhibition that would be expected from the Bliss independence model.[37] Differences between observed and expected inhibition were plotted in three dimensions and the surfaces of the 3D plots were inspected for areas of synergy (positive, Fig. 6) and antagonism (negative, Fig. 6). In additional control experiments, we observed no reduction in cell viabilities in the presence of any concentration of any antiviral agent analyzed. None of the combinations affected the activity of *Renilla* luciferase present in crude cell lysates, and only HPI affected rates of NS3-catalyzed DNA or RNA unwinding (data not shown).

Prichard & Shipman define significant differences from Bliss additivity as those that yield volumes under the surfaces that are greater than 25 (μM^2 %) in such a three-dimensional analysis, with minor but significant effects yielding volumes between 25 and 50, moderate effects yielding values between 50 and 100, and strong effects yielding values greater than 100.[37] Based on these definitions, no significant deviations from additive effects were observed when HPI was combined with telaprevir, or boceprevir (Fig. 6A, B). Minor, insignificant synergy was observed between HPI and danoprevir (Fig. 6C, where the volume under the plot is 17), but when HPI was combined with grazoprevir, strong synergy was

observed (Fig. 6D, where the volume under the plot is 101). The finding that HPI interacts with grazoprevir, but not with telaprevir and boceprevir supports the notion that HPI might alter the conformation of NS3 so that the enzyme is more sensitive to danoprevir and grazoprevir.

Discussion

This study shows that HPI (Fig. 1), a specific HCV antiviral (Fig. 2) that inhibits both the helicase and protease activity of the HCV NS3 protein, does not function like the peptidomimetic inhibitors in clinical use today. Instead of binding to the protease active site, HPI appears to exert its action by binding to an allosteric site between the helicase and protease domains (Fig. 5). HPI has a higher barrier to resistance (Fig. 3), is active against common protease inhibitor resistance alleles (Fig. 4), and can be used to enhance the efficacy of macrocyclic protease inhibitors (Fig. 6). This is also the first study to show that it is possible to target both the NS3 protease and helicase activities at the same time with different small molecules.

Hepatitis C virus NS3 is a multi-functional viral protein that plays at least two distinct roles in the virus life cycle, with the N-terminal domain cleaving viral and host proteins, and the C-terminal helicase unwinding duplex nucleic acids. This relationship provides a unique drug target because, other than NS3 encoded by HCV and related viruses, no other proteins are known to combine both protease and helicase activities in the same polypeptide. The two activities are tightly coupled with residues in the protease region promoting the helicase activity,[38] residues in the helicase region enhancing the proteolytic activity,[39] and the protease co-factor NS4A modulating RNA-stimulated helicase-catalyzed ATP hydrolysis.[40]

Numerous compounds that inhibit NS3-catalyzed peptide cleavage have been developed as antivirals,[6] three of which (telaprevir, boceprevir, and simeprevir) have been already approved by the FDA. Telaprevir and boceprevir are linear peptidomimetics, but they preferentially inhibit certain HCV genotypes, and they must be administered with interferon and ribavirin because relatively fit drug-resistant HCV variants evolve rapidly. Newer protease inhibitors, represented here by danoprevir and grazoprevir, are macrocyclic peptidomimetics that are pan-genotypic, more potent, and more active against known resistant HCV variants (Fig. 1). Part of this enhanced activity derives from interactions with the helicase domain when the protein is in the compact conformation. P1-P3 macrocyclic protease inhibitors similar to danoprevir interact with residues Val524, Gln526, His528, and Met485.[29] Compounds resembling grazoprevir with a P2-P4 macrocycle were designed to engage residues Gln526 and His528 on the helicase in addition to residues in the protease region.[30] Interactions with the helicase domain also enhance the ability of macrocyclic compounds to inhibit the drug-resistant R155K and A156T variants.[31, 41] A relevant observation here is that the mutation of residues suspected to interact with danoprevir or grazoprevir (*i.e.* Val524, Gln526, and His528) do not affect the ability of HPI to inhibit NS3-catalyzed peptide cleavage (Fig. 5), nor do telaprevir-resistant substitutions (R155K, V36A) influence the ability of HPI to inhibit subgenomic HCV replicons (Fig. 4). These data suggest that HPI does not bind in the same site as the peptidomimetic inhibitors.

In a prior study, Ndjomou *et al.* reported that HPI[10] inhibits the ability of NS3 to unwind DNA, unwind RNA, and cleave polypeptides, with a similar potency. However, much higher HPI concentrations are needed to inhibit NS3-catalyzed ATP hydrolysis.[10] In a later study, Sweeney *et al.*[11] used molecular modeling and site-directed mutagenesis to show that a compound similar to HPI (*i.e.* CID #50930756) binds NS3 helicase perpendicular to the known RNA binding cleft to trigger the movement of a so-called spring helix needed for RNA to reorient the ATP binding site and stimulate ATP hydrolysis. A similar binding site was observed here for HPI, although our new model of HPI bound to NS3 (Fig. 5) suggests that HPI bridges the helicase RNA-binding cleft to the cleft between the helicase and protease. In such a pose, the fluorinated part of HPI resides in the same site occupied by allosteric NS3 protease inhibitors reported by Astex pharmaceuticals, which also bind near Met485 in the NS3 helicase domain and Asp79 in the protease domain.[12] Our observation that mutation of Met485 affects the inhibitory potential of HPI supports this binding model (Fig. 5). Nevertheless, molecular modeling of the binding of HPI to the NS3 protein is, by definition, speculative and does not alone explain the mode of HPI action. We are pursuing structural studies to better understand how HPI interacts with NS3 on a molecular level.

Another noteworthy observation is that scNS4A-NS3 fusion proteins are more sensitive to HPI inhibition than NS3 not fused to NS4A (Fig. 5B). These data could be explained by the fact that NS3 must adopt different conformations to cleave its diverse cellular substrates. To cleave the junction between NS3 and NS4A, NS3 must assume a compact conformation where the NS3 C-terminus resides in the protease active site. However, to cleave other substrates, NS3 must shift to a more extended conformation, in which the C-terminus of NS3 is no longer bound in the protease active site as a product inhibitor.[26] An extended conformation is needed to cleave the other sites in the HCV polyprotein[42] and cellular targets like the mitochondrial antiviral signaling protein MAVS[43] and the Toll-like receptor 3 adaptor protein TRIF.[44]

We propose that HPI is a better inhibitor of scNS4A-NS3 than NS3 because HPI must interact with the NS3 compact conformation to inhibit NS3-catalyzed peptide cleavage. All HCV scNS4A-NS3 proteins crystallized to date adopt a compact conformation,[12, 26, 28, 29] but native NS3 without covalently tethered NS4A has been shown to more readily assume an extended conformation.[45] In our lab, all scNS4A-NS3 proteins migrate more rapidly on gel filtration columns than lower mass NS3 proteins, suggesting that NS3 is less compact than scNS4A-NS3. The substrate for assays used here to monitor NS3-catalyzed peptide cleavage is a short fluorescent depsipeptide, which could be cleaved when the protein is in either conformation. However, if HPI only inhibits the compact conformation, less HPI should be needed to inhibit recombinant purified NS3 constructs that primarily assume the compact conformation. Inhibiting NS3 by stabilizing a compact conformation is not new concept, and other compounds binding the helicase-protease interface, like the Astex inhibitors[12] noted above, have also been proposed to lock the NS3-NS4A complex in a compact conformation. Unlike HPI, however, the Astex compounds have not been reported to influence the NS3 helicase activity. To date, only the compact conformation of HCV NS3 has been captured in crystal structures, although the homologous complex from dengue virus (DENV) has been observed in an extended conformation with x-ray crystallography.[46]

Interestingly, HPI has no activity against dengue virus replicons (Fig. 2). Synergy between grazoprevir and HPI could therefore be explained if HPI stabilizes NS3 in a compact conformation so that NS3 will be more likely to interact with grazoprevir.

The final key observation reported here is that HCV appears to be less able to avoid the effects of HPI than other telaprevir (Fig. 2). Again, this is not a new observation for helicase inhibitors, as only one helicase inhibitor resistance allele has been reported in the literature, a T477A mutation resistant to a tropolone helicase inhibitor.[47] The higher barrier of resistance to helicase inhibitors could make them useful additions to DAA therapies that lack a nucleotide NS5B inhibitor because circulating HCV strains already include many variants with alleles encoding resistance to DAAs targeting NS5A, the NS3 protease, and non-nucleotide NS5B inhibitors.[48]

Simultaneously targeting both the NS3 protease and helicase functions with small molecules is a novel therapeutic design approach that could be critical in future combination therapies. With its high barrier to resistance, novel mechanism of action, synergy with the latest macrocyclic protease inhibitors in development, HPI could be a valuable new agent in the DAA arsenal available to design more cost effective all-oral therapies to eradicate HCV.[5]

Methods

Materials

HPI (PubChem CID #50930749) was synthesized and purified as described.[9] Telaprevir, danoprevir, and grazoprevir (MK-5172) were synthesized and purified as described.[41] Boceprevir was purchased from MedChem Express (Princeton, NY). All recombinant proteins were expressed in *E. coli* and purified as described for full-length NS3,[49] scNS4A-NS3 from genotype 1b (gt1b),[50] and scNS4A-NS3 from genotype 1a (gt1a) and scNS4A-NS3 mutants D79A, S483A, M485A, V524A, Q526A, and H528A.[13]

The sub genomic HCV genotype 1b(con1 strain) *Renilla* luciferase replicon (HCVsg 1b(con1)-*R*luc) and its stably transfected Huh7.5 cell line was the same as described before.[9, 10] Plasmid S52/SG-Feo(AII), which encodes the HCVsg 3a(S52) replicon, and plasmid ED453/SG-FEO(VYG), which encodes the HCVsg 4a(ED43) replicon, were obtained from Dr. Charles Rice (Rockefeller University),[23] Plasmid pYSGR-JFH-1,[21] which encodes a J6/JFH1 infectious clone, was obtained from Brett Lindenbach (Yale University).

The HCVsg 2a(JFH1)-*R*luc expression plasmid was constructed using a stepwise three-fragment PCR-fusion strategy. First, the HCV 5′UTR was amplified from pYSGR-JFH-1[21] with the forward primer RI-T7: 5′-GCC AGT <u>GAA TTC</u> TAA TAC GAC TCA CTA TAG-3′ (EcoRI restriction site underlined) and the reverse primer Core-R: 5′-GGG CGA CGG TTG GTG TTT CTT T-3′. The *R*luc gene was amplified from HCVsg 1b(con1)-Rluc with the forward primer Core-*R*Luc-F: 5′-CAA CCG TCG CCC AAT GGC TTC CAA GGT GTA C-3′ and the reverse primer *R*luc-FMDV2A-R: 5′-CGC AAG CTT AAG AAG GTC AAA ATT CAA CAG CTG CTG CTC GTT CTT CAG CAC-3′. The neomycin gene was amplified from pYSGR-JFH-1 with the forward primer FMDV2A-Neo-F: 5′-CTT CTT AAG CTT GCG GGA GAC GTC GAG TCC AAC CCT GGG CCC ATG ATT GAA CAA

GAT GGA TTG C-3′ and the reverse primer Neo-PmeI: 5′-GG GTT TAA ACT CAG AAG AAC TCG TCA AG-3′ (PmeI restriction site underlined). Second, the 5′UTR and *R*luc fragments were fused using primers RI-T7 and RLuc-FMDV2A-R to make 5′UTR-*R*luc fragment. Third, 5′UTR-*R*luc fragment was fused to the neomycin fragment using primers RI-T7 and Neo-*Pme*I to make the final PCR fragment 5′UTR-*R*Luc-Neo that has the foot and mouth disease virus 2A (FMDV2A) peptide cleavage sequence between *Renilla* luciferase and neomycin genes. The resulting PCR product (5′UTR-*R*luc-Neo) was then digested with *Eco*RI and *Pme*I and cloned into *Eco*RI/*Pme*I digested pYSGR-JFH-1 backbone to yield pRluc-HCVsg 2a(JFH1), the sequence of which was confirmed by sequencing.

Site-directed mutagenesis was performed on the HCVsg 1b(con1) plasmid using the QuikChange II kit (Agilent Technologies, La Jolla, CA). The R155K mutation was introduced in NS3 using the forward primer 5′-GCA CGC TGT GGG CAT CTT TAA GGC TGC CGT G-3′ and the reverse primer 5′-CAC GGC AGC CTT AAA GAT GCC CAC AGC GTG C-3′. The NS3 protease double mutant R155K/V36A was constructed by introducing the V36A mutation in the R155K plasmid backbone with primers 5′-GGG AGG TCC AAG CGG TCT CCA CCG C-3′ and 5′-GCG GTG GAG ACC GCT TGG ACC TC CC-3′. Mutations were confirmed by DNA sequencing.

HCV subgenomic replicon assays

All subgenomic replicon containing Huh-7.5 cells were isolated and maintained as previously described,[10] To assess the ability of each compound to inhibit HCV replication, Huh7.5 cells harboring HCV *R*luc subgenomic replicons were seeded at 10×10^3 cells per well in 96-well plates and incubated 4–5 hours to allow the cells to attach to the plate. Two-fold serial compound dilutions were made in dimethyl sulfoxide (DMSO), and diluted into media, such that the DMSO final concentration was 0.5% after adding dilutions to cells. Compounds and cells were incubated at 37 °C in 5% CO_2. After 3 days, *Renilla* luciferase was measured using Promega's *Renilla* luciferase assay kit. Quantitative reverse transcriptase PCR using Taqman probes specific to conserved sequences in the HCV 5′UTR was used to measure relative RNA levels as previously described.[10] PCR data were normalized to RNA levels observed in cells incubated with DMSO only.

Dengue virus subgenomic replicon assay

Baby hamster kidney (BHK-21) cells were stably transfected with the replicon described by Stahla-Beek *et al.*[22] using a replicon plasmid obtained from Brian J. Geiss (Colorado State). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, LifeTech) containing 4.5 g/l D-glucose, L-glutamine, and 110mg/L sodium pyruvate and supplemented with 10% FBS, 100 U/mL each of penicillin and streptomycin and 3 μg/mL puromycin to maintain replicons stability, seeded in clear 96-well culture plates (Corning #3599) without puromycin at a concentration of 1,000 cells per well. Cells were allowed to adhere, and HPI dissolved in DMSO and diluted in DMEM was added to cells such that the final volume in the well was 100 μl, and the final DMSO concentration was 1%. The effect of compounds on replication was assessed by measuring *Renilla* luciferase reporter gene activity as described above.

Cell Viability Assays

To assess compound effects on Huh-7.5 or BHK DENV-Rluc cell viability, cells were plated and treated as above for compound inhibitory activity and the effect of compound on cell viability was tested using the CellTiter-Glo luminescent cell viability kit (Promega).

Colony formation assays

Huh-7.5 HCVsg 1b(con1)-*R*luc replicon cells were plated at 2×10^5 cells per well in 6-well plates in complete DMEM in the presence of 350 μg/ml G418. Cells were treated with HPI (25 μM and 100 μM) or telaprevir (1 μM and 10 μM) or the DMSO control and incubated for 3 days after which a fresh dose of compounds or DMSO was added to the cells. Media containing fresh aliquots of compounds (HPI or telaprevir) or DMSO was added every 3 days for two weeks. The cells were washed with PBS, fixed with 4% PFA and stained with 0.5% crystal violet solution for 30 min at room temperature, then washed extensively with ddH$_2$O until no colored crystal solution was observed.

Resistance selection

Huh7.5 cells bearing HCVsg 1b(con1)-*R*luc replicons were exposed to various concentrations of HPI, telaprevir (positive control) or DMSO alone (negative control) corresponding to 0.25×, 0.5×, 1×, 2.5×, 5×, and 10× the IC$_{50}$ value for the compound and incubated in the presence of 350 μg/ml G418 selection. Media containing fresh aliquots of HPI or telaprevir was replenished every 3 to 4 days and upon cell splitting. After 3 or 6 weeks of continued selection, the susceptibility of evolved cells lines to HPI and telaprevir was tested by measuring *Renilla* luciferase remaining after exposure to various amounts of each compound.

Molecular Modeling

HPI was docked into PDB file 1CU1[26] that was stripped of its ligands. Water molecules and counterions were removed from each PDB file, and incomplete side chains were filled using UCSF Chimera 1.6.2.[34] Using UCSF Chimera's Dock Prep module, histidine protonation states were calculated, and incomplete side chains were automatically filled. A three-dimensional conformation of HPI was generated using Open Babel GUI, was saved as a PDB file, and positioned using a rigid body orienting code in UCSF DOCK 6.5.[27] The ligand-binding site of the protein was constrained to be the largest cluster of spheres surrounding the molecular surface of the protein as generated by UCSF DOCKS's sphgen module.

Protease assays

HCV NS3 depsipeptide cleavage assays[18] were performed with the 5-FAM/QXL™520 substrate (Anaspec), which is based on the NS4A/NS4B cleavage site. Each 20 μl assay contained 50 mM Tris–HCl pH 7.0, 50% glycerol, 2% CHAPS, 30 mM DTT, 5% DMSO, and 0.5 μM 5-FAM/QXL™520 substrate. When NS3 lacking a covalently tethered NS4A was used, reactions were supplemented with 6 μM pep4AK (Anaspec). Reactions were first performed with various concentrations of each NS3 protein to determine its specific activity. A unit of enzyme was defined as the amount needed to cleave 0.05 μM of substrate/min. To

determine the inhibitory potential of HPI with each enzyme, reactions were performed with 1 unit of each enzyme with various concentrations of HPI. Reactions containing HPI were performed in the presence of a 16-pt two-fold dilution series of HPI starting at 100 μM. Assays were performed in duplicate, and rates normalized to reactions performed in the absence of HPI. Initial rates of substrate cleavage were then plotted verses HPI concentration and fitted to a concentration response equation to calculate the concentration of HPI needed to reduce initial rates by 50% using GraphPad Prism (v.6).

Helicase assays

Molecular-beacon based helicases assays[32] were performed and data analyzed as described by Hanson et al.[19] Reactions were performed in the presence of 50 nM NS3, 12.5 nM substrate, and 12 different HPI concentrations ranging from 1 to 100 μM. Unwinding rates were calculated from the slopes immediately following ATP addition.

Combination studies

Two-fold serial dilutions, starting at ~4 times the EC_{50} values obtained in concentration-response assays with each compound alone, were prepared in 96-well plates. 50 μl of each dilution of the first compound to be tested (at twice the final compound concentration) was added horizontally and 50 μl of each dilution of the second compound was added vertically to make a $1\times$ final concentration of each compound. After 3 days at 37 °C, luciferase activity was measured. At least four separate experiments (in triplicate) were performed for each combination.

Data analysis

Half-maximal inhibitory concentrations were calculated from concentration response curves using nonlinear regression to fit data to a log(inhibitor) vs. normalized response equation with variable slope. The effects of drug-drug combinations were evaluated using the MacSynergy II (http://www.uab.edu/medicine/peds/macsynergy).[37, 51] MacSynergy II uses the Bliss independence model to estimate synergy and/or antagonism. Additive inhibition is calculated in MacSynergy using the equation $Z = X + Y (1−X)$, where X and Y correspond to the inhibitory effects of compound 1 and 2 respectively, and Z is the effect produced when the two compounds are combined. Resulting values are then subtracted from the normalized inhibition observed at each drug combination to estimate synergy (positive values) or antagonism (negative values). Synergy/antagonism values calculated using the MacSynergy II Excel spreadsheet at the 95% confidence levels were plotted in three dimensions using DeltaGraph 6 (Redrock Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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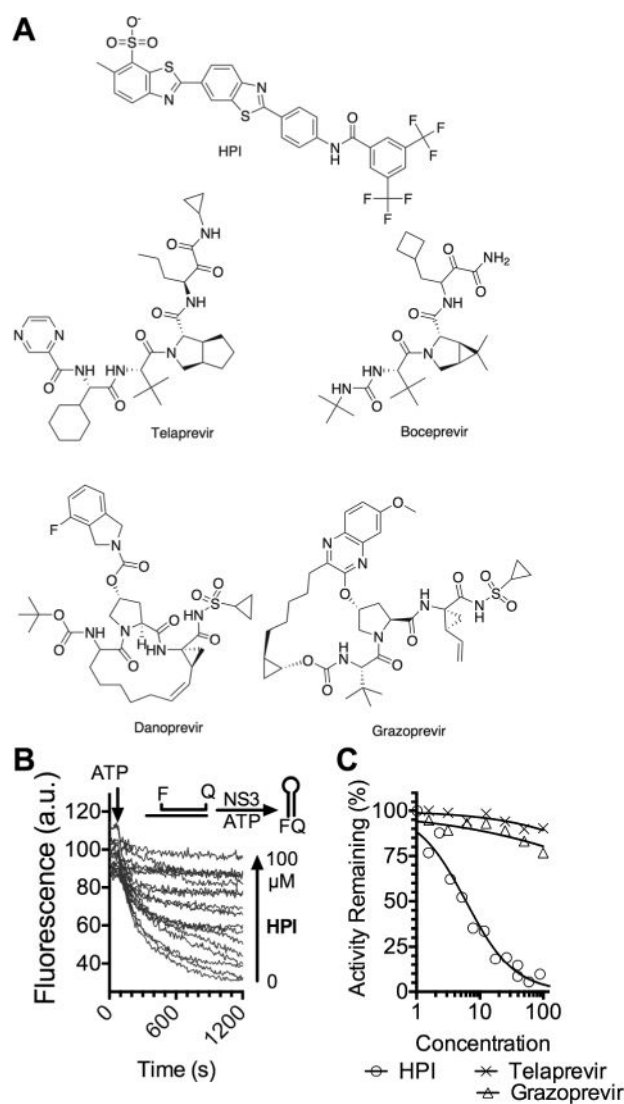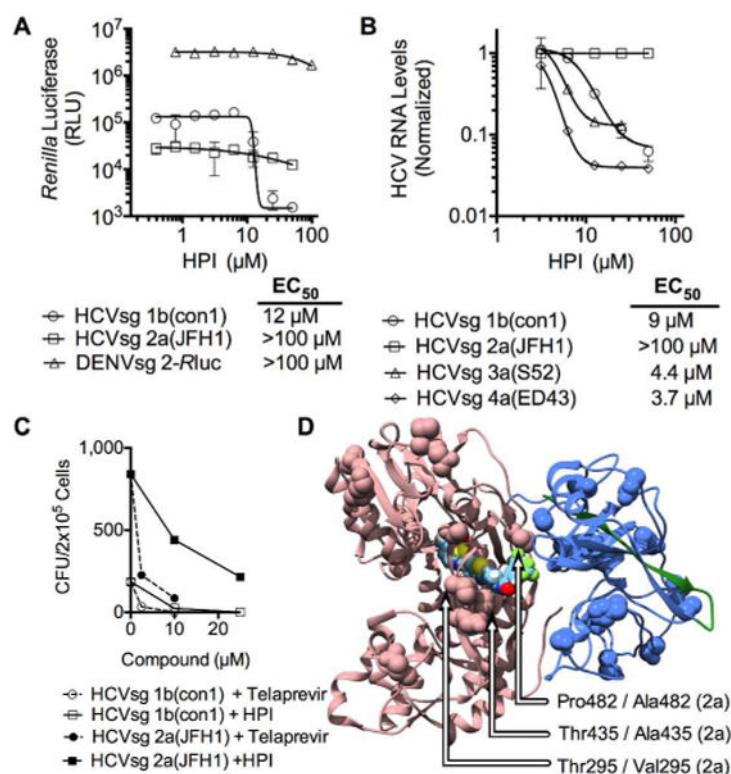
Figure 1.
Effect of HPI and other protease inhibitors on the ability of NS3 to unwind DNA. (A) Direct acting antivirals used in this study. (B) Ability of HPI to inhibit molecular beacon-based helicase assays (MBHAs). In MBHAs, NS3 separates a molecular beacon from a complementary strand so that fluorescence decreases with time. (C) Activity remaining, calculated from normalized initial velocities, in the presence of various NS3 protease inhibitors (data for boceprevir and danoprevir are omitted for clarity. Neither inhibit the reaction). Data were fitted to a normalized concentration-response equation using Graphpad Prism v.6. The IC_{50} value for HPI was 6 ± 2 μM.

Figure 2.
HPI specificity. (A) The ability of HPI to reduce cellular content of *Renilla* luciferase tagged subgenomic replicons made from HCV genotype 1b (HCVsg 1b(con1), circles), HCV genotype 2a (HCVsg 2a(JFH1), squares) and dengue virus strain 2 (DENVsg 2, triangles) (B) Effect of various HPI concentrations on relative levels of subgenomic replicon RNA, as measured by quantitative reverse-transcriptase PCR, with data normalized to RNA levels seen in cells treated with DMSO only. (C) Colony formation units (CFU) of Huh7.5 cell cultures harboring the HCVsg 1b(con1) or the HCVsg 2a(JFH1) replicon. Cells were initially plated at 2×10^5 cells/dish, and G418-resistant colonies were stained with crystal violet after 3 weeks of antibiotic selection. Note CFUs for the HCVsg 2a(JFH1) replicon were about 10 times higher than CFUs observed with HCVsg 1b(con1) in the absence of HPI or telaprevir. (D) Unique residues in genotype 2a(JFH1) are highlighted on the scNS4A-NS3 structure in which HPI is docked. Residues pesent in 2a(JFH1) NS3 but not genotypes 1a(H77), 1b(con1), 3a(S52), or 4a(ED42) are highlighted as spheres with unique amino acids within 5 Å of HPI noted with arrows. Sequence alignments are shown in Figure S1 (Supporting Information).
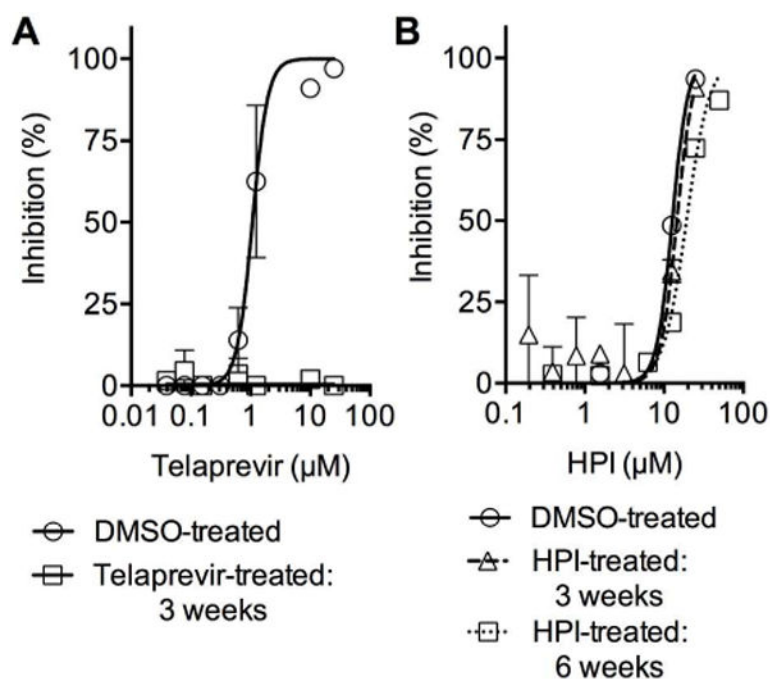
Figure 3.
Evolution of HCV resistant to telaprevir and HPI. (A) Sensitivity of the HCVsg 1b(con1)-*R*luc replicon to telaprevir after incubation with DMSO (circles) or telaprevir (squares) for 3 weeks. (B) Sensitivity of the HCVsg 1b(con1)-*R*luc replicon to HPI after incubation with DMSO (circles) or HPI for 3 weeks (trangles) or 6 weeks (squares). After 3 or 6 weeks of selection, the susceptibility of evolved cells lines to each compound was determined by measuring *Renilla* luciferase activity remaining after exposure to various amounts of each telaprevir or HPI for 3 days.
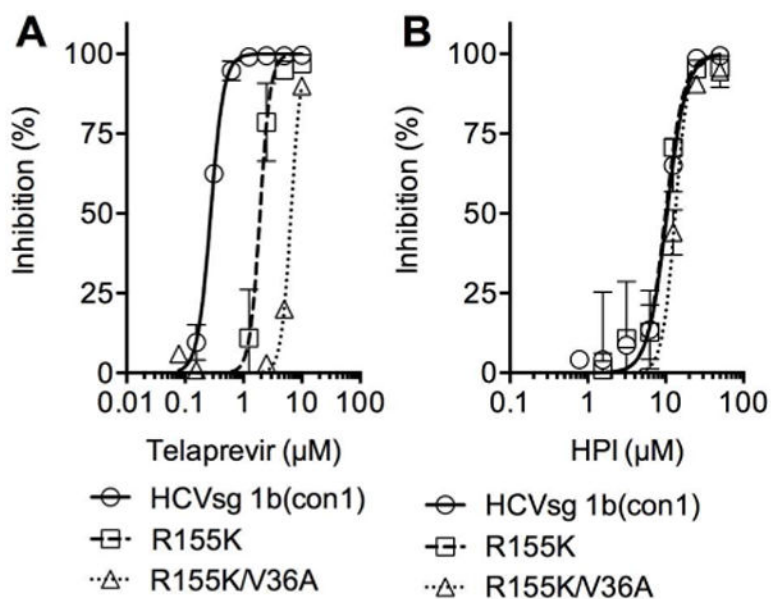
Figure 4.
Sensitivity of HCVsg 1b(con1)-*R*luc replicons carrying telaprevir-resistance mutations. (A) Normalized *Renilla* luciferase remaining after exposure of HCVsg 1b(con1) (circles), HCVsg 1b(con1) carrying the NS3 R155K substitution (squares), or HCVsg 1b(con1) carrying both a R155K and V36A substitutions (triangles) to various amounts of telaprevir for 3 days. (B) Normalized *Renilla* luciferase remaining after exposure of HCVsg 1b(con1) (circles), HCVsg 1b(con1) carrying the NS3 R155K substitution (squares), or HCVsg 1b(con1) carrying both a R155K and V36A substitutions (triangles) to various amounts of HPI for 3 days.
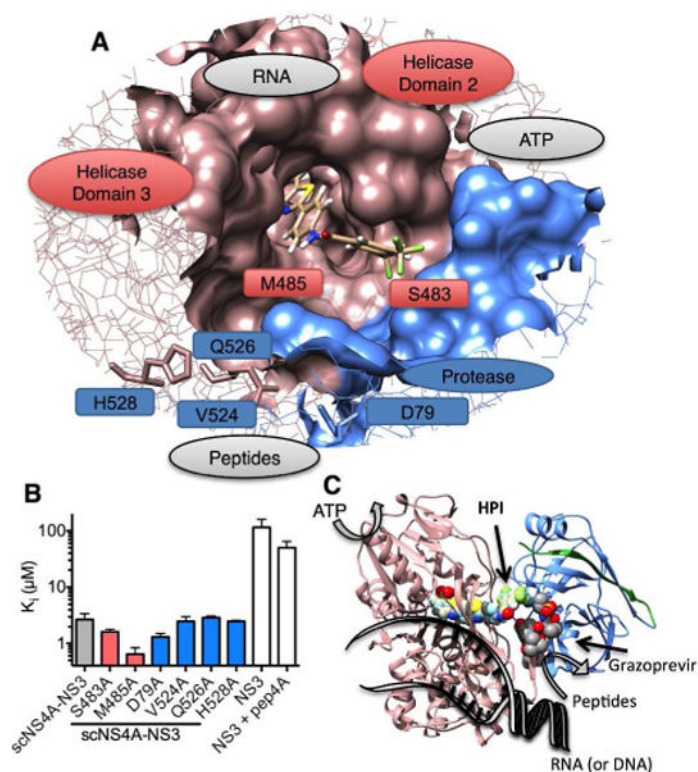
Figure 5.
The possible HPI-binding site on NS3. (A) Position of HPI when docked in the full-length HCV NS3 structure (PDB 1CU1). The putative HPI binding site is highlighted as a surface on a wireframe NS3 model with the helicase red and protease blue. Residues targeted for site-directed mutagenesis are shown as sticks. Natural ligand-binding sites are labeled in grey. (B) Inhibitory constant (K_i) describing the ability of HPI to inhibit NS3-catalyzed peptide hydrolysis. Wildtype scNS4A-NS3 is grey, amino acid substitutions in the putative HPI-binding site are red, and amino acid substitutions in the cleft that binds protease substrates are blue. Full-length NS3 constructs lacking the covalent tether to NS4A are white. (C) Position of docked HPI in realtion to the various NS3 active sites, ligand binding clefts, and the peptidomimetic protease inhibitor-binding site. Position of grazoprevir was determined by aligning the grazoprevir costructure with the NS3 protease (PDB file 3SUD)[31] with the structure of full-length NS3 (PDB file 1CU1).[26] The protease domain is blue, NS4A is green, and the helicase is red. Models were rendered using UCSF Chimera 1.8.[34]
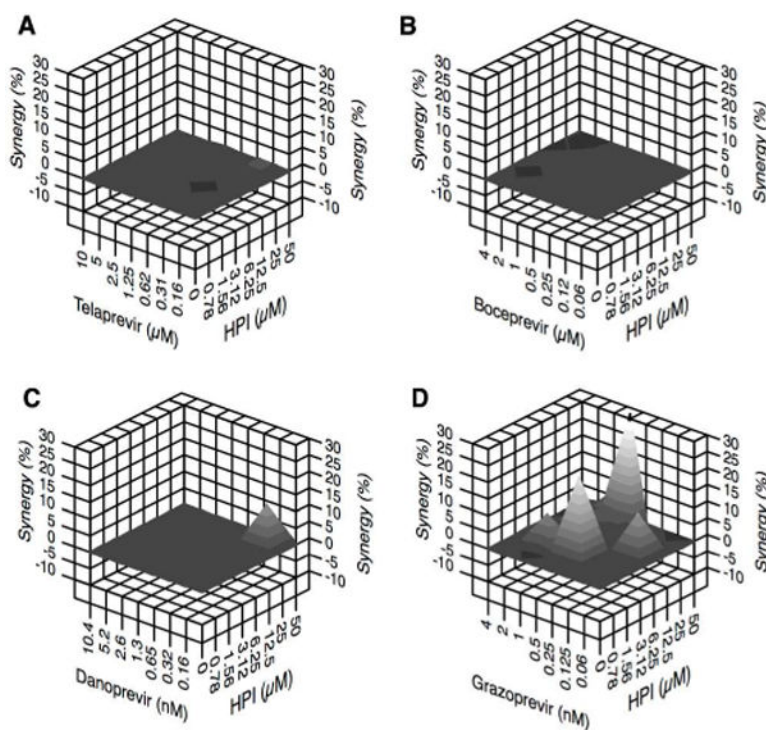
Figure 6. Interactions between HPI and other protease inhibitors
Points on the z-axes represent the difference in replicon inhibition observed from the inhibition that would be expected if the two agents produced additive effects in the Bliss independence model when compounds inhibited the HCVsg 1b(con1)-*R*luc replicon with the IC_{50} values listed in Table 1. In each 3D plot, combinations yielding synergy are progressively lighter grey. Data were analyzed using MacSynergy II.[37]

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Table 1

Ability of various DAAs to inhibit the genotype 1b subgenomic replicons and Huh7.5 cell viability

Compound	EC_{50} $(\mu M)^a$	CC_{50} $(\mu M)^b$
HPI	15 ± 4	>100
Telaprevir	0.81 ± 0.4	45 ± 4
Boceprevir	0.64 ± 0.1	> 100
Danoprevir	0.002 ± 0.0005	100
Grazoprevir	0.00076 ± 0.00013	54 ± 5

[a]EC_{50} values represent the concentration of each compound needed to reduce cellular HCVsg 1b(con1)-*R*luc replicon content by 50% relative to cells treated with DMSO alone.

[b]CC_{50} values are concentrations needed to reduce cell viability by 50%.