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Evaluating the Role of Macrocycles in the Susceptibility of Hepatitis C Virus NS3/4A Protease Inhibitors to Drug Resistance

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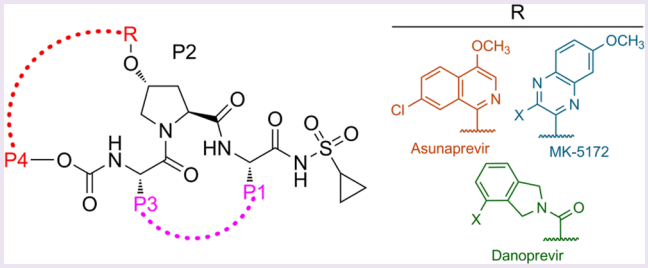
Supporting Information

ABSTRACT: The hepatitis C virus (HCV) infects an estimated 150 million people worldwide and is the major cause of viral hepatitis, cirrhosis, and liver cancer. The available antiviral therapies, which include PEGylated interferon, ribavirin, and one of the HCV NS3/4A protease inhibitors telaprevir or boceprevir, are ineffective for some patients and cause severe side effects. More potent NS3/4A protease inhibitors are in clinical development, but the long-term effectiveness of these drugs is challenged by the development of drug resistance. Here, we investigated the role of macrocycles in the susceptibility of NS3/4A protease inhibitors to drug resistance in asunaprevir, danoprevir, vaniprevir, and MK-5172, with similar core structures but varied P2 moieties and macrocyclizations. Linear and macrocyclic analogues of these drugs were designed, synthesized, and tested against wild-type and drug-resistant variants R155K, V36M/R155K, A156T, and D168A in enzymatic and antiviral assays. Macrocyclic inhibitors were generally more potent, but the location of the macrocycle was critical for retaining activity against drug-resistant variants: the P1−P3 macrocyclic inhibitors were less susceptible to drug resistance than the linear and P2−P4 macrocyclic analogues. In addition, the heterocyclic moiety at P2 largely determined the inhibitor resistance profile, susceptibility to drug resistance, and the extent of modulation by the helicase domain. Our findings suggest that to design robust inhibitors that retain potency to drug-resistant NS3/4A protease variants, inhibitors should combine P1−P3 macrocycles with flexible P2 moieties that optimally contact with the invariable catalytic triad of this enzyme.



The hepatitis C virus (HCV) infects an estimated 150 million people worldwide and is the major cause of viral hepatitis, cirrhosis, and liver cancer.[1] The current standard of care for HCV-infected patients is a triple combination therapy with PEGylated interferon α (Peg-IFN), ribavirin (RBV), and telaprevir or boceprevir,[2] recently approved direct-acting antiviral agents targeting the viral NS3/4A protease. This new treatment regime is effective for most patients,[3−7] but a combination of viral and host-cell genetic factors, including a human polymorphism at the IL28B gene associated with poor interferon response,[8] causes treatment failure in some patients. In addition, severe side effects associated with Peg-IFN [9] and the rapid emergence of drug resistance against protease inhibitors limit both patient adherence and the effectiveness of triple combination therapy.[10,11] Thus, more robust new direct-acting antivirals are needed against a broader spectrum of resistant HCV variants and genotypes.

The HCV NS3/4A is a bifunctional protein with a chymotrypsin-like serine protease and an ATP-dependent RNA helicase.[12,13] The NS3/4A protease is essential for viral replication playing a pivotal role in polyprotein processing and circumventing host immune response.[14−16] Pharmaceutical companies have invested significant effort in developing NS3/4A protease inhibitors, leading thus far to the discovery of two FDA approved drugs, boceprevir[17] and telaprevir,[18] both of which contain a ketoamide group that form a reversible, covalent bond with the catalytic serine of the protease. In addition, several non-covalent NS3/4A inhibitors are at a variety of stages of clinical development, and include both linear (asunaprevir,[19] BI 201335[20]) and macrocyclic compounds, containing either a P1−P3 (danoprevir,[21] TMC435[22]) or a P2−P4 (vaniprevir,[23] MK-5172[24]) macrocycle. Most of these drug discovery efforts primarily focus on the protease domain alone. However, a recent co-crystal structure of the full-length NS3/4A with an inhibitor interacting with both the helicase and protease domains[25] supports exploring inhibitor-helicase interactions in the design of NS3/4A protease inhibitors.

The NS3/4A protease inhibitors rapidly reduce HCV RNA levels when administered as monotherapy[26−29] and significantly improve treatment outcomes when given in combination with Peg-IFN/RBV.[3−7,30−32] However, HCV evolves very quickly

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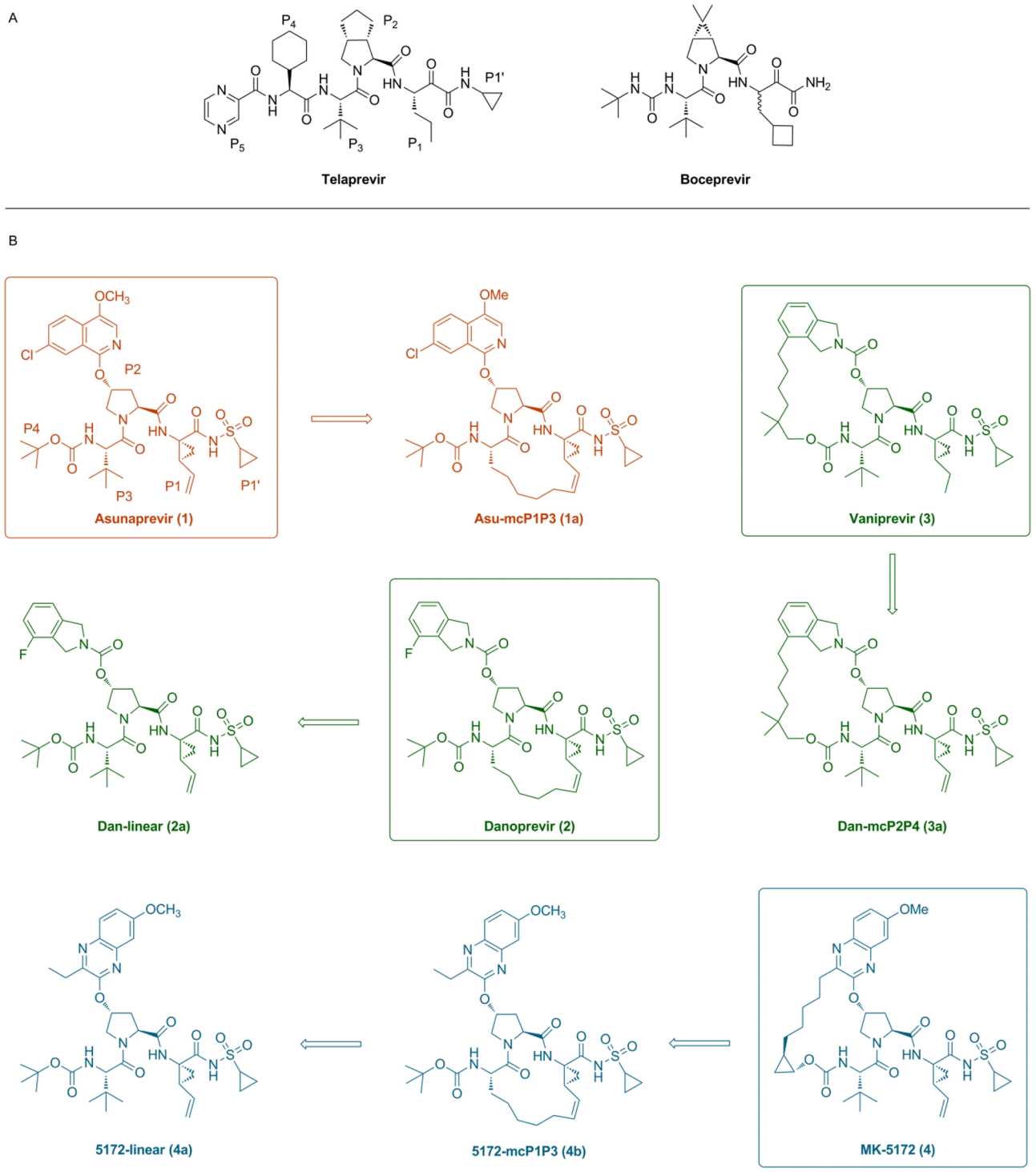

Figure 1. Structures of NS3/4A protease inhibitors: (A) FDA-approved drugs telaprevir and boceprevir. (B) Asunaprevir (**1**) and P1−P3 macrocyclic analogue Asu-mcP1P3 (**1a**); danoprevir (**2**), linear analogue Dan-linear (**2a**), vaniprevir (**3**), and P1 olefin analogue Dan-mcP2P4 (**3a**); MK-5172 (**4**), linear analogue 5172-linear (**4a**), and P1−P3 macrocyclic analogue 5172-mcP1P3 (**4b**). The canonical nomenclature for drug moiety positioning is indicated using telaprevir and asunaprevir; arrows indicate corresponding analogues. Asunaprevir, danoprevir/vaniprevir, and MK-5172 inhibitor series, which share the same P2 moiety but have different macrocylization status, are indicated by orange, green, and blue, respectively.

and drug resistance can emerge against direct-acting antiviral agents.[10,33,34] In patients undergoing protease inhibitor therapy, resistance develops due to overlapping but distinct sets of mutations in the NS3/4A protease.[10,34] Notably, single-site mutations at protease residues R155, A156, and D168 confer resistance to nearly all inhibitors in clinical development.[10,34] Mutations at V36, T54, and V36/R155 are also associated with resistance, particularly to ketoamide inhibitors.[35,36] Thus, despite the therapeutic success of NS3/4A protease inhibitors their long-term effectiveness is challenged by drug resistance.

We have previously shown that the NS3/4A protease inhibitors are particularly vulnerable to resistance where the inhibitors contact protease residues beyond the substrate-binding region, defined as the substrate envelope.[37] These

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residues are not essential for substrate binding and proteolysis, and mutations at these sites can selectively disrupt drug binding with minimal effect on protease function.[37] The non-covalent NS3/4A protease inhibitors protrude from the substrate envelope in the S2 subsite where large P2 inhibitor moieties extensively interact with residues R155, A156, and D168,[37,38] which mutate to confer multi-drug resistance.[10] Recently, we elucidated the molecular basis of drug resistance by determining co-crystal structures of several NS3/4A protease inhibitors in complex with wild-type protease and major drug-resistant variants.[38] Danoprevir and vaniprevir are highly susceptible to mutations at R155 and D168 as these changes disrupt favorable stacking interactions of the isoindoline moiety with R155.[38] However, the P2 quinoxaline moiety in MK-5172 does not directly interact with R155 and D168 but instead the catalytic residues H57 and D81. This unique binding mode explains the retained potency of MK-5172 against R155K and D168A variants.[38] Thus, various P2 moieties appear to differentially impact inhibitor activities against major drug-resistant variants.

The NS3/4A protease inhibitors with macrocycles at either P1−P3 or P2−P4 are reported to have enzyme inhibitory activities better than those of their linear analogues against the wild-type protease, with corresponding enhancements in antiviral potencies.[39,40] However, the role of macrocycles in the susceptibility to drug resistance has not been thoroughly evaluated. In this study, we investigated the effect of varying macrocyclization state on inhibitor activity against drug-resistant variants for four representative protease inhibitors, asunaprevir, danoprevir, vaniprevir, and MK-5172, containing similar core structures but different P2 moieties. We designed and synthesized linear and macrocyclic analogues of these drugs and tested their activities in enzymatic and replicon-based antiviral assays against wild-type and drug-resistant variants. Inhibitor activities against variants of the full-length NS3/4A and the isolated protease domain were compared to assess modulation of inhibitor binding by the helicase domain. The macrocyclic inhibitors were generally more potent than their linear analogues, but the location of the macrocycle significantly affected the potency against drug-resistant variants. The heterocyclic moiety at P2 was the major determinant of inhibitor resistance profiles, susceptibility to drug resistance, and the extent of modulation by the helicase domain. This study elucidates the role of macrocycles and P2 moieties in HCV NS3/4A protease inhibitor resistance and suggests strategies for designing robust drugs against this rapidly evolving virus.

RESULTS AND DISCUSSION

Mutations in NS3/4A protease, particularly at residues R155, A156, and D168, confer resistance to nearly all inhibitors in clinical development. However, the impact of drug resistance mutations on potency varies greatly between the various inhibitors. Elucidating the role of different structural features that affect inhibitor potency against drug-resistant variants is essential for designing more robust NS3/4A protease inhibitors.

Synthesis of Protease Inhibitors with Varied P2 Moieties and Macrocyclizations. The non-covalent NS3/4A protease inhibitors, such as asunaprevir (**1**), danoprevir (**2**), vaniprevir (**3**), and MK-5172 (**4**), contain similar core structures, including an acylsulfonamide-linked P1 cysteine mimic, a substituted P2 proline, a hydrophobic P3 side chain, and a P4 capping group (Figure 1). However, these inhibitors are structurally distinct as heterocyclic moieties at P2 are different and inhibitors are either linear or macrocyclic; macrocyclization is either between the P1 side chain and the P3 side chain (mcP1P3) or the P2 heterocyclic moiety and the P4 capping group (mcP2P4). To evaluate the effect of P2 moieties and macrocylization status on inhibitor activity against drug-resistant variants, we designed and synthesized linear and macrocyclic analogues of asunaprevir, danoprevir, vaniprevir, and MK-5172 (Figure 1). Danoprevir and vaniprevir have many key components in common, and their analogues are clustered together, hence three inhibitor series with the same P2 group but different macrocylization status.

For asunaprevir, a linear acylsulfonamide compound with an ether-linked isoquinoline moiety at P2 proline, a P1−P3 macrocyclic analogue Asu-mcP1P3 (**1a**) was designed. Danoprevir and vaniprevir are both macrocyclic compounds containing a carbamate-linked isoindoline moiety at P2 proline, but the location of the macrocycle is different: danoprevir contains a P1−P3 macrocycle, whereas vaniprevir contains a P2−P4 macrocycle and a saturated P1 side chain. For direct comparison with danoprevir, a danoprevir linear analogue Dan-linear (**2a**) was designed along with a vaniprevir P1 olefin analogue Dan-mcP2P4 (**3a**). For MK-5172, which is a P2−P4 macrocyclic compound containing an ether-linked quinoxaline moiety, a linear analogue 5172-linear (**4a**) and a P1−P3 macrocyclic analogue 5172-mcP1P3 (**4b**) were prepared; in both analogues the hydrocarbon chain of the P2−P4 macrocycle was replaced with an ethyl group at the quinoxaline.

The designed macrocyclization analogues were prepared using synthetic sequences illustrated in Figure 2. Analogous to asunaprevir synthesis, Asu-mcP1P3 (**1a**) was prepared from the intermediate **5**, obtained by the reaction of Boc-*trans*-4-hydroxy-L-proline and 1,7-dichloro-4-methoxyisoquinoline.[41] Coupling of **5** with the P1−P1′ fragment **6**[42] provided the P2−P1′ intermediate **7**. Boc deprotection and coupling of the resulting free amine with the amino acid **9** provided the precursor bis-olefin **10** for ring-closing metathesis. Cyclization of the bis-olefin intermediate **10** was accomplished using a highly efficient ring-closing metathesis catalyst Zhan 1B and provided the target compound Asu-mcP1P3 (**1a**) (Figure 2A).

Dan-linear (**2a**) was synthesized using the reaction sequence analogous to danoprevir.[37] Vaniprevir (**3**) and its P1 olefin analogue Dan-mcP2P4 (**3a**) were synthesized following the synthetic methods reported by McCauley et al.[43]

The MK-5172 analogues 5172-linear (**4a**) and 5172-mcP1P3 (**4b**) were synthesized using the reaction sequences shown in Figure 2B. The intermediate **11** was prepared from 3-chloro-7-methoxyquinoxalin-2-ol and the bosylated Boc-*cis*-4-hydroxy-L-proline using Cs_2CO_3-mediated S_N2 displacement reaction.[24] Palladium-catalyzed vinylation followed by hydrogenation of the double bond provided the common P2 intermediate **12**. The acyclic analogue 5172-linear (**4a**) was assembled from **12** by a four-step reaction sequence, including Boc deprotection, P2−P3 amide coupling, ester hydrolysis, and coupling with the P1−P1′ fragment **6** to provide the target compound 5172-linear (**4a**). The precursor for 5172-mcP1P3, bis-olefin **18**, was synthesized from the P2 fragment **12** in an analogous reaction sequence. Finally, cyclization of the bis-olefin intermediate **18** using ring-closing metathesis catalyst Zhan 1B provided the 5172-mcP1P3 (**4b**).

Macrocyclization Enhances Inhibitor Potency against Wild-Type and Drug-Resistant Variants. The enzyme

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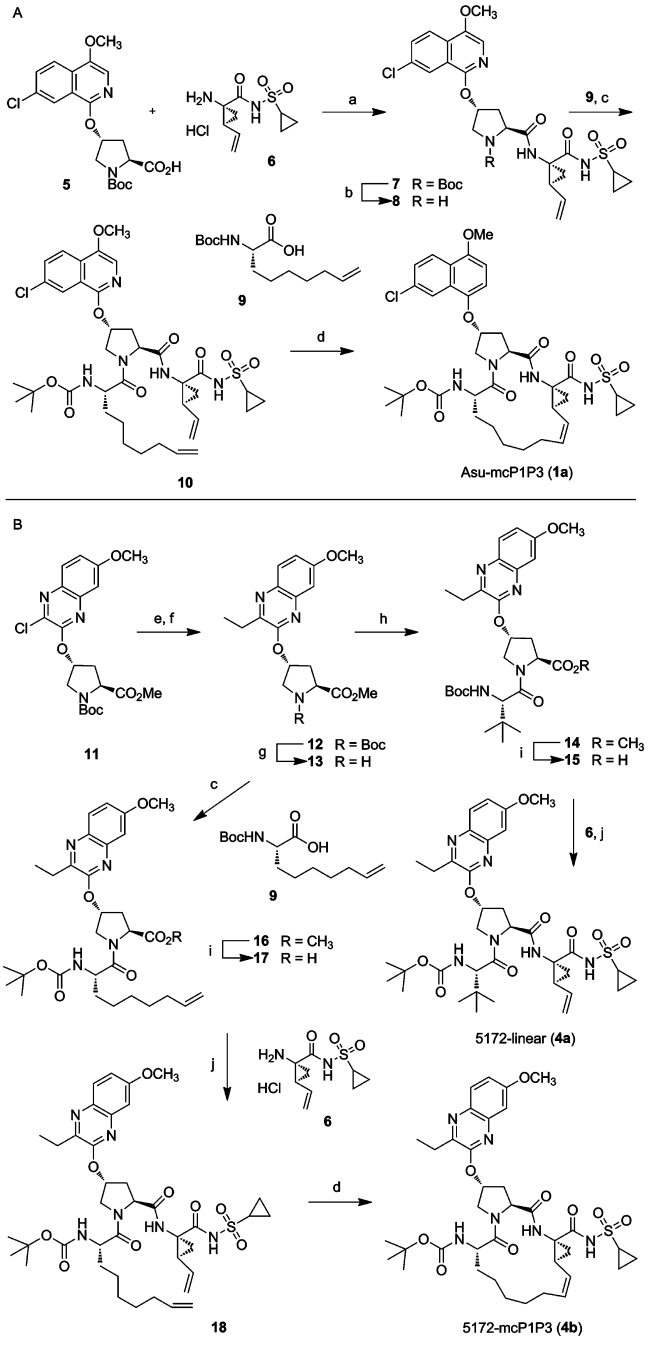

Figure 2. Synthesis of designed protease inhibitors. Reagents and conditions: (a) HATU, DIEA, DMF, CH_2Cl_2; (b) HCl, MeOH; (c) HATU, DIEA, DMF; (d) Zhan 1B catalyst, 1,2-DCE; (e) potassium vinyltrifluoroborate, TEA, $PdCl_2(dppf)$-CH_2Cl_2, EtOH; (f) H_2, 10% Pd/C, MeOH, dioxane; (g) 4 N HCl, dioxane; (h) Boc-Tle-OH, HATU, DIEA, DMF; (i) LiOH, THF, H_2O; (j) HATU, DMAP, DIEA, DMF, CH_2Cl_2.

inhibition constants (K_i) of asunaprevir, danoprevir, vaniprevir, MK-5172, and their respective analogues were determined against genotype 1a full-length NS3/4A, the isolated protease domain, and drug-resistant variants R155K, V36M/R155K, A156T, and D168A of both full-length enzyme and protease (Figure 3a; Supplementary Tables S1 and S2, Figure S1). In addition, half-maximal inhibitory concentrations (IC_{50}) were determined using viral replicon-based inhibition assays with wild-type genotype 1b HCV and drug-resistant variants R155K, V36M/R155K, A156T, and D168A; telaprevir was used as a control (Figure 3b; Supplementary Table S3, Figures S2–S6).

In each inhibitor series, macrocyclic compounds exhibited K_i's lower than those of the corresponding linear analogues against wild-type protease and, in most cases, drug-resistant variants. The P2–P4 macrocyclic compounds were highly active against wild-type protease, with K_i values in the mid to high pM range (K_i = 0.46 nM for vaniprevir, K_i = 0.22 nM for Dan-mcP2P4, and K_i = 0.06 nM for MK-5172), and retained low to high nanomolar activity against resistant variants. The P1–P3 macrocyclic compounds exhibited slightly lower enzymatic activities against wild-type, but overall retained better activities against drug-resistant variants. Notably, 5172-mcP1P3 (A156T K_i = 74.3 nM) retained 3-fold better enzymatic activity against A156T variant than the parent compound MK-5172 (A156T K_i = 251.3 nM). Interestingly, compared to vaniprevir, Dan-mcP2P4, though only slightly better against wild-type protease, was significantly more active against drug-resistant variants. This signifies the importance of the terminal olefin in the P1 moiety where potential π stacking interactions with F154 are possible,[25,37] likely reducing the off-rate of the P1 olefin analogue compared to vaniprevir.

The antiviral activities of macrocyclic compounds in replicon assays against wild-type HCV and resistant variants correlate with protease binding affinities, and further demonstrate enhanced potency of macrocyclic compounds compared to linear analogues (Figure 3). In general, the P1–P3 macrocyclic compounds exhibited better antiviral potencies against wild-type and drug-resistant variants than the corresponding P2–P4 analogues. Against wild-type virus, P1–P3 macrocyclic inhibitors danoprevir, Asu-mcP1P3, and 5172-mcP1P3 exhibited antiviral potencies in the subnanomolar range with IC_{50} of 0.24, 0.23, and 0.26 nM, respectively. The antiviral potency of Asu-mcP1P3 was significantly better than that of the parent drug, showing a 4-fold enhancement against wild-type and 4–16-fold against resistant variants. Interestingly, 5172-mcP1P3 (WT IC_{50} = 0.26 nM) was almost equipotent to MK-5172 (WT IC_{50} = 0.29 nM) against wild-type virus and R155K, V36M/R155K, and D168A variants but exhibited 12-fold improved potency against A156T variant (A156T IC_{50} = 3.96 nM for 5172-mcP1P3 versus 46.6 nM for MK-5172).

The macrocyclic inhibitors are more potent compared to their linear analogues likely due to the restriction of flexibility of the molecule core around the P2–P3 proline amide bond, which is expected to reduce the conformational entropic penalty for binding thus increasing the overall binding energy.[39,44] The P1–P3 macrocycle restricts the P1 terminus and rotation around the P2–P3 amide bond, which skews the *cis-trans* equilibrium, favoring the *trans* geometry, which is a conserved feature in all protease structures in complex with viral substrate peptides.[37,45] In contrast, the P2–P4 macrocycle restricts the flexibility of the large heterocyclic moiety attached to P2 proline and probably restricts the rotation around the P2–P3 amide bond, in concert with the bulky *tert*-butyl group at P3. In addition, the long hydrophobic chain of the macrocycles reduces the overall polarity of the molecule and makes additional interactions with the protease. Thus, macrocyclization provides several advantages, including preorganization of the molecule in a binding-competent conformation and restriction of the inhibitor geometry to stabilize protease–inhibitor interactions.[44]

P2 Moiety Also Determines Inhibitor Susceptibility to Drug Resistance. The susceptibilities of linear and macro-

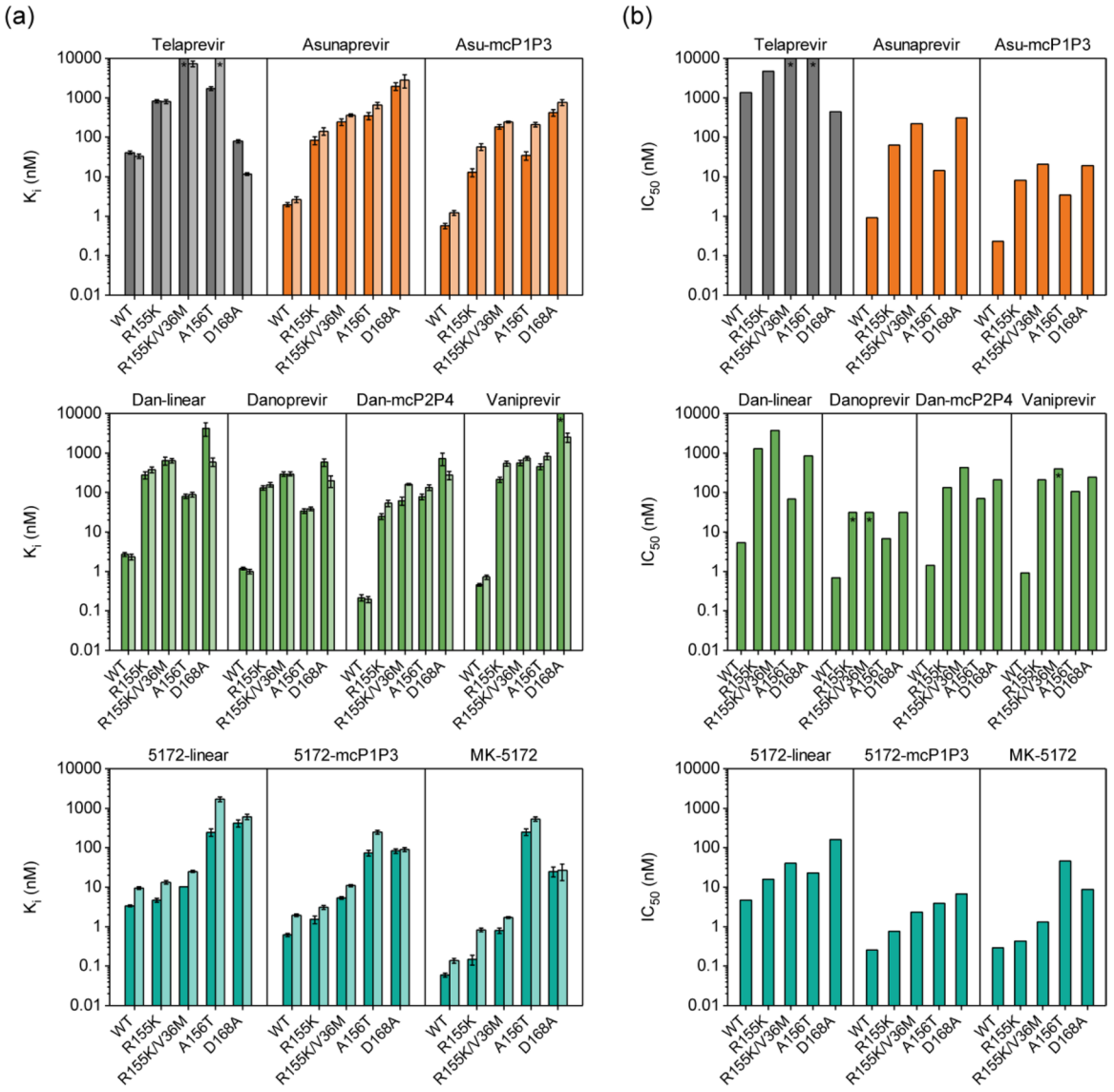

Figure 3. (a) Enzyme inhibition constants for HCV genotype 1a full-length NS3/4A and isolated protease domain (dark and light bars) and (b) replicon-based half maximal inhibitory concentrations for genotype 1b HCV NS3/4A and drug-resistant variants for (first row) telaprevir and asunaprevir series, (second row) danoprevir/vaniprevir series, and (third row) MK-5172 series. Error bars represent standard errors of the mean (n = 4); * indicates K_i and IC_{50} values are greater than the highest inhibitor concentration tested.

cyclic protease inhibitors to drug resistance were evaluated by normalizing K_i and IC_{50} values with respect to the corresponding wild-type enzyme (Figure 4) to better ascertain the relative changes. The K_i's against drug-resistant variants generally correlated with IC_{50}'s, but the fold potency losses in replicon assays were in most cases lower than those observed from in vitro enzymatic assays. However, we focused on the fold changes in enzymatic inhibition constants as these reflect the direct effects of drug resistance mutations on inhibitor binding, while antiviral potencies in cellular replicon assays may reflect changes in inhibitor binding when the protease is part of an active replication complex.

Within each inhibitor series of same P2 moiety but different macrocyclization (Figure 4, individual rows) the drug resistance profiles are similar, indicating that the identity of the P2 moiety largely determines the susceptibility to drug resistance. Notably, activity losses for the linear and P1−P3 macrocyclic analogues were similar against all resistant variants. The P2−P4 macrocyclic inhibitors displayed higher fold losses compared to those of linear and P1−P3 macrocyclic compounds against A156T variants and, to a small extent, D168A variants, but the overall resistance profiles are similar in each series. Thus, regardless of the macrocyclization, inhibitors with different P2 moieties exhibit distinct resistance profiles.

The P2−P4 macrocyclic inhibitors are more susceptible to drug resistance possibly because the P2 moiety is constrained to allow stronger interactions with the protein, reducing the inhibitor's ability to adapt to changes in binding environment due to mutations. In all inhibitors, the large heterocyclic P2 moieties bind to the protease in the S2 subsite but vary in how they interact with the primary sites of drug resistance mutations: R155, A156, and D168.[37,38] These mutations lead to disruption of stacking interactions (R155K), pronounced steric clashes (A156T), or disruption of the electrostatic network around the S2 subsite (D168A), but the rigidity of the P2−P4 macrocycle prevents the inhibitor from adjusting to these changes.[38] In contrast, the P1−P3 macrocyclic inhibitors are less susceptible to drug resistance as the location of the

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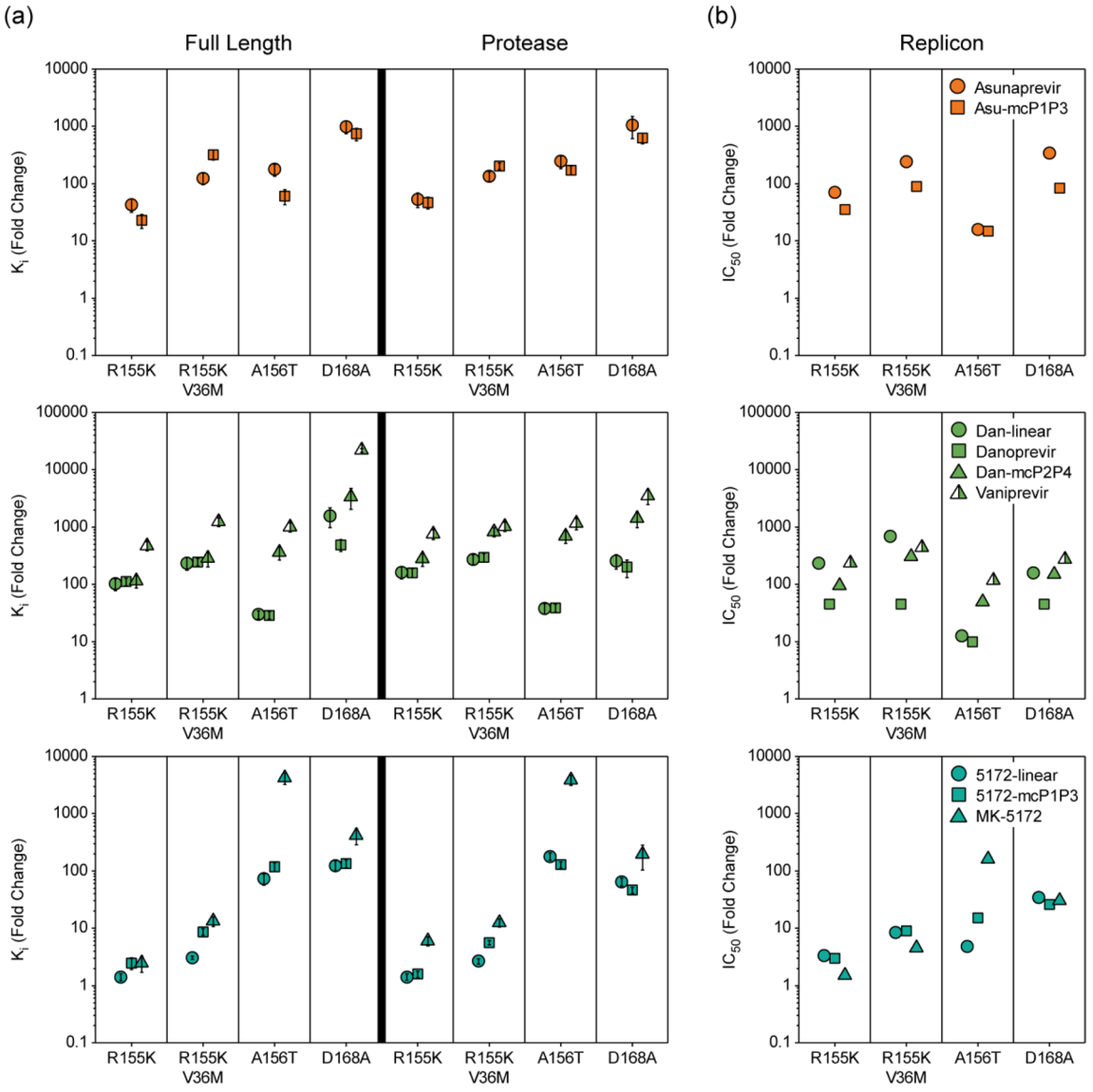

Figure 4. Resistance profiles of protease inhibitors in (a) enzyme inhibition and (b) cell-based replicon assays for (first row) asunaprevir series, (second row) danoprevir/vaniprevir series, and (third row) MK-5172 series. Enzyme inhibitory and antiviral activities against mutants were normalized with respect to the wild-type full-length HCV NS3/4A protease, NS3/4A protease domain, or wild-type HCV clone. Error bars represent propagated standard errors.

macrocyclization is distal from the sites of primary drug resistance mutations. Thus, the P1−P3 macrocyclization, although stabilizing the inhibitor's interactions with the protease, allows the P2 moiety to be flexible.

Specific interactions of the P2 moiety with known positions of drug-resistant mutations explain the mechanism behind their distinct resistance profiles. In the danoprevir/vaniprevir series, the aromatic ring of P2 isoindoline stacks on R155 side chain, making favorable cation-π stacking interactions.[38] As R155 conformation is stabilized by hydrogen bond interactions with D168; mutations at R155 and D168 destabilize this packing thereby lowering the affinity of these inhibitors.[38] For asunaprevir and analogues, although there is no reported co-crystal structure, the substituted isoquinoline moiety is expected to interact similarly with R155 and D168. Future co-crystal structures will shed more light on the molecular mechanisms of resistance for these inhibitors.

The in vitro resistance profiles of NS3/4A protease inhibitors generally correlate with resistance observed in patients failing protease inhibitor therapy. Asunaprevir resistance-associated variants mainly include protease variants D168A/E/V/Y,[46] in agreement with significant activity loss against D168A variant in vitro. Danoprevir resistance is primarily associated with R155K variant,[29] while vaniprevir selects for both R155 and D168 variants.[47] In in vitro assays, danoprevir was highly susceptible to R155K mutation but maintained relatively better activity against A156T and D168A variants, while vaniprevir was highly susceptible to all three variants.

We have previously shown that MK-5172 retains relatively better potency against R155K and D168A variants but is highly susceptible to A156T variant, due to steric clashes with the P2−P4 macrocycle.[38] Both the linear and P1−P3 macrocyclic analogues of MK-5172 retain better potency against drug-resistant variants compared to the parent compound. In fact, the P1−P3 macrocyclic analogue 5172-mcP1P3 displays single

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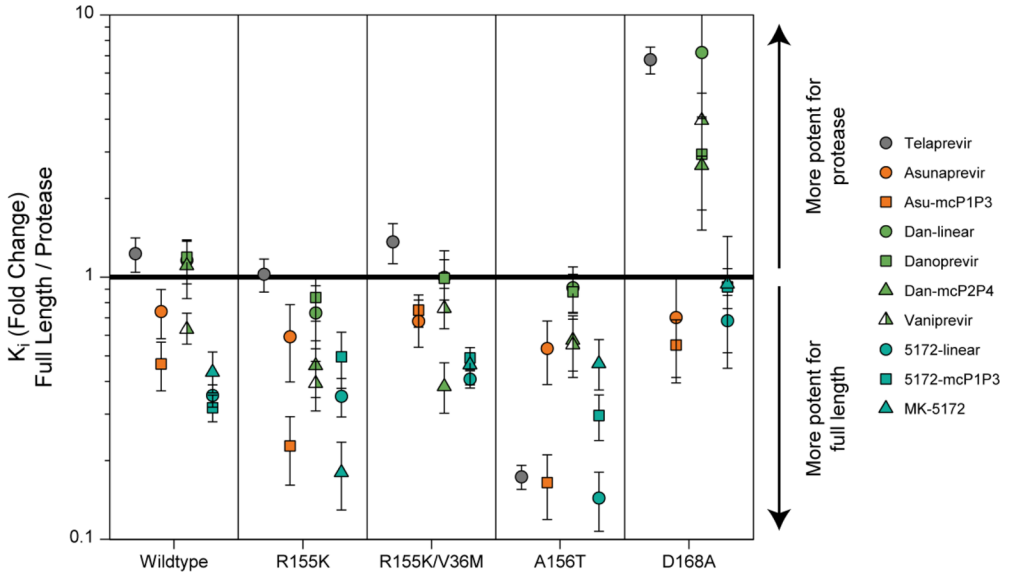

Figure 5. Fold-change in inhibitor potencies against full-length NS3/4A compared to the isolated HCV NS3/4A protease domain; values lower than 1 indicate higher inhibitor potency against the full-length protein compared to protease alone and *vice versa*.

digit nanomolar antiviral potency against all four drug-resistant HCV variants. Thus, compared to MK-5172, 5172-mcP1P3 exhibits a flatter resistance profile. Inhibitors with similar or slightly reduced potency but flatter resistance profiles may prove to be better than those with high potency against wild-type and pivotal resistant variants but reduced activity against other variants such as A156T. These variants may be less competent for replication but could select for secondary mutations that restore viral fitness, rendering the inhibitors highly susceptible to these variants, e.g., R155K versus R155K/V36M.

The activity profile of MK-5172 analogues suggest that even in the absence of P2−P4 macrocycle, the P2 quinoxaline moiety maintains the unique binding conformation observed in MK-5172.[38] In fact, the flexible P2 quinoxaline moiety lacking the P2−P4 macrocycle can be easily modified at the 3-position for structure−activity exploration and, together with changes at the P4 capping group, provide opportunities for improving potency against drug-resistant variants and for optimizing pharmacokinetic properties.

Helicase Domain Modulates Protease Inhibitor Potency. The overall drug resistance profiles for the full-length enzyme versus the isolated protease domain did not change significantly (Figure 4). Thus, relative to the effect of drug-resistant mutations, the helicase does not strongly modulate the protease inhibitor activity. Nevertheless, up to an-order-of-magnitude variations in inhibitor potency were observed relative to the isolated protease domain (Figure 5). This observed modulation by the helicase domain was dependent on the identity of the P2 moiety.

For the wild-type enzyme, modulation by the helicase domain was minimal (up to 3-fold preference for the full-length protein). However, with drug-resistant variants R155K and A156T, preference for the full-length protein was more pronounced, with up to 8-fold enhancement in activity for asunaprevir, MK-5172, and analogues, whereas limited modulation was observed for danoprevir and analogues. This trend was not observed for D168A for the asunaprevir and MK-5172 series. Conversely, danoprevir and analogues exhibited significant preference for the isolated protease domain (up to an-order-of-magnitude). Even though none of these protease inhibitors were designed to make any interactions with the helicase domain, the presence of the helicase domain is able to influence their potency. In a full-length NS3/4A crystal structure with an inhibitor similar to danoprevir, the heterocyclic P2 moiety occupies the groove formed between the helicase and the protease active site.[25] Although no specific interactions between the inhibitor P2 moiety and the helicase were observed, solvent exclusion from the otherwise exposed hydrophobic P2 extension may be the reason for the observed potency increase in the asunaprevir and MK-5172 classes. Recently, a new class of allosteric inhibitors were reported, which were shown to interact specifically with the helicase groove to lock the protein in the crystallographic conformation, disallowing substrate access and protein activation by rotation of the helicase domain away from the protease.[48] Further optimization of the heterocyclic P2 moiety to specifically contact the helicase domain may provide inhibitors with better activities and less susceptibility to drug resistance without sacrificing inhibitor potency.

Conclusions. We have found profound influences of macrocyclization status and P2 moieties on the potency of NS3/4A protease inhibitors and their susceptibility to drug resistance. Macrocyclization significantly enhances inhibitor potency, but location of the macrocycle is critical in avoiding drug resistance. Our results provide strong rationale for (1) using P1−P3 macrocyclization to restrict inhibitor geometry and stabilize interactions with the protease thereby improving inhibitor potency and (2) using a flexible P2 moiety to accommodate mutations in the S2 subsite thereby retaining potency against drug-resistant variants. Specifically, protease inhibitors with flexible P2 moieties that avoid direct contact with the S2 subsite, but instead exploit interactions with the essential catalytic residues, are highly promising to retain potency against drug-resistant HCV variants. Additionally, modulation of protease inhibitor potency by the helicase domain suggests that interactions with the helicase domain could be leveraged in future inhibitor development. These strategies may lead to the design of more potent HCV NS3/4A protease inhibitors that are robust against drug resistance.

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METHODS

NS3/4A Protease Constructs. The HCV genotype 1a NS3/4A protease domain described previously in a Bristol-Myers-Squibb patent[49] was synthesized (GenScript) and cloned into a pET28a bacterial expression vector. The single-chain NS3/4A protease (scNS3/4Apro) contains the protease domain (181 amino acids) and the 12-aa cofactor beta-sheet of the NS4A (residues 12−23) tethered to the N-terminus of the protease. The codon-optimized genotype 1a NS3 helicase (H77, provided by David Frick) was cloned downstream of the protease domain to generate the full-length NS3/4A construct. Similar to the protease domain, the single-chain full-length NS3/4A construct (scNS3/4Afl) contains the NS4A cofactor peptide (residues 12−23) ligated to the N-terminus of the protease. Multi-drug-resistant variants R155K, V36M/R155K A156T, and D168A for both the isolated protease and full-length constructs were generated using site-directed mutagenesis kit QuikChange II (Stratagene).

Expression and Purification of NS3/4A Protease Constructs. The expression and purification scheme for the isolated protease domain is detailed elsewhere.[37] Briefly, transformed BL21(DE3) *E. coli* expression cells were grown to an A_{600} of 0.6, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and incubated with shaking for 5 h at 37 °C. Cells expressing the protein were harvested by centrifugation and stored at −80 °C. Frozen pellets were resuspended in resuspension buffer (50 mM phosphate buffer, 500 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol (β-ME), pH 7.5), lysed, and centrifuged to pellet the cell debris, and the resulting supernatant was applied to a nickel column (Qiagen). The column was washed with resuspension buffer, and the protein was eluted with resuspension buffer, containing 200 mM imidazole. The eluate was supplemented with thrombin and dialyzed overnight to cleave the His-tag and remove the imidazole. The purified protein was flash frozen in liquid nitrogen and stored at −80 °C.

For the expression and the purification of the full-length NS3/4A, transformed BL21(DE3) *E. coli* expression cells were grown to an A_{600} of 0.6 at 37 °C, transferred to 20 °C, induced by 0.5 mM IPTG, and incubated with shaking for 4 h. Cells were then harvested via centrifugation; cell pellets were washed with 1X phosphate-buffered saline (PBS), repelleted, and stored at −80 °C. Frozen pellets were resuspended in Buffer HT (25 mM HEPES, 500 mM NaCl, 10% glycerol, 0.1% OβG, 2 mM TCEP and 20 mM imidazole, pH 8.0) supplemented with DNase I (Roche) and homogenized using a cell disruptor (Micro Fluidics). Lysed cells were centrifuged to clear the cell debris and applied to a 1 mL HisTrap HP column (GE Life Sciences) using an AKTA Purifier (GE Life Sciences). The protein was washed with Buffer HT supplemented with 40 mM imidazole and eluted with Buffer HT supplemented with 250 mM imidazole. The eluate was dialyzed overnight against Buffer S (25 mM MES, 150 mM NaCl, 10% glycerol, 0.1% OβG, 2 mM TCEP, pH 6.0). Dialyzed protein was applied to a Mono S Column (GE Life Sciences) and eluted with a linear gradient of NaCl up to 1 M. The eluate was judged >90% pure by polacrylamide gel electrophoresis, concentrated, flash frozen, and stored at −80 °C.

Determination of Michaelis−Menten Constants. Protease cleavage assays were performed to determine Michaelis−Menten constants (K_M), which were then used to obtain inhibition constants through Morrison's equation (see below). The assays were done in a final volume of 60 μL containing protease assay buffer (50 mM Tris, 2.5% glycerol, 0.1% OβG, 5 mM TCEP, 1% DMSO, pH 7.5) and up to 20 μM concentration of a modified HCV NS3/4A protease substrate Ac-DE-Dap(QXL-520)-EE-Abu-ψ-[COO]AS-C(5-FAMsp)-NH2 (Anaspec) in black 96-well flat bottom nonbinding surface half-area plates (Corning) at RT. The reaction was initiated by the rapid injection of 10 μL of HCV NS3/4A protease to a final concentration of 20 nM. The fluorescence output from the depsipeptide cleavage product was measured kinetically for at least 1 h using an EnVision plate reader (Perkin-Elmer) with excitation wavelength at 485 nm and emission at 530 nm. End point readings were also taken after 6 h. For each construct, three independent experiments were performed.

Inner filter effect in higher concentrations of substrate was corrected using the following scheme: End point readings were subtracted from the background fluorescence values to obtain reaction amplitudes, which were plotted against substrate concentration. The initial linear portion of this curve was fitted with linear regression and extrapolated to maximum substrate concentration to obtain the ideal amplitude line. Corresponding amplitude values in the ideal amplitude line were divided by the actual amplitudes to obtain inner filter effect correction parameters which were then multiplied by initial velocities to obtain corrected velocities. Corrected velocities were plotted against substrate concentrations, and all replicates were fitted to Michaelis−Menten equation globally to obtain Michaelis−Menten constants (K_M), sharing K_M between different data sets. Except full-length A156T mutant, K_M values for all the constructs were similar, which signifies that the substrate recognition was not altered significantly with respect to different multi-drug-resistant mutations and helicase mutations.

Enzyme Inhibition Assays. All enzyme inhibition assays were performed in nonbinding surface 96-well black half-area plates (Corning) in a reaction volume of 60 μL. The NS3/4A protease (~1 nM) was preincubated with increasing concentration of drugs in protease reaction buffer for an hour. The reaction was initiated by the rapid injection of 5 μL of HCV NS3/4A protease substrate to a final concentration of 200 nM and kinetically monitored using a Perkin-Elmer EnVision plate reader (excitation at 485 nm; emission at 530 nm) for 1 h with 30 s between data points. At least four independent data sets were collected for each inhibitor with each protease construct. Each inhibitor titration included at least 12 inhibitor concentration points, which were globally fit to the Morrison equation to obtain the K_i value.

Initial velocities were obtained from the progress curves and plotted against inhibitor concentrations to get inhibition curves. Resulting curves were fitted to Morrison's equation:

$$\frac{V_i}{V_0} = 1 - [\{([E]_T + [I]_T + K_i^{app}) - \sqrt{([E]_T + [I]_T + K_i^{app})^2 - 4[E]_T[I]_T}\}/2[E]_T]$$

$$K_i^{app} = K_i\left(1 + \frac{[S]}{K_M}\right)$$

where $[E]_T$ is the total enzyme concentration, $[I]_T$ is the total inhibitor concentration, $[S]$ is the substrate concentration, K_M is the Michaelis−Menten constant (obtained from protease cleavage assays), V_0 is the initial velocity when there is no drug, V_i is the initial velocity at $[I]_T$, K_i^{app} is the apparent inhibition constant, and K_i is the inhibition constant. Nonlinear regression analyses were performed where independent replicates from different protease constructs with different drugs were fitted globally, sharing K_i.

Cell-Based Drug Susceptibility Assays. Mutations (R155K, V36M/R155K D168A, or A156T) were introduced into the NS3 region of genotype 1b HCV Con1 luciferase reporter replicon using the mega-primer method of mutagenesis.[50] Replicon RNA of each protease variant was introduced into Huh7 cells by electroporation. Replication was then assessed in the presence of increasing concentrations of protease inhibitors (telaprevir, asunaprevir, danoprevir, vaniprevir, MK-5172 and respective analogues) by measuring luciferase activity (relative light units) 96 h after electroporation. The drug concentrations required to inhibit replicon replication by 50% (IC_{50}) were calculated directly from the drug inhibition curves.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): A.N., C.J.P., and W.H. are employees of Monogram Biosciences. C.A.S. has received small "Sponsored Research Grants" from Avila (CelGene) and Merck; however, the work described in this manuscript is supported only by the National Institutes of Health. This does not alter our adherence to all ACS policies on sharing data and materials..

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