Molecular Mechanism by Which a Potent Hepatitis C Virus NS3-NS4A Protease Inhibitor Overcomes Emergence of Resistance

Background: Antivirals must often be given in combinations to avoid rapid emergence of resistance. Results: We identified and structurally characterized protease inhibitors that maintain potency against genotype and resistant variants. Conclusion: Pan-variant potency was achieved by targeting invariant regions and incorporating flexibility where pocket variability occurs. Significance: Such inhibitors may yield simplified and/or more successful treatments for hepatitis C infections.

Although optimizing the resistance profile of an inhibitor can be challenging, it is potentially important for improving the long term effectiveness of antiviral therapy. This work describes our rational approach toward the identification of a macrocyclic acylsulfonamide that is a potent inhibitor of the NS3-NS4A proteases of all hepatitis C virus genotypes and of a panel of genotype 1-resistant variants. The enhanced potency of this compound versus variants D168V and R155K facilitated x-ray determination of the inhibitor-variant complexes. In turn, these structural studies revealed a complex molecular basis of resistance and rationalized how such compounds are able to circumvent these mechanisms.

The hepatitis C virus (HCV) was identified in 1989 as the causative agent of a life-threatening liver disease (1) that today infects approximately 2% of the worldwide population or 170 million individuals (2). Until recently, HCV infection was treated with pegylated interferon-α (IFN-α) combined with the broad spectrum antiviral ribavirin. Although ~80% of genotype 2 and 3 patients achieve sustained virologic response with this regimen, the success rate for genotype 1, which is the most prevalent in industrialized nations, is less than 50% (3).

HCV is a small enveloped virus containing a positive-strand RNA genome. This genome encodes for a polypeptide that is processed by host and viral proteases into 10 proteins that are required for viral replication. One of these proteins, the NS3 protease-helicase, is a bifunctional protein with a chymotrypsin-like serine protease located at the N terminus (4). The short NS4A protein is an integral part of the active protease (5), which we refer to as NS3-NS4A. Efforts to discover inhibitors of the viral enzymes began shortly after discovery of the virus, and in 2002, a clinical trial with the NS3-NS4A protease inhibitor BILN 2061 (ciluprevir) clinically validated the NS3-NS4A protease as an attractive direct antiviral target (6). In 2011, 22 years after the discovery of the virus, the first two direct acting antivirals boceprevir (7) and telaprevir (8), which also target the NS3-NS4A protease, were approved by the Food and Drug Administration to be used in combination with IFN-α and ribavirin. In phase III trials, these drugs improved the sustained virologic response for the previously untreated genotype 1-infected patients to ~67–75% (9, 10).

Second generation NS3-NS4A protease inhibitors are chemically distinct from the approved drugs. Although telaprevir and boceprevir contain α-keto amide warheads that form covalent reversible interactions with the protease catalytic serine (Ser-139), the second generation inhibitors form ionic interactions with the active site. Two second generation inhibitors are currently completing phase III clinical trials as follows: faldaprevir (BI 201335), which has a C-terminal carboxylic acid (11), and simeprevir (TMC 435), which has an acylsulfonamide as a carboxylic acid isostere (Fig. 1) (12).

HCV replicates rapidly using an error-prone polymerase, which in part contributes to the rapid emergence of directly acting antivirus-resistant variants, and current clinical stage NS3-NS4A protease inhibitors have substantially overlapping resistance profiles. As a result, these HCV protease inhibitors currently must be administered in combination with IFN-α and ribavirin (13). The most common substitutions that confer high levels of resistance to second generation inhibitors are different in genotypes 1a and 1b (Table 1). For faldaprevir, genotype 1b mutations encode changes in Asp-168V, most frequently to valine (D168V), whereas the most common substitution observed in genotype 1a is Arg-155 to lysine (R155K), with substitutions of Asp-168, usually to valine, more common at higher doses (14). These same substitutions, along with several others, are also frequently observed for simeprevir (15) and are consistent with in vitro resistance studies performed in genotype 1a and 1b replicons (16, 17). Both inhibitors are much less active.
Several strategies have been successfully applied to the discovery of inhibitors with improved resistance profiles. For example, incorporation of conformational flexibility may allow the inhibitor to adapt to structural and/or electronic alterations in a binding pocket that harbors resistance associated with amino acid substitutions. Furthermore, analogs that predominantly interact with the highly conserved residues and avoid strong interactions with portions of the binding pocket susceptible to significant quasi-species variation typically demonstrate an enhanced barrier to resistance (21–24). Either of these approaches is more likely to be successful if guided by a fundamental understanding of the three-dimensional structural features of the binding site and of the impact of the amino acid differences present in the most common variants.

We have recently reported a comprehensive analysis of the interaction of faldaprevir with HCV NS3 protease based on combined x-ray, NMR, and kinetic data (25). We described the induced formation of a salt bridge between residues Arg-155 and Asp-168 (Fig. 2). In forming this salt bridge, Arg-155 becomes desolvated such that the aliphatic portion of its side chain forms part of the hydrophobic surface that binds the quinoline moiety of the inhibitor. Similarly, in forming the salt bridge, the side chain of Asp-168 rotates such that a small hydrophobic pocket is formed that can accommodate the inhibitor’s N-terminal cyclopentyl carbamate capping group.

From this structural information, sensitivity of faldaprevir to clinically observed substitutions at Arg-155 and Asp-168 can be rationalized in two ways. Both residues are in close proximity to bound faldaprevir, and it is quite plausible that substitutions directly interfere with compound binding. Moreover, the existence of the induced salt bridge and its role in forming two important binding surfaces also provides an intriguing possibility for Arg-155 and Asp-168 substitutions to elicit indirect effects. Unfortunately, because of its significantly reduced affinity, we were unable to further clarify the mechanisms of resistance with x-ray structures of faldaprevir bound to either of the common variants, R155K or D168V. Nevertheless, the structural information suggested that analogs making less rigid contact with both Arg-155 and Asp-168 might adapt better to substitutions at these positions. We therefore developed a strategy to reduce inhibitor interactions in the area of the Arg-155–Asp-168 salt bridge to discover inhibitors with improved potency versus these key resistant variants. This strategy was successful, yielding an interesting series of pan-active NS3-NS4A protease inhibitors, which in turn yielded x-ray co-crystal structures in complex with both wild-type and drug-resistant proteases. Analysis of these structures has provided a deeper understanding of the mechanisms of resistance observed for clinical stage HCV NS3-NS4A protease inhibitors.

**EXPERIMENTAL PROCEDURES**

**Inhibitors**—Inhibitors 1–4 were prepared as outlined in Scheme 1 from common intermediate 6 (26). Displacement of the brosylate with hydroxyquinoline 5a or 5b, followed by
hydrolysis and acylsulfonamide formation with 7 or 8 (27), yielded compound 1 or advanced intermediates 10 or 11, respectively. Removal of the t-butoxycarbonyl-protecting group and a subsequent coupling reaction with 1-propyl chloroformate or the appropriate carboxylic acid (7) afforded the inhibitors 2–4. The full synthesis of these inhibitors will be reported elsewhere.3

**NS3 Protease Substrate Synthesis**—The depsipeptide fluorogenic substrates used in this study are both derived from the NS5A-NS5B cleavage site. The substrate anthranilyl-DDIVP-Abu-(C(O)–O)-AMY(3-NO2)TW-OH was synthesized as described previously (28), whereas the substrate AF555-DDIVP-Abu-(C(O)–O)-ASK(QSY7)-NH2 was custom-synthesized at Cambridge Research Biochemicals Ltd. (Billingham, Cleveland, UK).

**Proteins**—The cloning, expression, and purification of full-length NS3-NS4A proteins from the HCV genotypes 1a-H77, 1b-CON-1, 2a-JFH1, 3a, 4a, 5a, and 6a (29, 30), as well as the genotype 1b protease domain in complex with NS4A peptide used for crystallography (28), were previously described. Point mutations encoding resistant variants were introduced using the QuikChange™ II site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene). The resistant variants were expressed and purified as described for the wild-type NS3-NS4A proteins.

**NS3 Protease Assays**—The fluorogenic depsipeptide NS3 protease substrate AF555-DDIVP-Abu-(C(O)–O)-ASK(QSY7)-NH2 (where AF555 is the proprietary moiety AlexaFluor555 (Invitrogen)) was used for IC50 determination against full-length NS3-NS4A proteins from genotypes 2a, 3a, 4a, 5a, and 6a. Different concentrations of compound 4 and 0.025 mM NS3-NS4A protease of the tested genotype were incubated for 15 min at 23 °C in a reaction buffer composed of 50 mM Tris, pH 7.5, 0.25 mM sodium citrate, 0.01% n-dodecyl-β-D-maltoside, 1 mM DTT. Then the enzymatic reaction was initiated by the addition of 0.1 μM substrate followed by a 60-min incubation at 23 °C. The final DMSO content was 5%. The reactions were terminated by addition of 1 M MES, pH 5.8. Fluorescence of the N-terminal product AF555-DDIVP-Abu was measured using an EnVision® multilabel reader (PerkinElmer Life Sciences) equipped with a 530-nm excitation filter and a 595-nm emission filter. For IC50 measurement against resistant variants, the substrate anthranilyl-DDIVP-Abu-(C(O)–O)-AMY(3-NO2)TW-OH was used as described previously (29). Briefly, different concentrations of inhibitor, 5 mM substrate and 0.5 mM GT1b (WT, A156V, or D168V NS3-NS4A), or 1 mM GT1a (WT, R155K or D168V NS3-NS4A), or 10 mM GT1a D168V NS3-NS4A were incubated at 23 °C for 70 min in a buffer composed of 50 mM Tris-HCl, pH 8.0, 0.25 mM sodium citrate, 0.01% n-dodecyl-β-D-maltoside, 1 mM tris(2-carboxyethyl)phosphine hydrochloride, and 5% DMSO. The reactions were terminated by addition of 1 M MES, pH 5.8. Fluorescence of the N-terminal product AF555-DDIVP-Abu was measured using a POLARStar Galaxy plate reader (BMG Labtech) equipped with a 320-nm excitation filter and a 405-nm emission filter. Calculated percent inhibition at each inhibitor concentration was then used to determine the median effective concentration (IC50) (Assay Explorer version 3.2, Symyx Technologies).

**Cellular Assays**—Compounds were tested against genotype 1a (H77) and genotype 1b (CON-1) replicon (NS2-NS5B) cell lines as described previously. Resistant variants were engineered into genotype 1a (R155K) or 1b (A156V and D168V) backgrounds by site-directed mutagenesis (16). Cytotoxicity was evaluated as described previously (11). Resistance was raised to compound 4 by long term incubation of genotype 1a and 1b replicon cell lines in the presence of multiple inhibitor

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3 B. Moreau, J. Bordeleau, M. Garneau, C. Godbout, V. Gorys, M. Leblanc, J. A. O’Meara, J. Naud, E. Villemure, P. W. White, and M. Llinàs-Brunet, manuscript in preparation.
Table 2
Data collection and refinement statistics

|                      | WT       | R155K    | D168V    |
|----------------------|----------|----------|----------|
| **Data collection**   |          |          |          |
| Space group          | P6₁      | P6₁      | P6₁      |
| Cell dimensions      | 94.2, 94.2, 82.1 Å | 93.5, 93.5, 81.4 Å | 93.7, 93.7, 81.8 Å |
| a, b, c              | 90, 90, 120° | 90, 90, 120° | 90, 90, 120° |
| Resolution           | 57.9 to 1.93 Å | 57.4 to 1.9 Å | 57.61 to 2.3 Å |
| Overall              | 2.0 to 1.93 Å | 1.97 to 1.90 Å | 2.38 to 2.30 Å |
| Resolution           | 4.0 (31.7) | 6.6 (47.2) | 12.0 (54.1) |
| Completeness         | 99.8% (99.1%) | 98.7% (100%) | 98.9% (100%) |
| Redundancy           | 4.6 (3.9)  | 7.1 (7.0)  | 8.3 (8.5)  |
| **Refinement**       |          |          |          |
| Resolution           | 1.93 Å    | 1.90 Å    | 2.30 Å    |
| No. of reflections   | 30,772    | 31,261    | 17,937    |
| Rmerge               | 21.6/24.7% | 21.6/25.1% | 19.7/24.8% |
| Rfree, test set size | 5.1%      | 5.0%      | 5.1%      |
| Bond lengths         | 0.008 Å   | 0.007 Å   | 0.008 Å   |
| Bond angles          | 1.37°     | 1.30°     | 1.27°     |

Selectivity Assays—The human leukocyte elastase and human liver cathepsin B selectivity assays were performed as described previously (11). Activity against bovine pancreatic chymotrypsin (Roche Applied Science) was evaluated using 0.2 nm enzyme and 25 μM succinyl-Ala-Ala-Pro-Phe 4-methylcoumaryl-7-amide (Bachem) (31) in a buffer similar to the one used to measure NS3 protease activity (50 mM Tris, pH 8.0, 250 mM sodium citrate, 0.01% n-dodecyl-β-D-maltoside, 1 mM DTT, and a final content of 5% DMSO). Activities against caspase I, cathepsin G, matrix metalloproteinase 1, and trypsin were tested at a single concentration (10 μM) at Ricerca Biosciences (Taipei, Taiwan).

X-ray Crystallography—Wild-type and mutant NS3 protease domain (with NS4A peptide) crystals complexed with compound 4 were prepared as described previously (25). Because of limited compound solubility in the soaking buffer, soaking times were between 8 and 10 weeks. Diffraction data from mutant NS3 protease crystals were collected at 100° K on an FR-E x-ray generator equipped with Osmic HiRes optic and a MAR345db image plate detector, although data from the WT NS3 protease crystal was collected on the same generator equipped with Rigaku VariMax-HF confocal optical system and a Rayonix SX165 CCD detector. Data reduction and scaling were performed using d*TREK (32). Preliminary models for the structures were obtained via rigid body refinement in PHENIX (33) using a model originally based on the PDB structure of 1DY9 (34). Further iterations of PHENIX refinement followed by manual model building using COOT (35) yielded final models containing residues 1–182 with C-terminal residues Arg-180 and Ser-182 being artificially truncated to alanine. Despite the presence of reducing agent, long soaking times led to the oxidation of several cysteine residues, including those that bind the structural zinc atom. In the context of the crystal, cysteine oxidation did not structurally impact the NS3 active site as evidenced by comparison of oxidized zinc-containing crystal structures with oxidized structures that have partially or completely lost their zinc. 4 The data processing statistics and model refinement statistics are listed in Table 2. Stereochemical quality was verified with the program MolProbity (36) prior to deposition at the Protein Data Bank under the codes 4I31 (wild-type compound 4), 4I32 (D168V compound 4), and 4I33 (R155K compound 4). The asymmetric unit of the P6₁ crystal form is composed of two independently refined NS3 protease monomers, both of which are capable of binding active site inhibitors. The high degree of agreement between the monomers of these structures further supports their validity.

RESULTS

Our search for a next generation NS3 protease inhibitor began with an evaluation of compounds from our in-house HCV protease inhibitor collection. We selected inhibitors that possessed smaller, more compact carbamates and/or smaller P2 proline substituents relative to faldaprevir and profiled them versus two distinct replicons encoding the targeted resistance variants R155K in genotype 1a and D168V in genotype 1b. The goal was to identify inhibitors with enhanced cell-based potency, relative to faldaprevir, versus these resistant variants. Initial testing identified compound 1 (Fig. 3), a P1-P3 macrocycle with an acylsulfonamide active site binding group and a somewhat simplified P2-hydroxyquinoline substituent. Compound 1 exhibited a significantly improved potency against both WT and variants, as well as a smaller fold-change in EC50 for the variants (see below). We have described previously how P1-P3 macrocyclization improves potency by stabilizing the bound conformation, and the location of the aliphatic macrocycle linker within the binding pocket fit well with the strategy of avoiding interaction with variant residues (37). Furthermore, it is well documented that the acylsulfonamide binds in a similar fashion to the carboxylic acid but is capable of forming additional interactions with highly conserved active site residues (38). It is noteworthy, however, that simple incorporation of an acylsulfonamide active site binding group in either linear

4 C. T. Lemke, unpublished observation.
inhibitors, like faldaprevir, or P1-P3 macrocyclic inhibitors that possessed larger P2 tricyclic quinolines does not provide enhancements in variant potency comparable with compound 1. Compound 1 therefore represented an attractive lead structure, and an extensive SAR campaign was initiated in which its structural features were systematically modified, and the impact on potency versus the variant replicons was measured.

The full extent of this investigative SAR campaign will be published separately; however, Table 3 highlights some key observations. Further diminishing the bulk of the carbamate N-terminal capping group as in compound 2 led to significant improvements versus D168V, without significant impact on the R155K potency. This observation prompted a focused SAR evaluation of a variety of N-terminal capping groups. Carbamates, ureas, and amides were evaluated. Compound 3 exhibited potencies of less than 10 nM versus both variants and highlighted two very important SAR trends identified during the course of this study. First, we observed that N-terminal capping groups composed of small heterocyclic amides like pyrazole in compound 3 could provide substantial improvements in potency versus both variants. Second, extensive SAR around this promising molecular framework therefore represented an attractive lead structure. The full extent of this investigative SAR campaign will be published separately; however, Table 3 highlights some key observations. Further diminishing the bulk of the carbamate N-terminal capping group as in compound 2 led to significant improvements versus D168V, without significant impact on the R155K potency. This observation prompted a focused SAR evaluation of a variety of N-terminal capping groups. Carbamates, ureas, and amides were evaluated. Compound 3 exhibited potencies of less than 10 nM versus both variants and highlighted two very important SAR trends identified during the course of this study. First, we observed that N-terminal capping groups composed of small heterocyclic amides like pyrazole in compound 3 could provide substantial improvements in potency versus both variants. Second, extensive SAR around this promising molecular framework therefore represented an attractive lead structure.

Further SAR around this promising molecular framework led to the identification of 4. This inhibitor has subnanomolar potency against wild-type genotype 1a and 1b replicons (0.11 ± 0.03 nM for both), but more importantly, the fold-change in activities against GT1a R155K and GT1b D168V are only 10- and 1.4-fold, respectively, a major improvement over faldaprevir and simeprevir (Table 1). Furthermore, this compound also maintained acceptable in vitro and in vivo drug-like properties.

**Profile of Compound 4**—Optimization of these macrocyclic acylsulfonamide inhibitors was driven by cell-based potency against genotype 1-resistant variants, but as shown in Table 4,
in biochemical assays compound 4 is a potent inhibitor of all tested forms of the HCV NS3-NS4A protease, including proteins from each of the six HCV genotypes as well as several clinically observed genotype 1-resistant variants. The compound maintains at least single- or double-digit nanomolar biochemical potency against each of these variants. It is least active against D168V variants and also against genotype 3, which has a different wild-type residue at amino acid 168 and is less sensitive to many other NS3-NS4A protease inhibitors, including faldaprevir and simeprevir. Variants at Ala-156 (to Val or Thr) have also been observed clinically, albeit less frequently, and in vitro studies have shown that these impart significant resistance to all classes of NS3-NS4A protease inhibitors. Consistent with its good observed biochemical activity against Ala-156 variants, compound 4 was found to have a similarly low EC50 value against the A156V replicon (0.29 ± 0.09 nM). Along with this potent inhibition of NS3-NS4A protease, compound 4 maintains high specificity, with no activity detected against any other protease tested (Table 5). Also consistent with its good selectivity, in cell culture assays cytotoxicity was observed only at high micromolar concentrations, with a CC50 value of 49 μM observed in Huh7 cells.

Although compound 4 is highly active against all resistant variants tested, it was conceivable that prolonged inhibitor treatment may select for previously unreported variants specifically resistant to this inhibitor. To test for this, replicon-dependent Huh7 cells were cultured with 0.45–75 nM compound 4 (~4–700-fold EC50 for GT1a/1b wild type). Compared with results reported for other NS3-NS4A protease inhibitors (16), relatively little breakthrough resistance was observed at concentrations severalfold greater than the EC50 value (Fig. 4).

### TABLE 5

| Enzyme                  | EC50 (nM) |
|-------------------------|-----------|
| Caspase I               | >10       |
| Cathepsin B             | >30       |
| Cathepsin G             | >10       |
| Chymotrypsin            | >25       |
| Elastase, leukocyte      | >30       |
| MMP-1                   | >10       |
| Trypsin                 | >10       |

*All values are in μM. Value given is the highest concentration tested, which in all cases gave <50% inhibition.

![FIGURE 4. Compound 4 resistance selection.](image)

Huh7 cells harboring GT1a (top) or GT1b (bottom) cells were treated with increasing concentrations of compound 4 as indicated. Colonies growing out in the presence of compound 4 were visualized by staining with crystal violet. The number of colonies observed is indicated.
online binding surface, suggests that R155K resistance is not primarily attributable to a direct structural effect. Instead, this variant appears to have a significant indirect effect via its abrogation of the 155–168 salt bridge. In the absence of a salt bridge partner, Arg-155 remains in an apo-like solvated conformation, whereas the P2 moiety of the inhibitor, which is typically well ordered in x-ray structures, becomes significantly more mobile. The best fit of this group with the observed electron density is shifted some 3 Å toward the active site. This significant perturbation strongly suggests that desolvation of the Arg-155 guanidinium is highly energetically unfavorable for the D168V variant. Compound 4 limits the impact of Arg-155 solvation with a smaller and more mobile P2 and greater reliance on interactions elsewhere in the pocket. In contrast, NS3 protease inhibitors that are dependent on rigid P2 interactions for potency are particularly sensitive to the D168V-resistant mutants as they must pay the entire Arg-155 desolvation penalty to maintain this cornerstone binding interaction.

It is interesting to consider the effect of combining these two variants. The effects of distinct resistant mutants are often observed to be additive, which can be highly detrimental to efficacy. In this case, however, complete additivity is not expected given the intertwined relationship between Arg-155 and Asp-168; each variant yields, at the site of the other, a structural perturbation that is primarily responsible for the observed resistance. The genotype 1b NS3-NS4A R155K/D168V double variant was tested in parallel with the single variants. The IC_{50} of faldaprevir shifted 150- and >530-fold against the individual variants, respectively, relative to WT but only 50-fold against the double variant. Replicon data consistent with these results were recently reported (39). The structural arguments presented herein help to explain the fact that the double variant is rarely observed in the clinic.
Mechanism of HCV NS3-NS4A Protease Inhibitor Resistance

DISCUSSION

A thorough understanding of the binding mode of the second generation NS3 protease inhibitor faldaprevir permitted the development of a rational approach toward the identification of compound 4. In turn, the improved potency of compound 4 facilitated its co-crystallization with the variant NS3 proteases, thus enabling further structural characterization of these next generation inhibitors. The wild-type and variant structures indicate that the reduced potencies for faldaprevir against the variants arise principally from secondary structural effects, i.e. they are not a direct contact effect of the mutant amino acids that reduce potency, but they are rather due to the loss of the key Arg-155–Asp-168 salt bridge that causes a major indirect effect elsewhere in the inhibitor-binding site. This premise elaborates upon a recent work in which similar mutant-inhibitor structures were used to define a molecular basis of drug resistance for several clinical stage HCV NS3 protease inhibitors (40). Although that interpretation focused on the direct steric effects elicited by the variants, the structural and biochemical data presented also provided clear evidence supporting the indirect effects described above, thus extending the relevance of this concept across a variety of inhibitor chemotypes.

Taken together, our data provide a more complete understanding of the robust antiviral profile of compound 4 and a rationale for the reduction in faldaprevir sensitivity to these variants. This analysis has suggested multiple factors that make compound 4 a potent inhibitor of both WT protease and all tested resistant variants as follows: conformational flexibility (of the P2 hydroxyquinoline substituent), interaction with conserved residues (acylsulfonamide active site binding group), as well as the avoidance of strong interactions with portions of the HCV NS3 protease binding pocket that have natural polymorphisms (N-terminal capping group pocket). HCV NS3-NS4A inhibitors with a profile similar to compound 4 might provide a significant therapeutic advantage in the future treatment of HCV infections.

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Mechanism of HCV NS3-NS4A Protease Inhibitor Resistance

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