Title

Discovery of New Inhibitors of Hepatitis C Virus NS3/4A Protease and Its D168A Mutant

Journal

ACS Omega, 4(16)

ISSN

2470-1343

Authors

Meewan, Ittipat
Zhang, Xingquan
Roy, Suchismita
et al.

Publication Date

2019-10-15

DOI

10.1021/acsomega.9b02491

Peer reviewed

ACS OMEGA

Article

Cite This: ACS Omega 2019, 4, 16999−17008

http://pubs.acs.org/journal/acsodf

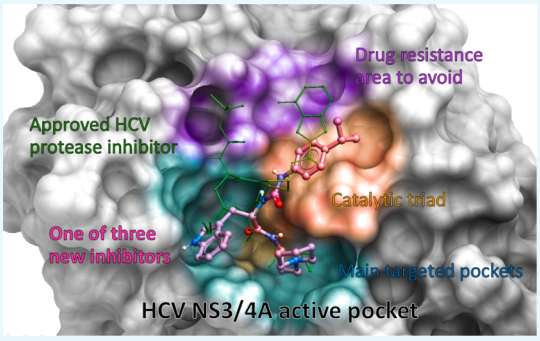
Discovery of New Inhibitors of Hepatitis C Virus NS3/4A Protease and Its D168A Mutant

Ittipat Meewan,[†,‡] Xingquan Zhang,[§] Suchismita Roy,[†] Carlo Ballatore,[†] Anthony J. O'Donoghue,[†] Robert T. Schooley,[§] and Ruben Abagyan*[,†]

[†]Skaggs School of Pharmacy and Pharmaceutical Sciences, [‡]Department of Chemistry and Biochemistry, and [§]School of Medicine, University of California San Diego, La Jolla, California 92093, United States

Supporting Information

ABSTRACT: Hepatitis C virus (HCV) is a human pathogen with high morbidity. The HCV NS3/4A protease is essential for viral replication and is one of the top three drug targets. Several drugs targeting the protease have been developed, but drug-resistant mutant strains emerged. Here, we screened a library and synthesized a novel class of small molecules based on a tryptophan derivative scaffold identified as HCV NS3/4A protease inhibitors that are active against both wild type and mutant form of the protease. Only the compounds with predicted binding poses not affected by the most frequent mutations in the active site were selected for experimental validation. The antiviral activities were evaluated by replicon and enzymatic assays. Twenty-two compounds were found to inhibit HCV with EC_{50} values ranging between 0.64 and 63 μM with compound **22** being the most active. In protease assays, **22** had a comparable inhibition profile for the common mutant HCV GT1b D168A and the wild-type enzyme. However, in the same assay, the potency of the approved drug, simeprevir, decreased 5.7-fold for the mutant enzyme relative to the wild type. The top three inhibitors were also tested against four human serine proteases and were shown to be specific to the viral protease. The fluorescence-based cell viability assay demonstrated a sufficient therapeutic range for the top three candidates.



INTRODUCTION

An estimated 170 million people around the world are infected with hepatitis C virus (HCV). HCV is transmitted through patient exposure to infected blood. Without treatment, chronic HCV infection can cause serious liver disease including cirrhosis and hepatocellular carcinoma. According to the World Health Organization record, approximately 400 000 deaths occur annually because of HCV-related complications, making it a serious health threat.[1−3] The nonstructural proteins of HCV play important roles in the viral production and replication. Three viral proteins, namely, NS3/4A serine protease, NS5A IFN resistance protein, and NS4B polymerase, are the major drug targets for the existing antiviral therapeutics.[4−6]

The NS3/4A protease is responsible for selective cleavage of polyproteins into individual viral proteins (NS4A, NS4B, NS5A, and NS5B).[7] The first NS3/4A HCV protease inhibitors, boceprevir and telaprevir, approved in 2011, were prescribed to patients with genotype 1 (GT1) viral strain along with pegylated interferon alpha (PEG-IFNα) and ribavirin. A second generation of orally available NS3/4 HCV protease inhibitor simeprevir (Olysio, Sovriad) and nucleoside analogue NS5B polymerase inhibitor sofosbuvir (Sovaldi) received FDA approval in 2013.[8,9] Between 2013 and 2016, several noncovalent peptidomimetic protease inhibitors including linear (asunaprevir), P_1−P_3 macrocyclic compounds (danoprevir, simeprevir, and paritaprevir), and P_2−P_4 macrocyclic compounds (vaniprevir and grazoprevir) were also approved in combination with NS5A interferon resistance inhibitor and/or NS5B polymerase inhibitor.[10−17] For these inhibitors, P_1, P_2, P_3, and P_4 correspond to the substrate amino acid side-chain equivalents in the inhibitor that interact with the S_1, S_2, S_3, and S_4 substrate binding pockets of the protease, respectively.

There are six well-characterized HCV genotypes (GT1−GT6), and the P_2−P_4 macrocyclic compounds have broad cross-genotype specificity.[1] Although GT1 is the most studied and targeted genotype,[18] several common mutants of the HCV protease have emerged, which include Q80K/R, R155K/Q, A156T, and D168A/V/T/H.[19,20] These mutations are in the S_2 and S_4 pockets[17,20−22] and may cause drug resistance. Several studies have shown that these single amino acid substitutions confer the resistance to all linear, P_1−P_3 macrocyclic, and P_2−P_4 macrocyclic compounds.[21−26] The most prevalent mutants causing drug resistance to all the current NS3/4A-targeting drugs are D168A and R155K.[19]

Received: August 6, 2019
Accepted: September 2, 2019
Published: October 2, 2019

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

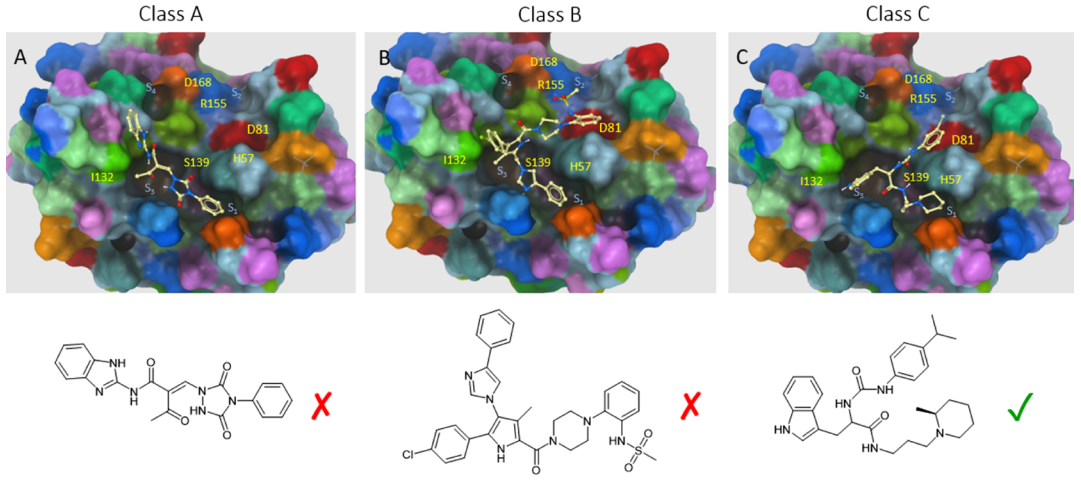


Figure 1. Binding poses of compounds from class A, B, and C in the HCV GT1a NS3/4A crystal structure represented by molecular surface colored by the side-chain types (PDB code 5EQR).[29] Binding mode from class A and class B have interactions in drug resistance susceptible subpockets S_2 and S_4, while class C compounds show preferred interaction with the receptor.

In this study, we chose a subpocket to reduce mutation-related drug resistance, three-dimensional (3D) modeling, computational docking, cell-based assays, and enzymatic assays to identify compounds that inhibit both the wild-type NS3/4A protease and the D168A mutant. This was achieved by optimizing the binding efficiency to S_1 and S_3 pockets within the HCV NS3/4 protease active site and avoiding the S_2 and S_4 pockets used by the current drugs and susceptible to mutations. The screening, identification, synthesis, and activity validation of compounds from the UC San Diego CDIPD library against the GT1 wild-type virus and the HCV protease D168A virus resulted in new promising candidates with desired properties.

RESULTS AND DISCUSSION

Identification of a Druggable Binding Pocket on HCV NS3/4A. HCV NS3/4 protease inhibitors that are approved or in clinical trials are effective against the wild-type GT1 strain, but mutations close to S_2 and S_4 pockets, in particular, R155K and D168A[17,20,21] (see Figure 1), significantly reduce the efficacy of these drugs. The X-ray crystal structure of NS3/4A from GT1a wt strain shows that the pocket conformation is relatively stable in part because of the inter-side-chain interactions between R155, D168, and R123. The side chains of R155 and D168 provide a substrate-friendly surface for the P_2 moiety of peptidomimetic protease inhibitors.[27,28] Therefore, mutations of either of these amino acids affect the P_2−S_2 interactions and drug binding.[28] The X-ray crystal structures of R155K, A156T, or D168A mutants clearly illustrate this mechanism.[27] In particular, the D168A mutation occurs frequently in viruses that have been treated with inhibitors that bind to S_2 or an adjacent S_4 patch. This mutation reduces the efficacy of drugs such as the linear inhibitor (asunaprevir) P_1−P_3 macrocyclic compounds (danoprevir, simeprevir, and paritaprevir), and P_2−P_4 macrocyclic compounds (vaniprevir and grazoprevir).[11−17]

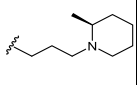
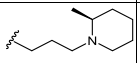
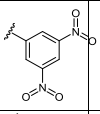
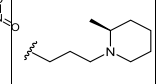
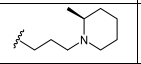
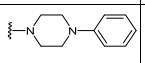
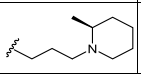
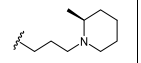
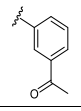
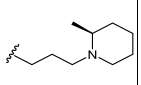
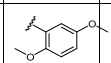
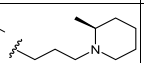
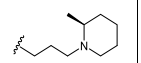
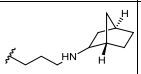
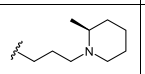
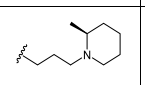
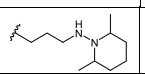
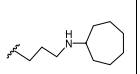
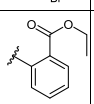
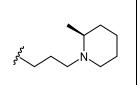
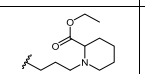
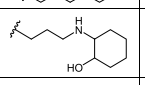
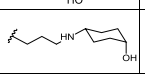
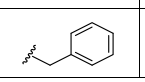
Because the S_2 and S_4 pockets are known as multidrug resistance sites, our goal was to discover a new class of small-molecule inhibitors that bind to the active site of the HCV protease but have no interactions with the S_2 and S_4 pockets. A model based on high-resolution co-crystal structure of HCV NS3/4A GT1a/GT3a protease (PDB code: 5EQR)[29] was mutated to the wild-type sequence (L132I, Q168D), converted to fully protonated models, and optimized. The selection of HCV NS3/4A crystal structure as the docking simulation receptor was based on the high-resolution, well-defined active site surrounded by secondary S_1 and S_3 pockets. The obtained models were used for virtual ligand screening with the Molsoft ICM software[30,31] against the structures of 26 743 compounds that are available at the UC San Diego Center for Discovery and Innovation in Parasitic Diseases (CDIPD).

For each compound, the binding free energy with the active site of HCV was estimated,[31] and these docking scores were used for ranking. The docking scores were only calculated for compounds that interact with the catalytic triad of H57, D81, and S139 and with the nearby residues. By setting the threshold score at −32 (arbitrary units), we identified three classes of candidate compounds likely to bind to the active site of the target enzyme (Figure 1). Within these classes, related compounds had consistent docking poses. For class A molecules, we selected five compounds with docking scores ranging from −38 to −32 (Table S1). These molecules contain *N*-phenylsulfonamide or aminosulfonamide functional groups that were predicted to interact with the S_1, S_2, and S_3 pockets. For class B molecules, two compounds with docking scores ranging from −34 to −32 were selected (Table S2). These compounds bind to the active site of the HCV protease in a different orientation from the class A molecules and primarily interact with S_1, S_3, and S_4 pockets. They consist of phenyl imidazolidine or phenyl triazolidin. The docking poses of compounds from these two classes show interactions with either the S_2 pocket (class A) or the S_4 pocket (class B) and are therefore not ideal compounds for perusing biochemical and cell-based studies using a protease with mutations in both the S_2 and S_4 pockets.

The class C molecules contain a tryptophan derivative scaffold that binds to a smaller patch compared to the class A and class B molecules. Importantly, the 14 molecules in this class (**1**−**14**) do not interact with the S_2 and S_4 pockets of HCV protease. They primarily bind to the S_1 and S_3 pockets (Figure 1) and have binding scores ranging from −32 to −38. Within the library of 26 743 compounds, we selected five additional compounds that were structurally similar to the members of the class C group (**15**−**19**); however, their

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

Table 1. Inhibition of Viral Replication (EC_{50}) in the HCV Replicon Assay for GT1b wt Selected 20 Compounds with the Same Chemical Scaffold Suggested by a Docking Screen[a]

Compound	R_1	R_2	R_3	Binding score[b]	EC_{50}±SD (μM)[c] Gt 1b wt	LC_{50}±SD (μM)[d]	Therapeutic Index[e]	Polar surface area (Å^2)	Lopophilicity (cLogP)
1			H	-38	0.95±0.26	23.52±1.89	24.73	71.05	5.10
2			F	-37	6.12±2.30	6.94±1.09	1.13	70.35	5.83
3			H	-37	14.54±0.51	36.82±13.58	2.53	137.8	5.59
4			H	-36	0.87±0.11	10.94±3.099	12.57	70.35	6.16
5			H	-36	4.78±0.40	>100	>20.92	63.27	4.50
6			H	-35	63.44±10.990	>100	>1.58	74.41	4.37
7			CH_3	-35	6.35±1.11	35.74±15.59	5.62	91.52	5.96
8			H	-34	10.92±4.93	>100	>9.16	84.88	4.00
9			H	-34	51.36±15.20	>100	>1.95	85.53	4.31
10			OCH_3	-34	33.25±3.17	>100	>3.03	70.35	5.65
11			H	-34	5.99±2.61	6.67±1.56	1.11	95.18	5.26
12			H	-33	1.50±0.07	45.91±13.01	30.61	71.05	5.37
13			H	-33	35.36±7.71	11.89±1.79	0.34	78.65	5.40
14			H	-32	5.15±0.63	92.37±45.25	17.94	94.32	4.55
15			H	-26	6.82±0.74	24.82±0.58	3.64	77.9	5.88
16			OH	-26	0.75±0.07	2.24±0.28	2.99	94.04	4.67
17			H	-24	2.98±1.08	2.71±0.11	0.91	79.36	4.60
18			H	-20	4.58±0.32	37.13±7.95	8.11	71.05	4.36
19			H	-16	32.63±14.83	>100	>3.06	82.85	4.25
20			H	-40	14.51±0.399	>100	>6.89	67.78	5.52

[a]The compounds are labeled from **1** to **20**. For each compound, the R groups are shown along with EC_{50} values, median lethal concentration at 50% (LC_{50}) for BM4-5 human liver cells, and three calculated descriptors. [b]Binding score is calculated using ICM-Pro v3.8.[30] [c]HCV replicon assay. [d]CellTiter-Blue cell viability assay. [e]Therapeutic index is the ratio of LC_{50} to EC_{50}.

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

binding scores were below the −32 cutoff. These compounds were selected for the downstream cell-based studies. In addition, libraries of commercially available compounds were searched for class C-related structures, docked and scored. From this search, we found a compound with high structural similarity and a lower binding score of −40. For our studies, we named this compound as analogue **20** (Table 1) (Figure 2).

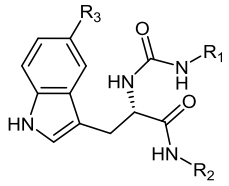


Figure 2. Common structure of class C that contains a tryptophan derivative scaffold identified as HCV NS3/4A protease inhibitors.

One important feature of this class of compounds is the ureido group that connects the core structure and R_1 group. A bidentate bond between the two secondary amines of the ureido group with the carbonyl oxygen of R155 backbone (Figure 3) stabilizes the ligand−enzyme interaction. In GT1a

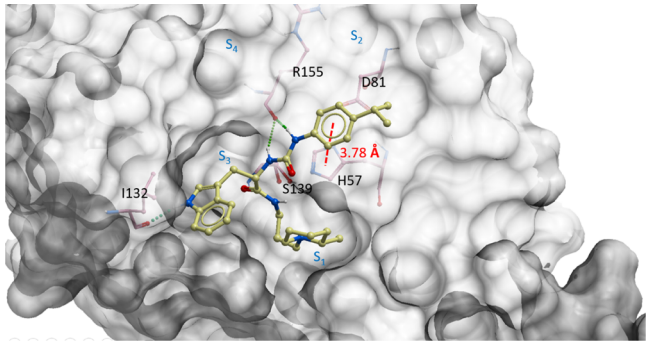


Figure 3. Structural modeling of compound **12** in the HCV NS3/4A active site. The binding mode of **12** (light yellow) in the HCV GT1a NS3/4A crystal structure is represented by multicolor skin (PDB code 5EQR).[29] Compound **12** is occupied in S_1 and S_3 pockets and forms hydrogen bond interactions (dashed green) with R155 and I132 (pink), and hydrogen bonding interactions are shown as dashed green and orange lines. Pi-stacking interaction (dashed red) between the isopropylphenyl group of **12** and H57 (pink) with a distance of 3.78 Å.

drug resistance mutants such as R155K and D168A, there are no salt bridges between the R155, D168, and R123 triads; thus, an additional backbone stabilization of R155 may be needed for a strong binding of the inhibitor to the mutant. The docking poses of superimposed structures of 20 compounds in NS3/4 GT1a (Figure S1) show consistency of binding modes among the compounds in this class. Substituted R_2 and indole groups of class C compounds are predicted to have interactions in the S_1 and S_3 subpockets, the main targeted pockets, in HCV protease active site. In addition, the H57 residue is involved in pi-stacking (3.78 Å) interactions with the substituted aromatic rings in R_1 for all the 20 inhibitors.

The 20 compounds from class C were tested in vivo for inhibition of viral replication (GT1b wt) in BM4-5 FEO cells and for mammalian cell cytotoxicity. The number of virus particles was evaluated by quantifying the luciferase produced during the virus life cycle,[32] while toxicity was evaluated using a cell viability assay. For viral replication, the 48 h EC_{50} of these compounds ranged from 0.64 to 63.44 μM (Table 1), while the 48 h cell toxicity ranged from 2.24 μM to more than 100 μM. Prior to additional biochemical studies, several key chemical properties for each of the class C molecules were determined. The polar surface area (PSA), the predicted lipophilicity (clog *P*), and the molecular weight for each compound (Table 1) were in agreement with the general criteria for drug-like molecules.

The efficacy and toxicity of class C compounds were found to be diverse with the therapeutic index, defined as the ratio of 50% lethal concentration for uninfected to 50% viral growth inhibition concentration of infected cells, for antiviral activity relative to cell toxicity ranging from 0.3 to 31. We were interested in pursuing biochemical studies for compounds **1** and **12**, the two candidates with the highest therapeutic index. These compounds also had favorable properties, such as molecular weight, water solubility PSA, and permeability within an acceptable range for a drug-like molecule.

Design of New Compounds for Improved Binding to HCV Protease. In compounds **1** and **12**, the R_2 functional group consisted of 1-propyl-2-methyl-piperidine. On the basis of the predicted docking poses, removal of the methyl group was expected to improve the interaction between the catalytic triad and the adjacent residues in the HCV protease active site. Therefore, we designed analogues of compounds **1** and **12** that lacked the methyl group at R_2 and called them compounds **21** and **22**, respectively. The binding score of **21** was unchanged from **1** (both −38), while the binding score of **22** was slightly better than **12** (−34 vs −33) (Table 2). The lowest energy binding pose of compound **22** in the active site of HCV NS3/4A is shown in Figure 4.

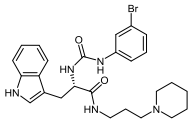
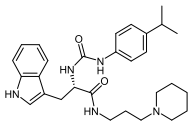
Synthesis of Compounds 21 and 22. As highlighted in Scheme 1, the tryptophan-derived HCV NS3/4 protease inhibitors, **21** and **22**, were synthesized starting from 3-(piperidine-1-yl)propan-1-amine (**23**), which could be accessed in two steps from commercially available *N*-(bromopropyl)phtalimide and piperidine, and *N*-Boc-protected L-tryptophan (**24**). Thus, the HBTU-mediated coupling of **25** and **24**, followed by *N*-Boc deprotection and carbamoylation reaction with the appropriate isocyanate, provided the desired compounds, **21** and **22**. These test compounds were characterized by ^{1}H NMR, ^{13}C NMR, and HRMS. In addition, the experimental pK_a values, lipophilicity (clog *P*), and log $D_{7.4}$ of **22** were determined via potentiometric titrations using a Sirius T3 instrument. Compounds **21** and **22**, as well as the 20 compounds from the primary screen, were then tested in cell-based assays to evaluate activity against HCV and toxicity to the host cells (Table 2).

Evaluation of Compounds in Viral Replication Assays and HCV Protease Activity Assays. Compounds **21** and **22** were evaluated in the HCV replicon assay and for cytotoxicity using BM4-5 FEO cells. Compound **21** had slightly weaker antiviral activity than **1** but lower cytotoxicity; therefore, the therapeutic index was unchanged for these two analogues. The IC_{50} value for compound **22** was 2-fold more potent than the related compound **12** and the cytotoxicity was unchanged. Therefore, the therapeutic index for **22** improved 2-fold over **12** from 30.6 to 73.4.

In order to validate that this group of molecules elicit their cellular effects via inhibition of the viral protease, we incubated compounds **1**, **12**, and **22** with recombinant NS3/4A protease

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

Table 2. Inhibition of Viral Replication (EC_{50}) in the HCV Replicon Assay for GT1b wt by Two Synthesized Compounds (21, 22)[a]

Compound	Structure	Binding score[b]	EC_{50}±SD (μM)[c] Gt 1b wt	LC_{50}±SD (μM)[d]	Therapeutic Index[e]	Polar surface area (Å^2)	Lopophilicity (cLogP)
21		-38	1.30±1.25	29.52±1.48	22.72	71.6	4.93
22		-34	0.64±0.13	46.97±6.98	73.39	71.6	5.19 (4.1)[f]

[a]For each compound, the R groups are shown along with EC_{50} values, median lethal dose (LC_{50}) for BM4-5 human liver cells, and three calculated descriptors. [b]Binding score calculated using ICM-Pro v3.8.[30] [c]HCV replicon assay. [d]CellTiter-Blue cell viability assay. [e]Therapeutic index is the ratio of LC_{50} to EC_{50}. [f]Experimental log *P*.

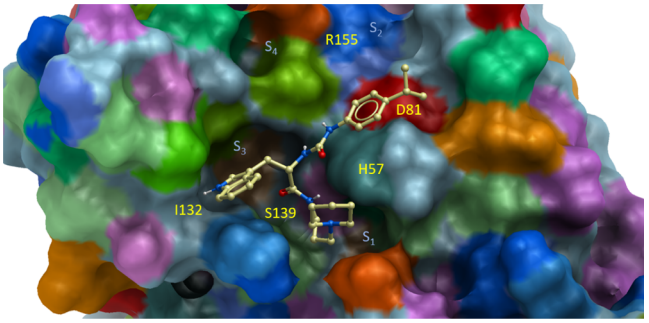
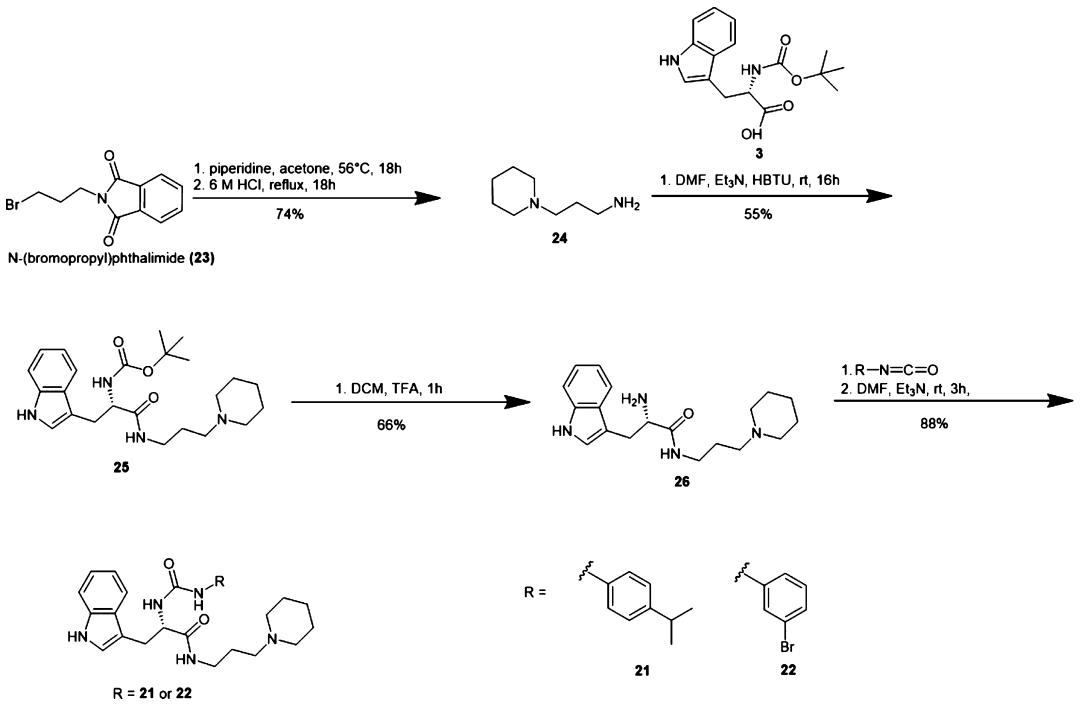


Figure 4. Structural modeling of compound **22** in HCV NS3/4A active site. Docking pose of compound **22** in the HCV GT1a NS3/4A crystal structure represented by molecular surface colored by the side chain types (PDB code 5EQR).[29]

GT1b and an inhibitor-resistant mutant containing a D168A mutation. D168 is located in the S_2 and S_4 subpockets, and mutations in these subpockets can cause significant resistance to the inhibitors, resulting in decreased activity of peptidomimetic inhibitors.[3,33,34] The enzyme activity was evaluated using a fluorogenic peptide substrate, Ac-Glu-Glu-Val-Val-Ala-Cys-AMC, that has previously been described as a HCV protease substrate.[35] No enzyme assays were performed with compound **21** because it showed no improvement in antiviral activity or selectivity when compared to the parent molecule, **1**. As a control, potency was evaluated with an approved HCV protease inhibitor, simeprevir.

The HCV protease was incubated with 0.625−40 μM of compounds **1**, **12**, and **22** and the IC_{50} values were calculated. These studies show that the wt protease activity is directly

Scheme 1. Synthesis of 21 and 22



DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

inhibited by compounds derived from the tryptophan scaffold. The potency of compound **22** was lower than simeprevir (IC_{50} 4.60 μM vs 0.043 μM) (Table 3); however, when inhibition was evaluated using the D168A mutant, the potency of simeprevir decreased by 5.7-fold, while no significant reduction of potency was observed for compound **22** (Figure 5). Likewise, compounds **1** and **12** that showed high efficiency and acceptable toxicity profile from the HCV replicon assay were assayed with wt and mutant HCV protease and found to have no significant change in potency. Therefore, our initial prediction that these tryptophan-containing inhibitors do not interact with the S_2 and S_4 subpockets is likely to be correct. Other HCV protease inhibitors, such as glecaprevir, paritaprevir, and grazoprevir, have been shown to have 4-fold to 154-fold reduced potency in cell-based assays when comparing the wild-type viruses with viruses that have a mutation at D168.[36]

Table 3. NS3/4A Inhibitory Assay of Compounds 1, 12, and 22[a]

compound	IC_{50} (μM) GT1b wt	IC_{50} (μM) GT1b D168A	IC_{50}_D168A/IC_{50}_wt ratio
1	14.68 ± 1.23	11.37 ± 0.45	0.8
12	13.08 ± 2.77	11.45 ± 0.55	0.9
22	4.60 ± 1.26	5.98 ± 0.43	1.4
simeprevir	0.043 ± 0.79	0.247 ± 0.39	5.7

[a]The identified compounds inhibit both the wild type and the D168A mutant form of protease at similar IC_{50} values.

To determine the HCV NS3/4A selectivity of compounds **1**, **12**, and **22**, we assayed these analogues with trypsin, elastase, and chymotrypsin at the HCV IC_{50} concentrations. In addition, we tested these compounds for inhibition against the fungal serine protease, proteinase K that is structurally related to several mammalian serine proteases such as the proprotein convertases.[37] Using a concentration of 40 nM for each enzyme, we found that 13.08 and 14.68, 4.60 μM of **1**, **12**, and **22**, respectively, did not decrease the activity of trypsin, elastase, chymotrypsin, and proteinase K significantly, while, at this concentration, these three compounds decreased HCV NS3/4A activity by about 50% (Figure 6). Taken together, these data indicate that compounds with the tryptophan scaffold derivative were selective from the viral protease over other serine proteases.

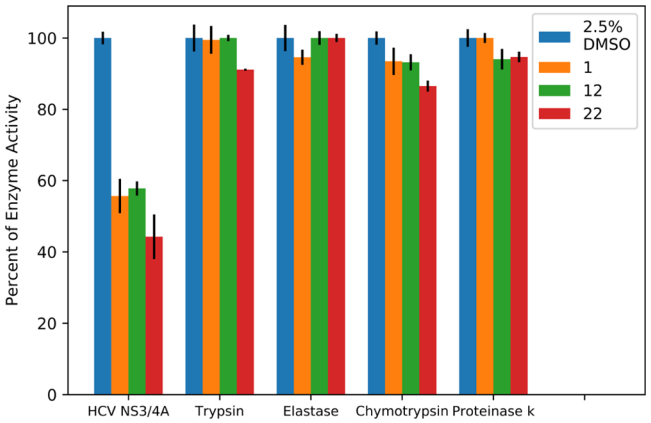


Figure 6. HCV protease selectivity of top three compounds against four human serine proteases. Counter-screening on of **1**, **12**, and **22** at 13.08, 14.68, 4.60 μM, respectively, to 40 nM concentration of HCV NS3/4A, trypsin, elastase, chymotrypsin, and proteinase K shows high HCV-NS3-specificity of **1**, **12**, and **22** inhibitors.

CONCLUSIONS

In this study, we identified and synthesized a new class of inhibitors of HCV protease that shows similar potency for the wild-type and D168A mutant enzymes. The inhibitory activity against the mutant was achieved by targeting subpockets not affected by the D168A mutation. The compounds were dissimilar to the common HCV protease inhibitors that contained groups mimicking P_1, P_2, P_3, and P_4 substrate side chains. In particular, **22** had an EC_{50} of 0.64 μM in a viral replication assay and an LC_{50} of 47 μM for mammalian cells, resulting in an overall therapeutic index of 73.4. This compound directly inhibited the protease with IC_{50} = 4.60 μM and no significant reduction if potency occurred when assayed with the D168A mutant. Three of the top compounds were tested against a panel of four nonviral serine proteases and no significant inhibition was noted. The new NS3/4A protease inhibitors are promising development candidates for treatment of emerging drug-resistant HCV strains.

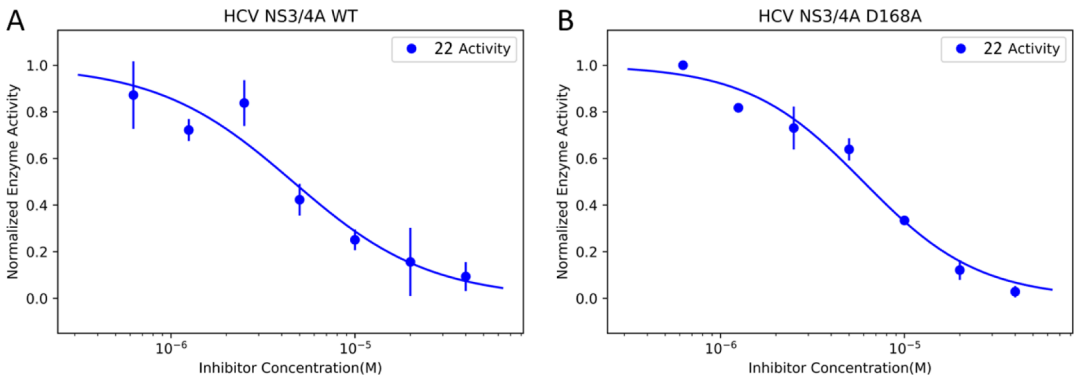


Figure 5. HCV NS3/4A protease inhibition assay. Dose response curve of **22** with HCV protease GT1b wt (A) and D168A mutant (B) derived from the relative fluorescence absorption that represents the concentration of the cleavage products of fluorogenic peptide substrate (Ac-Glu-Glu-Val-Val-Ala-Cys-AMC) at 60 μM by GT1b wt protease or by the D168A mutant in the presence of **22** at inhibitor concentrations between 0.625 and 40 μM; the errors calculated from three measurements at each concentration.

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

EXPERIMENTAL SECTION

All solvents were of reagent grade. All reagents were purchased from Aldrich, Enamine, or Fisher Scientific and used as received. Thin-layer chromatography (TLC) was performed with 0.25 mm E. Merck precoated silica gel plates. Silica gel column chromatography was performed with silica gel 60 (particle size 0.040−0.062 mm) supplied by Silicycle and Sorbent Technologies. TLC spots were detected by viewing under a UV light (254 nm). Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a 600 MHz Bruker AVANCE III spectrometer. Chemical shifts were reported relative to the residual solvent's peak. High-resolution mass spectra were measured using Thermo LCQdeca-MS. Unless otherwise stated, all final compounds were found to be >95% pure as determined by HPLC/MS and NMR.

3-(Piperidine-1-yl)propan-1-amine (23). To a stirring solution of piperidine (1 g, 11.74 mmol) in acetone (11 mL), *N*-(bromopropyl)phthalimide (0.739 g, 2.935 mmol) was added. The mixture was heated at 56 °C and stirred for 18 h.[38] Then, the solvent was evaporated obtaining a dark yellow powder, which was redissolved in ethyl acetate, washed with 2 M of K_2CO_3, and extracted with 2 M of HCl. The aqueous layer was adjusted to pH 11 and re-extracted with dichloromethane (DCM). The solvent was evaporated, and the resulting dark yellow brown crude was added with 6 M HCl (40 mL) and then heated to reflux for 18 h. The reaction mixture was then washed with DCM, added with NaOH to adjust the pH to 12, and extracted again with DCM. The organic layer was dried over sodium sulfate, filtered, and evaporated under reduced pressure to obtain the desired product as a brown solid and evaporated to get a brown crude of intermediate **24** in 74% yield (0.309 g). ^{1}H NMR ($CDCl_3$): δ (ppm) 2.752 (t, J = 6.9 Hz, 2H), 2.363 (m, 6H), 1.663 (m, 2H), 1.660 (m, 6H), 1.451 (m, NH_2). MS for $C_8H_{19}N_2$ [M + H^+], 143.15.

***tert*-Butyl (3-(1*H*-Indol-3-yl)-1-oxo-1-((3-(piperidin-1-yl)propyl)amino)propan-2-yl)carbamate (24).** To a solution of *Nα*-(*tert*-butoxycarbonyl)-L-tryptophan (0.643 g, 2.112 mmol) in anhydrous dimethylformamide (DMF, 8 mL), HBTU (1.602 g, 4.225 mmol) and Et_3N (1.068 g, 10.56 mmol) were added, followed by a dropwise addition of **24** (0.309 g, 2.176 mmol) in DMF (1.6 mL). The reaction mixture was stirred at room temperature for 22 h. During the reaction, the solution color changed from yellow to dark green in 2 h. The completion of the reaction was checked by TLC using 10% MeOH in dimethyl sulfoxide (DMSO) as the solute. The reaction was quenched by addition of saturated aqueous solution of $NaHCO_3$, which caused the mixture to turn yellow with white cloudy precipitates. The aqueous layer was extracted with ethyl acetate. The organic layer was dried over sodium sulfate, filtered, and evaporated under reduced pressure. Purification via silica gel column chromatography (15% MeOH in DCM) furnished the title compound in 26% yield (0.240 g). ^{1}H NMR ($CDCl_3$): δ (ppm) 8.785 (s, 1H), 7.635 (d, J = 6.6 Hz, 1H), 7.368 (d, J = 6.6 Hz, 1H), 7.289 (s, 1H), 7.181 (t, J = 7.8 Hz, 1H), 7.112 (t, J = 7.2 Hz, 1H), 7.082 (s, 1H), 4.410 (t, J = 3.6 Hz, 1H), 3.332 (d, J = 13.2 Hz, 2H), 3.186 (t, J = 6.0 Hz, 2H), 2.245 (m, 4H), 2.193 (t, J = 6.0 Hz, 2H), 1.510 (m, 4H), 1.453 (s, 9H), 1.402 (m, 2H). ^{13}C NMR ($CDCl_3$): δ (ppm) 171.697, 155.378, 136.217, 127.648, 123.339, 122.066, 119.514, 119.042, 111.232, 110.551, 79.830, 57.225, 55.454, 54.148, 38.980, 28.906, 28.885, 25.130, 24.206, 23.755. MS for $C_{24}H_{36}N_4O_3$ [M + H^+], 429.29.

2-Amino-3-(1*H*-indol-3-yl)-*N*-(3-(piperidin-1-yl)-propyl)propanamide (25). To a solution of **25** (50 mg, 0.17 mmol) in DCM (1.6 mL), trifluoroacetyl (0.35 mL) was added dropwise and the resulting mixture was stirred for 1 h. Evaporation of volatiles under reduced pressure followed by trituration in diethyl ether furnished the desired compounds in 88% yield (51 mg, 0.15 mmol) as a brown powder. ^{1}H NMR (MeOD): δ (ppm), 8.484 (s, NH_2), 7.641 (d, J = 7.8 Hz, 1H), 7.413 (d, J = 7.4 Hz, 1H), 7.263 (s, 1H), 7.168 (t, J = 7.2 Hz, 1H), 7.091 (t, J = 7.2 Hz, 1H), 4.127 (t, J = 4.2 Hz, 1H), 3.334 (d, J = 12 Hz, 4H), 3.209 (dq, J = 6.6, 23.4 Hz, 2H), 2.687 (m, 4H) 1.881 (m, 2H), 1.747 (m, 2H), 1.488 (m, 4H), ^{13}C NMR (MeOD): δ (ppm) 169.233, 136.632, 127.073, 124.126, 121.485, 118.886, 117.888, 111.262, 106.881, 53.866, 52.875, 52.684, 36.036, 27.186, 23.372, 22.759, 21.202 MS for $C_{24}H_{36}N_4O_3$ [M + H^+], 329.23.

2-(3-(3-Bromophenyl)ureido)-3-(1*H*-indol-3-yl)-*N*-(3-(piperidin-1-yl)propyl)propenamide (21). To a solution of **26** (50 mg, 0.1166 mmol) in DMF (1.17 mL) and the solution was added by 3-bromophenyl isocyanate (20.98 mg, 0.106 mmol). The reaction was started by adding the catalyst Et_3N (32.4 mg, 0.318 mmol), followed by stirring for 3 h, and then the solution turned yellow. The reaction was quenched by adding water (12 mL), extracted by ethyl acetate (4 × 15 mL), and washed by water (15 mL). The organic layer was purified by column chromatography (10−15% MeOH in DCM). The solvent was evaporated to obtain the remaining powder of **6** at a yield of 79% (44 mg, 0.084 mmol). ^{1}H NMR ($CDCl_3$): δ (ppm), 8.498 (s, NH), 8.235 (s, NH), 7.607 (d, J = 7.8 Hz, 1H), 7.561 (s, 1H), 7.290 (m, 1H), 7.198 (d, J = 7.2 Hz, 1H), 7.101 (m, 5H), 6.976 (s, NH), 4.656 (d, J = 6.6 Hz, 1H), 3.207 (m, 4H), 2.228 (m, 2H), 2.144 (m, 2H), 1.497 (m, 4H), 1.395 (p, J = 6 Hz, 4H) 1.292 (m, 2H). ^{13}C NMR ($CDCl_3$): δ (ppm), 173.145, 155.411, 140.939, 136.149, 130.102, 127.498, 125.092, 125.061, 123.505, 122.450, 122.021, 121.799, 119.472, 118.724, 117.460, 111.277, 110.411, 57.546, 55.300, 54.324, 40.017, 29.812, 25.865, 24.165. MS for $C_{26}H_{32}BrN_5O_2$ [M + H^+], 526.18.

3-(1*H*-Indol-3-yl)-2-(3-(4-isopropylphenyl)ureido)-*N*-(3-(piperidin-1-yl)propyl)propenamide (22). To a solution of **26** (51 mg, 0.1189 mmol) in DMF (1.2 mL), 4-isopropylphenyl isocyanate (17.4 mg, 0.108 mmol) was added, followed by the addition of a catalytic amount of Et_3N (32.8 mg, 0.324 mmol). The reaction mixture was stirred for 3 h at room temperature, which caused the solution to turn yellow. The reaction was quenched by adding water (12 mL) and extracted with ethyl acetate (4 × 15 mL). The combined organic layers were then washed with water (15 mL), dried over sodium sulfate, filtered, and evaporated under reduced pressure. Purification via silica gel column chromatography (15% MeOH in DCM) furnished the title compound in 66% yield (35 mg, 0.070 mmol). ^{1}H NMR ($CDCl_3$): δ (ppm), 9.071 (s, NH), 8.311 (s, NH), 7.898 (s, NH), 7.635 (d, J = 12 Hz, 1H), 7.290 (m, 3H), 7.103 (t, J = 7.2 Hz, 1H), 7.036 (m, 4H), 6.960 (s, NH), 3.244 (s, 2H), 3.116 (d, J = 27 Hz, 2H), 2.825 (t, J = 6.6 Hz, 1H), 2.217 (m, 1H), 2.128 (m, 4H), 1.466 (m, 4H), 1.364 (d, J = 9 Hz, 4H), 1.291 (s, 2H), 1.214 (d, J = 6.6 Hz, 6H), ^{13}C NMR ($CDCl_3$): δ (ppm) 173.287, 156.011, 142.890, 137.097, 136.162, 127.616, 126.690, 123.636, 121.781, 119.664, 119.245, 118.900, 118.866, 111.243, 110.503, 57.079, 55.240, 54.155, 39.333, 33.456, 29.765,

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

29.512, 25.561, 24.521, 24.171. MS for $C_{29}H_{39}N_5O_2$ [M + H^+], 490.32.

Replicon Construct. The BM4-5 replicon is a subgenomic HCV GT1b replicon that contains adaptive mutation of serine in the NS5A region.[32,39] The firefly luciferase gene was added into the BM4-5 replicon to generate BM4-5 FEO replicon. The construction procedures have been previously described.[40]

Luciferase Antiviral Efficacy and Cell Viability Assays. The BM4-5 FEO replicon of approximately 10 000 cells in 100 μL of medium was seeded into 96-well plates and incubated for 6 h for attachment. Then, the compounds were added to the wells in a specific concentration and incubated for 48 h. The luciferase assay (Bright-Glo; Promega) was carried out according to the manufacturer's instructions. The luciferase activity was determined using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems). For cell viability assays, BM4-5 FEO at the density of 10 000 cells in 100 μL medium was incubated at 37 °C for 6 h for attachment, and then the compounds were added and incubated for another 48 h. The number of living cells was measured by Cell Titer-blue assay (Promega). The fluorescence absorption ($560_{Ex}/590_{Em}$) for a 96-well plate was read by Benchmark Scientific MR9600. The activity and toxicity of each compound were measured by at least six different concentrations in triplicate.

Plasmid Construction and Purification of HCV NS3/A Protein. The drug-resistant mutant D168A was generated by mutagenesis using NS3-containing recombinant plasmid (pETDuet-1 Hepatis C Virus NS3/4A, a gift from Michael Johnson, Addgene plasmid #16196) as a template. The single mutation of aspartic acid (D) to alanine (A) was introduced by mutagenesis at position 168 using specific primers (forward: 5′-GTTGCCAAAGCCGTTGCTTTCGTTCCGGTGGAA-3′ and reverse: 5′-TTCCACCGGAACGAAAG-CAACGGCTTTGGCAAC-3′). The overexpression and purification were preformed following the method previously described.[41] Rosetta 2(DE3) cells (Novagen) containing the modified recombinant plasmid with a histidine tag were grown in a lysogeny broth medium. The overexpression was introduced by addition of isopropyl β-D-1-thiogalactopyranoside to the final concentration of 0.5 mM and the bacteria were incubated at room temperature for 16 h. The cell pellet was lysed by sonication in buffer A [50 mM HEPES (pH 7.6), 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol (β-MCE), 0.2% Triton X-100, 15% glycerol, and protease inhibitor cocktail (Sigma-Aldrich)]. The solution was passed through an Ni-NTA column and washed by buffer B (50 mM HEPES, pH 7.6, 500 mM NaCl, 50 mM imidazole, 5 mM β-MCE, 0.2% Triton X-100, and 15% glycerol). The His-tag-fused protein was eluted by buffer C (50 mM HEPES, pH 7.6, 500 mM NaCl, 500 mM imidazole, 5 mM β-MCE, 0.2% Triton X-100, and 15% glycerol), followed by dialysis using buffer (50 mM HEPES, pH 7.6, and 500 mM NaCl). Purified NS3/4A protein was concentrated and buffer-exchanged into buffer D (50 mM HEPES, pH 7.6, 500 mM NaCl, 5 mM β-MCE, 0.2% Triton X-100, and 50% glycerol) using a protein concentrator 10k (Amicon)

HCV NS3/4A Enzymatic Inhibition Assay. The activities of two proteases (recombinant HCV NS3/4A GT1b wt and full-length HCV NS3/4A GT1b D168A) were measured using a fluorogenic substrate, Ac-Glu-Glu-Val-Val-Ala-Cys-AMC (Sigma-Aldrich), where AMC corresponds to 7-amino-4-methylcourmarin. Assays were performed in 50 mM HEPES, pH 7.4, 15 mM NaCl, 0.01% Triton X-100, and 10 mM dithiothreitol in a black 384-well microplate (BD Falcon). The final concentrations of the enzyme, substrate, and DMSO were 40 nM, 60 μM, and 2.5%, respectively, in a total volume of 30 μL. Fluorescence was monitored at excitation and emission wavelengths of 360 and 460 nm, respectively, in a Synergy HTX Multi-Mode Microplate Reader (BioTek). Data were visualized using Gen5 Software (BioTek). The activity of the enzyme was calculated from the change in relative fluorescence units over time. For inhibition assays, compounds were preincubated with the enzyme for 240 min prior to adding the enzyme/inhibitor mixture to the substrate. All assays were performed in triplicate wells.

Counter-Screening Assay. Counter-screening assays were performed with 40 nM bovine trypsin (Sigma), pig pancreatic elastase (Sigma), human chymotrypsin, and proteinase K, and 60 μM of the appropriate fluorogenic substrates for trypsin, elastase, chymotrypsin, and proteinase K are Boc-Leu-Arg-Arg-AMC (trypsin), Me-Arg-Arg-Pro-Val-AMC (elastase), and Suc-Arg-Arg-Pro-Phe-AMC (chymotrypsin and proteinase K). Assays were performed in 50 mM HEPES, pH 7.4, 15 mM NaCl, and 0.01% Triton X-100 in black 96-well microplates (BD Falcon) at a final volume of 50 μL. The final concentration of DMSO in each well was 2.5%. Fluorescence was measured as outlined above.

Computational Modeling. Docking screening was performed by docking an in-house library of approximately 27 000 small molecules and ranked based on their docking scores, which represent Gibbs free energy.[30] The algorithm for conformational sampling 3D structures of ligands and pockets is generated randomly by biased probability Monte Carlo.[31] All scoring functions and pharmacokinetic property predictions were performed by the method implemented in ICM-Pro v3.8.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02491.

Structure and computational calculation data of class A and class B compounds; superimposition of the best docking structure of compounds in class A in HCV NS3/4A wt; docking pose of **22** with the HCV NS3/4A D168A mutant; SDS-PAGE of the purified protein; and ^1H and ^{13}C NMR and mass spectra of synthesized compounds and the intermediates (PDF)

3D coordinates of top three compounds (ZIP)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rabagyan@ucsd.edu.

ORCID

Carlo Ballatore: 0000-0002-2718-3850

Anthony J. O'Donoghue: 0000-0001-5695-0409

Ruben Abagyan: 0000-0001-9309-2976

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Huijun Zhang (School of Life Sciences, Tsinghua University, Beijing), Dr. Mohsin Khan, and

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

Dr. Hasan Imam (School of Medicine, University of California, San Diego) for the valuable advice and assistance in HCV replicon and cell viability experiments. The authors thank Dr. Jair Lage de Siqueira-Neto and Dr. James McKerrow for their valuable help with the CDIPD chemical library and Dr. Killian Oukoloff for advising about the chemical synthesis experiments in this study. The authors are especially grateful to Kirti Kandhwal Chahal and Dr. Irina Kufareva (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego) for the suggestions about protein purification.

REFERENCES

(1) Blach, S.; Zeuzem, S.; Manns, M.; Altraif, I.; Duberg, A.-S.; Muljono, D. H.; Waked, I.; Alavian, S. M.; Lee, M.-H.; Negro, F.; Abaalkhail, F.; Abdou, A.; Abdulla, M.; Rached, A. A.; Aho, I.; Akarca, U.; Al Ghazzawi, I.; Al Kaabi, S.; Al Lawati, F.; Al Namaani, K.; Al Serkal, Y.; Al-Busafi, S. A.; Al-Dabal, L.; Aleman, S.; Alghamdi, A. S.; Aljumah, A. A.; Al-Romaihi, H. E.; Andersson, M. I.; Arendt, V.; Arkkila, P.; Assiri, A. M.; Baatarkhuu, O.; Bane, A.; Ben-Ari, Z.; Bergin, C.; Bessone, F.; Bihl, F.; Bizri, A. R.; Blachier, M.; Blasco, A. J.; Mello, C. E. B.; Bruggmann, P.; Brunton, C. R.; Calinas, F.; Chan, H. L. Y.; Chaudhry, A.; Cheinquer, H.; Chen, C.-J.; Chien, R.-N.; Choi, M. S.; Christensen, P. B.; Chuang, W.-L.; Chulanov, V.; Cisneros, L.; Clausen, M. R.; Cramp, M. E.; Craxi, A.; Croes, E. A.; Dalgard, O.; Daruich, J. R.; de Ledinghen, V.; Dore, G. J.; El-Sayed, M. H.; Ergör, G.; Esmat, G.; Estes, C.; Falconer, K.; Farag, E.; Ferraz, M. L. G.; Ferreira, P. R.; Flisiak, R.; Frankova, S.; Gamkrelidze, I.; Gane, E.; García-Samaniego, J.; Khan, A. G.; Gountas, I.; Goldis, A.; Gottfredsson, M.; Grebely, J.; Gschwantler, M.; Pessôa, M. G.; Gunter, J.; Hajarizadeh, B.; Hajelssedig, O.; Hamid, S.; Hamoudi, W.; Hatzakis, A.; Himatt, S. M.; Hofer, H.; Hrstic, I.; Hui, Y.-T.; Hunyady, B.; Idilman, R.; Jafri, W.; Jahis, R.; Janjua, N. Z.; Jarčuška, P.; Jeruma, A.; Jonasson, J. G.; Kamel, Y.; Kao, J.-H.; Kaymakoglu, S.; Kershenobich, D.; Khamis, J.; Kim, Y. S.; Kondili, L.; Koutoubi, Z.; Krajden, M.; Krarup, H.; Lai, M.-s.; Laleman, W.; Lao, W.-c.; Lavanchy, D.; Lázaro, P.; Leleu, H.; Lesi, O.; Lesmana, L. A.; Li, M.; Liakina, V.; Lim, Y.-S.; Luksic, B.; Mahomed, A.; Maimets, M.; Makara, M.; Malu, A. O.; Marinho, R. T.; Marotta, P.; Mauss, S.; Memon, M. S.; Correa, M. C. M.; Mendez-Sanchez, N.; Merat, S.; Metwally, A. M.; Mohamed, R.; Moreno, C.; Mourad, F. H.; Müllhaupt, B.; Murphy, K.; Nde, H.; Njouom, R.; Nonkovic, D.; Norris, S.; Obekpa, S.; Oguche, S.; Olafsson, S.; Oltman, M.; Omede, O.; Omuemu, C.; Opare-Sem, O.; Øvrehus, A. L. H.; Owusu-Ofori, S.; Oyunsuren, T. S.; Papatheodoridis, G.; Pasini, K.; Peltekian, K. M.; Phillips, R. O.; Pimenov, N.; Poustchi, H.; Prabdial-Sing, N.; Qureshi, H.; Ramji, A.; Razavi-Shearer, D.; Razavi-Shearer, K.; Redae, B.; Reesink, H. W.; Ridruejo, E.; Robbins, S.; Roberts, L. R.; Roberts, S. K.; Rosenberg, W. M.; Roudot-Thoraval, F.; Ryder, S. D.; Safadi, R.; Sagalova, O.; Salupere, R.; Sanai, F. M.; Avila, J. F. S.; Saraswat, V.; Sarmento-Castro, R.; Sarrazin, C.; Schmelzer, J. D.; Schréter, I.; Seguin-Devaux, C.; Shah, S. R.; Sharara, A. I.; Sharma, M.; Shevaldin, A.; Shiha, G. E.; Sievert, W.; Sonderup, M.; Souliotis, K.; Speiciene, D.; Sperl, J.; Stärkel, P.; Stauber, R. E.; Stedman, C.; Struck, D.; Su, T.-H.; Sypsa, V.; Tan, S.-S.; Tanaka, J.; Thompson, A. J.; Tolmane, I.; Tomasiewicz, K.; Valantinas, J.; Van Damme, P.; van der Meer, A. J.; van Thiel, I.; Van Vlierberghe, H.; Vince, A.; Vogel, W.; Wedemeyer, H.; Weis, N.; Wong, V. W. S.; Yaghi, C.; Yosry, A.; Yuen, M.-f.; Yunihastuti, E.; Yusuf, A.; Zuckerman, E.; Razavi, H. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol. Hepatol.* **2017**, *2*, 161−176.

(2) World Health Organization. *Global Hepatitis Report 2017*; Geneva, Switzerland, 2017.

(3) Lu, L.; Pilot-Matias, T. J.; Stewart, K. D.; Randolph, J. T.; Pithawalla, R.; He, W.; Huang, P. P.; Klein, L. L.; Mo, H.; Molla, A. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro. *Antimicrob. Agents Chemother.* **2004**, *48*, 2260−2266.

(4) Keating, G. M. Ledipasvir/Sofosbuvir: a review of its use in chronic hepatitis C. *Drugs* **2015**, *75*, 675−685.

(5) Geddawy, A.; Ibrahim, Y. F.; Elbahie, N. M.; Ibrahim, M. A. Direct Acting Anti-hepatitis C Virus Drugs: Clinical Pharmacology and Future Direction. *J. Transl. Intern. Med.* **2017**, *5*, 8−17.

(6) Lawitz, E. J.; O'Riordan, W. D.; Asatryan, A.; Freilich, B. L.; Box, T. D.; Overcash, J. S.; Lovell, S.; Ng, T. I.; Liu, W.; Campbell, A.; Lin, C.-W.; Yao, B.; Kort, J. Potent Antiviral Activities of the Direct-Acting Antivirals ABT-493 and ABT-530 with Three-Day Monotherapy for Hepatitis C Virus Genotype 1 Infection. *Antimicrob. Agents Chemother.* **2016**, *60*, 1546−1555.

(7) Shiryaev, S. A.; Thomsen, E. R.; Cieplak, P.; Chudin, E.; Cheltsov, A. V.; Chee, M. S.; Kozlov, I. A.; Strongin, A. Y. New Details of HCV NS3/4A Proteinase Functionality Revealed by a High-Throughput Cleavage Assay. *PLoS One* **2012**, *7*, No. e35759.

(8) Zhang, X. Direct anti-HCV agents. *Acta Pharm. Sin. B* **2016**, *6*, 26−31.

(9) Foster, G. R.; Pianko, S.; Brown, A.; Forton, D.; Nahass, R. G.; George, J.; Barnes, E.; Brainard, D. M.; Massetto, B.; Lin, M.; Han, B.; McHutchison, J. G.; Subramanian, G. M.; Cooper, C.; Agarwal, K.; Group, B. S. Efficacy of sofosbuvir plus ribavirin with or without peginterferon-alfa in patients with hepatitis C virus genotype 3 infection and treatment-experienced patients with cirrhosis and hepatitis C virus genotype 2 infection. *Gastroenterology* **2015**, *149*, 1462−1470.

(10) Majumdar, A.; Kitson, M. T.; Roberts, S. K. Systematic review: current concepts and challenges for the direct-acting antiviral era in hepatitis C cirrhosis. *Aliment. Pharmacol. Ther.* **2016**, *43*, 1276−1292.

(11) Scola, P. M.; Sun, L.-Q.; Wang, A. X.; Chen, J.; Sin, N.; Venables, B. L.; Sit, S.-Y.; Chen, Y.; Cocuzza, A.; Bilder, D. M.; D'Andrea, S. V.; Zheng, B.; Hewawasam, P.; Tu, Y.; Friborg, J.; Falk, P.; Hernandez, D.; Levine, S.; Chen, C.; Yu, F.; Sheaffer, A. K.; Zhai, G.; Barry, D.; Knipe, J. O.; Han, Y.-H.; Schartman, R.; Donoso, M.; Mosure, K.; Sinz, M. W.; Zvyaga, T.; Good, A. C.; Rajamani, R.; Kish, K.; Tredup, J.; Klei, H. E.; Gao, Q.; Mueller, L.; Colonno, R. J.; Grasela, D. M.; Adams, S. P.; Loy, J.; Levesque, P. C.; Sun, H.; Shi, H.; Sun, L.; Warner, W.; Li, D.; Zhu, J.; Meanwell, N. A.; McPhee, F. The discovery of asunaprevir (BMS-650032), an orally efficacious NS3 protease inhibitor for the treatment of hepatitis C virus infection. *J. Med. Chem.* **2014**, *57*, 1730−1752.

(12) Jiang, Y.; Andrews, S. W.; Condroski, K. R.; Buckman, B.; Serebryany, V.; Wenglowsky, S.; Kennedy, A. L.; Madduru, M. R.; Wang, B.; Lyon, M.; Doherty, G. A.; Woodard, B. T.; Lemieux, C.; Do, M. G.; Zhang, H.; Ballard, J.; Vigers, G.; Brandhuber, B. J.; Stengel, P.; Josey, J. A.; Beigelman, L.; Blatt, L.; Seiwert, S. D. Discovery of danoprevir (ITMN-191/R7227), a highly selective and potent inhibitor of hepatitis C virus (HCV) NS3/4A protease. *J. Med. Chem.* **2014**, *57*, 1753−1769.

(13) Venables, B. L.; Sin, N.; Wang, A. X.; Sun, L.-Q.; Tu, Y.; Hernandez, D.; Sheaffer, A.; Lee, M.; Dunaj, C.; Zhai, G.; Barry, D.; Friborg, J.; Yu, F.; Knipe, J.; Sandquist, J.; Falk, P.; Parker, D.; Good, A. C.; Rajamani, R.; McPhee, F.; Meanwell, N. A.; Scola, P. M. P3-P4 ureas and reverse carbamates as potent HCV NS3 protease inhibitors: Effective transposition of the P4 hydrogen bond donor. *Bioorg. Med. Chem. Lett.* **2018**, *28*, 1853−1859.

(14) Bogen, S. L.; Pan, W.; Ruan, S.; Nair, L. G.; Arasappan, A.; Bennett, F.; Chen, K. X.; Jao, E.; Venkatraman, S.; Vibulbhan, B.; Liu, R.; Cheng, K.-C.; Guo, Z.; Tong, X.; Saksena, A. K.; Girijavallabhan, V.; Njoroge, F. G. Toward the Back-Up of Boceprevir (SCH 503034): Discovery of New Extended P4-Capped Ketoamide Inhibitors of Hepatitis C Virus NS3 Serine Protease with Improved Potency and Pharmacokinetic Profiles. *J. Med. Chem.* **2009**, *52*, 3679−3688.

(15) Manns, M. P.; Gane, E.; Rodriguez-Torres, M.; Stoehr, A.; Yeh, C.-T.; Marcellin, P.; Wiedmann, R. T.; Hwang, P. M.; Caro, L.; Barnard, R. J. O.; Lee, A. W.; Lingua::EN::Titlecase, M. K. P. S. Vaniprevir with pegylated interferon alpha-2a and ribavirin in

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008

treatment-naive patients with chronic hepatitis C: a randomized phase II study. *Hepatology* **2012**, *56*, 884−893.
(16) Manns, M. P.; Bourlière, M.; Benhamou, Y.; Pol, S.; Bonacini, M.; Trepo, C.; Wright, D.; Berg, T.; Calleja, J. L.; White, P. W.; Stern, J. O.; Steinmann, G.; Yong, C.-L.; Kukolj, G.; Scherer, J.; Boecher, W. O. Potency, safety, and pharmacokinetics of the NS3/4A protease inhibitor BI201335 in patients with chronic HCV genotype-1 infection. *J. Hepatol.* **2011**, *54*, 1114−1122.
(17) Svarovskaia, E. S.; Martin, R.; McHutchison, J. G.; Miller, M. D.; Mo, H. Abundant drug-resistant NS3 mutants detected by deep sequencing in hepatitis C virus-infected patients undergoing NS3 protease inhibitor monotherapy. *J. Clin. Microbiol.* **2012**, *50*, 3267−3274.
(18) Chen, Y.; Yu, C.; Yin, X.; Guo, X.; Wu, S.; Hou, J. Hepatitis C virus genotypes and subtypes circulating in Mainland China. *Emerging Microbes Infect.* **2017**, *6*, 1.
(19) Romano, K. P.; Ali, A.; Aydin, C.; Soumana, D.; Özen, A.; Deveau, L. M.; Silver, C.; Cao, H.; Newton, A.; Petropoulos, C. J.; Huang, W.; Schiffer, C. A. The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors. *PLoS Pathog.* **2012**, *8*, No. e1002832.
(20) Sarrazin, C.; Zeuzem, S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* **2010**, *138*, 447−462.
(21) Vermehren, J.; Sarrazin, C. The role of resistance in HCV treatment. *Best Pract. Res. Clin. Gastroenterol.* **2012**, *26*, 487−503.
(22) Belfrage, A. K.; Abdurakhmanov, E.; Åkerblom, E.; Brandt, P.; Alogheli, H.; Neyts, J.; Danielson, U. H.; Sandström, A. Pan-NS3 protease inhibitors of hepatitis C virus based on an R(3)-elongated pyrazinone scaffold. *Eur. J. Med. Chem.* **2018**, *148*, 453−464.
(23) Lin, C.; Gates, C. A.; Rao, B. G.; Brennan, D. L.; Fulghum, J. R.; Luong, Y.-P.; Frantz, J. D.; Lin, K.; Ma, S.; Wei, Y.-Y.; Perni, R. B.; Kwong, A. D. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J. Biol. Chem.* **2005**, *280*, 36784−36791.
(24) Kurt Yilmaz, N.; Swanstrom, R.; Schiffer, C. A. Improving Viral Protease Inhibitors to Counter Drug Resistance. *Trends Microbiol.* **2016**, *24*, 547−557.
(25) Cubero, M.; Esteban, J. I.; Otero, T.; Sauleda, S.; Bes, M.; Esteban, R.; Guardia, J.; Quer, J. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* **2008**, *370*, 237−245.
(26) Romano, K. P.; Ali, A.; Aydin, C.; Soumana, D.; Özen, A.; Deveau, L. M.; Silver, C.; Cao, H.; Newton, A.; Petropoulos, C. J.; Huang, W.; Schiffer, C. A. The molecular basis of drug resistance against hepatitis C virus NS3/4A protease inhibitors. *PLoS Pathog.* **2012**, *8*, No. e1002832.
(27) Soumana, D. I.; Ali, A.; Schiffer, C. A. Structural analysis of asunaprevir resistance in HCV NS3/4A protease. *ACS Chem. Biol.* **2014**, *9*, 2485−2490.
(28) Ali, A.; Aydin, C.; Gildemeister, R.; Romano, K. P.; Cao, H.; Ozen, A.; Soumana, D.; Newton, A.; Petropoulos, C. J.; Huang, W.; Schiffer, C. A. Evaluating the Role of Macrocycles in the Susceptibility of Hepatitis C Virus NS3/4A Protease Inhibitors to Drug Resistance. *ACS Chem. Biol.* **2013**, *8*, 1469−1478.
(29) Soumana, D. I.; Kurt Yilmaz, N.; Ali, A.; Prachanronarong, K. L.; Schiffer, C. A. Molecular and Dynamic Mechanism Underlying Drug Resistance in Genotype 3 Hepatitis C NS3/4A Protease. *J. Am. Chem. Soc.* **2016**, *138*, 11850−11859.
(30) Neves, M. A. C.; Totrov, M.; Abagyan, R. Docking and scoring with ICM: the benchmarking results and strategies for improvement. *J. Comput.-Aided Mol. Des.* **2012**, *26*, 675−686.
(31) Abagyan, R.; Totrov, M. Biased Probability Monte Carlo Conformational Searches and Electrostatic Calculations for Peptides and Proteins. *J. Mol. Biol.* **1994**, *235*, 983−1002.
(32) Guo, J.-T.; Bichko, V. V.; Seeger, C. Effect of alpha interferon on the hepatitis C virus replicon. *J. Virol.* **2001**, *75*, 8516−8523.
(33) McPhee, F.; Friborg, J.; Levine, S.; Chen, C.; Falk, P.; Yu, F.; Hernandez, D.; Lee, M. S.; Chaniewski, S.; Sheaffer, A. K.; Pasquinelli, C. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. *Antimicrob. Agents Chemother.* **2012**, *56*, 3670−3681.
(34) Courcambeck, J.; Bouzidi, M.; Perbost, R.; Jouirou, B.; Amrani, N.; Cacoub, P.; Pèpe, G.; Sabatier, J. M.; Halfon, P. Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations. *Antivir. Ther.* **2006**, *11*, 847−855.
(35) Po, A. Enzymatic Properties of Hepatitis C Virus NS3 Serine Protease and Bio-Engineering of Serine Protease Inhibitors (Serpins) Against the NS3 Protease and Elastase. Master's Thesis, University of British Columbia, Vancouver, British Columbia, Camada, 2003.
(36) Ng, T. I.; Tripathi, R.; Reisch, T.; Lu, L.; Middleton, T.; Hopkins, T. A.; Pithawalla, R.; Irvin, M.; Dekhtyar, T.; Krishnan, P.; Schnell, G.; Beyer, J.; McDaniel, K. F.; Ma, J.; Wang, G.; Jiang, L. J.; Or, Y. S.; Kempf, D.; Pilot-Matias, T.; Collins, C. In Vitro Antiviral Activity and Resistance Profile of the Next-Generation Hepatitis C Virus NS3/4A Protease Inhibitor Glecaprevir. *Antimicrob. Agents Chemother.* **2017**, *62*, No. e01620.
(37) Betzel, C.; Gourinath, S.; Kumar, P.; Kaur, P.; Perbandt, M.; Eschenburg, S.; Singh, T. P. Structure of a Serine Protease Proteinase K from Tritirachium album limber at 0.98 Å Resolution. *Biochemistry* **2001**, *40*, 3080−3088.
(38) Apelt, J.; Ligneau, X.; Pertz, H. H.; Arrang, J.-M.; Ganellin, C. R.; Schwartz, J.-C.; Schunack, W.; Stark, H. Development of a New Class of Nonimidazole Histamine H3 Receptor Ligands with Combined Inhibitory Histamine N-Methyltransferase Activity. *J. Med. Chem.* **2002**, *45*, 1128−1141.
(39) Wyles, D. L.; Kaihara, K. A.; Schooley, R. T. Synergy of a hepatitis C virus (HCV) NS4A antagonist in combination with HCV protease and polymerase inhibitors. *Antimicrob. Agents Chemother.* **2008**, *52*, 1862−1864.
(40) Wyles, D. L.; Kaihara, K. A.; Vaida, F.; Schooley, R. T. Synergy of small molecular inhibitors of hepatitis C virus replication directed at multiple viral targets. *J. Virol.* **2007**, *81*, 3005−3008.
(41) Lee, H.; Torres, J.; Truong, L.; Chaudhuri, R.; Mittal, A.; Johnson, M. E. Reducing agents affect inhibitory activities of compounds: results from multiple drug targets. *Anal. Biochem.* **2012**, *423*, 46−53.

DOI: 10.1021/acsomega.9b02491
ACS Omega 2019, 4, 16999−17008