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Synthesis and antiviral evaluation of novel peptidomimetics as Norovirus protease inhibitors

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Abstract

A series of tripeptidyl transition state inhibitors with new P1 and warhead moieties were synthesized and evaluated in a GI-1 norovirus replicon system and against GII-4 and GI-1 norovirus proteases. Compound **19**, containing a 6-membered ring at the P1 position and a reactive aldehyde warhead exhibited sub-micromolar replicon inhibition. Retaining the same peptidyl scaffold, several reactive warheads were tested for protease inhibition and norovirus replicon inhibition. Of the six that were synthesized and tested, compounds **42**, **43**, and **45** potently inhibited the protease in biochemical assay and GI-1 norovirus replicon in the nanomolar range.

Graphical Abstract

Keywords

Norovirus; Antiviral; Protease inhibitor; Peptide

Human noroviruses are single stranded, positive sense RNA viruses from the *Caliciviridae* family. Out of the six existing genotypes, genotypes I, II and IV are highly contagious pathogens that are the primary cause of acute gastroenteritis. In the US, there is an estimated 20 million cases of gastroenteritis each year resulting in more than 50,000 hospitalizations and nearly 570–800 deaths. Even though there is no therapeutic approved for the treatment

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of norovirus (NoV) infections, peptidomimetic inhibitors of the NoV protease (Figure 1) have emerged recently as a promising option.¹ Indeed, NoV 3C-like cysteine protease is essential to generate mature nonstructural proteins by cleavage of the viral polyprotein. Transition state inhibitors, such as compound **1**,² in which the peptidic moiety is selectively recognized by the 3CL protease while the electrophilic warhead reacts with Cys139 to form a covalent bond, can block the protease enzymatic activity (Figure 2). The general structure of these inhibitors can be divided into 5 distinct portions: P1, P2, P3, cap and warhead (Figure 1). Modifications of the P2 and P3 portions have been explored in the past but due to their key role in the specificity of these compounds, only minimal modifications seem to be tolerated at these positions. Chemical modification of the P1 position has only been partially explored³ and a (S)- γ -lactam P1 seems to remain the group of choice. Alternatively, the warhead moiety has been largely studied and several groups such as aldehydes, α -ketoamides, α -hydroxyphosphonates, and bisulfite adducts have been successfully evaluated.²

Herein, we report our effort to identify more effective peptidomimetics, inhibitors of NoV protease, using the structure of reported compound **1** as a starting point (Reported anti-NoV EC₅₀ = 0.04 μ M).² More specifically, we explored the chemical space around the P1 position and evaluated new potential warheads.

Evaluation of new P1 moieties

Based on several NoV PIs in which the P1 lactam moiety was successfully replaced by acyclic amides,³ replacement of compound **1**'s pyrrolidinone P1 moiety with an azetidine amide (compound **15a**), an azetidine carbamate (compound **15b**) or a 1,2,3-triazole (compound **15c**) was explored. In a similar manner, since (S)- δ -lactam segments at P1 position have been proven to be an effective replacement for (S)- γ -lactam groups in the case of peptidomimetic aldehydes, inhibitors of the human enterovirus protease,⁴ we decided to apply the same strategy and prepared analog **19**. Amino acid precursors bearing the desired P1 moieties were prepared according to the chemistry described in Scheme 1. Compound **3** was synthesized by reacting acid derivative **2**⁵ with azetidine in presence of HOBt, EDC and DIPEA while intermediate **5** was obtained from amine **4**⁶ by first, reaction with *N,N'*-disuccinimidyl carbonate (DSC) followed by treatment with azetidine in presence of DIPEA. Triazole derivative **7** was obtained under classical copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) conditions by reaction of azido compound **6**⁷ with trimethylsilylacetylene. Piperidinone intermediate **9** was prepared from natural glutamic acid **8** by following previously published procedures.⁸

Targeted peptidomimetic inhibitors **15a–c** were finally obtained from commercially available amino acid **10** (Scheme 2). Coupling of **10** with L-leucine methyl ester in presence of HOBt, EDC and DIPEA gave ester **11** which was then saponified in presence of LiOH and coupled with either intermediates **3**, **5** or **7**. Final aldehyde derivatives **15a–c** were obtained through a reduction/oxidation sequence using LiBH₄ and a sulfur trioxide pyridine complex. On the other hand, deprotection of intermediate **9** under acidic conditions followed by coupling with Boc-L-leucine gave **16**⁹ which was then deprotected and coupled with

amino acid **10**. As for the other analogs, a final reduction/oxidation sequence gave access to the desired peptidomimetic **19** (Scheme 3).

Evaluation of new warheads

The chemical reactivity of the warhead is essential and if too reactive, it can lead to a lack of selectivity and undesired off target effects. Therefore, we evaluated several groups as replacement for the highly reactive aldehyde warhead moiety in reference compound **1**. These groups include electrophiles such as a vinyl aldehyde (compound **26**), a halogen (compound **46**), epoxides (compounds **30** and **41**), a trifluoromethyl ketone (Compound **45**) or an acrylonitrile-based Michael acceptor, known to react with cysteines (Compound **43**).¹⁰ The replacement of the aldehyde warhead by a thiol in order to potentially form a disulfide bond with NoV protease Cys139 was also evaluated.

Peptidomimetic **26**, containing a vinyl aldehyde warhead, was prepared from known vinyl ethyl ester derivative **20**¹¹ (Scheme 4). After deprotection in presence of TFA, compound **21** was coupled with Boc-L-leucine under classical peptidic coupling conditions to give intermediate **22**. **22** was then deprotected and coupled with amino acid **10** to access vinyl ester derivative **24**. Final reduction of the ester group with DIBAL-H followed by oxidation of alcohol derivative **25** afforded the desired vinyl aldehyde peptidomimetic **26**.

Methylsulfonyl epoxide derivative **30** was obtained according to the chemistry described in Scheme 5. Aldehyde **27**¹² was reacted with diethyl (methylthiomethyl) phosphonate in presence of *n*-BuLi to give unsaturated sulfone derivative **28**. Boc removal and peptidic coupling with **10** gave access to compound **29** which was then further oxidized in presence of *t*-BuOOH and MeLi to give epoxide **30**.

Compound **35** was prepared following the chemistry described in scheme 6. Activation of compound **31**¹² with MsCl and further displacement of the mesylate group with KSAc gave the thioacetate intermediate **32** which, after deprotection in presence of TFA, was coupled with amino acid **10**. Final treatment of **34** with LiOH gave access to the desired thiol derivative **35**.

Aldehyde **27** was reacted in a Wittig type reaction with CH₃PPh₃Br in presence of LiHMDS to give **36** which was deprotected in presence of HCl and then, coupled with Boc-L-leucine. Removal of the Boc group under acidic condition, coupling of the resulting amine with amino acid **10** and final epoxidation with *t*-BuOOH and MeLi afforded the desired epoxide peptidomimetic **41** (Scheme 7).

Finally, cyanoacrylamide, hydroxyl sulfonate, trifluoromethyl ketone, and chloro analogs of compound **1** were prepared according to the chemistry described in schemes 8 and 9. Thus, reaction of aldehyde **19** with 2-cyanoacetamide or sodium bisulfate gave compounds **42** and **43** respectively. Trifluoromethyl keto derivative **45** was obtained by reaction of **19** with Me₃SiCF₃ in presence of CsF followed by oxidation with Dess-Martin periodinane. In contrast, reaction of hydroxyl intermediate **18** with CCl₄ and PPh₃ chlorinated the peptidomimetic **46**.

Anti-norovirus activity of newly synthesized compounds was evaluated in an HG23 cell-based replicon assay containing Norwalk virus replicon (prototypic GI.1).¹³ Briefly, HG23 cells were seeded at a density of 10,000 cells/well in 96-well plates along with testing compounds at concentrations ranging from 0.01 to 10 μ M followed by a four-day incubation at 37 °C and 5% CO₂. Total cellular RNA was isolated from the cells with Directzol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA). The replicon RNA was amplified with qScript XLT 1-Step RT-qPCR ToughMix (QuantaBio, Beverly, MA) in LightCycler 480 II (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocols. Results were reported as EC₅₀ and EC₉₀ values that represent 50% and 90% reduction in the replicon RNA levels, respectively. *In vitro* IC₅₀ and IC₉₀ analysis of the compounds against active Norovirus GI.1 and GII.4 proteases applied a FRET-based kinetic assay to test for enzymatic activity.

The FRET substrate is based on the P5-P2' residues of the NoV NS2/NS3 cleavage site. Enzyme and substrate were optimized to an emission wavelength of 488 nm and an excitation of 520nm (substrate: HiLyte Fluor 488 – DFELQGPK (QXL520), purchased from Anaspec, Inc.). Assays were conducted at 37 °C for 90 minutes with a total well volume of 100 μ L. The fluorescence emitted by the cleaved substrate is directly related to enzymatic activity. The fluorescent signal is monitored by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Readings are taken every minute for 20 mins. In addition, cytotoxicity was determined in primary human peripheral blood mononuclear (PBM) cells, human lymphoblastoid CEM, African Green monkey Vero cells and Huh7 cells.¹⁴ Results are shown in Table 1.

It is worth noting that, in our hands, reference compound **1** was 28 times less potent than previously reported (EC₅₀ = 0.04 μ M vs 1.13 μ M).² Replacement of the P1 lactam moiety in compound **1** with either an acyclic amide (**15a**) or carboxamide (**15b**) group was evaluated. Unfortunately, **15a** only displayed an EC₅₀ of 6.2 μ M in our NoV replicon while **15b** was inactive at concentration up to 10 μ M at both the enzymatic and cellular level. In a similar manner, introduction of an 1,2,3-triazole at this position was counterproductive and lead to the highly toxic compound **15c**. More interestingly, replacement of the (S)- γ -lactam with a (S)- δ -lactam segments at P1 position was more effective. Indeed, even though compound **19** was 2.5 times less potent at the enzymatic level than the reference compound **1** (IC₅₀ = 0.15 μ M); with an EC₉₀ of 0.92 μ M, it was ten times more active than **1**, in our NoV GT-1 replicon system. This may be due to improved cell penetration since compound **19** is slightly less polar (QlogP = 4.7) relative to **1** (QlogP = 4.4) by QikProp calculation.¹⁵

Thio derivative **35**, chloro derivative **46** and epoxide derivative **41** did not show activity against the protease itself nor the virus at concentrations up to 10 μ M. On the other hand, compounds **30**, and **26**, with, respectively, a methylsulfonyl epoxide or a vinyl aldehyde warhead, displayed EC₅₀s in the low micromolar range. However, since none of them really showed potency against the NoV protease, one can hypothesized that the activity observed *in vitro*, could result from cellular toxicity. Replacement of the aldehyde warhead by either a hydroxyl sulfonate, a cyanoacrylamide or a trifluoromethyl ketone function lead to the discovery of compounds **42**, **43** and **45**, respectively, which displayed submicromolar activities against NoV. If the hydroxyl sulfonate group has already been established as a

valid warhead,² to the best our knowledge, compound **43** and **45** represent the first examples of a NoV protease inhibitors with a cyanoacrylamide or a trifluoromethyl ketone warhead.

To determine molecular contributions to inhibition, compounds **43** and **19** were modeled into the NoV protease active site using Glide Covalent Docking module (Maestro, Schrodinger Suite 2016-3).¹⁶ In brief, the crystal structure of NoV protease covalently bound to a potent dipeptide inhibitor (PDBID 4XBB)¹⁷ was prepared using the protein preparation wizard. The P1 ring of the co-crystallized ligand was manually corrected to alkyl, the hydrogen bonding network was optimized at neutral pH with Epik, and the hydrogen positions were refined using Prime minimization with the OPLS3 forcefield. As a control, these methods recaptured the bioactive pose of the co-crystallized covalently bound ligand (3ZR) upon re-docking in the absence of water molecules (data not shown).

Compound **19** was covalently docked to the active site in a nucleophilic addition to a double bond reaction type resulting in the formation of a bond between Cys139 and the terminal aldehyde group for compound **19** (Figure 3A). The P1 sub-pocket accommodates the six-membered (S)- δ -lactam ring and maintains hydrogen bond accepting interactions with His157 and Thr134. The isobutyl substitution positions into the P2 sub-pocket formed by His30, Ile109, and Gln110. The P3 naphthyl ring fits into the “cap” sub-pocket (occupied by the 2-chloro-benzyl ring of the 4XBB co-crystallized 3ZR ligand) sandwiched between Ile109, Thr161 and Lys162. The benzyl cap of compound **19** points outwards into solution making weak interactions with Lys162 and stacking against the P1 (S)- δ -lactam moiety. The primary alcohol incurs no steric clash upon warhead reaction with Cys139, and docking suggests the alcohol accepts hydrogen bonds from His30 and the backbone amides of Gly137 and Asp138.

For compound **43**, the cyanoacrylamide warhead was reacted using the Michael addition reaction type in Glide covalent docking which formed a bond between Cys139 and the β -carbon (relative to the cyano group). This pose predicts the similar placement of P1, P2, and P3 moieties as observed for compound **19** (Figure 3B). However, unlike compound **19**, the benzyl cap of compound **43** extends outwards to solution and makes no significant interactions with the enzyme. This pose shows that the warhead incurs no steric interactions in the active site. The cyano group accepts hydrogen bonds from the backbone amides of Gly137 and Asp138. The amide group accepts a hydrogen bond from His30 and donates a hydrogen bond to Ser14. Interestingly, the amide group forms an intramolecular hydrogen bond with the P1 backbone amide. This interaction repositions the peptidomimetic backbone (relative to the pose of **19**) likely contributing to the differential positioning of the benzyl cap.

In conclusion, we have synthesized and evaluated several new peptidomimetics as potential NoV protease inhibitors. Among them, we discovered four new compounds that displayed anti-NoV activity in the sub-micromolar range. We also established cyanoacrylamides or trifluoromethyl ketones as valid warhead candidates. Despite some toxicity observed in certain cell systems, further modifications are currently being investigated.

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Figure 1.
General structure of known NoV cysteine protease inhibitors

Figure 2.
Interaction of a NoV cysteine protease with known peptidyl transition state inhibitor **1**.

Figure 3.

Model of A) compound **19** or B) compound **43** covalently bound to NoV protease. All modeling was conducted using Schrodinger's Maestro (2016-3) with the OPLS3 force-field. The crystal structure of NVpro covalently bound to a potent dipeptide inhibitor was selected for docking (4XBB). The structure was prepared using the protein preparation wizard, the P1 ring of the co-crystallized ligand was corrected to be alkyl, the hydrogen bonding network was optimized at neutral pH with Epik, and the hydrogen positions were refined using Prime minimization. The Glide Covalent Docking module was used to dock compounds **19** and **43** into the active site. Cys139 was selected as the reactive residue, and a covalent bond was formed with the aldehyde of **19** or the cyanoacrylamide warhead of **43**.

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Scheme 2.

Reagents and conditions: a) H-*L*-Leu-OMe.HCl, HOBt, EDC.HCl, DIPEA, DCM, rt, 52%;
 b) LiOH, THF-H₂O, rt, 2 h, 95%; c) 1). **3**, **5** or **7**, TFA/DCM, rt.; 2). HOBt, EDC.HCl,
 DIPEA, DCM, rt.; d) LiBH₄, THF; e) SO₃.Py, DIPEA, DCM-DMSO, rt.

Scheme 3.

Reagents and conditions: a) 1) 4 N HCl in dioxane, DCM, rt; 2) Boc-*L*-Leu-OH, HOBt, EDC.HCl, DIPEA, DCM, rt, overnight, 61%; b) 1) 4 N HCl in dioxane, DCM, rt; 2) **10**, HOBt, EDC.HCl, DIPEA, DCM, rt, overnight, 54%; c) LiBH₄/THF, 0 °C to rt, 2 h, 93%; d) Dess-Martin periodinane, rt, 4 h, 58%.

Scheme 4.

Reagents and conditions: a) TFA/DCM (1:1), rt, 2 h; b) Boc-*L*-Leu-OH, HOBt, EDC.HCl, DIPEA, DCM, rt, overnight, 61% over two steps; c) TFA/DCM (1:1), rt; d) **10**, HOBt, EDC.HCl, DIPEA, DCM, rt, overnight, 63% over two steps; e) DIBAL-H, THF, 27%; f) SO₃.Py, DIPEA, DCM-DMSO, rt, 5 h, 75%.

Scheme 5.

Reagents and conditions: a) $\text{CH}_3\text{SO}_2\text{CH}_2\text{PO}(\text{OEt})_2$, BuLi, THF, -78°C to rt, 1 h, 19%; b) 1). TFA/DCM (1:1), rt, 5 h, 2). **10**, HOBT, EDC.HCl, DIPEA, DCM, rt, overnight, 53% over two steps; c) *t*-BuOOH, MeLi, THF, -78°C 30 min then 0°C overnight, 54%.

Scheme 6.

Reagents and conditions: a) 1). MsCl, Et₃N, DCM, 0°C, 1 h; 2). KSAc, DCM, rt, overnight, 78% over two steps; b) TFA/DCM, rt., 1 h; c). **10**, HOBt, EDC.HCl, DIPEA, DCM, rt., overnight, 46% over two steps; d) LiOH, THF-H₂O, rt, 5 h, 79%.

Scheme 7.

Reagents and conditions: a) $\text{CH}_3\text{PPh}_3\text{Br}$, LiHMDS, THF, -78°C 30 min and then 0°C overnight, 31%; b) 4 N HCl in dioxane, DCM, rt., 3 h; c) Boc-L-Leu-OH, HOBT, EDC.HCl, DIPEA, DCM, rt., overnight, 67% over two steps; d) 4 N HCl in dioxane, DCM, rt.; e) **10**, HOBT, EDC.HCl, DIPEA, DCM, rt., overnight, 60% over two steps; f) *t*-BuOOH, MeLi, THF, -78°C 20 min and then 0°C overnight, 30%.

Scheme 8.

Reagents and conditions: a) 2-cyanoacetamide, piperidine, EtOH, MW, 80 °C, 25 min, 16%; b) sodium bisulfite, EtOAc/EtOH/H₂O (1:0.6:0.25), 55 °C 3 h, 67%; c) 1) Me₃SiCF₃, CsF, THF, -78 °C to rt, 2 h, 28%; 2) 1N HCl, rt, 1 h; d) Dess-Martin periodinane, DCM, rt, 15%.

Scheme 9.

Reagents and conditions: a) CCl_4 , PPh_3 , DCM, rt., overnight, 76%.

Table 1

Activity of compounds against NoV 3CL protease and replicon harboring cells.

Cpd	Anti-norovirus activity (μM)							Toxicity CC_{50} (μM)				
	IC_{50} (GIL4)	IC_{90} (GIL4)	IC_{50} (GL1)	IC_{90} (GL1)	EC_{50} (GL1)	EC_{90} (GL1)	EC_{50} (GL1)	Huh7	PBM	CEM	Vero	
1	0.060 ± 0.016	0.205 ± 0.047	0.032 ± 0.006	0.248 ± 0.070	1.13 ± 0.5	8.36 ± 0.24	17.34 ± 0.22	>10	28.4	39.5	>100	
15a	0.502	5.54	>1	ND	6.19 ± 1.35	ND	ND	ND	18.4	2.2	8.6	
15b	>100 (4%)	ND	>100	ND	>10	ND	ND	33.0	21.2	2.2	>100	
15c	>10	ND	>1	ND	ND ^a	ND	ND	2.7	1.9	0.3	3.2	
19	0.150 ± 0.002	1.19 ± 0.444	0.080 ± 0.023	0.155 ± 0.008	0.42 ± 0.15	0.92 ± 0.03	2.45 ± 0.75	25.7	23.6	31.6	19.8	
26	>100	ND	>10	ND	4.7 ± 0.18	>10	>10	ND	3.9	1.9	77.7	
30	30.0 ± 1.8	>100	>10	ND	4.33 ± 4.20	>20	>20	ND	>100	63.9	>100	
35	>10	ND	>10	ND	>10	ND	ND	93.5	>100	>100	>100	
41	>100	ND	>100	ND	>10	ND	ND	ND	15.7	14.0	30.4	
42	2.63 ± 1.04	14.03 ± 5.55	0.654 ± 0.285	2.241 ± 0.438	0.39 ± 0.08	2.45 ± 0.75	7.82 ± 0.01	11.2	43.8	18.3	>100	
43	1.17 ± 0.333	3.60 ± 0.501	0.593 ± 0.124	1.697 ± 0.332	0.75 ± 0.01	2.45 ± 0.75	7.82 ± 0.01	8.4	45.4	9.1	>100	
45	2.63 ± 1.04	14.03 ± 5.55	0.654 ± 0.285	2.241 ± 0.438	0.39 ± 0.08	2.45 ± 0.75	7.82 ± 0.01	11.2	43.8	18.3	>100	
46	>100	ND	>10	ND	>10	ND	ND	ND	75.0	26.6	51.7	

ND: Not determined

^aNot determined due to high cytotoxicities observed in both our replicon system (99% rRNA inhibition at 10 μM) and in our panel of cell lines.