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Macrocyclic inhibitors of 3C and 3C-like proteases of picornavirus, norovirus, and coronavirus

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ABSTRACT

The design, synthesis, and in vitro evaluation of the first macrocyclic inhibitor of 3C and 3C-like proteases of picornavirus, norovirus, and coronavirus are reported. The in vitro inhibitory activity (50% effective concentration) of the macrocyclic inhibitor toward enterovirus 3C protease (CVB3 Nancy strain), and norovirus 3CLpro, and norovirus 3C-like proteases, was determined to be 1.8, 15.5 and 5.1 μM, respectively.

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The picornavirus-like protease supercluster includes viruses in the Picornaviridae, Coronaviridae, and Caliciviridae families. Many human pathogens of major medical and economic importance belong to these virus families. For instance, the family Picornaviridae includes enterovirus (enterovirus, EV; coxsackievirus, CV; poliovirus, PV), human rhinovirus (HRV), and hepatitis A virus (HAV).1,2 Non-polio enteroviruses are responsible for 10–15 million symptomatic infections in the US each year,3 while HRV is the major causative agent of upper respiratory tract infections.4 In the Coronaviridae family, severe acute respiratory syndrome (SARS) caused by SARS-coronavirus (SARS-CoV) is a recognized global threat to public health.5 Noroviruses belong to the Norovirus genus of the Caliciviridae family and are highly contagious human pathogens that are the most common cause of food borne and water borne acute viral gastroenteritis.6 Thus, norovirus infection constitutes an important public health problem. There are currently no vaccines (except for poliovirus) or specific antiviral agents for combating infections caused by the aforementioned viruses; thus, there is an urgent and unmet need for the discovery and development of broad spectrum small-molecule therapeutics and prophylactics for these important pathogens.7–10

The picornaviral genome consists of a positive sense, single-stranded RNA of ~7.5 kb in length that encodes a large precursor polyprotein that requires proteolytic processing to generate mature viral proteins.1,2 Processing of the polyprotein is primarily mediated by the viral 3C protease (3Cpro). Likewise, the ~30 kb genome of SARS-CoV comprises both nonstructural and structural regions. Two polyproteins (designated as pp1a and pp1ab) encoded by the viral genome undergo proteolytic processing by two proteases: a chymotrypsin-like cysteine protease (3C-like protease, 3CLpro) and a papain-like protease (PLpro), to generate functionally active proteins. Finally, the 7–8 kb RNA genome of noroviruses encodes a polyprotein that is processed by a 3C-like protease (3CLpro) to generate mature proteins.11 Although there is high genetic diversity among these viruses, 3Cpro and 3CLpro are highly conserved, as well as essential for virus replication.

Inspection of the crystal structures of picornavirus 3Cpro12–15 and norovirus 3CLpro,16–18 reveals that the proteases share in common a chymotrypsin-like fold, a Cys-His-Glu/Asp catalytic triad (EV and CV 3Cpro, and NV 3CLpro) or Cys-His dyad (SARS-CoV 3CLpro),20 an extended binding site, and a preference for cleaving at Gln-Gly (P1–P1′) junctions in protein and synthetic peptidyl substrates (vide infra). The confluence of structural similarities in the active sites, mechanism of action, and substrate specificity preferences of EV and CV 3Cpro,12,13 SARS-CoV 3CLpro,20,21 and NV 3CLpro11,17,22 (Table 1) suggests that a drug-like entity can be fashioned that displays inhibitory activity against all three

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proteases, making them appealing targets for the discovery of broad spectrum antiviral agents.\textsuperscript{16,23}

Picornavirus 3C\textsubscript{pro},\textsuperscript{A} SARS-CoV 3CL\textsubscript{pro}\textsuperscript{23} and NV 3CL\textsubscript{pro}\textsuperscript{24} have been the subject of intense investigations. We report herein the design, synthesis, and in vitro evaluation of a representative member of a new class of macrocyclic transition state inhibitors (I) (Fig. 1) that is effective against all three proteases. To our knowledge, this is the first report describing the inhibition of 3C\textsubscript{pro} and 3CL\textsubscript{pro} of pathogens belonging to the picornavirus-like protease supercluster, by a macrocyclic inhibitor.

The design of macrocyclic inhibitor (I) rested on the following considerations: (a) proteases are known to recognize their ligands in the β-strand conformation;\textsuperscript{25} (b) macrocyclization is an effective way of pre-organizing a peptidyl transition state mimic in a β-strand conformation suitable for binding to the active site of a protease;\textsuperscript{26–28} (c) in general, macrocyclization increases affinity by reducing the loss of entropy upon inhibitor binding, as well as cellular permeability, and proteolytic stability;\textsuperscript{29} (d) macrocyclization improves drug-like characteristics;\textsuperscript{30,31} (e) the plasticity of the S\textsubscript{1} subsite in the 3C and 3CL proteases was exploited in the design of macrocyclic inhibitor (I) by tethering the P\textsubscript{1} Gln side chain to the P\textsubscript{3} residue side chain; and, (e) computational and modeling studies suggested that a ring size corresponding to n = 3 would produce good receptor binding and minimal intra-ligand strain.

Based on the aforementioned considerations, inhibitor (I) was assembled in a convergent fashion by first constructing fragments 2 and 4, followed by subsequent coupling of the two fragments to generate acyclic precursor 5 (Scheme 1). Cyclization was subsequently accomplished using click chemistry.\textsuperscript{32–35} Thus, fragment 2 was synthesized by coupling (L) Boc-protected propargyl glycine with (L) leucine methyl ester using EDCI/HOBt/DIEA/DMF to yield the dipeptidyl ester which was subsequently treated with dry HCl in dioxane to remove the N-terminal Boc protecting group. Reaction with benzylchloroformate yielded the Cbz-protected ester which was hydrolyzed with LiOH in aqueous THF to yield the corresponding acid 6. EDCI-mediated coupling of commercially available (L) Boc-Glu-OCH\textsubscript{3} with NH\textsubscript{2}(CH\textsubscript{2})\textsubscript{n}N\textsubscript{3} (n = 3), followed by removal of the Boc group, yielded fragment 4.\textsuperscript{4} The amino alkyl azide was conveniently synthesized by converting BocNH(CH\textsubscript{2})\textsubscript{n}OH to the mesylate via treatment with methanesulfonyl chloride in the presence of triethylamine, followed by reaction with sodium azide in DMF and removal of the protective group. Coupling of fragment 2 with 4 using standard coupling conditions yielded acyclic precursor 5 which was treated with Cu(I)Br/DBU in dichloromethane to furnish compound 6 in 45% yield. Compound 6 was treated with lithium borohydride to yield alcohol 7 (84% yield) which, upon Dess–Martin periodinane oxidation,\textsuperscript{37} and subsequent purification gave macrocyclic aldehyde 8 (Scheme 1, structure (I), n = 3, R = isobutyl, X = CHO).

### Table 1

| Viral 3C\textsubscript{pro} or 3CL\textsubscript{pro} | P\textsubscript{1} | P\textsubscript{2} | P\textsubscript{3} | P\textsubscript{4} | P\textsubscript{5} | P\textsubscript{6} |
|------------------------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| EV71                                          | E                | A                | V/I/T            | L/F              | Q                | G                |
| CVA16                                         | E                | A                | L                | F                | Q                | G                |
| SARS-CoV                                      | S                | A                | V/I/K            | L                | Q                | A/S              |
| NV                                            | D/E              | F/Y              | H/Q/E            | L                | Q                | G                |

### Scheme 1

Reagents and conditions: (a) EDCI/HOBt/DIEA/DMF then (L) NH\textsubscript{2}CHRCOOCH\textsubscript{3}; (b) HCl/dioxane; (c) benzylchloroformate/TEA/DCM; (d) LiOH/aq THF; (e) EDCI/HOBt/DIEA/DMF then NH\textsubscript{2}(CH\textsubscript{2})\textsubscript{n}N\textsubscript{3}; (f) EDCI/HOBt/DIEA/DMF; (g) Cu(I)Br/DBU/DCM; (h) LiBH\textsubscript{4}/THF; (i) Dess–Martin periodinane.

![Figure 1. General structure of macrocyclic inhibitor (I).](image)

![Scheme 1.](image)
prepositioning of inhibitor 8 into each of the three protease receptors, which was accomplished in Pymol via manual docking. Pymol was then used to produce a computational framework for refining the docked conformation as follows: a ligand–receptor complex was generated by protonating the preliminary receptor–ligand complex (according to physiological pH with anionic aspartate and glutamate residues, and cationic lysine and arginine residues), then retaining only the ligand plus all complete residues with at least one atom located within no more than 6.0 Å from any ligand atom. The resulting complex models were then permitted to undergo 1000 molecular mechanics optimization steps in Avogadro66 using the MMFF94 force field and electrostatic charge model. The resulting complexes were then rendered in Pymol. The computational studies indicate that inhibitor 8 is capable of nestling snugly in the active site of the 3C and 3CL proteases.

In summary, we report herein for the first time the inhibition of the 3Cpro and 3CLpro of viral pathogens belonging to the picornavirus–like protease supercluster by a macrocylic inhibitor. A full account describing the exploration of R, linker, n (ring size), and the nature of warhead X, will be reported in due course.

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