Published in final edited form as:

Expert Opin Ther Pat. 2016 ; 26(3): 297–308. doi:10.1517/13543776.2016.1153065.

Anti-Norovirus Therapeutics: A Patent Review (2010–2015)

Anushka C. Galasiti Kankanamalage¹, Pathum M. Weerawarna¹, Yunjeong Kim², Kyeong-Ok Chang^{2,*}, and William C. Groutas^{1,*}

¹Department of Chemistry, Wichita State University, Wichita, Kansas 67260, USA

²Department of Diagnostic Medicine & Pathobiology, Manhattan, Kansas 66506, USA

Abstract

Introduction—Human noroviruses are the primary causative agents of acute gastroenteritis and are a pressing public health burden worldwide. There are currently no vaccines or small molecule therapeutics available for the treatment or prophylaxis of norovirus infections. An improved understanding of norovirus biology, as well as the pathogenic mechanisms underlying the disease, has provided the impetus for a range of intense exploratory drug discovery efforts targeting viral and host factors.

Areas covered—An overview of norovirus inhibitors disclosed in the patent literature (2010–present) and Clinicaltrials.gov is presented. The review is further enriched and supplemented by recent literature reports.

Expert opinion—Seminal discoveries made in recent years, including a better understanding of the pathobiology and life cycle of norovirus, the identification and targeting of multiple viral and host factors, the advent of a replicon system and a small animal model for the preclinical evaluation of lead compounds, and the availability of high resolution X-ray crystal structures that can be utilized in structure-based drug design and lead optimization campaigns, collectively suggest that a small molecule therapeutic and prophylactic for norovirus infection is likely to emerge in the not too distant future.

Keywords

norovirus infection; 3CL protease inhibitors; RdRp inhibitors; attachment and entry inhibitors; host factors

1. Introduction

Human noroviruses are the principal cause of non-bacterial acute gastroenteritis worldwide [1–4]. It is estimated that noroviruses are responsible for 19–21 million infections, 56000–

*Authors for Correspondence Kyeong-Ok Chang, Department of Diagnostic Medicine & Pathobiology, Kansas State University, Manhattan, Kansas, 66506, USA, Tel. (785) 532 3849, Fax (785) 532 4039, kchang@vet.ksu.edu., William C. Groutas, Department of Chemistry, Wichita State University, Wichita, KS 67260., Tel. (316) 978 7374, Fax: (316) 978 3431, bill.groutas@wichita.edu.

Financial and competing interests disclosure

The authors were supported by the National Institutes of Health under grant number R01AI109039. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

71000 hospitalizations, and 700–800 deaths annually in the US [5]. Although norovirus infection is self-limiting with gastroenteritis lasting 2–3 days in healthy adults, it can be serious among the young and elderly, as well as immunocompromised individuals [6–7]. The problem is more acute in developing countries where diarrhea caused by noroviruses is estimated to result in >200000 deaths among children <5 years old [8–9]. The problem is compounded by the facile spread of norovirus infection, primarily via person to person via aerosolized particles and foodborne and waterborne routes, arising from the high infectivity, genetic diversity, prolonged shedding of virus in the stool, and environmental stability of noroviruses [9,10–11]. Clearly, noroviruses have a major impact on public health worldwide, however, combating norovirus infection presents a challenge because no effective vaccines or norovirus-specific therapeutics or prophylactics are currently available [12–13]. Despite accelerated progress in norovirus research, many aspects of norovirus biology and pathogenesis are poorly understood [14–15]. This is primarily due to the fact that human noroviruses are non-cultivable and an animal model that recapitulates all aspects of the disease is not available [16]. Consequently, progress toward the discovery of vaccines and therapeutics has been gravely hampered. Nevertheless, the discovery and establishment of cell culture and animal model of murine norovirus (MNV) [16–17], and the development of norovirus replicon harboring cells [18] and animal models with human norovirus infections using gnotobiotic pigs, calves, and chimpanzees [19–22], have provided effective tools for norovirus research.

Noroviruses are icosahedral RNA viruses with a positive sense genome that belong to the *Caliciviridae* family which is comprised of the genera *Vesivirus*, *Lagovirus*, *Nebovirus*, *Sapovirus* and *Norovirus* [4]. Of the six genogroups (GI–VI) belonging to the genus *Norovirus*, genogroups GI, GII and GIV cause human acute gastroenteritis, with GII.4 variants being more prevalent and the cause of most norovirus outbreaks [23–24].

2. Noroviruses

2.1 Genomic organization

The norovirus genome is a single-stranded positive sense RNA that is ~7.5 Kb long and is comprised of three open reading frames (ORF1–3) [4,25–26]. The genome is polyadenylated at the 3' end and is covalently linked to the nonstructural protein VPg at the 5' end. ORF1, ORF2, and ORF3 encode a nonstructural protein (~200 kDa amino acid residue long), the major capsid protein (VP1), and the minor capsid protein (VP2), respectively [27]. Cleavage of the polyprotein by the viral-encoded 3C-like protease (3CL^{pro}) generates six proteins designated p48 (N-terminal protein), p41 (NTPase), p22, VPg (viral protein genome-linked), Pro (3CL^{pro}), and Pol (RNA dependent RNA polymerase, RdRp) [28]. The generated proteins display an array of functions, including facilitating the anchoring and assembly of the membrane-associated replication complex (p48, p41, p22), infectivity and viral genome translation initiation (VPg), polyprotein cleavage (3CL^{pro}), and genome replication (RdRp) [27]. 3CL^{pro} and RdRp play essential roles in viral replication, consequently, they are particularly well-suited as targets for antiviral drug development.

2.2 Life cycle

Because human noroviruses do not grow in cell culture, precise understanding of the life cycle of human norovirus has remained elusive. Nevertheless, a comprehensive review of current understanding of the norovirus life cycle has been published [27], and although many aspects of norovirus replication await sharper definition, the basic steps comprising the norovirus infection include the initial utilization of carbohydrate factors to attach to the surface of intestinal epithelial cells, followed by receptor binding and entry [29–31]. Histo-blood group antigens are oligosaccharides linked to membrane bound proteins or lipids that are present on the surface of red blood cells and mucosal epithelia. They serve as attachment factors or receptors for norovirus attachment and entry. Subsequent uncoating releases the viral genome which undergoes translation via the mediation of VPg and the cellular translation machinery. The polyprotein is co- and post-translationally cleaved by norovirus 3CL^{pro}. Formation of the replication complex is followed by genome replication. RdRp generates genomic and subgenomic RNA using both de novo and VPg-dependent initiation mechanisms [27]. The replicated genomes are then packaged into the capsid (VP1) for virion assembly and exit.

2.3 Norovirus infection and pathogenesis

Epidemic gastroenteritis mostly occurs in hospitals, nursing homes, schools, military barracks, and restaurants. The infection presents itself in the form of multiple symptoms, including diarrhea, vomiting, nausea, and abdominal pain [9]. The infection is incapacitating but self-limiting for healthy adults, lasting 2–3 days. However, viral shedding continues much longer even after the disappearance of symptoms and can be a source of further contamination and infection. As stated earlier, the disease can be life-threatening in children, the elderly, and immunocompromised patients [7].

There is a need for a better understanding of the mechanisms by which noroviruses infect the gastrointestinal tract. Previous biopsy results, as well as studies with gnotobiotic pigs and calves infected with human norovirus, suggest that human norovirus targets intestinal epithelial cells [4,19–22]. Recent studies suggest noroviruses can infect macrophages, dendritic cells and B cells [17, 32–34], however, it is not clear if human norovirus can target those cells in the natural host.

3 Drugs against known targets

3.1 Attachment, receptor binding, entry, and uncoating blockers

Structural studies have shown that the norovirus capsid has a T=3 icosahedral geometry and is composed of 180 VP1s that organize into 90 dimers [35]. Each major capsid protein (VP1) is comprised of two major domains, the interior-forming shell (S) domain and the exterior protruding (P) domain, that are tethered by a flexible hinge. The P domain is further divided into two subdomains, P1 and P2, with the latter located at the outermost surface of the capsid. P2 is the least conserved region of VP1 among norovirus strains and serves as the primary site of interaction with individual oligosaccharide residues of the HBGA receptors [36–38]. As mentioned earlier, human noroviruses recognize histo-blood group antigens (HBGAs) as binding ligands [31]. Recognition of histo-blood group antigens by noroviruses

is known to play a pivotal role in viral infection and tropism. Structural and electrospray ionization mass spectrometry (ESI-MS) studies have illuminated further the interaction of HBGAs with noroviruses [39–43]. Importantly, it has been shown recently using a catch-and-release electron spray ionization mass spectrometry (CaR-ESI-MS) assay [44] that human noroviruses recognize and bind to sialic acid-containing glycosphingolipids with comparable affinities to those of HBGA oligosaccharide receptors [45].

The HBGA-binding interfaces are conserved in human noroviruses, consequently, agents that block the HBGA binding site may abrogate viral infection in all norovirus strains. Toward that end, a CaR-ESI-MS assay has been used to screen carbohydrate libraries to identify norovirus ligands and potential inhibitors [46]. Related efforts involving the screening of small-molecule libraries using virus like particles (VLPs) resulted in the identification of several binding blockers [47–49]. Advances in this area are beginning to lay a solid foundation for the eventual development of virus entry inhibitors.

A computer-aided drug design approach involving a) the construction and validation of computational models of the target protein based on its crystal structures with known functional HBGA binding sites, b) virtual high throughput screening of compound libraries to identify hits and, c) subsequent biochemical validation of the identified inhibitors, resulted in the identification of several compounds that blocked the binding of norovirus to HBGAs with a high affinity and displayed low cytotoxicity (Figure 1) [49]. The structures of the identified compounds share in common a cyclopenta [a] phenanthrene scaffold which can be used as a launching pad for conducting structure-activity relationship studies.

3.2 Protease inhibitors

Following translation of the viral genome, the viral polyprotein is cleaved by a cysteine protease (3CL^{pro}) with a prototypical catalytic triad (Cys139, His30, Glu54) and a strong preference for a P1 Gln residue, to generate structural and nonstructural proteins [50–52]. Because of the central role 3CL^{pro} plays in virus replication, this enzyme is an appealing target for the design of norovirus-specific antiviral drugs; consequently, 3CL^{pro} has been the focus of intense studies. Using a robust FRET-based assay [53] and a cell-based replicon system [18], an array of inhibitors of the protease displaying anti-norovirus activity have been reported, including peptidyl transition (TS) inhibitors (aldehydes, α -ketoamides, α -ketoheterocycles) [54–58] or latent TS inhibitors [59], and TS mimics (α -hydroxyphosphonates) (*vide infra*) [60]. The availability of several high resolution X-ray crystal structures with bound ligands has been invaluable in using structure-guided approaches in the design of inhibitors of the enzyme [55,57,61–65].

3.2.1 Peptidyl transition state (TS) inhibitors and TS mimics—Peptidyl aldehydes and α -ketoamides have been shown to be highly effective inhibitors of norovirus 3CL^{pro} and norovirus in cell-based replicon cells. Peptidyl inhibitors of 3CL^{pro} have demonstrated efficacy in the murine model of norovirus infection and are currently in preclinical development [55]. The synthesis of peptidyl derivatives of general structure (I) (Figure 2A) can be readily accomplished as illustrated in Figure 2B and representative results are summarized in Tables 1–2. The corresponding bisulfite aldehyde adducts were

also found to be an effective latent form of the aldehyde functionality which, under the conditions used, revert to the aldehyde, the actual inhibitory species, as demonstrated by X-ray crystallography [57]. Peptidyl transition state mimics, such as α -hydroxyphosphonates are also effective inhibitors of norovirus 3CL^{Pro} and norovirus in cell-based replicon cells (Table 3) [60]. Finally, it should be noted that the substrate specificity of 3C and 3CL proteases of viruses in the picornavirus-like supercluster are very similar (Table 4), consequently, it should in principle be possible to design broad spectrum inhibitors [57]. In addition to TS inhibitors, rupintrivir, a peptidyl inhibitor with an α , β -unsaturated ester Michael acceptor originally developed for enterovirus 3C, has been shown to be effective against noroviruses [66]. It efficiently clears human cells from their Norwalk replicon and its anti-norovirus activity is extended to murine norovirus. An additive antiviral effect is observed when rupintrivir is used in combination with polymerase inhibitors. Importantly, the cross-genotypic anti-norovirus activity displayed by rupintrivir suggests that it will most likely be effective against clinically relevant GI and GII noroviruses.

3.2.2 Macrocyclic inhibitors—A series of novel macrocyclic transition state inhibitors (II) (Figure 3A) have been synthesized and shown to display broad-spectrum activity against viruses that belong to the picornavirus-like supercluster, which includes important human and animal pathogens such as noroviruses, enteroviruses, coronaviruses, rhinoviruses and others [67]. A representative synthesis of triazole-based macrocyclic aldehydes is shown in Figure 3B. The conformationally constrained macrocycles were found to display cellular permeability and are anticipated to possess improved stability and oral bioavailability.

3.3 Polymerase inhibitors

Numerous polymerase inhibitors including nucleoside analogs and prodrug variants have been approved as antiviral agents against an array of viruses, including hepatitis C virus (HCV) [68–70] and HIV and HBV viruses [71]. The mechanism of action of nucleoside analogs involves conversion of the inactive nucleosides into their phosphate or diphosphate forms, which are the actual inhibitory species. Consequently, the norovirus RdRp is an attractive target that is well-suited to the development of norovirus-specific therapeutics due to (a) the vital role RdRp plays in the norovirus replication cycle, (b) the availability of high resolution X-ray crystal structures of free and ligand-bound enzyme complexes [72–75] and (c) the absence of a human homolog which diminishes the likelihood of off-target effects. Several novel nucleoside inhibitors have been shown to inhibit norovirus via the inhibition of RdRp (Figure 4) [76–77]. Representative syntheses of these inhibitors and variants are illustrated in Figures 5–7.

Drug repurposing or repositioning, namely, the strategy of applying established drugs to new indications [78], has resulted in the identification of nucleosides with significant anti-norovirus activity [79]. For instance, the nucleoside analog 2'-C-methylcytidine has been found to completely block the transmission of norovirus, affords protection against norovirus-induced diarrhea and mortality in a mouse model, and may be suitable for use as a prophylactic (Figure 8) [80–81]. Moreover, the pyrazine carboxamide antiviral agent favipiravir (T-705) has been shown to inhibit norovirus replication but the mode of action is not well understood [82–83]. Figure 8 also shows the structure of the first nonnucleoside

inhibitor of norovirus RdRp [84]. Collectively, the aforementioned studies strongly suggest that the future armamentarium of anti-norovirus therapeutics is likely to include one or more nucleoside analogs that can be used individually or in combination with protease inhibitors.

3.4 Host factors inhibitors

The lower potential for the emergence of resistance associated with the targeting of host factors has provided the impetus for the exploration of host factors as potential therapeutic targets. These include interferons (IFNs) [18, 85], Hsp90 [86], deubiquitinase [87], eIF4F [88], and cholesterol pathway [89] inhibitors. While most of these studies are at an early stage, rapid advances in norovirus pathobiology are likely to identify additional host factors worthy of further investigation.

3.5 Anti-norovirus drugs against unknown targets

Several studies describing the inhibition of noroviruses by low molecular weight compounds via unknown mechanisms have been reported. These include cyclic and acyclic sulfamides [90–91] and structural variants [92–94], pyranobenzopyrones [95], and chromones [96] (Figure 9).

3.6 Norovirus drugs in clinical development

Currently the only compound that has been evaluated in clinical trials as an anti-norovirus therapeutic is nitazoxanide [97–98] (Figure 9), a broad spectrum antiviral agent for multiple viruses including HCV and influenza virus, which has shown efficacy against norovirus infection but the mechanism is not well understood [99].

4 Conclusion

Only a limited number of compounds capable of inhibiting norovirus have been disclosed thus far in the patent literature. These include peptidyl and macrocyclic transition state inhibitors, as well as transition state mimics, of norovirus 3CL protease; nucleoside or nonnucleoside inhibitors of RNA dependent RNA polymerase, and steroidal derivatives capable of blocking the binding of noroviruses to HBGA receptors. These studies are at a very early stage of development and further preclinical studies need to be carried out before a rigorous assessment of these classes of compounds can be made.

5 Expert Opinion

Despite the fact that norovirus infections exact a heavy toll in terms of morbidity and economic burden worldwide, an effective small molecule therapeutic or prophylactic for the control of the disease is not currently available. However, the increasing awareness of noroviruses on public health is likely to accelerate drug discovery efforts in this area. The adverse impact of the lack of a cell culture model for complete human norovirus replication is mitigated by the availability of virus-like particles (with binding assays), norovirus replicon harboring cells, and the MNV in vitro and in vivo model, which have provided excellent tools for drug discovery efforts.

Significant advances made in the field in recent years have greatly illuminated our understanding of norovirus biology and pathogenesis, and while many facets of the disease await a better understanding, viral and host factors identified thus far could serve as potential druggable targets [100]. These include viral targets, such as entry blockers, and 3CL^{pro} and RdRp inhibitors. There has been a keen interest in the use of new nucleoside or repurposed nucleoside inhibitors of RdRp inhibitors, as well as 3CL^{pro} inhibitors, with most studies being at the early preclinical stage. High throughput screening of libraries using enzyme assays and the norovirus replicon harboring cell system will likely result in the identification of drug-like hits that are amenable to optimization and further advancement along the development pipeline.

Obstacles that impede progress in the field in general, as well as norovirus outbreaks and control in particular, include the lack of a) highly sensitive, specific, and low cost diagnostic tests that can be optimally used for the early detection of norovirus infection [2], b) effective and safe decontaminating agents; c) an effective vaccine; d) a cell culture model, e) an animal model that recapitulates all (or most) aspects of human norovirus infection and, f) a better understanding of norovirus infection. However, despite these obstacles, great strides have been made in our understanding of norovirus biology and pathobiology which should buttress ongoing research endeavors, ultimately leading to the introduction of a norovirus-specific therapeutic in the clinic. Clearly, the prevalence and impact of norovirus infections warrants development of norovirus-specific drugs.

With respect to diagnosis, surveillance, and control of norovirus outbreaks, various antiviral compositions have been reported in the patent literature. Linear or branched alkyl 2-hydroxycarboxylic acid derivatives and sulfonated surfactants in ethanol or isopropanol which have been shown to inactivate non-enveloped viruses, such as noroviruses [101]. These compositions are suitable for providing rapid antimicrobial activity against noroviruses when used as topical skin applications, in hand sanitizers, and in eradicating norovirus contamination of hard surfaces. Currently used agents such as chlorine bleach, iodine preparations and others provide partial protection against norovirus infection. An anti-norovirus disinfectant composition comprised of a persimmon extract and various additives (an alcohol, vitamin C, and a surfactant) has been disclosed recently [102–103]. Interestingly, the presence of citric acid results in a synergistic anti-norovirus effect. The medicinal use of the persimmon juice has been known in Chinese and Japanese folk medicine for a long time and its effects may arise from the presence of tannins.

Finally, the level of resources devoted to research aimed at combating noroviruses does not currently reflect the severity and impact of norovirus infections worldwide. This is clearly evident by the near-total absence of any potential drug candidates being currently evaluated in clinical trials or undergoing advanced preclinical evaluation. The unmet need for effective treatments, coupled with continued studies describing the burden of disease in different target populations and highlighting its impact on the public health system will no doubt greatly accelerate research efforts in the area.

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Box 1**Attributes of an ideal norovirus drug**

- Specific treatment and/or prevention of norovirus infection
- Rapid onset of action
- Effective when administered within 12–24 h of onset of symptoms
- Oral, topical, or parenteral route of administration
- One week or less duration of treatment
- Virus-specific target-based mechanism of action
- Nontoxic
- Therapeutic safety window >10-fold
- Stability at room temperature for 3 years or more
- High barrier to emergence of resistance
- Low cost

Box 2**Article highlights**

- Acute non-bacterial gastroenteritis caused by noroviruses has a major impact on public health worldwide
- No norovirus-specific therapeutic or vaccine is currently available
- Drug discovery efforts in the norovirus field are handicapped by the lack of an ideal animal model and a human norovirus cell culture system
- Most drug discovery efforts involve early preclinical studies and are focused on viral (entry blockers, 3CL protease and RdRp inhibitors) and host factors (Hsp90 and deubiquitinase inhibitors)
- HTS using a cell-based replicon system may result in the identification of novel drug-like chemotypes
- Evaluation of repurposed drugs may be a fruitful avenue of investigation
- The availability of structural information and an increased understanding of the etiology of acute gastroenteritis are likely to accelerate drug discovery efforts

Figure 1.
Examples of norovirus entry inhibitors identified through binding of capsid P protein with HBGAs.

Figure 2.

A. General structure of dipeptidyl inhibitors (I) of norovirus 3CL protease. **B.** General scheme employed in the synthesis of peptidyl inhibitors represented by general structure (I).

Figure 3.**A.** General structure of macrocyclic transition state inhibitors (II) of norovirus 3CL protease.**B.** Representative synthesis of triazole-based macrocyclic aldehyde inhibitors.

Figure 4.

Representative examples of RdRp inhibitors against norovirus. EC_{50} values were determined by a reporter assay (5BR assay) with co-transfecting plasmids expressing RdRp, VPg and RIG-I (or MDA5) as well as the firefly and Renilla luciferase reporters in Huh-7 cells [75]

Figure 5.
Representative synthesis of an RdRp inhibitor.

Figure 6.
Representative synthesis of an RdRp inhibitor.

Figure 7.
Representative synthesis of an RdRp inhibitor.

Figure 8. Examples of RdRp inhibitors effective against noroviruses. The EC_{50} values were determined using NV replicon harboring cells. [81–82, 104].

Figure 9. Anti-norovirus compounds against unknown targets. The EC_{50} values were determined NV replicon harboring cells [89–93, 95–96].

Table 1

Activity of representative dipeptidyl inhibitors (I): Aldehydes and bisulfite adducts.

	R	R1	Z	IC₅₀ (μM)	EC₅₀ (μM)
1	H	Isobutyl	CHO	0.6	0.2
2			CH(OH)SO ₃ ⁻ Na ⁺	0.8	0.3
3		Cyclohexylmethyl	CHO	0.3	0.06
4			CH(OH)SO ₃ ⁻ Na ⁺	0.4	0.06
5		Butyl	CHO	0.87	ND
6	<i>m</i> -Cl	Isobutyl	CHO	0.9	0.1
7			CH(OH)SO ₃ ⁻ Na ⁺	4.3	0.1
8		Cyclohexylmethyl	CHO	0.1	0.02
9			CH(OH)SO ₃ ⁻ Na ⁺	0.1	0.02

Table 2Activity of representative dipeptidyl inhibitors (I): α -ketoamides

	R	R1	Z	IC₅₀ (μM)	EC₅₀ (μM)
10	H	Isobutyl	C(O)C(O)NHC ₃ H ₇	5.3	2.8
11			C(O)C(O)NHC ₃ H ₅	3.4	1.1
12			C(O)C(O)NHC ₄ H ₉ / (butyl)	4.1	1.3
13			C(O)C(O)NHCH ₂ C(O)OC ₂ H ₅	2.1	0.8
14			C(O)C(O)NHC ₆ H ₁₁	3.5	1.2
15			C(O)C(O)NHCH ₂ (C ₆ H ₅)	7.1	1.8
16			C(O)C(O)NHC ₄ H ₉ / (t-butyl)	2.8	1.1
17		Phenylmethyl	C(O)C(O)NHC ₃ H ₇	21.5	4.2

Table 3Activity of representative dipeptidyl inhibitors (I): α -hydroxyphosphonates

	R	R1	Z	IC ₅₀ (μ M)	EC ₅₀ (μ M)
18	H	Isobutyl	CH(OH)P(O)(OC ₂ H ₅) ₂	11.5	0.8
19			CH(OH)P(O)(O)(OCH ₃) ₂	20.2	3.5
20			CH(OH)P(O)(OH) ₂	ND	7.5
21		Cyclohexylmethyl	CH(OH)P(O)(OC ₂ H ₅) ₂	3.5	0.25
22			CH(OH)P(O)(O)(OCH ₃) ₂	8.3	2.8
23			CH(OH)P(O)(OC ₄ H ₉) ₂	ND	0.5
24			CH(OH)P(O)(O)(OCH ₂ CF ₃) ₂	6.5	0.35
25			CH(OH)P(O)(O)(OCH ₂ C ₆ H ₅) ₂	ND	0.6
26			CH(OH)P(O)(OC ₂ H ₅) ₂	>10	0.06
27	m-Cl			>10	0.2
28	o-F			>10	>2
29	m-Br			6.5	0.15

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Table 4
Substrate specificity of 3C and 3CL proteases of viruses in the picornavirus-like supercluster

Viral 3C or 3CL protease	P ₅	P ₄	P ₃	P ₂	P ₁	P ₁ '	P ₂ '
EV71	E	A	V/L/T	L/F	Q	G	P
CVA16	E	A	L	F	Q	G	P
SARS-CoV	S	A	V/T/K	L	Q	A/S	G
NV	D/E	F/Y	H/Q/E	L	Q	G	P