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Current Tools for Norovirus Drug Discovery

Sahani Weerasekara[1], **Allan M. Prior**[2], and **Duy H. Hua**[1,*]

[1]Department of Chemistry, 213 CBC Building, Kansas State University, Manhattan, KS 66506 USA

[2]Molecular Sciences Institute, School of Chemistry, University of the Witwatersrand, PO Wits 2050, Johannesburg, South Africa, 2000

Abstract

Introduction—Rapid transmission of norovirus often occurs due to its low infectious dosage, high genetic diversity and its short incubation time. The viruses cause acute gastroenteritis and may lead to death. Presently, no effective vaccine or selective drugs accepted by the United States Food and Drug Administration (FDA) are available for the treatment of norovirus. Advances in the development of norovirus replicon cell lines, GII.4-Sydney HuNoV strain human B cells, and murine and gnotobiotic pig norovirus models have facilitated the discovery of effective small molecule inhibitors *in vitro* and *in vivo*.

Areas covered—This review gives a brief discussion of the biology and replication of norovirus before highlighting the discovery of anti-norovirus molecules. The article coverage includes: an overview of the current state of norovirus drug discovery, the targeting of the norovirus life cycle, the inhibition of structural and nonstructural proteins of norovirus such as proteases and polymerase, and the blockage of virus entry into host cells. Finally, anti-norovirus drugs in the clinical development stage are described.

Expert opinion—The current approach for the counteraction of norovirus focuses on the inhibition of viral RNA polymerase, norovirus 3C-like protease and the structural proteins VP1 as well as the blockade of norovirus entry. Broad-spectrum anti-norovirus molecules, based on the inhibition of 3C-like protease, have been developed. Other host factors and ways to overcome the development of resistance through mutation are also being examined. A dual approach in targeting viral and host factors may lead to an effective counteraction of norovirus infection. Current successes in developing norovirus replicon harboring cells and norovirus infected human cells, as well as murine norovirus models and other animal models such as piglets have facilitated the discovery of effective drugs and helped our understanding of its mechanism of action.

[*]University Distinguished Professor, Kansas State University – Chemistry, 213 CBC Building Manhattan Kansas 66506-0100, United States, T: 785-532-6699 F: 785-532-6666, Contact: duy@ksu.edu.

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Keywords

Gastroenteritis; norovirus; proteases; protease inhibitors; 3CLpro; polymerase inhibitors; virus entry

1. Introduction

Noroviruses belong to the family of *Caliciviridae* and are currently recognized as the leading pathogens causing acute gastroenteritis among all ages worldwide [1,2]. In the United States, norovirus associated gastroenteritis accounts for about 20 million cases with more than 70,000 hospitalizations and about 800 deaths every year [3,4]. Moreover, noroviruses are considered as the major pathogen responsible for severe childhood diarrhea after rotavirus, and still being one of the top causes of death in children [1,5,6]. In developing countries, about 200,000 deaths have been estimated for children aged less than five years as a result of the diarrhea associated with norovirus infection every year [7]. When considering its overall impact in terms of fatality, suffering, economic losses, and disruption to services, norovirus infections are estimated to result in a loss of $2 billion per year [2,8]. Generally, gastroenteritis caused by norovirus is acute and self-limited among healthy adults, but in infants, elderly and immunocompromised patients it may be severe and prolonged resulting a significant morbidity and mortality [9]. The efficacy of antiviral drugs usually takes about 24 hours, and since the duration of norovirus illness can last around four to five days, effective drug treatment would be beneficial. Norovirus outbreaks usually occur in semi-closed environments such as cruise ships, nursing homes, schools and hospitals, where person to person transmission is more favorable and often difficult to control due to the factors such as high genetic diversity of noroviruses, their short incubation time and long persistence in the environment, as well as their very low infectious dose (17 virions are sufficient for infection) [10–13]. Despite the significant economic and health impacts of noroviruses, there's no effective vaccine or anti-norovirus drug approved by FDA for treatment or for prophylactic purposes. Studies have been carried out to identify potential vaccine candidates against noroviruses with the use of HuNoV virus-like particles (VLPs). One such double blind study performed with 18 – to 50-year-olds against norovirus GII.4 strain has found that vaccination resulted less frequent vomiting and diarrhea associated with the infected group compared to control group [14]. Recent advances have been achieved in this field such as the development of norovirus replicon cell lines and murine norovirus (MNV) have enabled the search of effective small molecule inhibitors *in vitro* and *in vivo* against noroviruses [2,15,16]. Previously, it was difficult to cultivate human noroviruses (HNV) in cell system, however, replicon cell systems and MNV that shares many of the biological properties of HNV can be replicated in cell culture providing a feasible small animal model of HNV infection [16]. Recently, a protocol for culturing the GII.4-Sydney HuNoV strain directly in human B cells and information on variables that contribute to the efficiency of viral replication have been reported [17]. Though it achieves a modest level of viral replication in its current state, the success demonstrates the first cultivation system for norovirus and may provide insight into identification of both host and viral factors contributing to norovirus replication and thereby facilitating the development of effective anti-norovirus therapeutics [17]. Herein, this review highlights recent efforts in the discovery

of active molecules against norovirus infections as well as the current tools that can be utilized for future directions in this area.

2. Noroviruses and Strategies for Counteraction

2.1. Biology of Noroviruses

Noroviruses are small, non-enveloped, single stranded, positive sense RNA viruses of 27 – 35 nm in diameter and belong to the genus *Norovirus* of the family *Caliciviridae* [9,10,18–21]. The family *Caliciviridae* comprises of four other genera called *Sapovirus*, *Lagovirus*, *Vesivirus,* and *Nebovirus,* and only the viruses in the *Norovirus* and *Sapovirus* genera cause acute gastroenteritis in humans and animals [9,10,19]. Based on molecular characterization of the major viral capsid VP1 gene, noroviruses are divided into five geno-groups, GI – GV, that share greater than 60% amino acid identity in the VP1 protein [19,22]. Noroviruses that cause gastroenteritis in humans belong to the geno-groups GI, II and IV, which are further subdivided into 26 or more genotypes that share greater than 80% identity in amino acid sequence of VP1 [20]. Viruses in the GII group are more common and GII.4 sub variants are associated with most of the recent major norovirus outbreaks [2,23]. The single-stranded RNA genome (7.4 – 7.7 kb) of noroviruses is covalently linked to a viral protein called VPg at the 5′ end and polyadenylated at the 3′ end [18,24]. The norovirus genome is organized into three open reading frames, ORF1 - ORF3 [18,19] (Figure 1). ORF1 encodes a polyprotein, which is cleaved by virus-encoded 3C-like cysteine protease (3CLpro) to generate six non-structural proteins: p48/N-terminal protein (NS1-2), NTPase/RNA helicase (NS3), p22 (NS4), VPg (NS5), viral protease (NS6), and viral RNA dependent RNA polymerase (RdRp or NS7) [18,24]. ORF2 encodes the major structural protein of norovirus, a capsid protein VP1. ORF3 encodes a small basic protein VP2, which presents in one or two copies per virion [18,19]. Factors such as rapid evolution and the generation of new antigenic variants of norovirus have led to the increased genetic diversity of noroviruses resulting in a rapid emergence of new strains capable of evading antiviral drugs [2,25].

2.2. Norovirus genome translation and replication

Understanding the mechanism of norovirus life cycle and viral replication processes assists in the design and optimization of selective anti-norovirus therapeutics. The first step in norovirus life cycle involves the attachment of virion to the host cell surface receptors such as individual oligosaccharide residues of human histo-blood group antigen (HBGA), sialic acid moieties, glycolipids, and heparan sulfate through the interaction of P2 subdomain of VP1 protein of the virus [18]. A detailed description of the norovirus entry and interaction of human norovirus with cell surface receptors has been described [26]. The studies performed with MNV have shown that norovirus enters the cell through a pH independent non-clathrin and non-caveolin mediated endocytic pathway, which is dependent on dynamin-2 and cholesterol [27,28]. Upon entry into the cytoplasm, viral genome is uncoated and it behaves as an mRNA template for the viral RNA translation [18]. Then prepackaged nonstructural protein (VPg) mediates translation of ORF1 of the viral RNA genome into a large polyprotein [18]. The mature polyprotein is then processed by a virus 3CLpro and releases a number of nonstructural proteins such as p48, NTP, p22, VPG, 3C, and RdRp. The replication of norovirus is believed to occur in a replication complex (RC), which is formed

by the recruitment of host membranes (endoplasmic reticulum, golgi, endosomes) and viral nonstructural proteins p48 and p22 [29–31]. RC is a membranous structure that contains the viral nonstructural proteins, viral RNA genome and host cell proteins, which facilitate viral replication. The synthesis of negative sense RNA is initiated from positive sense RNA by the action of RdRp followed by the use of negative sense RNA as a template for the synthesis of new genomic RNA and sub genomic RNA for packaging into new virion [32]. Sub genomic RNAs are translated into structural proteins VP1 and VP2, which assemble to form new virion capsids. After assembly of the structural proteins and packaging of new genomic RNA, the mature virions are released from cells. So far, the mechanisms involved in virus assembly, encapsidation, and release of assembled virion to complete the viral life cycle are poorly understood [18], which lead to the difficulty in growing caliciviruses in general.

3. Discovery of anti-norovirus therapeutics

3.1. Current state of drug discovery

To date, no specific anti-norovirus agent for NV treatment or for prophylactic purposes is available. The lack of a NV specific vaccine is in part due to the lack of a robust NV animal model or cell cultural assay specific for human norovirus [33]. MNV has been used as a model system to study HNV, because MNV is used to infect cells and produce an animal model [34]. Since MNV is closely related to HNV, it is often assumed that active compounds in MNV systems are similarly active against HNVs [35]. Recently however, MNV has been shown be an imperfect model to study HNV due to the differences in the capsids of the viruses. The enteric nature of HNV influences the cultivation of HNV in epithelial cells. The difficulty in producing HNV in cell lines likely due to problems associated with purification of virus particles and the internalization of virus particles into cells. For example, a cell-based FRET sensor to probe the specificities of various norovirus proteases and their activities at cleaving various norovirus cleavage sites were used [36], and showed that there are indeed differences in protease activities and specificities between genogroups I and II (HNV) with genogroup V (MNV). Work into developing robust HNV cell and animal models is thus highly relevant and will mark a breakthrough in the study of HNV life cycle and HNV drug development. In addition, animals such as gnotobiotic (Gn) pigs have been evaluated and used as a model to study the pathogenesis of HNV as well as to test antivirals against HNV since pigs and humans are closely resembled in their gastrointestinal anatomy, physiology and immune responses [37,38]. It has been shown recently however that HNV can replicate to moderate levels in immortalized B cells and in immunocompromised mice [17,39]. This promising research could open the door for development of HNV models, allowing for a more detailed understanding of the HNV lifecycle to be studied. It should also provide a basis for development of HNV cell culture models for screening new HNV inhibitors.

Computational docking experiments to search for anti-norovirus agents have been used with some successes. Computational docking studies have identified a polysulfonated naphthylurea (suramin) that inhibited RdRp of both MNV and HNV with IC_{50} values in the low micromolar range [40]. Due to the overall negative charge on the inhibitor, cell permeability is poor. The problem was addressed by employing a liposomal drug delivery

system. Effective inhibition of norovirus replication with an EC_{50} value of 0.3 μM and therapeutic index of more than 21.4 μM were found [41]. Microsecond molecular dynamics (MD) studies of RdRp from Norwalk virus in complex with natural nucleoside triphosphate substrates, cytidine triphosphate (CTP) and 2-deoxy-CTP (2dCTP) or chemically modified derivatives were reported [42].

The backbone and side-chain resonance assignments of the Norwalk virus, a norovirus strain, have been recently solved using solution NMR spectroscopy methods [43]. These structural assignments should aid in the study of mechanism of interaction of protease substrates and inhibitors with norovirus proteases. Fluorescence resonance energy transfer (FRET) assays, X-ray crystallography, and NMR spectroscopy analysis of three 3CLpro's from Norwalk virus (genogroup I), MD145 (genogroup II), and murine norovirus-1 (MNV-1, genogroup V) were performed in the presence of a hit protease inhibitor [44]. Protein X-ray crystallographic data has provided structural information regarding norovirus 3C protease and its binding interaction with peptidyl substrates [44]. A solution NMR structure of 3CL Norwalk virus protease has been studied and showed it exists predominantly as a monomer in solution [43]. The overall folding of norovirus 3C protease corresponded favorably to the reported protein X-ray crystal structures [44]. A fuller understanding of the structural dynamics of 3CLpro in solution may aid in further drug discovery efforts and may elucidate the mechanisms leading to drug resistance.

3.2. Design of inhibitors

3.2.1. Targeting norovirus life cycle—Anti-norovirus drug development efforts have targeted both host and viral factors including the blockage of virus entry into host cells and inhibition of certain stages of virus life cycle. Noroviruses are thought to gain entry into cells *via* the binding of virus capsid domains to HBGAs of host cells followed by internalization [45]. HBGAs exist as complex carbohydrates that are covalently linked to lipids or proteins on the surface of mucosal epithelial cells as well as red blood cells [46]. In bodily fluids such as blood, saliva, milk and intestinal liquid, they are found as free oligosaccharides [46]. Different types of HBGAs are categorized according to the carbohydrate structure of the non-reducing end of the oligosaccharide. For example, types A, B, H or Lewis antigens are known and each type is further subdivided into 6 subtypes. Since the host HBGAs are the main cellular receptors for viral entry into cells, inhibition of these virus capsid-HBGA binding interactions would result in the blockage of viral entry into the host cells [46]. In fact, B cells that expressed HBGAs in bacteria of the intestine are prone to infect by norovirus [47]. High-resolution protein X-ray crystal structures of the protruding (P) domains of norovirus capsid of GII.4 variant with a range of HBGA types have been reported [47]. A saliva-based enzyme immune assay that was efficiently used to screen a library of molecules for their inhibitory activities at blocking norovirus like particles with HBGA receptors has been reported [48]. A cross-sectional study in children was performed [49] to probe the association of HBGA antigen type with norovirus infection. The study examined 260 children that were hospitalized with diarrhea in northern Vietnam from 2010 – 2012, in which 61% were deemed HBGA secretors, 27% partial-secretors, and 12% non-secretors. The results showed a link between host cellular HBGA expression and norovirus infection [49].

Electrospray ionization mass spectroscopy (ESI-MS) has been used for identification and quantification of carbohydrate-protein interactions in solution. A method known as catch and release (CaR) ESI-MS was demonstrated [46] for identifying high affinity HBGA virus-like particle (VLP) interactions. The method involved incubating norovirus VLP with an oligosaccharide ligand library (a library of many molecules at once) followed by ESI-MS analysis. Ligands that bound with the virus VLP showed a molecular weight corresponding to the protein-ligand complex in the mass spectrum, and mass isomers of ligands could be distinguished from one another by allowing the ligand to dissociate from the complex (released) during a collision-induced dissociation followed by high-resolution mass analysis. In the case of ligands that bind to norovirus VLP with affinities (K_a) greater than 10^4 M^{-1}, more than 200 carbohydrates can be assessed in a single CaR-ESI-MS experiment. A sub-viral P particle of norovirus capsid as a model for studying virus-HBGA interactions was employed in this study [46]. After screening a 50-carbohydrate-molecule library having pre-identified binding affinities (to confirm assay reliability), a library of 146 compounds was screened to discover new carbohydrates that bind to norovirus VLP protein. The study found that a series of oligosaccharides normally found on the cell wall of mycobacteria and human milk bound to norovirus P-particle with similar binding affinities to known HBGA receptors [46]. Moreover, sialic acid-containing gangliosides also serve as ligands for HNV, as determined by CaR-ESI-MS experiments [50].

NMR spectroscopy provides a useful way to study the molecular interactions of HBGAs and viral capsid. To observe the interactions, synthesis of α-L-fucose ligand (Figure 2) covalently linked to a rigid lanthanide binding tag *via* a triazole linker was carried out [51]. The lanthanide tagged fucose allows the assignment of protein backbone, due to the paramagnetic nature of lanthanide ions [41]. The use of saturation transfer difference (STD) and nuclear Overhauser effect (NOE) based NMR experiments was employed to design viral entry inhibitors against whole virus-like particles [52]. Two polymeric inhibitors, polymer 1 and polymer 2 (Figure 2), were synthesized and screened in a competitive surface plasmon resonance experiment. Both polymer 1 and polymer 2 showed promise as entry-inhibitors and were found to have IC_{50} values of 80 μM and 0.61 μM respectively [52]. STD NMR spectroscopy in conjunction with transfer NOESY experiments was used to investigate the binding of VLP of a GII.4 norovirus strain to HBGAs [53]. The experiments involved combining synthetic HBGA saccharides with norovirus VLP and monitoring the overall binding patterns with STD NMR spectroscopy. Binding was confirmed by the presence of STD signals, while absence of STD signals indicated no binding interaction [53]. *L*-Fructose was recognized by a binding pocket within the norovirus VLP and this binding pocket is highly conserved across a broad range of norovirus GII.4 strains. This suggests that the HBGA-fructose binding pocket is a promising target for the design of inhibitors that block viral entry into the host cells for infections with GII.4 [53].

A saliva binding assay was used to access the ability of various small molecules at inhibiting the interaction of HBGAs with norovirus VLP and found that 14 compounds out of their 5,000-compound library inhibited the binding interaction effectively [48]. Three representative active molecules, **5** – **7**, are depicted in Figure 3. Hansman *et al.* demonstrated that citrate can mimic the binding normally observed between the terminal fucose saccharide

of HBGA and GII norovirus capsid [45]. Protein X-ray crystallography showed that citrate bound to norovirus GII capsid domain using binding interactions similar to that observed in fucose-HBGA binding, however, in the case of citrate binding, a water molecule was incorporated into the binding interaction and formed a ring-like structure that mimics the pyranoside ring of fucose. STD NMR experiments showed that citrate binds to norovirus capsid domain with an affinity of 460 μM which is similar to those of fucose binding (460 μM) and H-type 2 trisaccharide binding (390 μM). STD NMR experiments suggest that citrate binds competitively with HBGAs for norovirus capsid binding and citrates and other citrate derivatives could be used as anti-norovirus agents that interfere with HNV-HBGA binding, thus inhibiting viral entry into cells. Computational modelling experiments supported that citrate binds to GII.10 P domain in a high degree of mimicry to L-fucose [45]. An ESI mass spectrometry technique was used to determine the intrinsic binding affinities (per binding site) for various HBGA ligands with HNV VA387 VLPs and sub-viral P particle [54]. Binding affinities in the range of 1,000 – 4,000 M^{-1} were found for the interactions of VLP with A and B-type tri- and tetra-saccharides of HBGA. Binding affinities in the range of 500 – 2,300 M^{-1} were revealed for interactions between the sub-viral P particle and HBGA oligosaccharides of various epitopes such as A, B and H (di- and tetrasaccharides) and with different precursor chain types [54].

A single-crystal X-ray crystallography was achieved to identify two additional fucose binding pockets in the GII.10 HNV capsid protruding (P) dimer [55]. The additional sites are termed fucose-3 and fucose-4 sites and are located in-between the two previously determined L-fucose binding sites. A cooperative binding of fucose to the protruding (P) dimer in a dose dependent manner was observed, with fucose site-1 being occupied first, followed by fucose site-2 and lastly fucose-3/4 sites. The biological relevance of the newly identified fucose-3/4 sites remains unknown [55]. NMR experiments were used to confirm that norovirus protruding (P) domain does indeed bind HBGAs in a cooperative, multi-step process [56]. Native mass spectrometry data showed the presence of four HBGA binding sites per P-dimer. This was also confirmed through protein X-ray crystallographic data of the protruding (P) dimer of a related norovirus GII.10 strain. This data is highly relevant to those research groups studying the mechanism of norovirus entry into host cells. It is speculated that the mechanism of HBGA-capsid binding may be alike for the general *Caliciviridae* viral family.

A fragment-based molecular docking approach was adapted to find high-affinity fragments that bind to HBGA binding pockets [57]. Bound fragments that were close enough to be linked together and which occupied different parts of the binding site were proposed. Subsequently, four molecular structures, **1** – **4**, were proposed as shown in Figure 2. However, synthesis and biological evaluation have not been reported.

A computational docking and virtual screening approach (Autodock software) to discover new inhibitors of HBGA-capsid P protein (GII.4) binding were reported [58]. A library of >2 million molecules, generated from the ZINC molecule database, was screened using a multistage docking protocol. Of the 160 hit molecules, several molecules blocked HBGA binding in experimental oligosaccharide- and saliva-based assays with an IC_{50} value of less than 10 μM. Three most active small molecules, **8** – **10**, possess a

cyclopenta[a]dimethylphenanthren scaffold thus suggesting a new and promising scaffold to pursue further drug design efforts (Figure 3) [58]. The protein capsids of norovirus genoclusters GI.1 (Norwalk) and GII.4 (VA387) have been shown to also use different binding sites to accommodate various ligands. Computational modelling including molecular mechanics, molecular dynamics and Glide scoring was utilized to assess the clinical importance and broad binding specificity of the VA387 binding site and to provide structural insights into the binding of VA387 with different blood types (such as A, B, and O) saccharides. The study showed that the fucose binding site of VA387 can indeed favorably accommodate a variety of type 1 and type 3 extended ABO-saccharides [59].

The inhibitory effects of a variety of Chinese medicinal herbs on the binding of HBGAs to norovirus protrusion (P) protein was investigated [60]. Out of a series of 50 clinically effective herbs normally active for gastroenteritis disease, two were found to effectively prevent HBGA (P) protein binding. Chinese gall and pomegranate exhibited IC_{50} values of 5.4 μg/ml and 15.6 μg/ml against type A saliva, and 21.7 μg/ml and 66.7 μg/ml against type B saliva, respectively. Tannic acid was suggested to be the active component in the two herbs since it is a common component in both herbs. When pure tannic acid was tested for binding inhibition activity, a good inhibitory activity was observed, with an IC_{50} value of 0.1 μM against both A and B type saliva [60].

3.2.2. Design of inhibitors – targeting structural proteins of norovirus—The two structural proteins encoded by ORF2 and ORF3 of the norovirus genome are the capsid protein VP1 and minor structural protein VP2 [61–63]. VP1 is the major structural protein and contains a shell domain and a protruding domain (PD), which is further divided into two subdomains (P1 and P2) [63]. Subdomain P2 is located at the outermost surface of the viral capsid and comprises the binding surface for host HBGA receptors [62,64–66]. The large degree of conservation of capsid interacting surfaces has prompted antiviral approaches that focus on the design of specific molecules against them [2]. However, only a few reports on targeting VP1 structural protein have appeared, this may be due to the limited information associated with the attachment and host cell entry processes. The viral surface proteins can undergo mutations, which allow resistant strains to emerge [67].

3.2.3. Targeting nonstructural proteins—As described above, targeting structural proteins may result in the development of viral resistance, hence viral nonstructural proteins that have well conserved regions along with critical functions may represent a more beneficial target. A detailed review describing the function of norovirus nonstructural proteins has been reported [12]. Since humans lack RdRp enzymes, these enzymes are considered one of the promising targets for anti-norovirus drug development [35] including norovirus 3CLpro. Protease 3CLpro cleaves the long polypeptide chain that translates from ORF1 of viral RNA into multiple non-structural proteins which serve as important enzymes supporting norovirus life cycle. To date, numerous research groups have studied the cleavage of norovirus polyprotein by virus proteases. The most common approach involves the use of FRET peptide substrates in *in vitro* assays, analysis of infected cell lysates using Western blotting experiments [24,36,68–70], or a luciferase-based in-cell assay [71]. Despite results of substrate studies and the available protein X-ray crystal structures for HNV [72–74] and

MNV [75,76] protease enzymes, norovirus protease substrate specificity is not fully understood. Until recently, the properties of HNV NS6 protease from eukaryotic systems in context of a full polyprotein translated from ORF1 has been difficult to assess due to lack of a robust HNV cell culture system. An improved assay system that allows for the study of the protease cleavage at multiple cleavage sites of the complete ORF1 polyprotein expressing in a transient, yet simple system containing mammalian cells was reported [77]. In order to aid with the study of HNV replication in cell cultures, a novel Gaussia Luciferase-based HNV reporter system that can be also used as an efficient assay to screen for protease inhibitors has been shown [71].

3.2.3.1. Protease Inhibitors: X-ray crystallographic data shows that norovirus protease is a chymotrypsin-like protease, 3CLpro, with an active site consisting of three important amino-acid residues, Cys139, His30 and Glu54 [74]. A detailed mechanism of protease cleavage has been reported [78]. Development of peptidyl protease inhibitors having a Michael acceptor at the C-terminus has led to the discovery of many anti-viral compounds having promising *in vitro* and *in vivo* antiviral activities. The Michael acceptor moiety allows for a covalent and irreversible binding of the inhibitor with the enzymes active site *via* nucleophilic attack of a cysteine thiol function onto the Michael acceptor. In 2011, the first protein X-ray crystal structure of the Southampton norovirus protease bound with the polypeptide irreversible inhibitor **11** was published, and the Michael acceptor moiety employed was an ene ester (Figure 4) [73]. The structural binding information obtained from this data provided the foundation for current knowledge regarding the design of subsequent anti-norovirus inhibitors. Rupintrivir (**12**) possesses a similar ene ester function, is a known enterovirus protease inhibitor and also active against MNVs and HNVs thus showing an excellent broad-spectrum anti-norovirus activity [79].

The design, synthesis and biological activity for a series of optimized dipeptidyl inhibitors for norovirus 3CLpro have been reported [80]. A structure-activity-relationship approach along with X-ray crystallography was used to design inhibitors and a MNV *in vivo* assay was used to screen the activities. The general structure of the dipeptidyl inhibitors, **13**, is shown in Figure 4 containing a R^2 substituted benzyloxycarbonyl group as N-terminal cap, P2 amino acid residue with side chain R^1, a glutamine surrogate at the P1 position, and a C-terminal warhead X (aldehyde or α-ketoamide). One of the most active compounds, **13**, having R^2 = *m*-Cl, R^1 = cyclohexylalanine, and X = aldehyde, exhibited potent inhibitory activity in a 3CLpro enzyme assay (IC_{50} = 0.1 μM) and norovirus replicon-cell-based assay (EC_{50} = 20 nM). Compound **13** effectively reduced norovirus titers in mice. The small and large intestine of mice showed a 42.1- and 7.98-fold reduction, respectively, in viral titers 3 days post viral infection as compared with the control group.

The active site of 3CLpro (*Caliciviridae*) shares sequence and structural similarities with proteases from *Coronaviridae*, and *Picornaviridae*. A series of dipeptidyl inhibitors were evaluated for activities against various *Caliciviridae*, *Coronaviridae*, and *Picornaviridae* families using a protease enzyme assay and/or cell-based assay [81]. Two of the tested compounds, **14** (GC373) and **15** (GC376), showed excellent broad-spectrum antiviral activity (IC_{50} values in the low micromolar or high nanomolar range) against most of the viruses screened, with the exception of HAV and feline caliciviruses (FCV). A series of

novel tripeptidyl inhibitors, such as **16** and **17**, of norovirus 3C protease that effectively inhibited norovirus protease in enzyme assays and viral replication in a norovirus replicon-cell-based assay has been reported [82] (Figure 4). The general structure of the tripepidyl inhibitors contained an *N*-terminal Cbz cap, P3 arylalanine, P2 leucine, P1 glutamine surrogate and C-terminal warhead. Norovirus proteases are quite specific to the identity of the substrate P1 amino acid side chain and require either a Gln (Q) of Glu (E) in P1 position, but have higher tolerability for changes in substrate P2 and P3 positions. A number of potent inhibitors have been reported to have IC_{50} values ranging from 0.14 – 35.5 μM and EC_{50} values ranging from 0.04 – 4.2 μM [82]. Notably, the tripeptidy series required a hydrophobic arylalanine in the P3 position to necessitate good cell based inhibitory activity suggesting improved cell membrane permeability. The most promising compound was compound **16** having an IC_{50} value of 0.14 μM and EC_{50} value of 40 nM. The bisulfite adduct, **17**, was shown to act as a pro-drug, converting back into the aldehyde warhead functionality *in vivo*. This also explains the analogous EC_{50} value of compound **17** to compound **16** in cell the based assay (40 nM). A recent study showed that compound **16** and its structural analogues were highly efficacious antiviral agents against feline coronaviruses (FCoV) and FCV in cell culture with EC_{50} values in the low micromolar range [83]. It was suggested that these compounds have potential to be developed further as therapeutic agents against FCoV and FCV viruses in domestic and wild cats [83]. The structure-activity relationship study of a series of peptidomimetic protease inhibitors has been reported [78], and it was concluded that the role and structure of the P1 and P2 side chains are important for achieving good activity. The role of the P3 side chain only plays a minor role in binding. The most potent tripeptidyl inhibitors had an aldehyde moiety on the C-terminal end which acts as an electrophile, reacting with Cys139 to form a reversible-covalent bond. This class of inhibitors is therefore competitive with natural substrate for active site binding.

It is known that macrocyclic peptides may offer advantages over linear peptides in terms of drug-like characteristics and are less prone to proteolysis [84]. Representative macrocyclic peptidyl compounds **18** and **19** (Figure 4) were synthesized by utilizing the P1 and P3 side chains which has the effect of locking the peptide into a β-strand conformation. The macrocyclic peptides **18** and **19** were screened for anti-norovirus activity in enzyme and cell based assays. Molecule **19** showed a good inhibition activity in an enzyme assay (IC_{50} = 8.5 μM), however it did not show inhibitory activity below the concentration range tested >10 μM in cell-based assays. The ester **18** was not active in enzyme or cell-based assays, suggesting the ester functionality is a poor C-terminal warhead.

A series of macrocyclic protease inhibitors was further investigated [84,85], and two of which, compounds **20** and **21**, showed promising cell-based anti-norovirus activity with EC_{50} values of 6.1 μM and 4.5 μM, respectively. A derivative of compound **20**, triazole **22**, showed broad-spectrum anti-viral activity against 3CLpro (IC_{50} 5.1 μM), enterovirus (CVB3 Nancy strain), 3Cpro (IC_{50} 1.8 μM), and SARS-CoV 3CLpro (IC_{50} 15.5 μM) [85]. Computational docking studies were performed in order to gain further understanding into the binding of macrocyclic inhibitor **22** with different protease enzymes [85].

3.2.3.2. Polymerase Inhibitors: Virally encoded RNA dependent RNA polymerase, RdRp or NS7, is a key target in the designing of anti-norovirus therapeutics due to its vital role in the replication of norovirus genome and the absence of homologous human enzymes. RdRp carries out the synthesis of genomic and subgenomic RNA of noroviruses in infected hosts [21,86,87]. Recombinant RdRp was used to elucidate the biochemical properties and X-ray crystal structures of RdRps from human Norwalk viruses GI and GII and mouse GV noroviruses have been revealed [86,88,89]. Detailed information about active site of RdRp has been studied [86,89,90]. RdRp inhibitors can be divided into two categories, nucleoside inhibitors and non-nucleoside inhibitors [87]. Upon penetration into cells, nucleoside inhibitors undergo phosphorylation by triphosphate, giving nucleotides, which then are incorporated into the polymerized nucleic acid chain elongated by the RdRp and subsequently included into the growing RNA chain, resulting in the termination of the replication elongation step [87,91]. Non-nucleoside inhibitors have the potential to bind to one of several allosteric sites of RdRp enzyme and thereby induce a conformational change in the enzyme leading to the inhibition of the initiation step in the RNA replication [91]. *In silico* screening as well as *in vitro* enzyme assays have led to the identification of various inhibitors against norovirus RdRp. Suramin (Figure 5), an inhibitor for various DNA/RNA polymerases, and its analog NF023 inhibited MNV and HNVs RdRps with IC_{50} values in the nanomolar range [40]. However, these two compounds with naphthalene-trisulfonic acid heads display poor membrane permeability [35]. Subsequently, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt (PPNDS), a known specific P2X1 receptor antagonist with low micromolar IC_{50} value against MNV RdRp *in vitro* was discovered [35]. Crystal structures of the RdRp with suramin, NF023, and PPNDS have been determined, and it was found that PPNDS binds to a different site in RdRp, which is distinct from that occupied by suramin and NF023 [92]. In addition, 2′-C-methylcytidine (Figure 5), a hepatitis C virus inhibitor, also inhibits *in vitro* replication of MNV with an EC_{50} about 2 μM and of the Norwalk replicon cell system with an EC_{50} of 18 ± 4 μM [93]. Furthermore, 2′-C-methylcytidine reduces viral shedding and transmission to uninfected mice in an experiment performed with a mouse model with a value of about 270 $CCID_{50}$ (50% cell culture infectious dose) [94]. Several small molecule non-nucleoside inhibitors against GII.4 RdRp transcription were found from high throughput screening followed by radioactive nucleotide incorporation RdRp assay [21]. Phenylthiazole carboxamide NIC02, pyrazole acetamide NIC04, triazole NIC10, and pyrazolidinedione NIC12 with IC_{50} values of 5.0 μM, 5.5 μM, 9.2 μM, and 9.8 μM, respectively, were found [21] (Figure 5). In addition, 5-fluorouracil, favipiravir, 2-thiouridine and ribavirin also inhibited RdRp *in vitro* [88,93,95–97]. Moreover, the crystal structures of MNV polymerase bound to some of the most active compounds such as 5-fluorouracil, 2-thiouridine, ribavirin, suramin, and suramin analogs have been examined and thus providing useful insight into the binding site of these inhibitors. The anti-norovirus activities of 12 chromone and (*E*)-2-styrylchromones were assessed in MNV assays as a surrogate model for HNV [98], and the hit molecules, (*E*)-5-hydroxy-2-styrylchromone (**30**) and (*E*)-4′-methoxy-2-styrylchromone (**31**), exhibited promising IC_{50} values in the low micromolar range (≈ 7 μM) (Figure 5). Although the mechanism of action is not known, the authors hypothesize that these compounds may target the norovirus polymerase enzyme. Representative RdRp inhibitors that have been reported are listed in Figure 5.

3.2.3.3. Entry blocking molecules: Recently, a small molecule deubiquitinase (DUB) inhibitor was found to show promising anti-infective activity against HNV and MNV [99]. This study stemmed from earlier reports of DUB inhibitor, WP1130, showing anti-norovirus activity, anti-infective activity against other food-borne pathogens, as well as anti-cancer activity. WP1130 has limited use due to its poor oral bioavailability and low water solubility and therefore was subjected to a rational drug design approach in search of improved DUB inhibitors. The studies prompted the identification of a promising drug candidate, compound **32**, by screening libraries of molecules using the Norwalk virus replicon system [99]. Compound **32** decreased MNV-1 viral titers more than 2.5-logs when RAW264.7 cells were treated for 0.5 hours at a concentration of 2.5 μM when compared to the control. After quantification of the Norwalk virus genome using qRT-PCR, compound **32** decreased the level of detectible Norwalk virus genomes by 84.7%. Ubiquitination and DUB enzymes are becoming recognized as interesting targets for therapeutic drug development, however, no DUB inhibitors have reached clinical trials to date. It is hopeful that the hit molecule, **32**, can be further optimized to discover improved anti-infective agents for noroviruses with broad-spectrum activity [99]. The role that DUBs play in modulating noroviral infections remains poorly understood. It was suggested that DUB inhibition induces an unfolded protein response (UPR), which blocks norovirus infection [100].

Noroviruses have a highly variable genome with up to 100 distinct viruses already identified and up to 43% divergence in nucleotide sequence among the five known genogroups (G1 – G5) [101]. It has been shown however that the 5′-end of the norovirus genome is the most conserved region in all noroviral RNA and shares sequence similarity with 5′-end of the viral subgenomic RNA that interacts with the start of ORF2. Targeting the noroviral mRNA by designing phosphorodiamidiate morpholino oligomers (PMOs) as noroviral replication inhibitors due to the ability of PMOs to bind to noroviral mRNA through Watson-Crick base-paring causing steric blockade to inhibit replication was proposed [101]. The designed PMOs have a neutral backbone which is stable and use a morpholine ring in place of the deoxyribose sugar. PMOs can also be conjugated with an arginine rich cell-penetrating peptide (peptide-PMO or PPMO) to improve cellular uptake into cells [101]. The series of PPMOs presented a dose responsive uptake of PPMOs into RAW264.7 macrophages and exhibited zero cellular toxicity up to a concentration of 20 μM for the most toxic PPMO. Two of the tested PPMOs (B = CGTTGCCATCCTCATTTCAC and C = CGCAGAAGATGGCGTTGCCATCCTC) that were designed to target the 5′-end of MNV-1 ORF1 inhibited virus replication by at least 90% when their maximal non-toxic concentrations were used in the assay. The IC_{50} value for the most active PPMO was found to be 5 μM (B) and the IC_{70} for compound C was found to be 20 μM.

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) was found to be upregulated by more than 1.5-fold in norovirus replicon-harboring cells [102], and commercially available ACAT inhibitors were shown to inhibit norovirus infection. Hence, a series of pyranobenzopyrones for the inhibition of norovirus was investigated, and likely through ACAT inhibition in the host cells [103,104]. Two representative molecules, **33** and **34** (Figure 5), showed the best anti-norovirus activity with EC_{50} values of 3.4 and 2.4 μM, respectively, and therapeutic index values greater than 40. All compounds were screened for activity using a norovirus

replicon harboring cell-based assay, and the inhibition was revealed by a reduction in norovirus RNA replication. These results demonstrated that ACAT in the host cells could be a target for anti-norovirus drug development and optimization of these inhibitors may lead to hit molecules.

3.3. Anti-norovirus drugs in the clinical development stage

So far there is no specific anti-norovirus drug or vaccine to proceed through clinical trial phases. However, nitazoxanide (**35**, Figure 5), which was originally developed and commercialized as an anti-protozoal agent, has been shown to reduce the resolution of symptoms (the median time from first dose to resolution of symptoms was 1.5 days) compared to placebo (2.5 days) in 50 patients at least 12 years of age (mean 33.5 years) infected with rotavirus ($P = 0.0052$) and norovirus ($P = 0.0295$) [105,106]. Another case report indicated that the treatment of nitazoxanide in an immunocompromised patient with a 10-day history of norovirus infection had shown a decline in bowel movements from approximately 10 to 2 bowel movements per day after 24 hours, and the consistency and frequency of bowel movements returned to baseline after 4 days of treatment [107]. In addition, the molecule has shown an efficacy towards diarrhea caused by rotavirus infections [13,106]. These data provide a rational for the design of a new class of compounds (thiazolides) against norovirus infections.

4. Conclusion

An overview of the discovery of inhibitors for norovirus has been described including the inhibitions of structural and non-structural norovirus proteins and viral entry into host cells. A number low nanomolar inhibitors have been discovered. Though it has been passed more than a decade since the discovery of Norwalk replicon cell line, yet there is no FDA approved anti-norovirus drug or vaccine available. The lack of human norovirus cell line and understanding of mechanism of encapsidation and cell exiting pathway likely hinder the drug development.

5. Expert opinion

Currently, anti-norovirus drug discovery has been mainly focused on approaches related to viral targets such as RNA dependent RNA polymerase, norovirus 3C-like protease, and structural proteins such as VP1 to some extent that were able to identify several lead compounds with potential anti-norovirus activity *in vitro* and *in vivo* [84]. However, none of them have proceeded up to the level of clinical use as a treatment or prevention for norovirus infection in part due to bioavailability, efficacy, and possibly safety. These issues illustrate the necessity of developing safe and more efficacious anti-norovirus therapeutics with improved bioavailability. The fact that potential anti-norovirus molecules mainly target the pathogens in the intestine, the effective delivery of inhibitors to the site of action, and the tolerance of those compounds to rather harsh conditions make the need for better optimization necessary. There are a number of peptidyl compounds possessing strong anti-viral protease activities *in vitro* with nanomolar inhibitory activity, but issues such as low bio-availabilities *in vivo* due to rapid metabolism by various proteases in the body have hampered the progress of these molecules. Therefore, optimization of the efficacy of current

compounds through medicinal approaches like modification of inhibitors without peptidyl structures or possessing non-cleavable peptidyl structures appears to be a challenging and rewarding route. Notably, other key targets such as viral polymerases and viral entry host receptors along with host chaperone proteins belong to the Hsp family for proper protease folding and proteolysis [108] are important and need to be explored further to search for more potent drug candidates with low toxicity. We believe that a combination of small molecules that inhibit viral proteases or polymerases and blockage of the viral entry through the inhibition of viral entry proteins such as capsid protein VP1 would provide effective counteraction of virus infection. There is continuous interest in exploring other targets such as host factors and inherited host variabilities to overcome the development of resistances through mutations. Identifying and understanding host factors in details and their contribution to virus replication inside the host may provide new targets and facilitate the discovery of antiviral compounds with less chance of resistance to develop [109]. Since the treatment period is short (four to five days), the toxicity would be a minor issue for healthy adults infected with norovirus. Hence, targeting the viral factors and host factors are equally important. In fact, it can be critical in immune-compromised and elderly patients infected with noroviruses who need prolonged treatment periods, and therefore it is essential to develop safe and effective anti-norovirus therapeutics especially for those populations. Future prospects in searching for safe and efficacious drug candidates are likely based on the current tools available in this field. Key findings, such as the finding that human noroviruses can be replicated in B cells, MNV, other animal models and norovirus replicon cell lines have illuminated the aspects related to virus replication and infection *in vivo* and have laid the groundwork to understand the *in vivo* virus – host interactions, other fundamental aspects of immunity, and viral pathogenesis to some extent. Moreover, these cell systems and animal models are available for initial screening and confirmation of efficacy of discovered hit molecules. A hurdle in the field of norovirus drug discovery is to develop a fully permissive cell culture system and small animal models for human noroviruses, which can be used to study various aspects of the disease identified in humans. There are numerous research efforts going on to achieve these goals. Another major issue that is particularly important in this field is the proper elucidation of the molecular mechanisms associated with norovirus genome translation and replication and how they are used by noroviruses to elicit enteric pathology, an area that is not fully understood and would be of interest for study. Ongoing and future research may hopefully overcome limitations such as inability to cultivate human norovirus *in vitro* and will lead to the identification of successful drug candidates. The development of vaccines to counteract norovirus likely will be explored in coming years.

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Article highlights

- Noroviruses are the major pathogens responsible for acute gastroenteritis accounting significant morbidity and mortality in infants and elderly populations.
- Several steps in the norovirus life cycle such as virus assembly, encapsidation and release of virion from the infected cells remain to be studied.
- The lack of an effective HNV cell culture model has a significant influence on the discovery of anti-norovirus therapeutics.
- MNV, norovirus replicon cell lines, X-ray crystallographical data of viral proteases and polymerases, NMR spectroscopy and computational docking experiments have facilitated the discovery of potential anti-norovirus molecules.
- Several potential antiviral molecules have been discovered targeting viral factors as well as host factors.

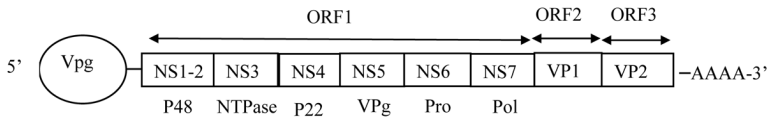

Figure 1.
Organization of human norovirus genome.

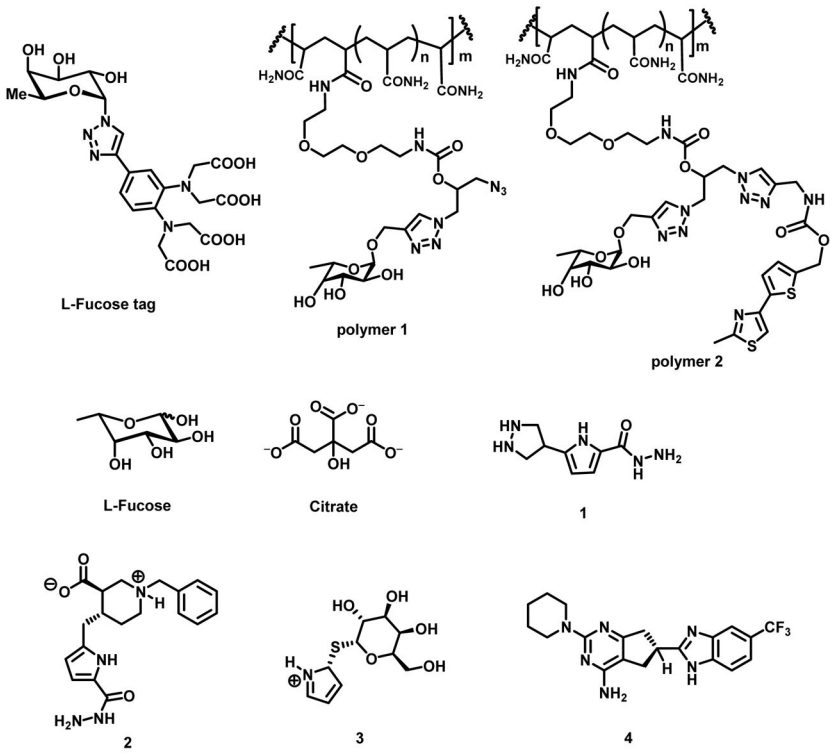

Figure 2.

Structures of L-fucose-tag, polymer 1, polymer 2, L-fucose, citrate, and four computer generated HBGA inhibitors **1** – **4**.

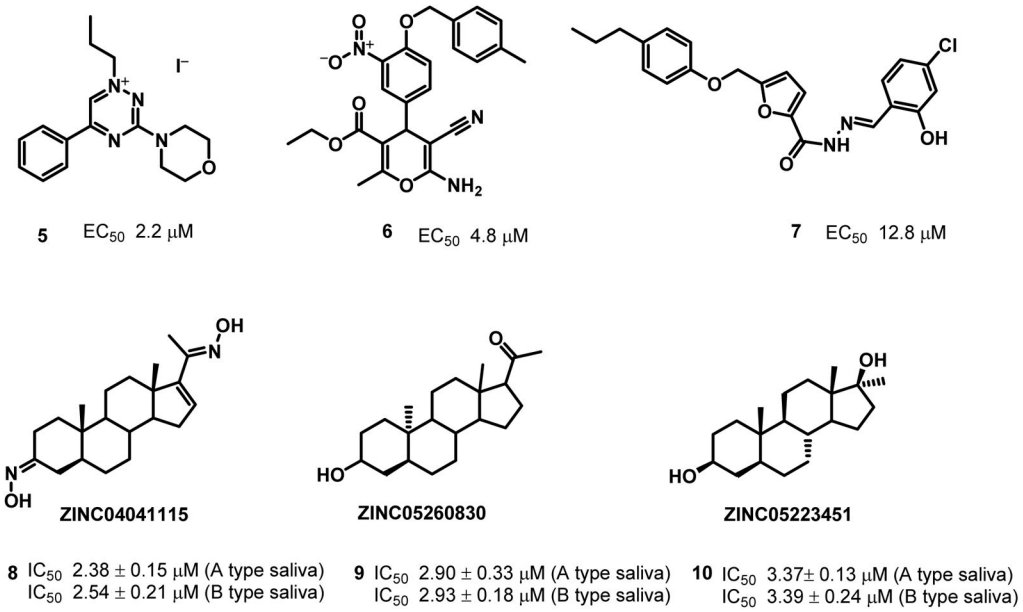

Figure 3.

Representative inhibitors against binding of noroviruses to the corresponding HBGA receptors.

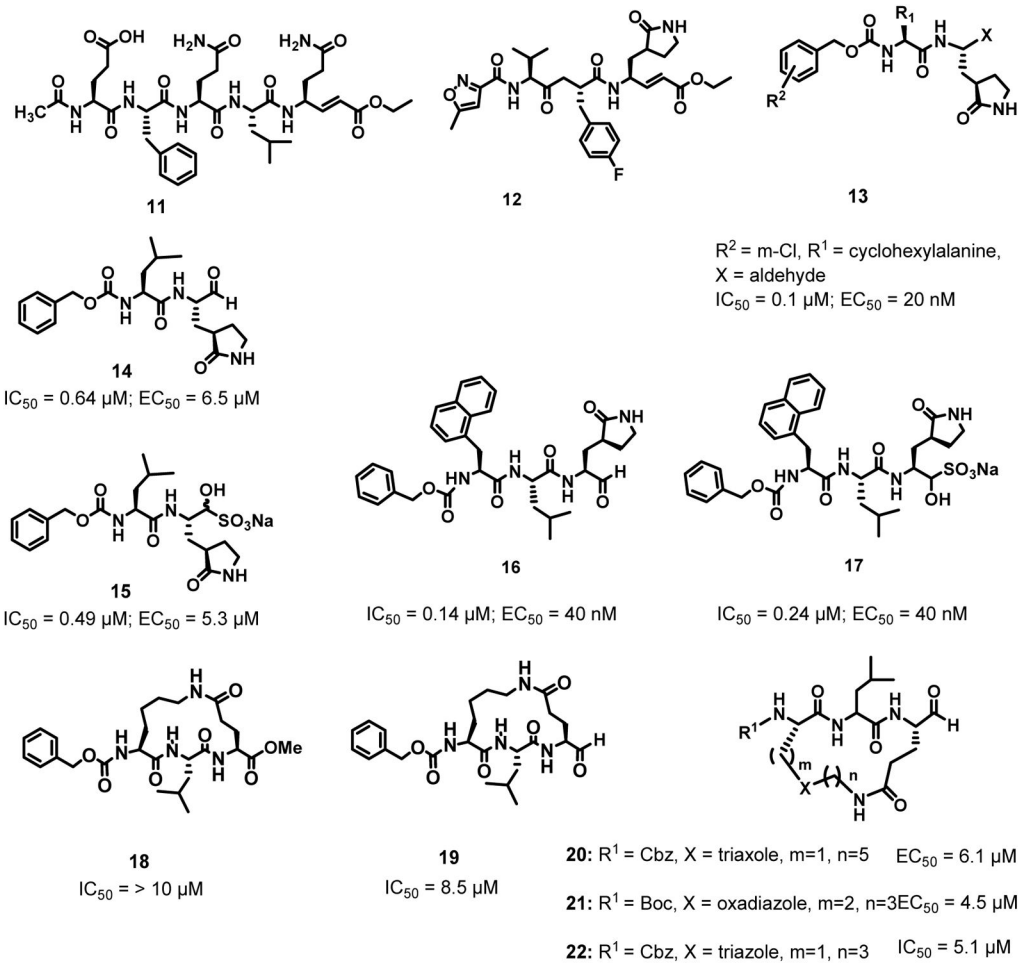

Figure 4.
Representative peptidyl norovirus protease inhibitors.

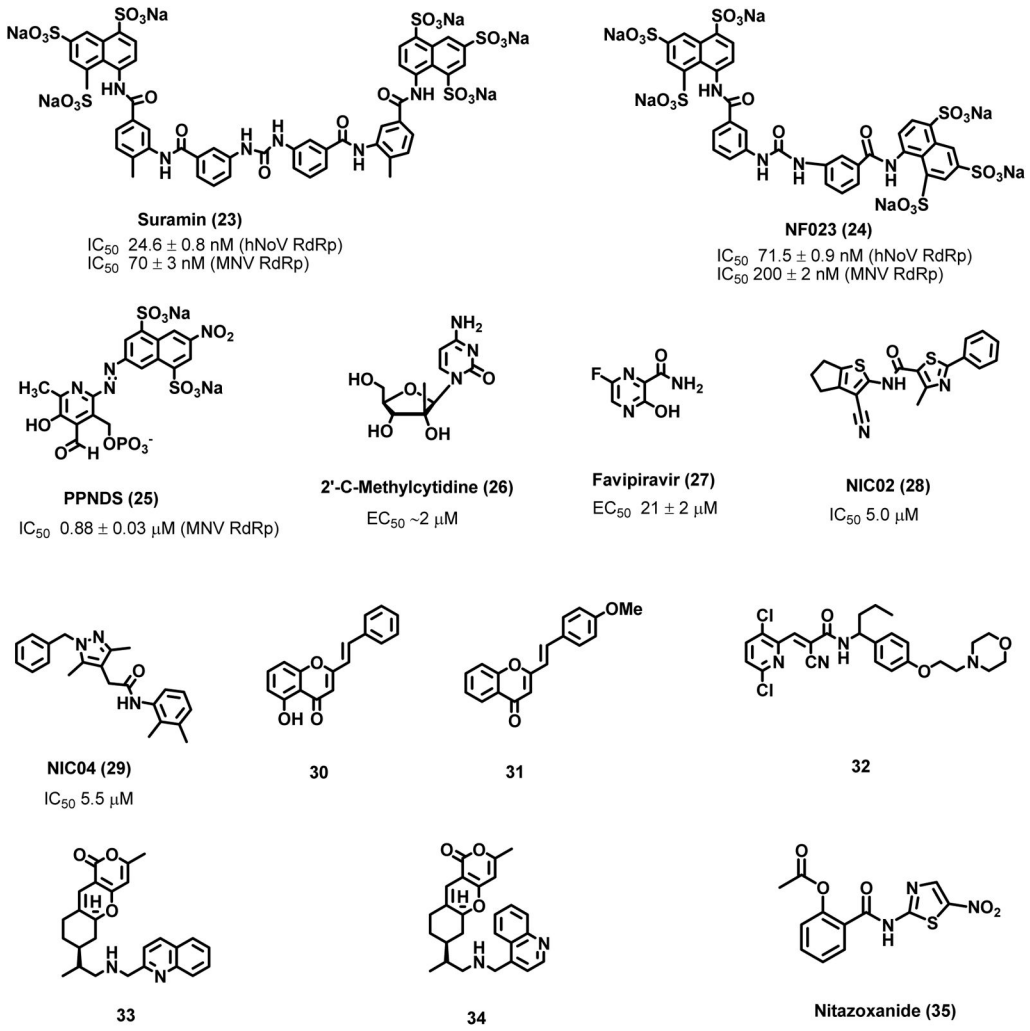

Figure 5.

Representative chemical structures of RdRp inhibitors **23** – **31**, entry blocking inhibitors **32** – **34**, and nitazoxanide.