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Antiviral Targets of Human Noroviruses

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Abstract

Human noroviruses are major causative agents of sporadic and epidemic gastroenteritis both in children and adults. Currently there are no licensed therapeutic intervention measures either in terms of vaccines or drugs available for these highly contagious human pathogens. Genetic and antigenic diversity of these viruses, rapid emergence of new strains, and their ability to infect a broad population by using polymorphic histo-blood group antigens for cell attachment, pose significant challenges for the development of effective antiviral agents. Despite these impediments, there is progress in the design and development of therapeutic agents. These include capsid-based candidate vaccines, and potential antivirals either in the form of glycomimetics or designer antibodies that block HBGA binding, as well as those that target essential non-structural proteins such as the viral protease and RNA-dependent RNA polymerase. In addition to these classical approaches, recent studies suggest the possibility of interferons and targeting host cell factors as viable approaches to counter norovirus infection. This review provides a brief overview of this progress.

Introduction

Human noroviruses (HuNoVs) are the most common cause of epidemic and sporadic cases of acute gastroenteritis worldwide [1]. In the US alone, HuNoVs cause approximately 19–21 million cases of acute gastroenteritis annually in all age groups [2*,3]. HuNoV infection can be life-threatening, especially in the elderly and immunocompromised transplant patients [4,5] who are at high risk for serious and prolonged chronic illness. In recent years, with the success of rotavirus vaccination in young children, HuNoVs have replaced rotaviruses as the most common cause of gastroenteritis in this age group [6,7*]. The economic burden of

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HuNoV infection in the US is estimated to be ~$5.5 billion [8]. In developing countries HuNoVs are estimated to cause more than 1 million hospitalizations and 218,000 deaths in children under 5 years of age occurring annually [9].

HuNoVs belong to the genus *Norovirus*, one of the five major genera in the *Caliciviridae* family. These ~400 Å icosahedral viruses have a positive-sense, single-stranded RNA genome. They exhibit enormous genetic diversity and are phylogenetically divided into at least six genogroups (GI-GVI). The GI, GII and GIV genogroups contain human pathogens. Each of these genogroups is further divided into several genotypes [10]. The HuNoVs belonging to genogroup II and genotype 4 (GII.4) are the most prevalent, and account for the majority of global outbreaks [11]. Epidemiological studies suggest that the GII.4 strains undergo epochal evolution with a new variant emerging every 2–4 years [12,13]. Recent studies also show outbreaks involving GI strains are becoming increasingly prevalent worldwide, with certain GI genotypes predominating in different geographical regions. The preponderance of global HuNoV outbreaks with periodic emergence of new variants poses a major health concern. Currently, there are no effective vaccines or antivirals available to counter HuNoV infection.

Vaccines against HuNoV infections

The genetic and antigenic diversity of HuNoVs and the lack of naturally-occurring longstanding immunity are possible significant challenges for the development of effective vaccines that can offer widespread cross-protection. However, significant effort has led to development of a bivalent vaccine, based on genotype GI.1 and a consensus GII.4 recombinant virus-like particles (VLPs) [14], which is in phase II clinical trials [15–17**]. The GII.4 VLP was designed by obtaining a consensus sequence from three GII.4 variants (Henry_2001, Yerseke_2006a, and Den Haag_2006b) using the Houston virus (Henry_2001 variant) as the backbone [18]. Point mutations were made to alter the amino acids into a consensus sequence. The consensus GII.4 VLP elicits antibody responses that recognize a wide array of GII.4 variants, including those that have yet to emerge [19*]. The HuNoV VLPs are produced by the expression of the major capsid protein VP1, which as 90 dimers forms the T=3 icosahedral capsid (Fig. 1) [20,21]. VP1 is encoded by the open reading frame (ORF) 2 of the HuNoV genome. A second minor structural protein, VP2, not present in the vaccine construct, is encoded by ORF3, whereas the ORF1 encodes a polyprotein that is processed by the virally-encoded protease into 6 non-structural proteins (NSPs). The VP1 exhibits a modular domain organization consisting of an S domain, formed by the N-terminal residues, that provides a scaffold for the protruding P domain, which is further subdivided into P1 and P2 subdomains (Fig. 1A and 1B). The distally located and surface-exposed P2 subdomain, which can be considered as a large insertion in the P1 subdomain, harbors the most sequence variations across the genogroups and genotypes and is responsible for many virus-host interactions. Recombinant VLPs are morphologically and antigenically similar to the authentic HuNoV capsid and are highly immunogenic. Such VLPs can be made from any HuNoVs genotype [22], suggesting the possibility of designing multivalent vaccines from selected multiple genotypes. In addition to the VLPs, recombinant P domain by itself elicits a strong immune response and has been suggested as a possible candidate for vaccine development efforts [23–25]. Even if an effective vaccine becomes

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available, there is a great interest in the development of antiviral drugs [26–28]. Antiviral treatment could be useful for therapy of chronic infection in immunocompromised patients; treatment and prophylaxis in outbreak situations where ongoing transmission continues to occur, as in a nursing home outbreak. As prophylaxis in certain circumstances, such as for travelers, if the medication is safe and vaccine is unavailable; and for treatment of acute illness, particularly in the young and old and hospitalized patients in whom symptoms may last for up to a week. What are the targets for the design and development of such anti-HuNoV drugs?

Glycan binding site as a potential target

Susceptibility to HuNoV infection is associated with expression of histo-blood group antigens (HBGAs) [1] that are found in mucosal secretions and on epithelial cells. These genetically-determined glycoconjugates function as initial cell attachment factors for HuNoVs [29,30]. The unique requirement of binding to polymorphic HBGAs may influence the evolution of NoV strains [13]. HuNoVs bind to HBGAs through the hypervariable P2 subdomain in the protruding P domain of VP1 [31–37]. Studies using VLPs or P domain constructs have shown that HuNoVs, as a result of variations in the P2 subdomain, exhibit strain-specific HBGA binding patterns. Crystallographic studies of P domain-HBGA complexes show that while the HBGA binding site is distinct between GI and GII, the sequence changes around the conserved HBGA site within each genogroup allow for modulations in the HBGA binding profiles [38] (Fig. 1C and Fig. 1D). These sequence changes result in significant alterations in the structural and electrostatic topography of the P2 subdomain consistent with strain-dependent antigenic variations [36,37]. The observation that these changes are in close proximity to the HBGA binding site is consistent with the notion that a coordinated interplay between variations in HBGA binding profiles and antigenicity are critical factors in driving the evolution of HuNoV.

Although there are differences in HBGA binding profiles between the genotypes within each genogroup, there are also conserved features. For example, all the genotypes in GI and GII genogroups primarily recognize a galactose and a fucose residue in the HBGA, respectively [38]. The structural elements including the amino acid residues in the respective P2 subdomains of these genogroups that coordinate the binding of the galactose and fucose moieties are highly conserved. In the case of a GII (GII.10) P domain, crystallographic studies have shown that fucose alone can bind the same set of P2 subdomain residues as the terminal fucose residue of HBGA [34]. Interestingly, a citrate together with a water molecule mimicking the pyranoside ring of fucose also can bind effectively to the same site [39]. Furthermore, recent studies have shown that oligosaccharides derived from human milk such as 2′-fucosyllactose and 3-fucosyllactose bind at the same site in the GII as HBGA, and they can block HBGA binding [40*]. These observations raise the possibility that HBGA binding sites can be potential targets for the design of glycomimetics or small molecules that inhibit HBGA binding. However, because these interactions with HBGA generally occur with low affinity, that is, in the low micromolar range, designing potent glycomimetics may be a challenging task.

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HBGA-blocking antibodies as therapeutic agents

A more feasible approach may be to design antivirals based on monoclonal antibodies (mAbs) that inhibit HuNoV-HBGA interactions [19,41–43], particularly considering recent studies that have identified such antibodies from the sera of HuNoV infected patients [44–46]. The design and development of suitable mAbs or their derivatives, single-chain antibody fragments (also called VHH or nanobodies) and disulphide-stabilized single-chain antibody fragments that particularly target the entry mechanism, have been in consideration for prophylactic and therapeutic use to counter viruses such as influenza virus, rabies virus, Ebola virus and hepatitis B virus [47]. Adaptive humoral immunity is also involved in resistance to HuNoV infection. Serum antibodies that block interactions between the virus and HBGAs are associated with lower risks of developing infection or illness following exposure to virus [44–46*]. Such antibodies have been proposed to be functionally similar to hemagglutination inhibiting or neutralizing antibodies of influenza virus [48,49]. HBGA-blocking antibodies can vary in specificity, from genotype-specific to variant-specific and even strain-specific among the globally prevalent GII.4 NoVs, further emphasizing a correlated interplay between antigenicity and HBGA specificity in the evolution of NoVs [12,13,50]. Human monoclonal antibodies that block HBGA binding have been isolated and produced from the peripheral blood mononuclear cells of blood donors, and the few that have been characterized to date appear to be genotype-specific [51]. In addition, nanobodies that block HBGA binding in GI.1 and GII.4 VLPs, have been identified and characterized [52*]. Currently the mechanism of how these antibodies block HBGA binding is unclear. Structural studies of the P domain in complex with Fabs of these mAbs or nanobodies should provide mechanistic details of their blockade activity that then can be leveraged to design antibody-scaffolds or antibody-like molecules as HuNoV-specific therapeutic agents.

Non-structural proteins as targets

As noted earlier, the ORF1 of HuNoVs encodes a polyprotein that is proteolytically processed by the virus-encoded protease into at least six NSPs [53]. These NSPs, from N- to C-terminus of the polyprotein, include p48, whose precise function is yet to be determined; p41, an NTPase with distinct highly conserved SF3 helicase motifs similar to picornavirus 2C; p22, which shares sequence similarities with picornavirus 3A, with a possible function as an antagonist of Golgi-dependent cellular protein secretion [54]; VPg that is covalently linked to the viral RNA; a protease that is similar to picornavirus 3C; and an RNA-dependent-RNA polymerase (RdRp) orthologous to picornavirus $3D^{pol}$ [55–58]. Of these six NSPs, protease and RdRp have been considered potential antiviral targets not only because their structures and functions are well characterized, but also because their picornavirus homologues have been studied extensively for the development of antivirals.

HuNoV Protease

Proteolytic processing of the polyprotein by the virally-encoded protease is a common essential step in the replication of the (+)RNA viruses, including HuNoVs. Unlike cellular proteases that generally target one site, these viral proteases can recognize and cleave at multiple specific sites in the polyprotein. Because of their critical function in viral

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replication, viral proteases including HuNoV protease [56,59–64*], have been attractive targets for the design and development of small molecule drugs that inhibit proteolytic processing. Typically, such protease inhibitors are short peptides that mimic N-terminal residues preceding the cleavage site (P1–P5) of the substrates in the polyprotein and modified further at the P1 position by attaching adducts such as aldehydes, ketones, esters or bisulfite as electrophilic warhead. These inhibitors bind to the active site irreversibly and covalently modify the active-site nucleophilic residue to inhibit proteolytic activity.

The HuNoV protease, similar to picornavirus 3C, is a cysteine protease with a chymotrypsin-like fold. It is comprised of two domains separated by a cleft where the active site is located [65–67]. The active site consists of a catalytic triad with cysteine as a nucleophile, histidine as the general base catalyst, and glutamic acid as the anion to orient the imidazole ring of histidine [66,68–70]. Crystal structures of HuNoV proteases in complex with substrates bearing P1–P4 residues or substrate-mimics have shown how these residues optimally interact with the S1–S4 pockets in the protease (Fig. 2A), respectively, and how the protease accommodates the varying residue compositions in the polyprotein by undergoing suitable conformational changes in the active-site cleft [68,71,72*]. These studies have provided valuable insights into the design of peptide-mimetics that effectively inhibit protease activity for both GI.1 and GII.4 proteases [62,72*]. Crystal structures of the Norwalk virus (GI.1) protease in complex with three of these substrate-based peptide inhibitors with a terminal aldehyde showed that in addition to the formation of the covalent adducts (Fig. 2B), these inhibitors prevent the conformational change necessary for the formation of the oxyanion hole. These studies further suggest that peptido-mimetics with suitable warheads with a Glu-like chemical entity at P1 for optimal interactions with S1 pocket, and an appropriate combination of hydrophobic residues at P2 and P4 that maximizes the interactions with S2 and S4 pockets, are factors to be considered for enhancing the potency of the inhibitors. More recently, based on the observation that protease-bound peptidyl inhibitors typically adopt a β-strand conformation between the P1 and P3 positions, Weerwarna et al., [73**] have designed and synthesized a novel set of triazole-based macrocyclic inhibitors in which these two positions are linked using a suitable linker such that the P1–P3 is pre-organized into a β-strand conformation for optimal interactions with the norovirus protease as demonstrated by structural studies. In addition to potentially higher stability to metabolic enzymes these inhibitors exhibit increased cellular permeability. Further studies should be anticipated that are directed at optimizing the design strategies and improving the pharmacokinetic properties and metabolic stability of HuNoV protease inhibitors using structural analysis and cell-based assays [74–76]. Considering that the active-site residues are highly conserved between the HuNoV proteases in various genogroups and picornavirus proteases, there is a distinct possibility of designing broad-spectrum protease inhibitors as antivirals [77].

HuNoV RdRp

The NoV RdRp, similar to picornavirus 3D, is critical for synthesizing both negative-sense RNA as well as newly made positive-sense genomic RNA and has been the target for developing small molecule inhibitors. X-ray structures of RdRps from several NoV genogroups including HuNoV (GII) [78,79], sapovirus [80], and murine NoV [81,82] have

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been determined. As observed in all RNA/DNA polymerases, this protein exhbits a typical "right hand" configuration of palm, finger, and thumb domains [83] (Fig. 2C). The active site is located in the thumb domain. It consists of three conserved Asp residues that are critical for mediating catalysis through a two metal-ion mechanism, and other key residues such as Arg, Asn and Ser that are required for substrate binding and catalysis. Crystallographic studies have further shown that NoV RdRp can exist in two principal conformations. An 'open' active site conformation that represents the inactive state of the RdRp [78,80,84], and a 'closed' active site conformation that is primed for catalyzing nucleotidyl transfer reaction [79] by optimally positioning the nucleotide, RNA and the metal ions for catalytic reaction.

There has been significant progress in identifying small molecule inhibitors of NoV RdRp using *in silico* screening, and in understanding the structural basis of inhibition by analyzing co-crystal structures of RdRp with some of these inhibitors [85,86*]. Non-nucleoside inhibitors such as suramin, a drug used in the treatment of sleeping sickness caused by the protozoan Trypanosoma, and its analogue NF023, consisting of naphthalene-trisulfonic acid moiety, have been shown to be effective in inhibiting NoV RdRp with IC_{50}s in low nanomolar range [86*]. Both these inhibitors bind RdRp along the nucleotide access pathway between the fingers and thumb domains (Fig. 2D). Nucleoside-analogs such as 2′-C-methylcytidine (2CM-C) and ribavirin, hepatitis C virus polymerase inhibitors, and 6-fluoro-3-hydroxy-2-pyrazinecarboxamide (T-705; favipiravir), a nucleoside precursor which was originally developed against influenza viruses, have been shown to be effective in inhibiting *in vitro* replication of murine norovirus (MNV), and also HuNoV replication using a Norwalk virus replicon model [87*–89]. More recent studies have shown that triphosphates of 2CM-C and T-705 also inhibit MNV and HuNoV RdRp activities with IC_{50}s in the low micromolar range [90]. These studies found that 2CM-C triphosphate inhibited RdRp by directly competing with CTP during primer elongation whereas T-705 triphosphate competed mostly with ATP and GTP at the initiation and elongation steps. Further structure-based techniques coupled with high throughput screening [91] will likely lead to design and development of more potent and perhaps even broad-spectrum RdRp inhibitors with the necessary pharmacokinetic properties.

Interferons and targeting host factors as antiviral approach

Interferons are a group of peptides that have antiviral activity against a variety of viruses. *In vitro* studies have demonstrated that type I interferons inhibit norovirus replication in a replicon system [88] although no human studies have been reported to date. MNV replication is inhibited by both type I and II interferons [92]. Recent studies have also demonstrated that chronic infection caused by MNV can be cleared with the administration of the type III interferon, interferon λ, in the absence of an adaptive immune response [93**]. In addition, a number of cellular factors such as La, PTB, DDX3, PCPB2, and hnRNPs have been identified as critical for MNV replication using RNAi methodology, suggesting that these molecules may be targets for antiviral drug development [94**]. Small molecule inhibitors of deubiquitinases, such as WP1130, inhibit MNV replication in addition to several other RNA viruses [95]. Whether such approaches are viable for HuNoV replication needs further studies.

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Conclusions

HuNoVs pose a significant global health concern. With the current lack of effective antiviral strategies, there has been an intense focus in vaccine development as well as antiviral drug discovery. Recent progress in both these fronts is encouraging. An immunogenic VLP-based candidate vaccine is in phase-II clinical trials and other candidate vaccines based on P domain, which elicit strong immune responses, also hold promise. These studies raise the possibility of designing multivalent vaccines to counter the antigenic and genetic diversity exhibited by HuNoVs. Recent progress in the isolation and characterization of HBGA-blocking human mAbs points to a distinct possibility of designing antibody-based scaffolds as immunotherapeutic agents. In parallel, several studies have focused on selected non-structural proteins such as protease and RdRp for small molecule drug discovery. Further studies are required to optimize their metabolic stability and pharmacokinetic properties. Progress made in recent years in producing human NoVs in cultured cells [96**,97**] may prove critical for robust optimization of such antiviral drugs and lead to a better understanding of the mechanisms that underlie virus replication, which in turn may pave way for discovering novel antiviral agents. Combined use of antivirals with an effective vaccine may indeed be realized in the near future to counter and control HuNoV infections.

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Highlights

- Development of capsid-based vaccines – a VLP-based vaccine in phase-II clinical trials –potential for multivalent vaccines
- Human monoclonal antibodies that block glycan binding - prospects for immunotherapeutic agents
- Viral protease and polymerase as targets for drug discovery
- Interferons and inhibitors of host cell factors as antivirals.

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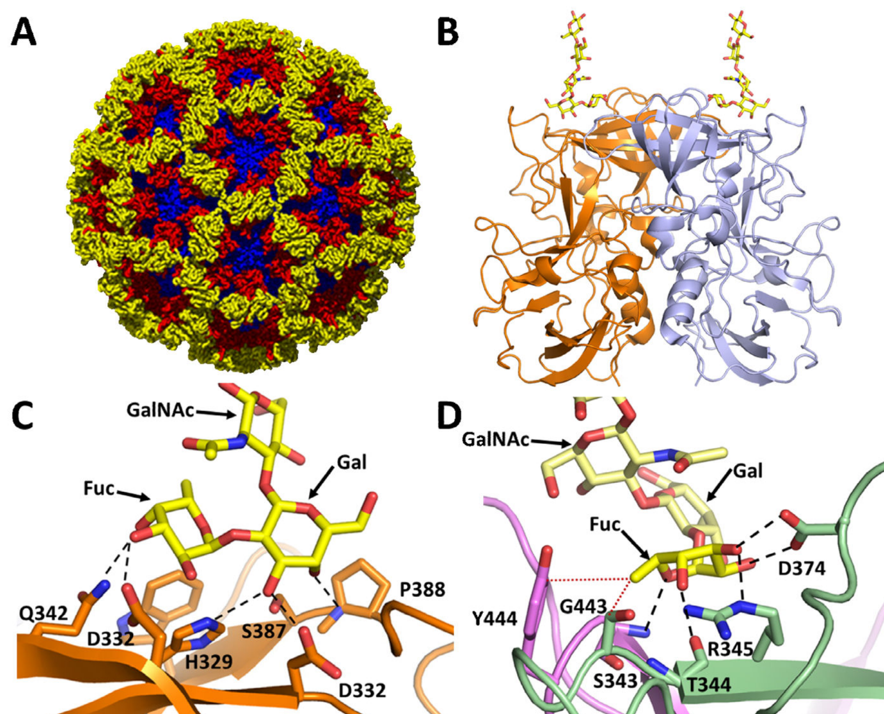

Fig. 1. NoV-HBGA binding site, a potential target for antivirals

A) Structure of the Norwalk virus-like particle (PDB ID: 1IHM) comprised of 90 VP1 dimers. The VPI S domain, P1 and P2 subdomains are shown in blue, red and yellow respectively. **B)** Cartoon representation of P-domain dimer (PDB ID: 2ZL6) bound to H type HBGA. The HBGA binding site is located on the top of the P domain. The individual subunits of the dimer are shown in orange and blue, respectively, and the H type HBGA is shown in yellow as a stick model. **C)** Close up of HBGA binding site in GI HuNoV showing the galactose dominant nature of HBGA binding. All the residues involved in hydrogen bond interactions with H type HBGA (yellow) are contributed by the individual subunits of the dimer shown in orange stick models with oxygen (red) and nitrogen (blue) atoms shown; hydrogen bonds are shown as black dashed lines. **D)** Close up of the HBGA binding site in GII.4 bound to H type HBGA (PDB ID: 3SLN) showing the fucose dominant nature of HBGA binding. The HBGA binding site in GII NoVs lies on the dimeric interface with both subunits of the dimer (green and pink) contributing to HBGA binding. Residues involved in hydrogen bond interactions (dashed lines) with HBGA (yellow) are labeled and bound HBGA is shown as a stick model following the same coloring scheme as in C.

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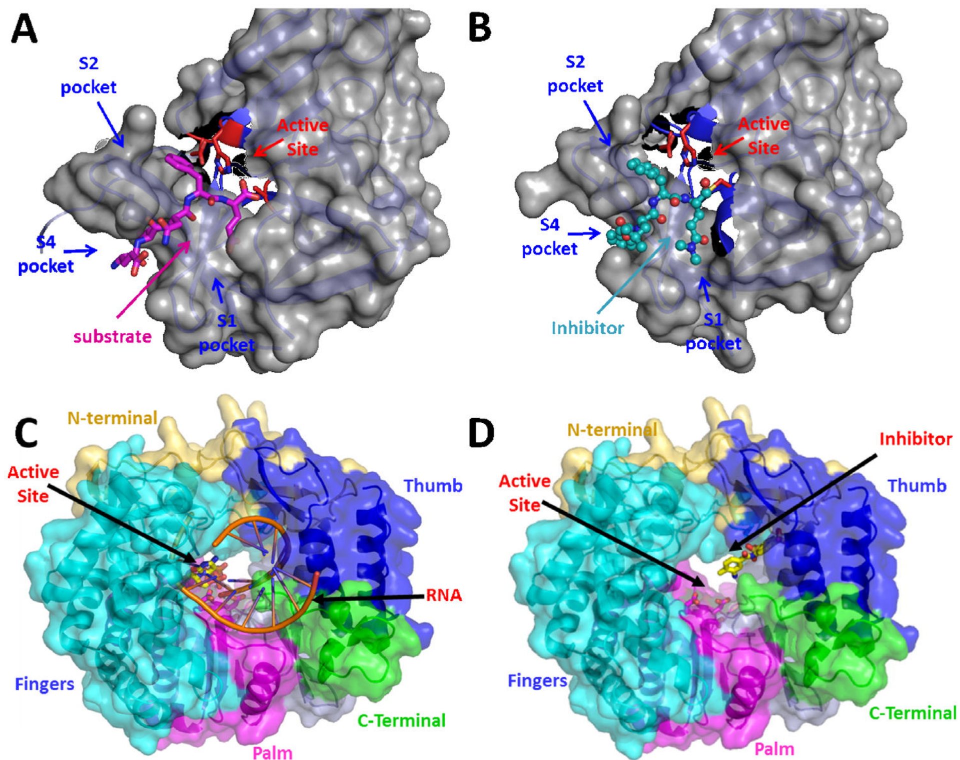

Fig. 2. HuNoV NSPs protease and RdRp as antiviral targets

A) Structure of the Norwalk virus protease bound to its natural substrate (PDB ID: 4IN1) (PDB ID: 4IMZ syc10). The protease is depicted in both surface (grey) and cartoon model (light blue). The substrate binding pockets are labeled S1, S2 and S4 and the residues forming the catalytic triad are shown as stick model in red and labeled active site. The substrate comprising residues INFE is shown in pink as stick model with oxygen and nitrogen atoms labeled in red and blue respectively. **B**) Structure of the Norwalk protease bound to substrate-based peptide inhibitor syc10 (PDB ID: 4IMZ). The protease is depicted similar to Fig. 2A. The inhibitor is represented as a ball and stick model in cyan and is shown to mimic substrate binding **C)** Structure of the norovirus polymerase, RdRp (PDB ID: 1SH2) bound to its primer-template RNA duplex. RdRp is depicted in both surface and cartoon model. The thumb (blue), fingers (cyan), palm (pink) domains, along with the N- (light yellow) and C-terminal (green) regions are indicated. The active site is labeled and is shown with bound primer-template RNA duplex (orange). D) Structure of the norovirus polymerase, RdRp (PDB ID 4NRT) bound to suramin inhibitor, which blocks RNA exit pathway, with each of the RdRp structural elements shown using the same coloring code as in Fig. 2C.