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REVIEW

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Computer-aided approaches for human norovirus drug discovery: a comprehensive review

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ABSTRACT

Introduction: Human norovirus is the leading global cause of viral acute gastroenteritis, with an estimated ~685 million cases and ~200,000 deaths annually. No licensed antivirals or vaccines are currently available. Despite historical limitations in robust in vitro models, structure and ligand-based computational approaches – supported by protease and polymerase crystal structures – have identified multiple chemotypes as potential antivirals.

Areas covered: This review provides an overview of all studies reported to date, indexed in public databases, in which computer-aided drug discovery (CADD) techniques have been employed. The authors report the computational methodologies used, the chemical structures of the identified compounds, and, if available, their biological activities. Where in silico results lack experimental validation, the authors highlight limitations and propose minimal validation assays.

Expert opinion: The absence of 3D structures for most viral proteins has limited the identification of novel chemotypes through CADD approaches. Furthermore, the lack of biological validation after *in silico* studies may slow down progress in this field, as researchers might focus on compounds that seem promising only at the computational level. Emerging systems such as human intestinal enteroids, together with AI/ML augmented CADD, can accelerate optimization and triage of non-nucleoside and covalent protease inhibitors.

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Norovirus; antiviral-research; computer-aided drug discovery; medicinal chemistry; structure-based drug design

1. Introduction

Human norovirus (HuNoV) is the leading cause of viral acute gastroenteritis (AGE), also referred to as 'winter vomiting disease,' with vomiting and diarrhoea being the predominant symptoms [1]. While adults generally recover within 2–3 days, immunocompromised patients may develop chronic gastroenteritis and, together with children under 5 years of age and the elderly, are considered the most at-risk groups [2]. Globally, HuNoV causes ~685 million cases of acute gastroenteritis annually, including ~200,000 deaths, predominantly in low- and middle-income countries [3]. Beyond the clinical burden posed by hospital outbreaks [4], norovirus infections also generate substantial economic costs, estimated at around US$65 billion in healthcare and social costs [5]. NoV is a non-enveloped, positive-sense single-stranded RNA virus belonging to the *Caliciviridae* family, with a genome made of three open reading frames (ORF1-3) [1]. ORF1 encodes a polyprotein that, after cleavage by the viral protease, produces six non-structural proteins: NS1-2 (p48), NS3 (NTPase), NS4 (p22), NS5 (VPg, covalently linked to the 5′ end), NS6 (the viral protease), and NS7 (the RNA-dependent RNA polymerase, RdRp) [1]. ORF2 encodes VP1, the major structural capsid protein [1], whereas ORF3 encodes VP2, an additional capsid protein [1]. Based on the VP1 sequence, NoV strains are classified into 10 genogroups (GI-GX), which are further subdivided into genotypes according to capsid sequence [6]. Additionally, each strain is assigned to one of sixty P-types (P1-P60) based on RdRp sequence [6]. Not all of them infect humans. Until recently, the GII.4 variant had been the most prevalent in humans; however, according to the data provided by public health agencies, GII.17 became the most frequently detected variant from July 2023 and June 2024 in Germany and France (accounting for 64% and 52% of outbreaks, respectively). It showed a proportional increase in Austria, England, and the United States, with rises of 39%, 30%, and 14%, respectively, when comparing the 2017–2023 period (excluding 2019/20, 2020/21, and 2021/22 seasons, during which surveillance was impacted by COVID-19 pandemic) with the 2023/2024 season [7]. Currently, no approved therapeutics exist for HuNoV infections. Five VP1-based vaccines are currently undergoing clinical trials. The most studied is Takeda's candidate (TAK-214), which demonstrated effectiveness of 61.8% against moderate to severe AGE caused by different genotypes (GI.1, GI.7, GII.2, and GII.4) in a phase 2b study [8]. Vaxart's candidate (VXA-G1.1-NN) was shown to be well tolerated and to elicit a substantial systemic immune response in a phase 1 clinical trial [8], and more recently completed the enrolment for a phase 1b trial to evaluate safety, tolerability, immunogenicity, and efficacy has been completed [9]. The most recent Moderna's candidate (mRNA-1403) showed to be safe and induced antigen-specific immune responses in a phase 1/2 study [10] and a phase 3 study to evaluate safety and

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Article highlights

- No vaccines or antivirals are currently available to treat norovirus infections, which are responsible for approximately 200,000 deaths annually.
- Several compounds with antiviral activity have been identified through screening campaigns, synthetic chemistry, and drug repurposing, yet no promising clinical candidate has emerged.
- Computer-aided drug discovery (CADD) techniques have been employed over the past decade to identify multiple compounds with potential anti-noroviral activity, whose optimisation could yield a clinical candidate.
- Preclinical research has been and remains hampered by the lack of a robust *in vitro* cellular model supporting efficient viral replication.
- The integration of emerging cellular models, such as human intestinal enteroids (HIEs), with *in silico* approaches based on AI/ML may accelerate the development of novel antivirals.

efficacy has been announced [11]. By contrast, the vaccine candidate developed by the National Vaccine and Serum Institute (NVSI) of China (Hansenulapolymorpha) underwent a phase I clinical trial to evaluate safety and immunogenicity, but the results have not been published yet [12]. Analogously, a phase I/IIa clinical trials was designed by the Anhui Zhifei Longcom Biologic Pharmacy Company to evaluate safety and tolerability of their vaccine candidate (Longkoma), but the study results have not been submitted [13]. Regarding antivirals, only two compounds have progressed to clinical trials: the antiparasitic nitazoxanide (NTZ) and the nucleoside polymerase inhibitor CMX521. NTZ has produced inconsistent results: symptomatic resolution was reported in a small cohort of transplant patients and/or undergoing chemotherapy or immunotherapy [14], as well as in two isolated case reports involving a 13-year-old male transplant recipient [15] and a 43-year-old adult transplant recipient [16]; improvement in the time of resolution of the symptoms was also observed in 13 norovirus-positive patients enrolled in a randomised, double-blind, placebo-controlled clinical trial [17]. However, NTZ demonstrated no clinical efficacy in a subsequent phase II trial conducted in transplant patients [18]. Conversely, no further updates about CMX521 have been reported after the encouraging results of a phase I study, where the drug candidate showed to be safe and well-tolerated in healthy volunteers [19]. Therefore, at present, no promising novel antivirals are in advanced development stages. This is largely due to the historical lack of robust *in vitro* cellular and animal models, which has significantly limited preclinical studies [20]. However, several molecules with potential antiviral activity have been identified through traditional medicinal chemistry approaches, including screening of in-house compound libraries and subsequent structure-activity relationship (SAR) studies, drug repurposing campaigns, and rational design based on empirical considerations. The main classes of compounds identified with these approaches are listed in Table 1. Their biological activity has been assessed in some cases using murine norovirus (MNV) as a surrogate, as it replicates efficiently in murine macrophage cell lines (RAW 264.7 cells) [54] and its inhibition can be readily evaluated via plaque reduction assays [55] or cytopathic effect (CPE) inhibition assays [56]. In other cases, a hepatocellular carcinoma cell line (Huh-7) bearing a HuNoV GI.1 replicon system (HG23 cells) has been employed [33]. Only in one example the antiviral activity was measured in stem cell-derived human intestinal enteroids (HIEs), a more recent and innovative *in vitro* model introduced by Ettayebi et al., which can also be infected with HuNoV GII and GIII genotypes [57]. Furthermore, for polymerase and protease inhibitors in particular, specific enzymatic inhibition assays have been used [58–62]. Robust *in vivo* animal models, such as Balb/c mice [63] and zebrafish larvae [64], have only emerged in recent years. Consequently, most of these inhibitors have not been tested in these *in vivo* systems.

In recent decades, the resolution of 3D protein structures via NMR or X-ray crystallography, the increase in computational power, and the availability of large on-demand compound libraries have led to a surge in the use of *in silico* approaches for hit compound identification, commonly referred to as computer-aided drug design (CADD) [65]. In particular, the availability of crystallographic structures within the Protein Data Bank (PDB) (https://www.rcsb.org/) enables techniques such as structure-based virtual screening (SBVS), in which a library of compounds is docked into the active or allosteric site of a target protein and ranked according to the predicted binding energy released during the interaction [66]. Furthermore, even in the absence of a 3D protein structure, the same compound libraries can be employed for ligand-based methods, where the steric and electronic features of compounds with known activity on the target are used as queries to identify new molecules with similar features [66]. Additionally, molecular dynamics (MD) simulations enable the temporal analysis of interactions within the binding site, providing insights into target affinity [66]. These approaches significantly reduce the time and cost required to identify compounds that will subsequently undergo biological evaluation and iterative medicinal chemistry process. In the context of human norovirus drug discovery, computational methods have been applied to identify compounds with potential antiviral activity. All studies reported to date where CADD was applied to discover potential antivirals for norovirus focused on three viral targets: the major capsid protein VP1, the viral 3CL protease, and the viral polymerase. The aim of this review is to summarise these efforts, highlighting both their potential and limitations. For this review, all relevant articles were systematically retrieved, without date restrictions, from three public databases (Google Scholar, Scopus, and PubMed) searching for the following terms in both titles and full texts: 'Norovirus', 'Norovirus AND compounds', 'Norovirus AND CADD', and 'Norovirus AND drug discovery'.

2. *In Silico* Approaches for norovirus Drug Discovery

2.1. *VP1*

VP1 is the major capsid protein and is divided in two domains: the shell (S) domain, which forms the inner layer protecting the viral RNA, and the protruding (P) domain, which is exposed to the surface and mediates the interaction with host cells [67]. When recombinant VP1 is expressed *in vitro*, 180 monomers self-assemble into virus-like particles (VLPs) with a T=3 icosahedral geometry analogous to that of infectious virions [67]. Viral entry is

Table 1. Anti-norovirus compounds identified following traditional methods.

Compound/Compound class	Source	Target	MNV	HuNoV (GI.1 replicon system)	Notes	Refs
Peptide-conjugated phosphoramidate morpholino oligomers (PPMOs)	Rational design	5′end of the viral genome	✓	✓	Target-complementary oligonucleotides	[21]
Di- and tri-peptidyl inhibitors	*In house* library screening	3CL protease (NS6)	✓	✓	Activity in a protease *in vitro* inhibition assay Activity in a Zebrafish larvae *in vivo* model	[22]
Rupintrivir	Drug repurposing	3CL protease (NS6)	✓	✓	Previously developed as HRV 3Cpro inhibitor	[23,24]
Nitazoxanide (NTZ)	Drug repurposing			✓	Previously commercialised as antiparasitic agent Inconsistent results from clinical trials	[14–18,25]
2′-C-Methylcytidine (2′-CMC)	Drug repurposing	RdRp (NS7)	✓	✓	Previously developed as HCV polymerase inhibitors Activity *in vitro* in HIEs Activity *in vitro* in B cells	[26–30]
Favipiravir (T-705)	Drug repurposing	RdRp (NS7)	✓		Previously developed for influenza virus infections Activity in an *in vitro* RdRp inhibition assay Activity in a Zebrafish larvae *in vivo* model	[28,31,32]
Ribavirin	Drug repurposing	RdRp (NS7)		✓	Used for the treatment of chronic HCV infection	[33]
NITD008	Drug repurposing	RdRp (NS7)	✓	✓	Previously developed for Dengue virus (DENV) infections Nucleosidic analogue (NA) of 2′-CMC	[34]
NIC02, NIC04, NIC10, NIC12	High-throughput screening (HTS)	RdRp (NS7)	✓	✓	Activity in an *in vitro* RdRp inhibition assay	[34]
5-Chloro-2-carboxamide derivatives	*In house* library screening	RdRp (NS7) Not confirmed		✓		[35]
Co-29	Synthetic exploration	RdRp (NS7)	✓			[36]
2′-Fluoro-2′-deoxycytidine	Drug repurposing		✓			[37]
Carbocyclic nucleosides	Synthetic exploration			✓		[38–40]
(*E*)-2-styrylchromones	Synthetic exploration		✓			[41]
Sulfamide-based derivatives	Synthetic exploration			✓		[42–45]
Vinyl-stilbene analogs	*In house* library screening/SAR studies			✓		[46]
Heterocyclic carboxamide derivatives	*In house* library screening/SAR studies		✓			[47]
Pyranobenzopyrone derivatives	*In house* library screening			✓	ACAT inhibitors ACAT-1 expression is increased in HG23 cells	[48]
Dasabuvir	Drug repurposing				Previously developed as HCV RdRp inhibitor Activity in GII.3 and GII.4 infected HIEs	[29]
Endoxifen, Netupitant, Pimozide, Regorafenib	*In house* library screening	3CL protease (NS6)			Activity in GII.4 infected HIEs Activity in a *in vitro* 3CL protease inhibition assay	[49]
Loxoribine, Resiquimod, Gardiquimod, Vesatolimod, Imiquimod	*In house* library screening	Toll-like Receptor 7 (TLR7) Host factor	✓		TLR7 agonism triggers immune system to produce cytokines with antiviral activity	[50]
WP1130 and derivatives	*In house* library screening	DUBs	✓	✓	Deubiquitinases (DUBs) are involved in viral replication Proteasome-associated USP14, fundamental for MNV replication in RAW 264.7 cells, is inhibited by WP1130	[51–53]

Abbreviations: HCV: Hepatitis C Virus; HRV: Human rhinoviruses; HIEs: Human Intestinal Organoids; ACAT: Acyl-CoA: Cholesterol acyltransferase.

mediated by the interaction of the P domain with human histo-blood group antigens (HBGAs), complex oligosaccharides located on the surface of gut cells [68], but also present in body fluids such as saliva and blood [69]. Accordingly, saliva-based enzyme immunoassays (EIA) [70], catch-and-release electrospray ionisation mass spectrometry assays [71], and saturation transfer difference nuclear magnetic resonance (STD-NMR) experiments [72] using VLPs generated from recombinant VP1 expression [73] have been employed to identify carbohydrates, small molecules, or polymers capable of blocking the VP1-HBGA interaction. However, none of these compounds have ever been evaluated for antiviral activity.

By contrast, although several X-ray crystal structures of the recombinant P domain from various HuNoV strains either alone or in complex with oligosaccharides, antibodies, or antibody fragments have been solved (Table 1) only few computational approaches have been applied to this target. This is mainly due to the lack of knowledge about the actual host receptor recruited by HuNoV, and the consequent absence of crystallographic structures bound to known inhibitors.

The X-ray crystal structure of the HuNoV GII.1 protruding (P) domain in complex with glycochenodeoxycholate (GCDCA), a bile acid which is an important co-factor for the cell entry [74], was used for a virtual screening of 24 natural compounds with known antiviral activity (retrieved from PubChem database [75]) using AutoDock Vina [76], with the aim of identifying potential novel inhibitors of the interaction with HBGAs [77]. Cyclocommunol was identified as a potential inhibitor, an MD simulation (100 ns) was performed to predict the binding status under physiological conditions, and its drug-likeness was further supported by *in silico* prediction of its ADMET properties [77]. This study was not supported by any antiviral assay, the number of compounds screened was relatively limited, and MD simulations should ideally have been conducted after *in vitro* activity has been confirmed, to rationalise the binding mode. Nonetheless, the study highlights the growing interest in plant-derived products as potential antiviral agents, and the increasingly prominent approach of phytochemical-based drug design [77].

In another study, a combination of molecular docking experiments (AutoDock 4.2 [78]) and MD simulations (100 ns) was employed to investigate the binding modes of 2′-fucosyllactose (2′-FL) and 3′-fucosyllactose (3′-FL) to the P domain of HuNoV GII.17 [79]. Experimental studies on these two trisaccharides, both part of the human milk oligosaccharides (HMOs) and known to inhibit the interaction between the virus and histo-blood group antigens (HBGAs) on gut epithelial cells by acting as decoy receptors, are limited by the high conformational flexibility of oligosaccharides and the difficulty of co-crystallising them with their target. The study elucidated the most likely binding modes of both compounds and indicated that 3′-FL exhibits superior binding efficacy. These insights may be valuable for future virtual screening of small-molecule libraries on these binding sites [79].

Another research group performed a virtual screening using AutoDock Vina [76], based on the X-ray crystal structure of VP1 in complex with a synthetic A-type trisaccharide (PDB ID: 2OBS), employing two small-molecule libraries: the Maybridge library [80] (500 compounds) and a selection of compounds from the ZINC database [81] (6423 compounds) [82]. The aim of the study was a fragment-based docking experiment to identify fragments that bind VP1 within the α-L-fucose binding pocket at distinct sites, but close enough to allow the linkage of the two fragments to generate higher molecular weight molecules potentially capable of inhibiting cell entry. The docking experiment was complemented by MD simulations of the top-ranked molecules and binding free energy (BFE) calculations employing the linear interaction energy (LIE) method [82]. Four compounds were proposed as fragments potentially able to inhibit the interaction with HBGAs [82]. However, no molecules resulting from the linkage of these fragments were synthesised, neither these fragments were evaluated for their ability to inhibit cell entry through STD-NMR experiments previously reported. The binding mode of A trisaccharide and the HuNoV VP1 P domain is depicted in Figure 1.

2.2. 3CL Protease

The protease belongs to the family of 3C-like cysteine proteases (3CLpro), which form a chymotrypsin-like fold and contain the catalytic triad Cys139, His30, and Glu54 within the active site. This motif is highly conserved not only in proteases of other caliciviruses but also in those of coronaviruses (3CLpro) and picornaviruses (3Cpro) [83]. Norovirus mediates cleavage of the ORF1-encoded polyprotein precursor through a nucleophilic attack by Cys139 at defined cleavage sites, while His30 functions as a general acid/base and Glu54 aligns His30 for deprotonation of the catalytic cysteine [83].

The first applications of computational methods to the design of protease inhibitors coincided with the first protease inhibitor series reported in the literature. Tiew et al. first demonstrated the anti-noroviral activity of a series of di- and tripeptidyl aldehydes [58], followed by a series of α-ketoamides and α-ketoheterocycles. These compounds bear an electrophilic

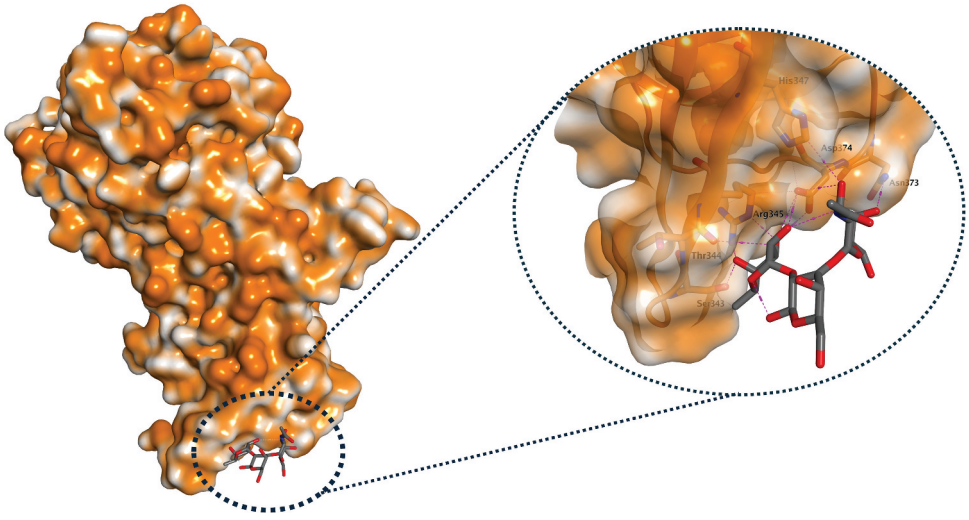

Figure 1. Surface representation of the HuNoV GII.4 VP1 P domain co-crystal structure with a synthetic A trisaccharide (PDB ID: 2OBS), showing polar/mildly polar surfaces in orange, hydrophobic surfaces in white and hydrogen bonds as dashed purple lines.

warhead that is likely targeted with a nucleophilic attack by the catalytic cysteine residue, leading to the formation of a tetrahedral intermediate that acts as a transition state (TS) inhibitor. Critical to this work was the first X-ray crystal structure deposited in the PDB of the Southampton norovirus 3C protease (SN3CP), bound to a peptide inhibitor specifically designed on the basis that the protein belongs to the (chymo)trypsin-like cysteine protease family, which is also conserved among other viruses of the picornavirus-like supercluster, in which caliciviruses are included [84]. These initial hit compounds were rationally designed starting from the co-crystallised inhibitor, and their binding modes within the active site were predicted using one representative compound of the series as a query to perform a genetic algorithm (GA) conformational search with SYBYL [85]. In this approach, the original inhibitor present in the crystallographic structure downloaded from the PDB was removed, and the new ligand was built in an analogous conformation. The subsequent conformational search identified the 20 energetically most favourable conformers, among which the top-ranked one was selected as the most probable binding pose of the ligand within the active site. Subsequently, a massive chemistry optimisation campaign was performed to enhance their activity and pharmacokinetic properties, to gain further insights on the mechanism of inhibition of the enzyme, to rationalise the binding modes through molecular mechanics (MM) simulations using the Avogadro program [86], and to generate additional X-ray crystal structures [23,42–45,87–93]. This hit-to-lead process represents the first example of a combined approach involving rational drug design and early-stage CADD techniques applied to norovirus drug discovery. Each compound generation was designed to enhance active site occupancy and optimise interaction patterns based on X-ray crystal structures from the previous series, occasionally supported by GA searches and MM simulations to rationalise the binding mode, as previously described. Generally, these di- or tri-peptidic inhibitors feature a glutamine residue at the P1 position, adjacent to the warhead, which is crucial for interaction with the S1 binding pocket of the enzyme. At the P2 position, residues such as phenylalanine, cyclohexylalanine, or leucine are typically employed, playing a key role in interactions with the S2 pocket. At P3, when present, greater modifications are tolerated and can be exploited to modulate pharmacokinetic properties. Various warheads have been explored: aldehydes, which represent the most reactive functional group towards the catalytic cysteine; bisulfite adducts, which generally display improved pharmacokinetic properties and act as 'pro-warheads,' converting to the aldehyde under physiological pH; α-ketoamides and α-ketoheterocycles, both exhibiting reduced reactivity compared to aldehydes but offering enhanced solubility and stability. In the case of α-ketoheterocycles, the ketone is incorporated within a heterocyclic scaffold connected to the P1 position, providing significant advantages in terms of lipophilicity and cellular uptake. An example of a co-crystal structure of in complex with a dipeptidyl inhibitor of this series is shown in Figure 2. Most of the inhibitors were active either in a cell-based GI.1 replicon model or against MNV in the micromolar/submicromolar range, one of them was effective *in vivo* in Balb/c mice [90] and also a gnotobiotic pig model (unpublished results) was employed [93]. Nevertheless, despite the promising *in vitro* and *in vivo* results, no recent updates have been reported on the lead compounds progressing to clinical trials.

Another significant contribution to the development of protease inhibitors derives from the release of the first crystal structure of a GII.4 protease, which enabled the *in silico* design of new classes of inhibitors specific for this genotype [94]. In this work, Muzzarelli et al. determined the crystal structure of GII.4 Minerva virus and tested in a FRET assay two compounds to make a comparative analysis between GI.1 and GII.4 PR [94]. These two molecules had been previously designed as selective GII.4 PR tripeptidic inhibitors using a GII.4 homology model based on the GI.1 template. One of these compounds showed to be 10-fold more active against GI.1 PR (GII.1 IC_{50} = 0.225 μM, GII.4 IC_{50} = 2.409), in contrast with the results of a molecular docking experiment, thereby underscoring the importance of having an actual crystal structure available for computer-aided drug design [94]. The same group had already designed and synthesised another series of tripeptidic inhibitors [95] using classic structure optimisation approaches starting from a previously identified hit [7], but the availability of the X-ray structure now offers the opportunity to design novel active scaffolds with CADD techniques. Moreover, the crystal

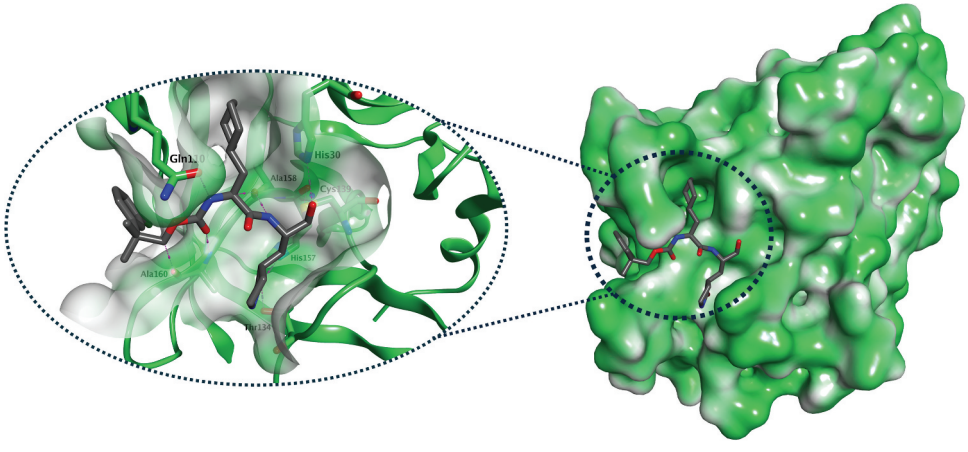

Figure 2. Surface representation of the HuNoV GI.I co-crystal structure with a dipeptidyl inhibitor (PDB ID: 6W5H), showing polar/mildly polar surfaces in green, hydrophobic surfaces in white, hydrogen bonds as dashed purple lines, and the covalent bond with Cys139 in yellow.

structures of the GII.4 Sydney 2012 norovirus, both in the apo state and in complex with an inhibitor, have been released and provide valuable platforms for *in silico* investigations [96].

A highly promising study was conducted to discover new protease inhibitors, employing a three-step SBVS protocol with AutoDock Vina [97] across five different compound libraries on the GII.4 norovirus 3CL protease (PDB: 8U1V) [60]. From an initial set of 58,836 compounds, which was used to perform a virtual high-throughput screening (HTS), the top 10% compounds were subjected to high-accuracy screening (HAS) and, finally, the top 10% of the second round underwent a refined screening, resulting in the selection of 17 compounds for evaluation in a FlipGFP assay [60]. Eight compounds (Figure 3), in particular the PI3K-AKT-mTOR pathway inhibitor Gedatolisib, showed activity and represent hits to be further optimised through medicinal chemistry campaigns, taking advantage of insights into key interactions within the protease active site predicted from their docking pose and MD simulations (100 ns) [60].

In a recent study, an alternative computational approach led to the identification of two peptidyl inhibitors, CIP-1 and its prodrug CIP-PD, capable of inhibiting HuNoV GI.1 replication in a replicon-based model (CIP-1 EC_{50} = 5.67 μM; TI = 17.64; CIP-PD EC_{50} = 0.46 μM; TI = 217.39). CIP-1 also demonstrated inhibition of HuNoV GII.4 3CL protease in a FRET assay (CIP-1 IC_{50} = 3.74 μM) [98]. These compounds were initially designed and evaluated as inhibitors of SARS-CoV-2 3CL protease and their antiviral activity was demonstrated in different cell lines. Subsequently, a search of the PDB25 database using the Distance matrix ALIgnment (DALI) server [99] with the first two domains of SARS-CoV-2 3CL protease as queries identified 24 structurally similar proteases. Several of these were selected for further investigation, including HuNoV GI.1 protease (PDB ID: 6W5H) and a hybrid model of HuNoV GII.4 3CL protease, generated using the 3D structures of HuNoV GII.1 3CL protease in complex with a dipeptidyl inhibitor (PDB ID: 6W5H) and unbound HuNoV GII.4 3CL protease (PDB ID: 6B6I) via the Bioluminate module of the Schrödinger suite [100]. These models were subsequently employed in docking experiments to predict the binding mode within the active site (Covalent Docking module of the Schrödinger suite [101]), with Prime MM-GBSA scoring used to estimate binding affinity and 100 ns molecular dynamics simulations to assess complex stability. The *in silico* predictions were subsequently confirmed through *in vitro* analysis of the compounds, suggesting a potentially useful workflow for drug repurposing.

A similar approach was employed in another study, where a multi-step VS protocol on the (PDB ID: 6W5H) was carried out using four different compound databases and the sequential application of three distinct docking modes with increasing precision starting with Glide [102] High-Throughput Virtual Screening (HTVS), followed by the more accurate Standard Precision (SP) and Extra Precision (XP) modes. This strategy enabled the identification of 6,827 ligands from an initial pool of more than 10 million commercially available compounds, subsequently reduced to 18 candidates through visual inspection and estimation of binding free energy using the Molecular Mechanics Generalized Born Surface Area (MM/GBSA) method. However, none of the selected compounds exhibited antiviral activity in a cell-based replicon system, highlighting

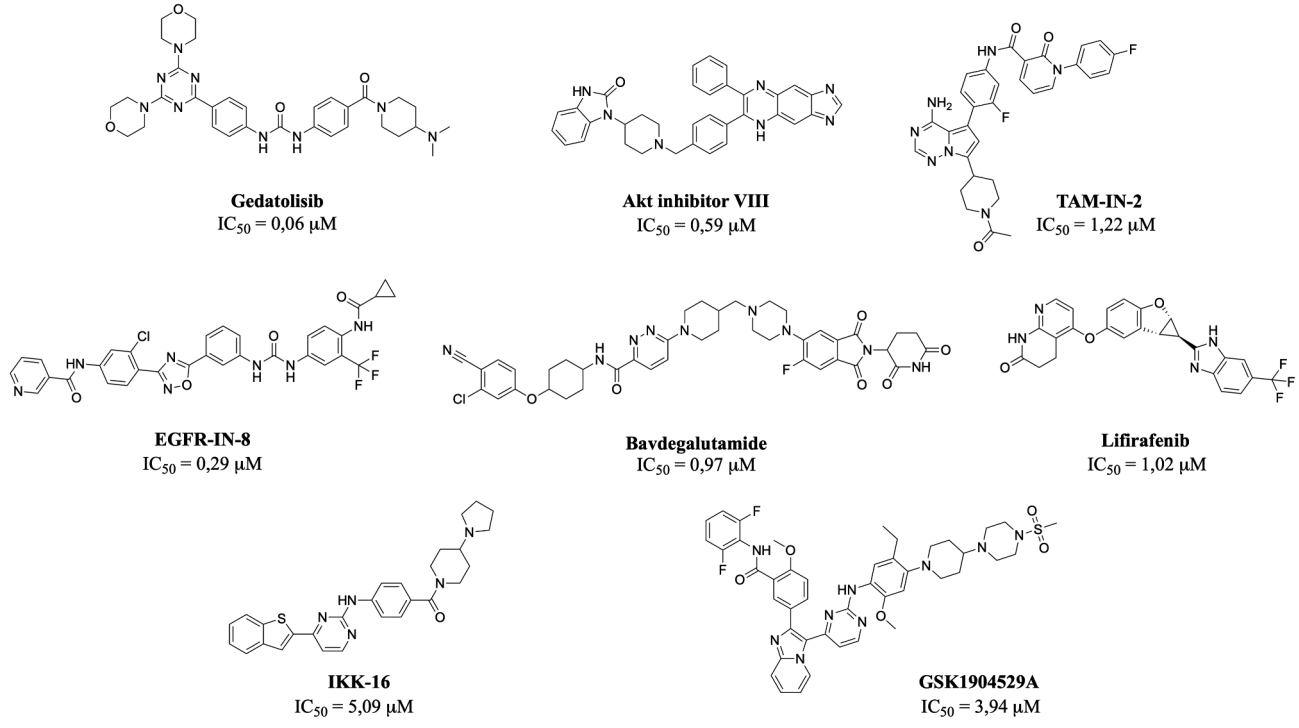

Figure 3. Chemical structures and enzymatic activity of protease inhibitors discovered with CADD.

the potential challenges related to cellular penetration that have also been reported for other compounds investigated for their anti-norovirus activity [103]. It should be noted that no enzymatic assays were conducted to at least assess the ability of these compounds to inhibit the enzyme activity.

Unfortunately, in several studies reported in the literature, the X-ray crystal structures of the norovirus protease have been employed in computational studies, which, however, were not followed by experimental biological evaluation.

In one of these, the protease crystal structure (PDB ID: 5T6F) was used for the virtual screening (PyRx 0.8) [104] of a small library of 700 compounds, including known HIV and HCV protease inhibitors, as well as integrase, reverse transcriptase, and other enzyme inhibitors [105]. The top 20 compounds were further subjected to a molecular docking experiment (AutoDock 4.2 [78]). Two potential hit compounds, the kinases inhibitors Sorafenib and YM201636, were identified based on their docking score, compared to the dipeptidyl inhibitor co-crystallised with the protease in the employed crystal structure, as well as interaction analysis and MD simulations (100 ns) [105].

An *in silico* study [106] was conducted to investigate the impact of two mutations in the protease on the binding of rupintrivir, a broad-spectrum protease inhibitor already known to inhibit HuNoV replication [23]. In addition to predicting the distinct interactions of rupintrivir with the wild-type protein (PDB ID: 2IPH) and the two mutants (generated with PyMol [107]) through docking experiments (AutoDock Vina [76]) and MD simulations (150 ns), the authors also performed a VS (AutoDock Vina) [97] of the Food Constituent Compound Collection (FOOD-lib) from the MTiOpenScreen platform [108] against the active site of both the wild type protease and a mutant. Amentoflavone was identified as a potential hit compound, but *in vitro* studies that validate these findings have not yet been reported [106].

Another group investigated the (PDB ID: 5T6F) as a target following a similarity search against the ChEMBL database [109] with the Extended-connectivity-fingerprint 4 (ECFP4) method (Python RDKit) [110] using five compounds in preclinical trials (2′-CMC and its prodrug Valopicitabine, Nitazoxanide, Suramin, and PPNDS) [111]. Ninety-seven molecules with a Tanimoto coefficient, the most widely used estimator of molecular similarity [112], of 0.6 or above were docked (SCORCH [113] and PLANTS [114]) in the active site of the protease. Three molecules (CHEMBL393820, CHEMBL2028556, and CHEMBL3747799) found in the top 10 compounds of both docking programs were also subjected to MD simulations (100 ns), free energy landscape (FEL) analysis, and MM/GBSA calculations to predict the binding stability. However, the study lacks *in vitro* validation, and the compounds were selected via a molecular fingerprint-based chemical similarity search, consequently no novel chemotypes with potential anti-norovirus activity were identified [111].

Recently, researchers constructed a machine learning-based 3D-QSAR model to identify potential inhibitors of the 3CL protease (PDB ID: 6W5L) [115]. The ML-based QSAR model was constructed and trained with 228 compounds retrieved searching 'Norovirus' in all Targets on the ChEMBL database [109]. This model was used for a screening of 9,699 compounds from the Bioactive Compound Library-I from Selleckchem (https://www.selleckchem.com/screening/chemical-library.html). The model identified 431 compounds as potentially more active than the original dipeptidyl inhibitor co-crystallised with the protease. This number was subsequently reduced to 64 through molecular docking experiments (AutoDock 4.2) [78] and, finally, three compounds with diverse chemical scaffolds according to Tanimoto similarity computation were selected. An MD simulation (300 ns) and MM/GBSA calculation highlighted one compound as a promising 3CL protease inhibitor. However, although the study is methodologically rigorous from a chemoinformatic perspective, no biological assays were carried out to validate the biochemical or antiviral activity of the selected scaffolds. Another potential limitation is that the compounds used to train the model act on multiple viral targets, rather than being specifically protease inhibitors [115].

2.3. RNA dependent-RNA polymerase

The RNA-dependent RNA polymerase (RdRp) catalyses the synthesis of the negative-sense RNA and, using the latter as a template, the viral RNA of new virions [116]. Similar to other polymerases of positive-sense RNA viruses, it adopts a partially closed right-hand conformation comprising the fingers, thumb, and palm subdomains [116]. The interaction between the fingers and thumb forms the template channel where the RNA template is located, and the template channel in turn merges with the central channel, where there is the exit of both the 3′-end of the template RNA and the growing chain [116]. At the junction of the template and central channels lies the NTP channel, through which nucleosides triphosphate (NTPs) are incorporated into the growing chain [116]. A key milestone for all *in silico* studies involving the polymerase was the discovery of two inhibitors of MNV and HuNoV RdRps, suramin and its analogue NF023, identified through a virtual screening of the Library of Pharmacologically Active Compounds (LOPAC, 1280 compounds) [117] with AutoDock4 [78] using a previously deposited X-ray crystal structure of MNV RdRp (PDB ID: 3BSN) [62]. Both suramin and NF023, bearing a polysulfonated naphtylamine central core, inhibit HuNoV (IC_{50} of 25 nM and 72 nM, respectively) and MNV polymerases (IC_{50} of 70 nM and 200 nM, respectively) in a fluorescence-based *in vitro* enzymatic inhibition assay and, importantly, the crystallographic structures of MNV RdRp in complex with the two inhibitors were solved, revealing their binding at what is now commonly referred to as Site A (Figure 4), the entry site for the NTP that is incorporated into the growing RNA strand [62]. Moreover, to overcome the poor pharmacokinetic profile of suramin, which is responsible for its lack of *in vivo* efficacy, few analogues possessing the same scaffold and lower molecular weight fragments were synthesised and evaluated [118]. Although these compounds retained inhibitory potency comparable to that of suramin against both MNV and HuNoV polymerases, this scaffold was not further investigated because it shows poor cell permeability and pharmacokinetic properties, as suramin and its derivatives have a high molecular weight and are polyanionic [119].

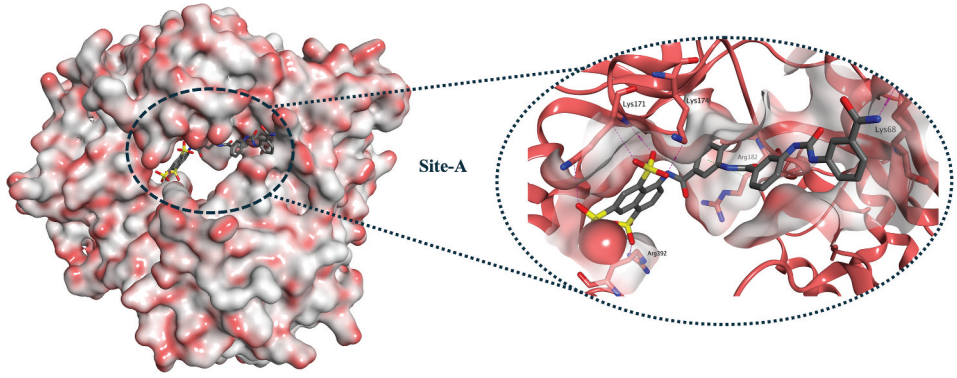

Figure 4. Surface representation of the MNV RdRp co-crystal structure with Suramin (PDB ID: 3UR0), showing polar/mildly polar surfaces in white, hydrophobic surfaces in dark pink and hydrogen bonds as dashed purple lines.

In a subsequent study by the same group, the crystallographic structure of HuNoV RdRp in complex with NAF2, the naphtalene-disulfonate portion of suramin/NF023 thought to represent the pharmacophoric moiety responsible for enzyme inhibition, revealed the existence of a second binding pocket for potential inhibitors, referred to as Site B [120]. An analogue of NAF2 with a higher molecular weight, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt (PPNDS), binds to this site with greater affinity, displaying submicromolar activity (IC_{50} = 0.45 μM) in the enzymatic inhibition assay [120]. Site B is located within a pocket along the exit channel through which the nascent RNA strand leaves the enzyme. The resulting X-ray co-crystal structures of HuNoV RdRp/PPNDS (Figure 5) and of MNV RdRp/PPNDS, subsequently deposited [121], have been widely used in later CADD studies.

Our research group has extensively investigated potential polymerase inhibitors, by combining *in silico* and *in vitro* approaches. In an initial study, the crystal structures of MNV RdRp in complex with suramin (PDB ID: 3UR0) and HuNoV RdRp in complex with PPNDS (PDB ID: 4LQ3) were used to perform, in parallel, a structure-based (PLANTS [114], LeadIT Flex [122] and Glide XP [102]) and a ligand-based virtual screening (ROCS 3D [123]), the latter selecting the occupational volumes and the pharmacophoric features of suramin and PPNDS as queries, respectively [124]. From the SPECS library of ~300,000 drug-like compounds [125], 62 candidates were selected and biologically assessed for their ability to inhibit HuNoV RdRp in two independent assays, from which two compounds were found to completely abolish enzymatic activity. The two active molecules were subsequently subjected to a SAR study, leading to the identification of a hit compound (**4**, Figure 6), bearing a furan-2-ylmethylene-pyrazolidine-3,5-dione, capable of inhibiting the polymerase of three different members of the *Caliciviridae* family (IC_{50} = 12.1 μM against MNV RdRp). Although the *in vitro* anti-noroviral activity against MNV-infected RAW 264.6 cells was limited (EC_{50} > 50 μM), the compound was shown to behave as a PPNDS antagonist in a combinational study and, therefore, to target RdRp, but at a distinct binding site, as supported by mutational analysis and molecular docking experiments [124]. Starting from this hit, we next employed an alternative *in silico* strategy to identify molecules with measurable activity in the cell-based assay. Because of the solubility issues of the initial compound, which likely were responsible for its lack of activity in RAW 264.7 cells, a series of more soluble analogues was synthesised, but these derivatives also showed to be inactive [126]. Therefore, the structure of the hit was used for a flexible alignment analysis using Flexible Alignment Tool in MOE [127], in which the lowest-energy conformer was kept rigid while searching for molecules with three-dimensional similarity with known inhibitors of other viral polymerases using MOE conformational tool [127]. This led to the design of a modified chemical scaffold, which enabled the synthesis of a series of hybrid molecules with

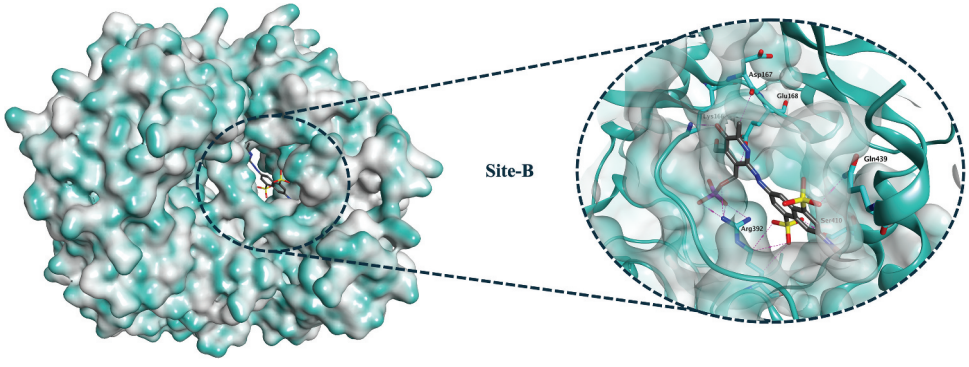

Figure 5. Surface representation of the HuNoV GII.4 RdRp co-crystal structure with PPNDS (PDB ID: 4LQ3), showing polar/mildly polar surfaces in white, hydrophobic surfaces in turquoise and hydrogen bonds as dashed purple lines.

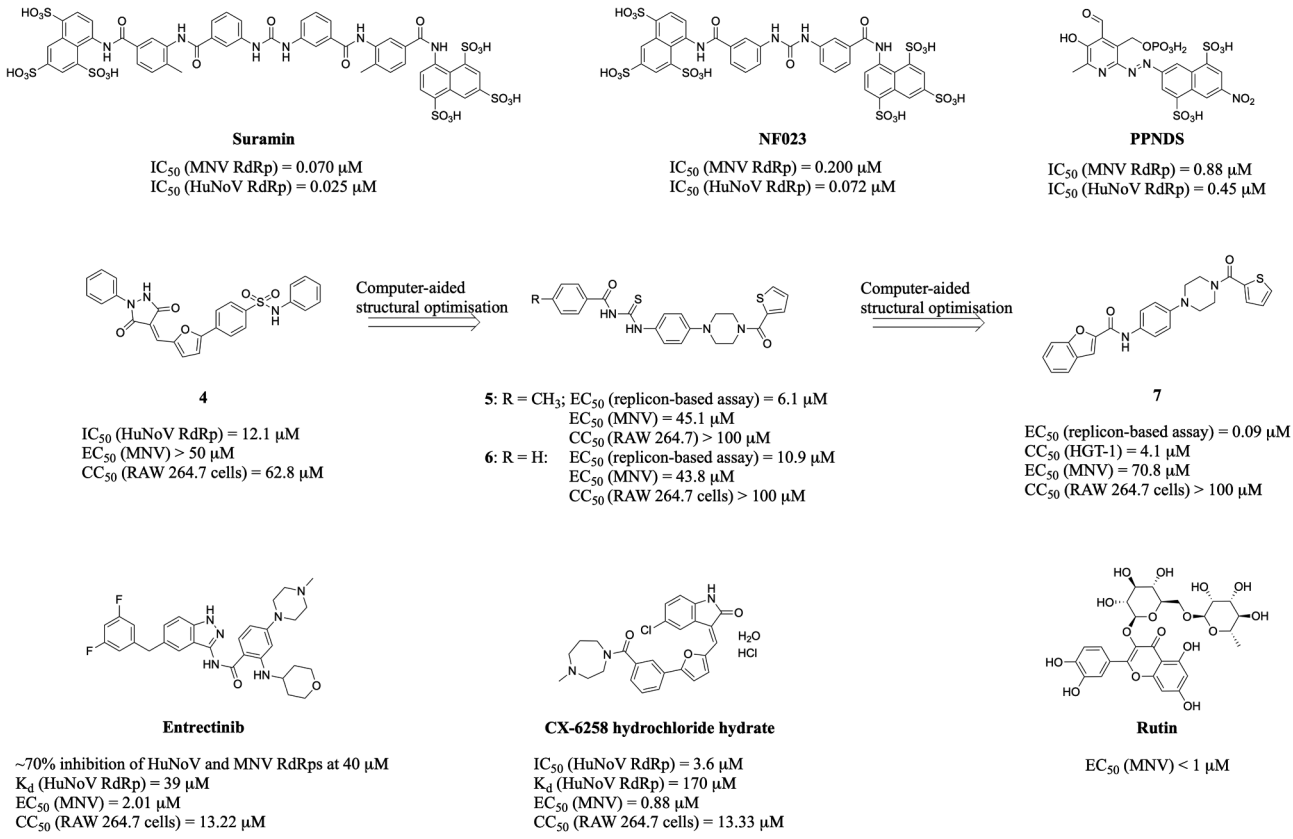

Figure 6. Chemical structures and biological activity of HuNoV RdRp inhibitors discovered with CADD.

activity in the MNV assay. Importantly, two of these new compounds (**5-6**, Figure 6), possessing a ((4-(piperazin-1-yl)phenyl) carbamiothioyl)benzamide central scaffold, also displayed low-micromolar activity in a replicon-based assay (HGT-1 cells) [126]. Starting from these compounds, a further effort in structural optimisation was undertaken in a third study, with the dual aim of discovering more potent analogues in the cell-based assays (**7**, Figure 6) and, importantly, of generating a dataset for the construction of an *in silico* 3D-QSAR model (using Forge V10 software [128]) of inhibitors targeting both MNV and HuNoV RdRp, which will guide the rational design of novel compounds [129]. In the final study of this series, five structurally diverse scaffolds, corresponding to the most active compounds from the enzyme-based assay previously carried out, were analyzed through molecular docking (Glide SP) [102] at Site B of the HuNoV RdRp (PDB ID: 4LQ3), to identify common interaction patterns and, importantly, unoccupied regions within the enzyme. This guided the rational design of 25 new compounds, two of which (**8-9**, Figure 6) achieved >50% inhibition of HuNoV RdRp activity, representing a further milestone in this hit-to-lead process [130].

Another virtual screening study (AutoDock Vina) [97] was conducted on site B of HuNoV RdRp (DPB ID: 4LQ3) and on the allosteric site of Dengue virus (DENV) RdRp (PDB ID: 5F3T), using the TargetMol's Bioactive Compound Library Plus [131] (7,568 compounds) and the 3D-Biodiversity Library [132] (32,248 compounds) [133]. Twenty chemical entities, selected through visual inspection among the top-ranked compounds of both targets, were evaluated in HuNoV and DENV RdRp through fluorescence-based enzymatic inhibition assays. One of them, entrectinib (RAI-13) (Figure 6), inhibited both enzymes (~70%) at 40 μM, as well as the replication of MNV in infected RAW 264.7 cells (EC_{50} = 2.01 μM: CC_{50} = 13.22 μM) and DENV2 in infected A549 cells (EC_{50} = 2.43 μM; CC_{50} = 20.75 μM). Moreover, it exhibited a good affinity for HuNoV RdRp (K_d = 39 μM) in a Bio-layer interferometry (BLI) binding assay and MD simulations (10 ns) followed by MM/GBSA calculations for both the complexes with DENV and HuNoV RdRp were carried out to predict the binding mode. Therefore, entrectinib represents a potential starting point for an optimisation campaign aimed at developing a broad-spectrum antiviral [133].

Following the previous methodology, the same research group performed a new virtual screening on the HuNoV RdRp using the MCE bioactive compound library HY-L001 [134] (13,980 compounds) [135]. The 23 top-ranked compounds were biologically evaluated for their ability to inhibit HuNoV RdRp. For the two compounds exhibiting over 75% inhibitory activity, IC_{50} values and dissociation constants (K_d) were determined using the BLI assay, along with assessments of antiviral activity against MNV in RAW 264.7 cells and cytotoxicity in the same cell line. CX-6258 hydrochloride hydrate (Figure 6), a pan-Pim kinases inhibitor, showed enzymatic inhibition in the micromolar range (IC_{50} = 3.61 μM) and antiviral activity at a sub micromolar concentration (EC_{50} = 0.88 μM), with moderated cytotoxic concentration (CC_{50} = 13.33 μM). The binding affinity was higher than entrectinib (K_d = 170 μM), but CX-6258 represents a novel non-nucleoside

polymerase inhibitor identified through computational techniques and a starting point for further structural optimisation [135]. In another work, a solvated molecular model of HuNoV RdRp was constructed in complex with CTP/ATP with homology-modelling feature in Molecular Operating Environment (MOE) [136] starting from a previously deposited HuNoV RdRp crystal structure in complex with CTP (PDB ID: 3BSO), which contained some unstructured residues [137]. These nucleotides were then replaced with different pyrimidinic and purinic NTPs previously identified as inhibitors, and the binding free energy was calculated using free energy perturbation (FEP) calculations [137]. The results were compared with EC_{50} values obtained from a replicon-based assay and/or with nucleotide incorporation efficiency determined experimentally. Despite some discrepancies with the experimental data, particularly for compounds displaying only minor differences in binding energy, the model appears effective in predicting whether a compound is active or completely inactive. A subsequent virtual screening of 121 NTPs, generated by attaching fragments to natural nucleotides (combinatorial builder part of Molecular Operating Environment package), was then performed running FEP simulations (NAMD [138]) with a supercomputer. The synthesis and biological evaluation of the most promising candidates warrant further experimental assessment [137].

Another example is a multi-step SBVS protocol performed on the MNV RdRp (PDB ID: 3UPF) using the 'Zinc In Man' library of compounds already used in humans [139] (11,421 compounds) [140]. Glide [102] SP and XP, progressively, were employed, and the best 37 molecules were docked with the QM-Polarised Ligand Docking Approach [141]. Of the top sixteen candidates identified through visual inspection, four were purchased based on commercial availability, and evaluated for antiviral activity against MNV in a cell-based assay, with one compound, rutin (Figure 6), displaying activity at concentrations as low as 1 μM and cytotoxicity at 100 μM. However, no EC_{50} was determined through a dose-response experiment, and although a time-of-addition assay demonstrated that rutin does not inhibit viral replication by blocking cell entry, the compound failed to show inhibitory activity in an RNA synthesis assay using recombinant GII.4 HuNoV RdRp, or in a FRET-based assay with MNV-1 and GII.4 HuNoV proteases [140]. Further investigations are therefore required.

As with other targets, the polymerase has also been investigated *in silico* without subsequent *in vitro* evaluation of the virtual hits identified. Given the structural similarity between the active site of HuNoV RdRp and that of Hepatitis C Virus (HCV), a machine learning (ML)-based model was developed using HCV RdRp inhibitors and non-inhibitors to predict potential HuNoV RdRp inhibitors [142]. The model was trained with 188 compounds from BindingDB [143] and PubChem [144] databases, divided in active ($IC_{50} \geq 50$ μM) and inactive ($IC_{50} \leq 50$ μM), while the virtual screening was conducted on the ChEMBL database [109] (1,766 compounds), based on molecular fingerprints and 1D/2D molecular descriptors generated with WEKA software [145]. The compounds were docked with OEDocking's FRED procedure [146], following filtration based on pharmacokinetic parameters. Two potential hits with a predicted better absorption than the native ligand and a similar binding landscape were evaluated with MD simulations (50 ns), leading to the identification of a potential inhibitor; however, experimental *in vitro* studies are required to confirm these findings [142].

In another study, a virtual screening on the HuNoV RdRp (PDB ID: 4LQ3) using the Antiviral Screen Database established by the National Cancer Institute (NCI) [147] branch of the National Institute of Health (NIH) (42,390 compounds in total) [148]. First, a pharmacophore query (e-pharmacophore) was generated capturing the most relevant features of the interaction between the co-crystallised ligand, PPNDS, and the RdRp [102], and then used to pre-filter the compounds database. The compounds matching the query were then screened in following stages of progressively increasing precision (Glide HTVS, SP, e-pharmacophore based-screening, Glide XP). The top-ranked candidates were further filtered using induced fit docking (IFD), MD simulations (100 ns), and QM/MM calculations, identifying two virtual hits bearing a scaffold with a pyrimidindinone core, but no experimental confirmation was provided [148].

In an earlier study employing a similar approach, the clinical candidate CMX521 [119] was used to construct a pharmacophoric model used as an input for a virtual screening of 26,682 compounds retrieved from the PubChem database on the basis of their structural similarity to CMX521 [149]. The compounds were first narrowed down to 706 compounds through docking on the MNV RdRp (PDB ID: 3SFU) (Glide HTVS mode) [102], after that using the receptor grid generated with CMX521, 18 virtual hits were individuated according to the fitness scores in the pharmacophoric sites (Glide XP mode). The candidates were further classified according to the docking scores and analyzed through MM/GBSA calculations and MD simulations (50 ns), leading to the identification of two compounds (CID-57930781 and CID-44396095) worthy of experimental investigation [149].

In a further study whose findings have not been validated experimentally, a SBVS (PyRx 0.8 [104]) of a library comprising 473 natural compounds from the ZINC database [81] was performed on the active site of HuNoV RdRp (PDB ID: 4LQ3), leading to the identification of 12 compounds with lower binding energy (BE) values than PPNDS, used as a reference [150]. The two most promising compounds (ZINC66112069 and ZINC69481850) were further analyzed with MD simulations (100 ns), which predicted the formation of stable complexes [150]. Finally, another SBVS was conducted (PyRx 0.8 [104]) using an internal library of 752 compounds with known antiviral activity against other viral enzymes and FDA-approved drugs, of which 10 exhibited better binding affinity than PPNDS [151]. In both these studies, the number of compounds screened was relatively small, and the absence of biological assays limits their chemoinformatic relevance. However, the presence of ribavirin, a compound already demonstrated to exert anti-noroviral

activity in previous studies [33], among the top five ranked compounds in the second study suggests that the reported compounds may be worth of experimental evaluation.

3. Practical guidance for validation

Most computational studies conducted on the three viral proteins for which structural data are available have not been followed by any *in vitro* biological validation. Despite the lack of a robust *in vitro* model, which has hindered the progression of potential anti-noroviral compounds into clinical studies, it remains essential to employ the *in vitro* assays developed thus far to confirm antiviral activity, or at least enzymatic inhibition, of compounds identified through CADD techniques.

The historically most employed surrogate, MNV, remains the fastest model for assessing the antiviral effect of potential hits. The activity of a compound can be rapidly quantified using a plaque-reduction assay [41]. In this assay, MNV replicates in a monolayer of murine macrophages (RAW 264.7 cells), forming plaques in the presence of agarose, which restricts viral diffusion so that replication occurs only in immediately adjacent cells, causing localised CPE in areas not stained by a dye that penetrates only viable cells. Cells are incubated for a defined period with a dilution of MNV that produces a known number of plaques per well and subsequently incubated for 24 h in the presence or absence of the test compound. The IC_{50} is calculated as the concentration of compound required to reduce plaque numbers by 50% relative to the control [41]. An alternative is the 50% tissue culture infectious dose ($TCID_{50}$) assay, in which RAW 264.7 cells are infected with serial dilutions of the virus, and the $TCID_{50}$ corresponds to the dilution causing CPE in 50% of cells observed microscopically. The IC_{50} of the inhibitor is defined as the concentration needed to reduce CPE by 50% relative to control [37]. Cellular viability can also be quantified via a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), with antiviral activity expressed as EC_{50}, the concentration of the compound needed to restore 50% of the viability of infected cells relative to control [35]. All these examples measure viral infectivity, but the effects of a compound can also be quantified on viral RNA levels via qRT-PCR, or on viral protein expression using western blotting or confocal microscopy fluorescence [37].

Conversely, the most commonly used HuNoV model is the HG23 cell line, Huh7 hepatocellular carcinoma cells bearing the HuNoV GI.1 replicon. In this model, the antiviral activity of a compound is determined by its ability to reduce HuNoV genome levels detected via qRT-PCR or protein levels analyzed via western blotting relative to control [33]. An advantage of this model is the possibility to perform sequence analysis of the HuNoV genome region after treatment with a potential antiviral, allowing the identification of mutations and therefore confirming target engagement [33]. More recently, the HuNoV model has shifted toward Human Intestinal Enteroids (HIEs), human induced pluripotent stem cell-derived intestinal organoids, in which the clinically relevant HuNoV GII.4 strain can replicate [57]. To date, a single drug screening of 326 compounds using J2 HIE 2D monolayers (derived from adult jejunal tissue) demonstrated the antiviral activity of dasabuvir (DSB), already approved for Hepatitis C virus (HCV) treatment, with antiviral activity being measured as the ability to reduce HuNoV genome levels detected via qRT-PCR [29]. More recently, HuNoV replication has been demonstrated in differentiated 3D HIEs (derived from fetal ileum), which are more physiologically relevant than 2D monolayers due to preservation of 3D spatial organization, and can be used as a platform for drug screening, as previously identified active compounds effectively reduce viral replication in this system [152]. Finally, HuNoV GII.4 has been found to replicate efficiently in a human Burkitt lymphoma B cell line (BJAB) [153], and this model has been used to confirm the antiviral activity of 2′-C-methylcytidine, which reduces HuNoV genome levels as measured through qRT-PCR [30]. Although this is the only example reported to date, it highlights the potential of BJAB-infected cells as a drug screening platform.

While the MNV model may be considered outdated, the GI.1 replicon also has limitations, including the absence of a complete viral cycle, lack of structural protein expression, absence of innate immune response typical of natural enterocyte infection, and accumulation of mutations during cell culture [154]. Conversely, organoids are costly to maintain in quantities sufficient for screening campaigns. Nonetheless, multiple biochemical assays are available to measure the activity of potential hits on various viral targets and should be used, given that most of the new hit compounds emerge from VS on the three proteins with known crystallographic structures.

For compounds potentially inhibiting the VP1-HBGA interaction, a saliva-based enzyme immunoassay (EIA) can be used: VLPs incubated with an inhibitor are unable to bind HBGAs commonly present in saliva coated on ELISA plates [70]. Subsequent addition of an anti-norovirus antibody and a secondary antibody catalysing a substrate-specific reaction results in low absorbance relative to control. An alternative is the catch-and-release electrospray ionization mass spectrometry (ESI-MS) assay. In this approach, protein-ligand complexes are ionized under 'soft' conditions, preserving noncovalent interactions, allowing the mass spectrometer to detect protein-ligand adducts [71]. Controlled collision-induced dissociation then releases ligands, which are detected as free ions, enabling identification and relative affinity quantification. Saturation transfer difference nuclear magnetic resonance (STD-NMR) experiments can also be conducted using VLPs: selective irradiation of the protein transfers saturation to bound ligands, and the difference between the two spectra highlights only ligand signals interacting with the P-domain, revealing inhibitor binding sites [72].

For protease inhibitors, the FRET-based assay is the most widely used [58,59]. A typical FRET assay for 3CL protease employs a peptide substrate bearing a fluorophore and quencher at opposite ends; protease cleavage separates the two, relieving quenching and yielding a fluorescence increase over time that directly measures enzymatic activity and

Table 2. Most frequently used crystallographic structures of norovirus viral proteins for virtual screening deposited in the PDB.

Macromolecule	Ligand	Genotype	PDB ID	Resolution
VP1 P domain	GCDCA	HuNoV GII.1	6GVZ	1.54 Å
VP1 P domain	HBGA type B	HuNoV GII.4	2OBT	2.00 Å
VP1 P domain	3-fucosyllactose	HuNoV GII.17	5LKK	1.49 Å
3CLpro	Peptide inhibitor	HuNoV GI.1	2IPH	1.75 Å
3CLpro		HuNoV GI.1	3UR6	1.50 Å
3CLpro		HuNoV GII.4	6B6I	2.44 Å
3CLpro		HuNoV GII.4	8U1V	2.79 Å
3CLpro	Dipeptidyl inhibitor	HuNoV GI.1	6W5H	1.85 Å
3CLpro	Dipeptidyl inhibitor	HuNoV GI.1	5T6F	1.90 Å
3CLpro	Dipeptidyl inhibitor	HuNoV GI.1	6W5L	2.10 Å
3CLpro	Peptide inhibitor	HuNoV GI.1	2IPH	1.75 Å
3CLpro	Dipeptidyl inhibitor	HuNoV GII.4	8U1W	1.84 Å
RdRp		MNV-1	3UQS	2.00 Å
RdRp	NF023	MNV-1	3UPF	2.60 Å
RdRp	PPNDS	MNV-1	4O4R	2.40 Å
RdRp	Ribavirin	MNV-1	3SFU	2.50 Å
RdRp	Cytydine-5′-triphosphate	HuNoV GI.1	3BSO	1.74 Å
RdRp	5-nitrocytidine	HuNoV GI.1	3BSN	1.80 Å
RdRp	PPNDS	HuNoV GII.4	4LQ3	2.60 Å
RdRp	NAF2	HuNoV GII.4	4LQ9	2.04 Å

inhibition. Alternatively, another study reported the use of a FlipGFP assay [60]. In this assay, the Green Fluorescent Protein (GFP) sequence is interrupted by a cleavage site specific to the norovirus 3CL protease. Depending on the presence or absence of an inhibitor, the protease is either able or unable to cleave the sequence and restore fluorescence emission. In both cases, IC_{50} is calculated as the concentration inhibiting 50% of protease activity.

Potential RdRp inhibitors can be tested using either a fluorescent RdRp activity assay or a gel-based elongation assay. In the fluorescent assay, poly(C) primed with oligoG_{12} is used as a template and GTP as a substrate in the presence of HuNoV or MNV RdRp. dsRNA synthesis is monitored via fluorescence with a specific stain (PicoGreen), and the enzyme pre-incubated with test compounds has a lower signal compared to the control if inhibition occurs [62]. In the gel-based assay, a hairpin-shaped RNA template undergoes primed transcription in the presence of GTP when incubated with the HuNoV or MNV RdRp, and dsRNA synthesis of known length is monitored using TBE-urea gel [155]. In both assays, IC_{50} is defined as the concentration reducing RNA synthesis by 50%.

4. Conclusions

Several *in silico* approaches have been employed to identify novel compounds with potential antiviral activity against norovirus, the causative agent of viral acute gastroenteritis (AGE). The major capsid protein, VP1, has been primarily investigated through molecular docking and molecular dynamics (MD) simulations to explore the binding mode of 2′-fucosyllactose and 3′-fucosyllactose, two known inhibitors of VP1-HBGAs interactions. The insights gained from these studies may support the rational design of novel entry inhibitors, although entry inhibitors are currently of limited interest, as the specific host receptor involved in the interaction with the virus remains unknown. In contrast, crystallographic structures of the 3C-like chymotrypsin protease (3CLpro) and the RNA-dependent RNA polymerase (RdRp) in complex with inhibitors have been exploited to perform structure-based and ligand-based virtual screenings, often complemented by MD simulations. These efforts led to the identification of several compounds showing activity against murine norovirus (MNV), commonly used as an *in vitro* surrogate, and in GI.1 HuNoV replicon-bearing Huh-7 cells (HG23 cells). On the other hand, a considerable proportion of computational studies have not been followed by any form of experimental evaluation of the virtual hits presented. Although the persistent lack of a robust cellular model continues to hamper preclinical research, and apart from Nitazoxanide and CMX521 no other small molecule has advanced to the clinical stages of development, computational studies should still always be followed by at least a biochemical evaluation.

5. Expert opinion

CADD techniques are nowadays fundamental for the discovery of novel compounds with therapeutic potential and compared with more traditional approaches such as high-throughput screening (HTS), in-house library screening, rational drug design, and synthetic exploration, they significantly reduce the time and cost required to identify new hit molecules. In the context of norovirus drug discovery, the promising compounds such as 2′-C-methylcytidine (2′-CMC), or less specific agents studied in clinical trials, such as nitazoxanide, were primarily discovered through drug repurposing campaigns. This can be attributed to two main reasons:

- The lack of crystallographic structures of certain viral proteins, either alone or in complex with inhibitors.
- The absence of robust models for cell-based antiviral assays.

Currently, only the crystal structures of the VP1 P domain, the protease, and the polymerase are available in the Protein Data Bank (PDB). The 3D crystallographic structures used in the studies listed in this review are summarised in Table 2 (see Table SI

for the list of all the proteins present in the PDB). While the latter two have been quite extensively exploited through virtual screening experiments, VP1 has attracted less interest, as the P domain is subject to strong evolutionary pressure and thus drives the emergence of new strains [156]. Moreover, the specific receptor for HuNoV remains unclear, making it difficult to determine which site should be targeted in virtual screening experiments, aside from the site involved in interactions with histo-blood group antigens (HBGAs). In this context, VP1 appears more suitable for vaccine development or, at most, for the design of antibodies, which can achieve higher specificity of interaction. Notably, all vaccines currently in clinical trials are either VLP-based (Takeda, NVSI of China), or consist of salt-adjuvanted VP1s (IPS), or employ recombinant adenoviral vectors carrying genes which encode for VP1s (Vaxart), or, alternatively, are mRNA vaccines encoding VP1s (Moderna). Moreover, a recent study demonstrated that a llama-derived nanobody is able to inhibit HuNoV GII.4 replication in HIEs by binding to an epitope conserved among different strains, distinct from the HBGAs binding site [157]. In contrast, the structure and catalytic motifs of the protease and polymerase are well characterized and share similarities with those of coronaviruses, picornaviruses, and other positive-sense RNA viruses. Consequently, known compounds have been repositioned as potential anti-norivirus agents once studied for other viruses considered more urgent clinical issues (HCV, DENV, coronaviruses, etc.), but they have not shown efficacy. Resolving the 3D structures of NS1/2, NS3, NS4, and NS5, which play key roles in viral replication, and performing *in silico* studies on these targets would be crucial to expand the chemical space of compounds specific for norovirus infections explored to date. The difficulty in solving the crystallographic structure of NS1/2 and NS5 lies in their disordered nature: NS1/2 is classified as an intrinsically disordered protein (IDP) [158], as its N-terminus (NS1) contains an intrinsically disordered region (IDR), namely a highly disordered proline-rich domain that does not adopt stable tertiary structures. Consequently, the protein exhibits aberrant migration during size exclusion chromatography [159]. NS5 (VPg) also contains disordered regions at both its N- and C-termini, and attempts to resolve its crystallographic structure concluded that NS5 is disordered in the crystals [158]. In contrast, NS3 and NS4 tend to associate with lipid structures [160,161] and do not appear in a soluble, monodisperse form, a prerequisite for crystallization. Even in the absence of experimentally resolved structures, alternative strategies can be employed to construct three-dimensional models of these proteins. However, homology modelling, which is typically employed to construct the 3D structure of a protein using another protein with high sequence homology whose structure has already been resolved, has not been applied in this case, as no proteins with significant sequence similarity to NS1/2, NS3, NS4, or NS5 are currently available. Instead, approaches such as threading, based on fold recognition, or *ab initio* methods, which rely exclusively on the amino acid sequence, may be employed to generate structural models suitable for *in silico*, structure-based drug design, albeit with generally lower accuracy.

The lack of a reliable cell-based model of the viral replication suitable for inhibitor evaluation is closely linked to the first limitation. Indeed, from one prospective, this hinders a comprehensive understanding of the virus pathophysiology, which is crucial for both elucidating the functions and mechanisms of proteins whose 3D structures have not yet been resolved and for identifying host factors that may represent additional targets for computational studies. Conversely, the absence of a robust HuNoV cellular system prevents reliable antiviral evaluation, which is essential once a set of compounds has been selected through computational approaches. While many compounds have been identified through virtual screening on the active sites of the protease and polymerase, a significant portion demonstrated activity against MNV but not in the HuNoV GI.1 replicon-based assay, or vice versa. The reasons for these discrepancies remain unclear. Besides the obvious biological differences between the two viruses, RAW 264.7 cells used to propagate MNV are less metabolically active than HG23 cells, potentially masking the efficacy of compounds that require metabolic activation. Conversely, the replicon-based model is less suitable for large-scale compound library screening, as using it is more time-consuming, and it evaluates activity against a single viral strain, GI.1, which is of less clinical relevance compared to the GII.4 and GII.17 genotypes. Moreover, the absence of key steps of the viral replication cycle (cell recognition, cell entry, and encapsidation) restricts screening campaigns to potential inhibitors of non-structural proteins alone, and adaptive mutations in these proteins may also occur. Finally, only two research groups have so far succeeded in establishing HuNoV infection in a human B cell line, and only for certain GII subtypes (GII.4 and GII.6). As a result, this model remains insufficiently characterised and not robust enough to be considered suitable for large-scale compound library screening. The most physiologically relevant model is currently represented by infected human intestinal enteroids (HIEs), which better reflect HuNoV pathophysiology. However, these are costly to maintain and are not suitable for high-throughput screening of extensive compound libraries, but more suitable for the *in vitro* validation of a limited number of compounds emerging from computational studies. These technical challenges represent the main reason why, apart from companies focused on vaccine development, there are currently few industries investing in the development of small-molecule antivirals against norovirus. At the same time, norovirus infections rarely lead to large-scale hospitalisations, and most fatalities occur in low-income countries. Consequently, there is limited incentive for significant investment in new antiviral drugs, due to the perceived low economic return.

Moreover, these technical issues partly explain why several *in silico* studies reported so far in the literature have not been followed by the biological evaluation of the virtual hits presented. Indeed, the availability of biochemical assays to evaluate protease and polymerase inhibition would enable the experimental validation of *in silico* studies. The extensive use of computational approaches without subsequent collection of biological data may even slow down the search for new potential antivirals, as researchers might begin to speculate on compounds that perform well from a computational

perspective but display no activity in biological assays. Conversely, research groups engaged in the development of novel protocols for antiviral assays employing HIEs, currently considered the most promising model, could potentially evaluate the main virtual hit compounds identified in the studies discussed in this review, or compounds that may emerge from future computational studies, possibly accelerating the norovirus drug discovery process. Furthermore, the accumulation of biological data on the widest possible range of chemically diverse compounds across multiple targets may facilitate the construction of artificial intelligence/machine learning (AI/ML) models for predicting the potential antiviral activity of new chemical entities, an approach increasingly adopted in contemporary medicinal chemistry.

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