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Norovirus drug candidates that inhibit viral capsid attachment to human histo-blood group antigens

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Abstract

Human noroviruses are the leading causative agents of epidemic and sporadic viral gastroenteritis and childhood diarrhoea worldwide. Human histo-blood group antigens (HBGA) serve as receptors for norovirus capsid protein attachment and play a critical role in infection. This makes HBGA-norovirus binding a promising target for drug development. Recently solved crystal structures of norovirus bound to HBGA have provided a structural basis for identification of potential anti-norovirus drugs and subsequently performed *in silico* and *in vitro* drug screens have identified compounds that block norovirus binding and may thereby serve as structural templates for design of therapeutic norovirus inhibitors. This review explores norovirus therapeutic options based on the strategy of blocking norovirus-HBGA binding.

Keywords

Norovirus; Human histo-blood group antigen; Norovirus capsid proteins; binding inhibitors; antiviral therapy

1. Introduction

Noroviruses are single-stranded, positive-sense RNA viruses that are the leading cause of acute viral gastroenteritis worldwide, being responsible for nearly 60% of all foodborne illness outbreaks (Arias et al., 2013; Changotra et al., 2009; Kocher and Yuan, 2015; Prasad et al., 2016; Rocha-Pereira et al., 2014; Scallan et al., 2011; Thorne et al., 2016). The human histo-blood group antigens (HBGAs) and in particular the Lewis, Secretor and ABO HBGA families, have been shown to serve as norovirus attachment receptors (Dabelsteen, 2002;

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Marionneau et al., 2001) with norovirus genotype-specific sequences determining recognition of specific HBGAs (Donaldson et al., 2010; Tan et al., 2009). The large strain diversity in noroviruses presents a major challenge for vaccine development and there are currently no approved vaccines against norovirus (Arias et al., 2013; Karst et al., 2014). Although anti-viral approaches targeting norovirus replication (Arias et al., 2013; Rocha-Pereira et al., 2014) and monoclonal antibodies have been attempted (Chen et al., 2013; Lindesmith et al., 2012) this has been with limited success.

Recent determination of the recognition requirements between norovirus attachment protein VP1 and host receptors and, in particular, solving of the crystal structure of the complex of norovirus P domain and HBGAs (Cao et al., 2007) have provided a new opportunity to develop drugs targeting the first attachment step in norovirus infection (Huang et al., 2005; Koppisetty et al., 2010; Lundborg et al., 2013). The idea of targeting viral binding is well established in antiviral drug development. For example, Palivizumab is an approved monoclonal antibody used to treat children with Respiratory Syncytial virus (RSV) infection that targets the RSV F protein receptor (Subramanian et al., 1998). Similarly, Maraviroc, a CCR5 inhibitor, prevents binding of HIV gp120 and thereby provides broad protection against a wide range of HIV-1 strains (Bocket et al., 2016). Other drugs that block HIV viral fusion include Enfuvirtide (Fuzeon), which binds to HIV gp41 and interferes with its ability to approximate the two membranes (Chong et al., 2016; Zhu et al., 2015). Pleconaril, a capsid-binding antiviral, prevents rhinoviruses from attaching to the host cell and successfully reduced infection in human trials (Hayden et al., 2003).

This review describes current norovirus drug candidates directed at inhibition of HBGA attachment.

1.1 Human histo-blood group antigens as norovirus attachment receptors

Human HBGA's serve as norovirus attachment receptors and co-receptors (Hutson et al., 2002; Marionneau et al., 2002; Tan and Jiang, 2005, 2007, 2008, 2011, 2014). HBGA's are complex carbohydrates located on the surfaces of erythrocytes and intestinal epithelial cells (Ravn and Dabelsteen, 2000) and are important markers used for blood typing. Blood group trisaccharides A and B (Fig. 1) serve as the main attachment receptors for norovirus strain VA387 (Hutson et al., 2002). The binding modes of HBGA-A (BGTA) and -B (BGTB) trisaccharides to a representative norovirus capsid protein are shown in Figure 2A and 2B, where, a predominant GII.4 norovirus VA387 strain has been characterised for its binding to HBGAs. Binding free energies of the HBGA A and B trisaccharides are -11.4 and -9.5 kcal mol⁻¹, respectively (Lundborg et al., 2013). HBGAs with a terminal fucose serve as attachment receptors for human norovirus in the gastrointestinal tract (Rydell et al., 2011).

While the question of whether HBGA molecules act as functional receptors that internalize norovirus into cells remains debated, it is clear that they act as attachment receptors and that this binding interaction is a precondition to internalization for most strains. For example, an in vitro study employing norovirus virus-like particles (VLPs) showed that norovirus VLP attachment to HBGA resulted in VLP internalization into the cell (Marionneau et al., 2002). Moreover, several studies, using X-ray crystallography, demonstrated the interaction of norovirus capsid protein with HBGA, and the fact that such interactions affect host

susceptibly in human volunteer studies indicate that HBGA act as attachment and/or internalization receptors determining host susceptibility (Cao et al., 2007; Hutson et al., 2002; Tan and Jiang, 2014). While several studies have reported that some human cell lines expressing HBGA did not support norovirus replication (Duizer et al., 2004; Karst and Wobus, 2015), this may simply be due to these cell lines lacking other factor or co-receptors required for viral infection and replication and does not diminish the importance of HBGA in viral infection (Duizer, E. et al. 2004). In addition to those drug candidates, serum HBGA blocking antibodies have recently been recognized as a correlate of protective immunity against norovirus-initiated illness (Czako et al., 2012; Reeck et al., 2010). In addition, virus-specific salivary IgA antibodies are also correlates of protection (Ramani et al., 2015). In a recent study, a monoclonal antibody, when incubated with virus prior to inoculation, was able to stop human norovirus infection in a chimpanzee (Chen et al., 2013). Nevertheless, secretor negative individuals remain susceptible to some norovirus strains, consistent with the existence of a non-HBGA receptor as well, a fact that needs to be consider when targeting HBGA binding as a norovirus antiviral strategy. Interestingly, human GII.4 norovirus VLP induces membrane invaginations during assembly of glycosphingolipids (Rydell et al., 2013); thus, binding to glycosphingolipids by human norovirus may also be involved in cellular entry (Karst and Wobus, 2015). Additional cofactors may also play a part in norovirus binding, entry and uncoating. For example, murine noroviruses bind to sialic acids and glycolipids (Taube et al., 2012; Taube et al., 2009) and recent studies suggest that enteric bacteria may bind human noroviruses (Miura et al., 2013) with human norovirus infection of human B cells shown to be expedited by enteric bacteria expressing a HBGA (Jones et al., 2015; Jones et al., 2014; Karst, 2015).

The fucosyltransferase gene is involved in catalyzing the addition of a terminal fucose to HBGA's. A polymorphic change in a single nucleotide (G428A) in the fucosyltransferase gene on chromosome 19 present in 20% of the Caucasian population reduces susceptibility to norovirus infection (Rydell et al., 2011). However, such protection is not complete, with some norovirus genotypes still capable of infecting people with this HBGA type (Nordgren et al., 2010). HBGA backbone structure is important for presenting the oligosaccharide antigen for recognition by norovirus (Lundborg et al., 2013; Tan et al., 2004; Tan and Jiang, 2005). The terminal residues and internal structures of HBGA's may assist norovirus-HBGA interaction (Harrington et al., 2004; Shirato, 2011). Different norovirus genotypes have variable HBGA-interaction patterns (Huang et al., 2005), due to the polymorphic characteristics of HBGA and the diverse recognition of HBGA receptors by noroviruses (Tan and Jiang, 2007). Because norovirus consists of antigenically diverse groups, identification of conserved norovirus-HBGA structures will be critical to the development of broadly-acting norovirus attachment inhibitors (Shirato, 2012).

1.2 Norovirus classification

Noroviruses are divided into genogroups I–VII depending on the identity of amino acids in the major structural protein (VP1) (Dang Thanh et al., 2016; Vinjé, 2015). Furthermore, based on >85% sequence similarity in the whole VP1 genome, noroviruses are further categorised into genotypes (Wang et al., 2005; Zheng et al., 2006). Noroviruses evolve over time through the changes in the sequence of VP1 (Lindesmith et al., 2008; Siebenga et al.,

2009). A diverse range of different norovirus genotypes have been shown to cause sporadic gastroenteritis outbreaks around the world (Supplementary Table 1). One particular norovirus genotype, termed genogroup II, 4 (GII.4) is the leading circulating genotype causing norovirus illness in developed countries for three decades (Hasing et al., 2013; Vega et al., 2014). The norovirus capsid protein of strain GII.4 has been widely used in functional and structural studies since it is a well-characterized human strain known to be of major clinical relevance (Cao et al., 2007; Lindesmith et al., 2012). Understanding the genetic diversity of norovirus is of paramount importance in the design of anti-norovirus strategies. A number of new GII.4 variants (genotypes can be further subdivided into variants) have appeared every two to three years (Kumazaki and Usuku, 2015). Some GII.4 variants such as US95/96, Den Haag 2006b and Sydney 2012 have been recognized as having pandemic potential (Eden et al., 2014).

1.3 HBGA structure and synthesis

HBGAs usually occur at the non-reducing ends of the oligosaccharide components of glycolipids or glycoproteins, located at the outermost part of N- or O-linked glycans on cell surfaces (Abeijon and Hirschberg, 1992). The synthesis of HBGA starts with a disaccharide precursor by sequential addition of a monosaccharide. Each step of the synthesis is catalysed by a glycosyltransferase with specific substrates (Tan and Jiang, 2007). The H-antigen (also called O antigen), an essential precursor oligosaccharide to the A and B antigens, has sugar groups connected to protein with a small percentage of the H antigen attached to ceramide. The H-antigen consists of a chain of β -D-galactose, β -D-N-acetylglucosamine, β -D-galactose, and 2-linked, α -L-fucose, attached to the protein or ceramide and thus having the form of (—Lipid—Glucose—Galactose—N-acetylglucosamine—Galactose—Fucose). The H-antigen can act as an oligosaccharide acceptor (Smolarek et al., 2008). The A and B alleles of the ABO gene complex express enzymes with glycosyltransferase activities that allow for addition of A and/or B antigens to the H antigen (Lodish et al., 2000; Shirato, 2011). The amino acid sequences of glycosyltransferases A and B differ by four residues, of which only two, Leu/Met266 and Gly/Ala268 in transferases A and B, respectively, cause a variation in enzyme specificity (Smolarek et al., 2008). It is the action of these glycosyltransferase enzymes that add either N-acetyl galactosamine or galactose to the H antigen, which changes the H-antigen into A or B antigen, respectively (Tan and Jiang, 2007; Yamamoto et al., 1990). In both cases, the new antigen forms a α -1, 3 linkage to the outermost galactose component of the H-antigen. Specifically, GalNAc transferase (transferase A) using a UDP-GalNAc donor adds the extra N-acetylgalactosamine to create the A antigen while Gal transferase (transferase B) using a UDP-galactose donor adds the extra galactose to create the B antigen. Blood group antigens are widely expressed on human cells including RBCs and the mucosal epithelium with potential roles in adhesion, membrane integrity and membrane transportation as well as acting as receptors for extracellular ligands and enzymes (Mohandas and Narla, 2005). HBGA's serve as host receptors for viruses most notably noroviruses but also rotaviruses (Bohm et al., 2015).

1.4 Norovirus-HBGA interaction

Using site-directed mutagenesis, it was shown that three residues (Q331, K348, and I389) on the VP1 protein of VA387, a predominant GII.4 norovirus, were involved in interactions

with the acetamide group at the terminal α -1,3-N-acetylgalactosamine sugar of HBGA A (Tan et al., 2008). While the acetamide group plays an important role in the norovirus-HBGA binding specificity (Tan et al., 2008), α -fucose monosaccharide of HBGAs has shown to play a central role in norovirus-receptor interactions as determined by X-ray crystallography (Cao et al., 2007), site-directed mutagenesis (Tan et al., 2008) or hemagglutination assays (Rademacher et al., 2011). While HBGA B lacks an acetamide group, its α -1,3-galactose sugar has been predicted to interact, via solvent molecules, with three amino acids (A346, S441, and K348) of VA378 capsid protein (Cao et al., 2007; Tan et al., 2008). It has also been suggested that direct contact of six VP1 amino acids (T344, R345, A346, D374, C440, and G442) with fucose on HBGA are required for norovirus-HBGA binding (Cao et al., 2007). Additionally, sequence alignment of VP1 proteins of GII.4 indicates that amino acids involved in the recognition of α -fucose on HBGA are highly conserved, consistent with a functional attachment role (Rademacher et al., 2011).

1.5 Physical Screening for Compounds Blocking Norovirus Host Cell Attachment

Although various agents including naturally derived plant products such as tannic acid (Zhang et al., 2012), carvacrol (Gilling et al., 2014) and re-purposed existing anti-virals such as nucleoside analogs or ribavirin have been investigated (Kocher and Yuan, 2015), there are currently no approved drugs for treating norovirus infections. Recent studies have utilised library screening using protein-based and cell-based methods as summarized in Table 2 to identify small molecule norovirus inhibitors. Other approaches have sought to identify potential anti-norovirus drugs based on their ability to bind to important structures involved in norovirus functions particularly host cell attachment. Examples of different categories of compounds inhibiting norovirus activity are listed in Table 1.

Computational biology methods including fragment-based drug/lead discovery (FBDD or FBLD), x-ray crystallography, surface plasmon resonance imaging, nuclear magnetic resonance structural studies and ELISA blocking assay also been employed to try and identify potential norovirus therapies (Feng and Jiang, 2007; Han et al., 2014; Jiang et al., 2015; Lundborg et al., 2013; Rademacher et al., 2011; Shang et al., 2013; Zhang et al., 2013), as summarised in Table 2.

As noroviruses recognize and bind HBGAs to gain entry into cells interruption of this interaction is a potential therapeutic target to block infection (Hutson et al., 2002). In 2006, using an ELISA-based screening approach, HBGA-containing hydrogels were shown to be able to trap norovirus *in vitro* (Zhang et al., 2006). This led to the suggestion that glycosylated hydrogel preparations could be given orally to treat norovirus-infected patients (Arias et al., 2013; Rohayem et al., 2010) and has spurred the search for inhibitors to this interaction. For example, a saliva-based enzyme immune assay was used to screen a library of 5000 synthetic compounds (Diversity screening set; Timtec, Inc. (<http://www.timtec.com>)) for blocking of HBGA-norovirus interaction, with 14 inhibitory compounds identified with an EC₅₀ value of < 15 μ M (Feng and Jiang, 2007). More specifically, 14 compounds showed strong inhibition of binding of VA387 to the A antigen, with an EC₅₀ value of <15 μ M, whereas 4 of the 14 compounds showed robust inhibition of binding of VA387 to the B antigen with an EC₅₀ value of < 15 μ M. These *in vitro* enzyme

based approaches to assess inhibitor molecules have been advanced by the structural analysis of the norovirus P domain-HBGA complex.

The molecular details of the interactions of norovirus GII.4 with fucosylated HBGAs were investigated using molecular dynamics (MD) simulations based on the crystal structure of HBGA trisaccharide B in complex with VA387 GII.4 norovirus P dimer, a region of the norovirus capsid protein that contains the HBGA binding site (Koppisetty et al., 2010). In their study, Koppisetty et al. (2010) showed that computational studies on the interaction of HBGA with the norovirus VA387 capsid protein are in agreement with experimental binding (systemic mutational) data as derived by Tan et al. (2008). This has provided a strong framework with which to investigate and develop inhibitors of this interaction further using structure based analysis techniques.

In a more recent study, 430 compounds from the Maybridge Ro5 fragment library of Thermo Fisher Scientific, Inc. were screened using saturation-transfer difference (STD) nuclear magnetic resonance (NMR) spectroscopy in conjunction with spin-lock filtering to investigate the ability of compounds to bind to the HBGA binding site of a VLP of norovirus GII.4 (Rademacher et al., 2011). In that compound library, 26 ligands (Supplementary Table 2) were shown to compete with L-fucose binding to norovirus capsid protein (as competitive binders, they bind to the L-fucose pocket of the HBGA binding site located on the capsid protein surface) and 4 aromatic ligands were categorized that bound to VLPs at sites close to the fucose binding site. With the demonstration that both univalent and multivalent compounds were capable of binding to the HBGA binding site, it was proposed that each of the 4 compounds (which bind to VLPs at sites close to the fucose binding site) could be linked to α -L-fucose to deliver monomeric ligands with increased binding affinities that, in turn, might be used as building blocks for the synthesis of multivalent inhibitors. Two multivalent prototype entry-inhibitors against norovirus infection were synthesized with IC₅₀ values of ~ 80 μ M (compound ID **16**) and 0.61 μ M (compound ID **17**) as measured using competitive surface plasmon resonance (Supplementary Table 2). Some hetero-bifunctional ligands were also synthesized on a polymeric scaffold to determine the effect of multivalency on binding avidity and demonstrated, by competitive SPR and STD-titration experiments, to have significant inhibitory activities against norovirus VLPs (compounds ID from **44** to **47**) (Guiard et al., 2011) (Supplementary Table 2). Moreover, citrate, having a high degree of mimicry to fucose, may have the potential to block human noroviruses from binding to HBGAs (Hansman et al., 2012). In an effort to improve the inhibitor capacity of the small molecules from Rademacher et al., binding epitopes and bioactive conformations of some synthetic HBGA fragments were determined (Fiege et al., 2012). More recently, surface plasmon resonance imaging (SPRi) competitive binding assays were used to demonstrate that a polyvalent neoglycoconjugate with assembled carbohydrate moieties was able to prevent norovirus capsid binding to host receptor glycan (Shang et al., 2013). A recent X-ray crystallography study showed that two human milk oligosaccharides, 2' - fucosyllactose (2' FL) and 3-fucosyllactose (3FL) block norovirus from binding to surrogate HBGA samples (Weichert et al., 2016).

1.6 *In Silico* Screening Approaches For Compounds With Anti-Norovirus Activity

Fragment-based lead discovery (FBLD) can be used to identify lead compound(s) whereby by combining two or three fragments, a higher affinity drug can be designed (Figure 2). Using an *in silico* FBLD screening approach based on the crystal structure of norovirus capsid protein, Lundborg et al. (2013) identified four small molecules that could prevent binding of HBGA to the norovirus VA387 capsid protein. For screening purposes, flexible ligand docking, molecular dynamics simulations and the linear interaction energy (LIE) (Hansson et al., 1998) methods were employed to study the absolute binding free energy of small molecules to the norovirus capsid protein. The screened small molecules were derived from the Maybridge 'Ro3' fragment library designed and computationally clustered by Thermo Fisher Scientific, UK Ltd. consisting of 500 compounds, plus 6423 compounds from the ZINC database. The physicochemical properties of the Maybridge Ro3 library of 500 compounds and the parameters used to create the ZINC subset used in the study of Lundborg et al. (2013) are detailed in Table 3.

The HBGA-A and -B trisaccharides were docked to the biological unit of the P domain of norovirus VA387, using the crystal structure of the protein binding the HBGA B trisaccharide (PDB ID 2OBT). It was concluded that the HBGA A trisaccharide in PDB ID 2OBS was not correct (Koppisetty et al., 2010), and therefore, the focus was put on HBGA B trisaccharide (Lundborg et al., 2013). The binding free energies of the HBGA trisaccharides and the top 20 fragments from each of the two libraries (Maybridge Ro3 and ZINC) were calculated. The linear interaction energy value obtained from MD simulations ranked the blood group trisaccharides B and A, respectively, to the second and first ranking position (Lundborg et al., 2013). As natural ligands, trisaccharides B and A are anticipated to have higher binding affinities than any small fragments identified from library screening. After LIE calculations, many small fragments showed poor binding affinities in comparison to the trisaccharide B and A natural ligands. However, considering the size of the fragments, they are expected to have rather low binding affinities (Lundborg et al., 2013). In this sense, the binding affinities of many of these small fragments are quite reasonable. Among top 40 fragments from 2 libraries, at least two different fragments were joined together to have higher binding affinities and ultimately four candidate (combined) molecules were identified. A REOS (Rapid Elimination of Swill) method was used (Walters and Murcko, 2002; Walters et al., 1998) by filtering in Canvas in order to avoid suggested molecules that might be unsuitable (e.g., containing known toxic features). The compounds were also assessed using the programme SYLVIA (<http://www.molecular-networks.com>) (Boda et al., 2007) to estimate the synthetic accessibility. The four identified compounds were identified to inhibit interaction of HBGA B trisaccharide with the norovirus P domain, be unlikely to have toxic properties and be of intermediate difficulty for synthesis. A final step will be to perform *in vitro* and *in vivo* studies to specifically assess their potential to block norovirus binding to HBGA expressing cells.

In another study, Virtual High Throughput Screening of 2 million compounds from the ZINC library identified 5 compounds with an ability to block the binding of norovirus to HBGA (Zhang et al., 2013). Four out of 5 of the identified compounds had a basic structure of cyclopenta [a] dimethyl phenanthren and were able to block the interaction of norovirus

VA387 P dimers with HBGA A and B trisaccharides at an $IC_{50} < 10 \mu M$ (Zhang et al., 2013). There was a good correlation between K_i values obtained by molecular dynamic simulations and IC_{50} values obtained in a physical blocking assay. The top five compounds identified showed low *in vitro* cell toxicity by a MTS (a tetrazolium salt) cytotoxicity assay and thus represent promising candidates for further investigation (Jiang et al., 2015; Zhang et al., 2013).

1.7 Drug Candidate Screening *in vivo*

The failure to culture human norovirus *in vitro* and the previous lack of a small animal model for oral infection (Duizer et al., 2004; Guix et al., 2007; Jones et al., 2014; Karst and Wobus, 2015; Wobus et al., 2004) has hampered development of norovirus drug candidates. But, the discovery of a murine-specific norovirus, that is homologous to human viruses and replicates well in cell culture offers a platform to study norovirus biology and test new drug candidates (Fantini et al., 1993; Farkas et al., 2008; Karst et al., 2003; Wobus et al., 2006). However, while HBGAs are the main carbohydrates to which human norovirus bind, murine norovirus binds to GD1a ganglioside, sialic acid and N-linked and O-linked cell surface glycoproteins (Karst and Wobus, 2015; Taube et al., 2012). Interestingly, with the mapping of a glycan-binding site in the MNV-1 capsid, a region topologically similar to the HBGA-binding sites of the human norovirus strain VA387 was identified (Taube et al., 2012). Human norovirus was recently found to be able to be grown in B cells (BJAB cells) thereby providing a culture system for testing of drug candidates (Jones et al., 2014). Very recently, a human intestinal enteroid culture system has been described to support human norovirus replication *in vitro*. Excitingly, this culture system also shows dependency on HBGA for infection and thus presents a new *in vitro* model to test the anti-viral potential of inhibitors of norovirus HBGA interaction (Crawford et al., 2016). Furthermore, there are now mouse models (Balb/c Rag- $\gamma c^{-/-}$ mice) (Taube et al., 2013) that have been successfully used to test the inhibitory effect of 2'-C-methylcytidine on human norovirus replication (Kolawole et al., 2016). Moreover, a recent study showed that canine noroviruses interact with histo-blood group antigens in the similar way of human noroviruses (Caddy et al., 2014). There are also now calf (Souza et al., 2008) and gnotobiotic pig (Bui et al., 2013) models of human norovirus infection. These advances should thereby help accelerate development of human norovirus drug candidates.

1.8 Potential Challenges

Although the candidates described in Table 2 have been rationally derived and are promising candidates, they do have some caveats. Firstly, the norovirus capsid protein of strain VA387 has been widely used in the above studies since it is a well-characterized human strain that is known to be of major clinical relevance and has an associated structural model. Although the P domain and HBGA binding site of norovirus strains is conserved (Tan et al., 2009), this strain represents only one type of the many circulating norovirus strains. Notably, different genotypes vary in their ability to bind to HBGAs (Huang et al., 2003; Huo et al., 2016). Further, VA387 may not only bind to HBGA trisaccharides A and B, but also to other natural carbohydrates (Bally et al., 2012; de Rougemont et al., 2011; Shang et al., 2013; Tamura et al., 2004). The significance of virus binding to other carbohydrates and the impact this will have on the ability to block norovirus-HBGA interaction needs to be addressed. Further,

ligand docking studies using MD may lack important physical characteristics of the compounds such as solvation, entropy or pH changes important to the gut microenvironment (Kitchen et al., 2004; Warren et al., 2006). Lastly, it is difficult to make a rank-ordered list of the ligand binding affinities using only molecular modeling data. For the determination of absolute binding free energy of ligands for the given target protein, the LIE method and Molecular mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method are best at predicting experimentally-obtained values (Duan et al., 2003). The LIE method alone is not rigorous, but its simplicity and low computational cost make it attractive. In the case of NMR, STD-NMR experiments do not differentiate between specific and nonspecific ligand binding (Ji et al., 2009; Viegas et al., 2011). Thus, candidates identified by molecular modeling or modeling followed by biophysical techniques must still be experimentally validated. A list of all potential active compounds that target norovirus-HBGA interaction, with their structures, half maximal inhibitory concentration (IC₅₀) and toxicity values are summarized in Supplementary Table 2.

1.9 Concluding remarks

HBGAs play a critical role in determining norovirus infection through binding of the outer shell of the capsid protein to HBGA's on cell surfaces. The norovirus-HBGA binding interaction is a promising target for antiviral strategies. Numerous candidates targeting this interaction have been identified through in vitro and in silico screening assays. A reliable correlation between IC₅₀ in blocking assay and K_i values obtained by molecular dynamic simulations indicates modeling approaches may be suitable to identify norovirus drug candidates. Several structural studies mentioned above have shown that the fucose moiety of HBGA plays an important role in norovirus-HBGA attachment suggesting that reduction of HBGA fucosylation, might be another potential approach to reduce the susceptibility to norovirus infection. Fragment-based lead discovery technology can be used to synthesize ligands with high efficiency, and used iteratively to increase the affinity of potential ligands. Once potential binding ligands are identified, combining LIE approximation and thermal conformational sampling methods can be used for evaluating binding free energies for small fragments to macromolecular targets of defined structure. This information can be used to further assess the binding affinities of the molecules in silico, prior to testing binding properties in vitro. Candidates that show promise in initial binding studies can now be tested for activity in recently developed human cell culture models, prior to further validation in recently developed animal models. Like all antiviral drugs, the potential for development of drug resistance will also need to be carefully studied. For example, human rhinoviruses with capsid protein mutations in amino acids at positions 152 and 191 were found to decrease the efficiency of pleconaril, a capsid-binding antiviral (Ledford et al., 2005). Nevertheless, pleconaril resistance was overcome by further chemical modifications (Schmidtke et al., 2009). Hence, drugs targeting norovirus binding to HBGA remain promising candidates, notwithstanding the challenges of all antiviral drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HBGA	Human histo-blood group antigen
FBLD	Fragment based lead discovery
K_d	Dissociation binding constant
LIE	Linear interaction energy
MD	Molecular dynamics
K_i	Inhibition constant

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Highlights

- Human histo-blood group antigens (HBGAs) serve as norovirus attachment receptors
- Inhibitors of HBGA-Norovirus capsid protein binding may be promising drug candidates.
- Conserved norovirus-HBGA structures may act as targets for broad antiviral activity.
- The role of HBGAs in norovirus infection warrants further study.

Figure 1.
Chemical structures of human blood group trisaccharide A and B that serve as norovirus receptors

Figure 2. Example of how therapeutic norovirus inhibitors can be constructed using a fragment based binding approach

Panel A. Alternative binding modes of blood group trisaccharide A to the norovirus capsid protein, BGTA shown in pose 2 (green carbons) and pose 5 (black carbons); Panel B: Binding mode of blood group trisaccharide B to norovirus capsid protein; Panel C: Binding mode to norovirus capsid protein of 3 potential inhibitory fragments (yellow, blue and red coloured); Panel D: Overlay of binding mode of trisaccharide B (green carbons) and 3 potential inhibitory fragments from panel C. This shows how the fragments might be able to prevent attachment of trisaccharide B to the norovirus capsid protein (surface representation of VA387 norovirus capsid protein). By joining together two or three such fragments it is possible to design a compound with higher binding affinity than the single parent fragment. In Lundborg et al. this technology was used to create candidate drugs (see also, compounds **48 to 51** in Supplementary Table 2). Colour conventions for atoms of trisaccharide B used: white for hydrogen atom, green for carbon, red for oxygen atom. The picture was prepared using PyMOL (<http://www.delanoscientific.com/>).

Table 1

Compounds shown or predicted to have anti-norovirus activity

Compound ID (Actual structures are shown in Supplementary Table 2)	Targeted moiety/structure	Predicted to target a selected moiety	Reference
1	Entrapment of the whole protein shell of norovirus (VLPs)	–	(Zhang et al., 2006)
16 to 43	Fucose-binding pockets on the VLP surface	–	(Rademacher et al., 2011)
44, 45, 46, 47	(For increasing binding avidity to) Fucose-binding pockets on the VLP surface	–	(Guiard et al., 2011)
48, 49, 50, 51	–	Competitive binding (of HBGA B) to VLP surface and covering up fucose-binding pocket	(Lundborg et al., 2013)
52	Binding specificities between human milk glycan epitopes and norovirus capsid	–	(Shang et al., 2013)
53, 54, 55, 56, 57	–	HBGA binding sites of VA387 norovirus (a predominant GII.4 norovirus)	(Jiang et al., 2015)
58, 59	HBGA binding pocket on the GII.10 norovirus capsid		(Weichert et al., 2016)

Table 2

Summary of methods used for identification of compounds that inhibit norovirus-HBGA interaction

(Number of) potential compounds/agents	Method (s) used for identification (or development)	References
Glycosylated hydrogel (see also Compound 1 in Supplementary Table 2)	VLP from VA387 strain was used to interact with HBGA B-containing hydrogel in solution. Concentration of VLPs determined by ELISA before and after trapping with glycosylated hydrogels	(Zhang et al., 2006)
14 compounds with an EC ₅₀ value of < 15 µM (see also, compounds 2 to 15 in Supplementary Table 2)	Saliva-based enzyme immune assay was used to measure the inhibitory activities of compounds against the binding of VA387 VLPs to HBGA receptors followed by a cytotoxicity assay in HeLa and Caco-2 cells	(Feng and Jiang, 2007)
2 polymeric compounds, 26 competitive binders of fucose (see also compounds 16 to 43 in Supplementary Table 2)	Saturation-Transfer Difference (STD) NMR spectroscopy followed by SPR-based binding assays	(Rademacher et al., 2011)
4 polymeric compounds (see also compound 44 to 47 in Supplementary Table 2)	'Double Click' protocol and competitive titration by using STD NMR spectroscopy	(Guiard et al., 2011)
4 candidates (compounds 48 to 51 in Supplementary Table 2)	Fragment-based lead discovery (FBLD) employing docking followed by molecular dynamics simulations	(Lundborg et al., 2013)
1 polyvalent neoglycoconjugate (with assembled carbohydrate moieties) (compound ID 52 in Supplementary Table 2)	Surface plasmon resonance imaging (SPRi) competitive binding assays	(Shang et al., 2013)
5 compounds (4 out of 5 having a basic structure of cyclopenta [a] dimethyl phenanthren) (see also compounds ID 53 to 57 in Supplementary Table 2)	Molecular simulations followed by validation of hits by norovirus-HBGA blocking assays, and subsequent MTS cytotoxicity assays. The cytotoxicity of the top 5 hits was tested in HeLa and LLC-MK2 cells	(Jiang et al., 2015; Zhang et al., 2013)
2' -fucosyllactose (2' FL) and 3- fucosyllactose (3FL) [compound 58 and 59 in Supplementary Table 2]	X-ray crystallography to check if 2'FL and 3FL bind equivalent HBGA pockets on the norovirus GII.10 P domain plus ELISA blocking assay to assess blocking of GII.10 VLPs binding to A-type and B-type saliva samples.	(Weichert et al., 2016)

Table 3

The physicochemical properties of the Maybridge Ro3 library of 500 compounds and the parameters used to create the ZINC subset used in the study of Lundborg et al. (2013). The ZINC database search was performed 10-09-2010 and retrieved 6423 molecules (Lundborg et al., 2013).

Parameter	Maybridge Ro3	ZINC
MW	300	125 – 225
Number of rotatable bonds	3	4
Calculated log <i>P</i>	3	3 ^a
Charge	– 2	– 2
Hydrogen bond donors	3	4
Hydrogen bond acceptors	3	4
Polar surface area	60 Å ²	

^aCalculated using XLOGP (Lundborg, 2011; Lundborg et al., 2013; Wang et al., 1997).