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Human norovirus inhibition by a human milk oligosaccharide

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ABSTRACT

Human noroviruses are the leading cause of outbreaks of acute gastroenteritis. Norovirus interactions with histo-blood group antigens (HBGAs) are known to be important for an infection. In this study, we identified the HBGA binding pocket for an emerging GII genotype 17 (GII.17) variant using X-ray crystallography. The GII.17 variant bound the HBGA with an equivalent set of residues as the leading pandemic GII.4 variants. These structural data highlights the conserved nature of HBGA binding site between prevalent GII noroviruses. Noroviruses also interact with human milk oligosaccharides (HMOs), which mimic HBGAs and may function as receptor decoys. We previously showed that HMOs inhibited the binding of rarely detected GII.10 norovirus to HBGAs. We now found that an HMO, 2′-fucosyllactose (2′FL), additionally blocked both the GI.1 and GII.17 noroviruses from binding to HBGAs. Together, these findings provide evidence that 2′FL might function as a broadly reactive antiviral against multiple norovirus genogroups.

1. Introduction

Noroviruses typically cause an acute illness of fever, vomiting, cramping, and diarrhea that persists for 2–5 days. Human noroviruses have a single-stranded, positive sense RNA genome of ~7.4–7.7 kbases. The genome contains three open reading frames (ORFs), where ORF1 encodes the non-structural proteins, which includes the protease and RNA dependent RNA polymerase (RdRp), ORF2 encodes the capsid protein (VP1), and ORF3 encodes a minor structural protein. Noroviruses can be divided into at least seven genogroups (GI-GVII), with GI, GII, and GIV causing infections in human and GV infecting mice. Each genogroup can be further subdivided into numerous genotypes based on the capsid amino acid sequences (Hansman et al., 2006). The capsid protein can be divided into two domains, shell (S) and protruding (P) domain (Prasad et al., 1999). The S domain surrounds the viral RNA, whereas the P domain, which can be further subdivided into P1 and P2 subdomains, contains determinants for cell attachment and antigenicity.

The prototype strain of human norovirus, GI genotype 1 (GI.1), was first discovered in 1972 from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio, USA (Kapikian et al., 1972). Human norovirus capsid genes often evolve into genetically and antigenically distinct variants, and frequent genetic recombination at the RdRp and capsid junction also increases their diversity (Eden et al., 2013). The GII.4 noroviruses have dominated over the past decade and caused the majority of pandemics (Eden et al., 2013). Large outbreaks usually follow the emergence of new GII.4 genetic variants, which can re-infect previously exposed individuals. Recently, the GII.17 noroviruses, which are genetically distinct from the GII.4 noroviruses, were found to cause an alarming number of epidemics in several countries (Zhang et al., 2015; Matsushima et al., 2015; Lee et al., 2015; Fu et al., 2015; Chan et al., 2015). Before this time, the GII.17 noroviruses were only a minor cause of infections, although they were first identified in 1978. Reports are now discovering that the GII.17 variants are emerging in other parts of the world.

Human norovirus interaction with histo-blood group antigens (HBGAs) is important for virus attachment to cells. HBGAs are found as soluble antigens in saliva and are expressed on epithelial cells. To date, based on the ABH- and Lewis-HBGA types, at least nine different HBGAs were found to interact with human noroviruses (Schroten et al., 2016). Earlier structural studies showed that noroviruses bind two HBGA molecules per P domain dimer, and the GI and GII noroviruses bind HBGAs in different regions on the capsid. Recently, two additional HBGA binding pockets on the GII norovirus capsid were identified, which suggested that HBGA binding interactions are more complex than previously recognized (Mallagaray et al., 2015; Koromyslova et al., 2015a; Singh et al., 2015a).

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The GII.4 variants are capable of binding an extensive panel of HBGA types (Singh et al., 2015a), which may be associated with their worldwide dominance. Less prevalent GI.1 noroviruses interact with some HBGA types, but do not bind to B-type HBGAs (Choi et al., 2008). Similarly, the earlier GII.17 noroviruses were thought to have a limited interaction with the HBGAs, whereas the newer GII.17 variants may have acquired additional HBGA binding capabilities (Zhang et al., 2015; Chan et al., 2015; Singh et al., 2015b). On the other hand, several GII genotypes that have caused numerous outbreaks in the past, i.e., GII.1 and GII.2, may not interact with any HBGA types (Singh et al., 2016). Overall, HBGA binding interactions with human norovirus significantly varies among the different genotypes. Deciphering the HBGA binding sites and interacting residues in newly emerging strains could provide insights into the mechanisms of virus evolution and persistence.

Despite their discovery over four decades ago, there are still no approved antivirals or vaccines for human noroviruses. The norovirus life cycle offers a number of potential targets for antiviral therapy. Three key targets for norovirus antivirals are the protease, the RdRp, and the capsid. Several candidate compounds and nucleoside analogues that are directed against the protease and RNA dependent RNA polymerase have been developed, but these have not advanced to clinical trials (Mandadapu et al., 2013; Muhaxhiri et al., 2013; Rocha-Pereira et al., 2015, 2012).

Most antiviral development is targeted towards the capsid protein (Feng and Jiang, 2006; Rademacher et al., 2011; Horm and D'Souza, 2011; Su et al., 2010a, 2010b, 2010c; Whitehead and McCue, 2010). Several compounds that bind at the HBGA pocket were identified, including cyclopenta-phenanthren derived molecules from drug-like compound libraries, citrate, and human milk oligosaccharides (HMOs) (Weichert et al., 2016; Koromyslova et al., 2015b; Hansman et al., 2012a; Zhang et al., 2013). HMOs consist of similar monosaccharide building blocks as HBGAs and structurally mimic HBGAs. HMOs are understood to act as a receptor decoy for certain carbohydrate binding pathogens, including human rotavirus (Etzold and Bode, 2014; Yu et al., 2014). All HMOs are composed from the precursor disaccharide lactose (Galβ1-4Glc), which is the most abundant milk oligosaccharide. A number of studies have suggested norovirus interactions with HMOs may decrease the capacity of norovirus to cause an infection (Jiang et al., 2004; Shang et al., 2013). One study showed that human milk provided some protection to infants with a norovirus infection (Morrow et al., 2004). HMOs can resist degradation in the gut and the majority is excreted intact in the feces. Therefore, HMOs likely travel the same path as noroviruses in the host.

The recognition of different HBGAs and HMOs types is often genotype specific, therefore emphasizing the need of inhibition studies with several norovirus variants. Recently, we have shown that HMOs bound and inhibited GII.10 norovirus from binding to HBGAs. However, the evidence that HMOs can similarly inhibit other norovirus genotypes and genogroups and function as broad norovirus antivirals has been lacking.

In this study, we determined the HBGA binding pocket for a most recent GII.17 variant (isolated in 2015) using X-ray crystallography. We showed that the GII.17 and epidemic GII.4 noroviruses bound HBGAs with an equivalent set of P domain residues. Moreover, we expanded our investigations of HMOs capability to inhibit multiple norovirus strains across GI and GII genogroups. We found that HMO 2′-fucosyllactose (2′FL) blocked GI.1 and GII.17 virus-like particles (VLPs) from binding to HBGAs and showed that the 2′FL bound at both GI and GII HBGA pockets. Overall, our data indicates that HMOs can inhibit multiple norovirus genogroups and genotypes by targeting the conserved HBGA binding pocket.

2. Materials and methods

2.1. P domain production

The 1968 GI.1 (Norwalk virus, M87661) and 2015 GII.17 (Kawasaki308, LC037415) P domains were expressed and purified (Singh et al., 2015b). Briefly, the codon optimized P domains were cloned into a modified expression vector pMal-c2X and transformed into BL21 cells for protein expression. Transformed cells were grown in LB medium supplemented with 50 μg/ml ampicillin for 4 h at 37 °C. Expression was induced with IPTG (0.75 mM) at an OD_{600} of 0.7 for 18 h at 22 °C. Cells were harvested by centrifugation at 6000 rpm for 15 min and disrupted by sonication on ice. His-tagged MBP fusion-P domain proteins were purified from a Ni-column and digested with HRV-3C protease overnight at 4 °C. The cleaved P domains were separated on the Ni-column and dialyzed in gel filtration buffer (GFB: 25 mM Tris-HCl and 300 mM NaCl) overnight at 4 °C. The P domains were purified by size exclusion chromatography, concentrated to 3–7 mg/ml, and stored in GFB at 4 °C.

2.2. VLP production

The GI.1 (AY502016.1) and GII.17 (Kawasaki308) VP1 were expressed in insect cells as previously described (Hansman et al., 2005, 2007). Briefly, the recombinant VP1 bacmids were transfected into Sf9 cells using Effectene. Sf9 cells were incubated for five days at 27 °C, after which the culture medium was clarified by low-speed centrifugation, and the supernatant containing the seed baculovirus was collected. H5 cells were infected with the baculovirus at 27 °C and harvested six days post-infection. The VLPs secreted into the cell medium were separated from the cells by low-speed centrifugation and then concentrated by ultracentrifugation at 35,000 rpm for 2 h at 4 °C. The VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 35,000 rpm for 18 h at 4 °C. The morphology of the GI.1 and GII.17 VLPs was confirmed using electron microscopy.

2.3. HBGA binding assay

The binding of GI.1 and GII.17 VLPs to porcine gastric mucin type III (PGM; containing HBGAs) and A-type saliva was measured using a surrogate HBGA-blocking assay (Weichert et al., 2016). Briefly, 96-well plates were coated with 10 μg/ml of PGM for 4 h at room temperature (RT). For the A-type saliva sample, the saliva was first heated at 95 °C for 10 min, briefly centrifuged, and then the supernatant was diluted 1:500 in PBS and added to wells overnight at 4 °C. The PGM and saliva plates were washed three times with PBS containing 0.1% Tween20 (PBS-T), and blocked with 5% skim milk (SM) in PBS overnight at 4 °C. The VLPs were two-fold serially diluted in PBS, added to the duplicate wells, and then incubated for 1 h at 37 °C. Plates were washed and incubated with α-VLP-biotinylated Nanobodies (NB60 for GI.1 and Nano-4 for GII.17) for 1 h at 37 °C. Following a wash step, horseradish peroxidase conjugated streptavidin was added to the wells and incubated for 1 h at 37 °C. Plates were washed and then developed with *o*-phenylenediamine and H_2O_2 in the dark for 30 min at RT. Finally, the reaction was stopped with 3 N HCl and absorbance was measured at 490 nm (OD_{490}). For the 2′FL blocking assay, an OD_{490} value that decrease after the maximum was selected for the blocking assay, i.e., for GI.1, we used 0.5 μg/ml and for GII.17, we used 5 μg/ml.

2.4. HMO blocking assay

The 2′FL inhibition assay was performed as previously described (Weichert et al., 2016). Briefly, 96-well plates were coated with PGM or A-type saliva sample. The GI.1 and GII.17 VLPs were pre-treated with