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Emerging gut microbial glycoside hydrolase inhibitors

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The human gut microbiota has been linked to numerous diseases through their metabolism of molecules in the gastrointestinal tract. Post-translational glycosylation is applied to many secreted proteins, including mucins and immunoglobulins, and glycosides are present in diet and generated by host metabolism systems. Thus, glycosides are key targets for degradation by gut microbial glycoside hydrolases (GHs). Indeed, diverse xenobiotic compounds, including therapeutics and dietary phytochemicals, along with endobiotics like neurotransmitters and hormones, are conjugated to monosaccharides making them substrates for GH enzymes. A range of GH inhibitors have been developed to study lysosomal storage diseases, treat viral infections, and to address type II diabetes. Recently, GH inhibitors have offered promising avenues for investigating gut microbial GHs and their influence on host health and disease. In this review we describe the growing classes of GH inhibitors and their applications in studying gut microbial GHs that target host-derived glycans and dietary and drug-xenobiotic molecules. We also review the use of GH-targeting activity-based probes to pinpoint specific proteins expressed by the gut microbiota that influence molecular and phenotypic outcomes. As we deepen our understanding of gut microbial GH function, we will further elucidate the roles played by the microbiota in host physiology and disease toward potential therapeutic interventions that target non-host factors in acute and chronic disorders.

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Introduction

The human gut microbiota is composed of trillions of bacteria, encoding approximately 150 times more genetic information than the human genome.[1,2] Gut microbiota composition has been associated to a variety of diseases including obesity,[3] inflammatory bowel disease (IBD)[4] and chronic kidney disease.[5] The microbiota plays a key role in the metabolism of diet-derived compounds, hormones, neurotransmitters, host-glycans and drug–molecules.[6–11] The metabolism of these molecules by the gut microbiota has implications in IBD,[12] diabetes,[13,14] drug efficacy and toxicity.[8,15] Many of these substrates exist as glycoconjugates and glycoproteins, making them targets of carbohydrate active enzymes (CAZymes) produced by gut bacteria.[16] A promising avenue for studying metabolism of these compounds is through CAZyme-targeting small-molecule inhibitors.

The gut microbiota encodes a diverse suite of CAZymes, including glycoside hydrolases (GHs), sulfatases, carbohydrate esterases, polysaccharide lyases, and glycosyltransferases. The largest category of CAZymes are GHs, with nearly 200 families annotated according to sequence homology.[16,17] GHs are active on glycosidic bonds, enabling the degradation of polysaccharides, oligosaccharides, sugar-conjugated small-molecules, and glycoproteins into monosaccharides to be used as an energy source for bacteria.[16,18–20]

Gut bacterial GHs are crucial in processing diet-derived plant polysaccharides and host-derived glycoproteins such as mucins and immunoglobulins, which are highly abundant in the gastrointestinal (GI) tract.[21–25] The human genome lacks much of the machinery to degrade these glycans, relying on the gut microbiota to accomplish this chemistry. Given these substantial roles of gut microbial CAZymes in these processes (reviewed by Wardman *et al.*[26]), CAZyme inhibitors provide a valuable resource for uncovering the gut microbiota's role in human physiology and disease, and could serve as potential avenues for therapeutic intervention.

Due to the abundance of glycoconjugates and their importance in biology, numerous GH inhibitors have been discovered and developed. However, to date, the focus of GH inhibitors has generally been applied to host disease states like lysosomal storage diseases,[27] type II diabetes,[13,14] and viral infections,[28] and has thus been outside the scope of the gut microbiota. This review examines small-molecule inhibitors and chemical probes that target GHs involved in *O*-glycan, *N*-glycan, dietary

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xenobiotic and drug xenobiotic metabolism in the gut microbiota.

Carbohydrate substrates for gut microbes

Mucin *O*-glycans

Mucin glycoproteins comprise the protective mucus layer that lines the GI tract, providing a barrier between the gut microbiota and intestinal epithelium. Mucins are highly glycosylated, with ~80% of their total mass coming from the oligosaccharides that decorate its structure.[29,30] The most abundant mucin in the gut is MUC2 and because of the high degree of glycosylation, MUC2 forms a gel-like matrix acting as a barrier separating the intestinal epithelium and intestinal microflora. The Ser- and Thr-linked *O*-glycans commonly contain the monosaccharides galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose, and sialic acid (Fig. 1).[31] Additionally, the individual sugar moieties in these glycans can be decorated with a sulfate group. The chemical complexity of the glycosylations and sulfations requires a collection of gut microbial enzymes to achieve mucin oligosaccharide degradation. Several gut bacteria have been shown to metabolize mucin *O*-glycans, notably the well-studied *Bacteroides thetaiotaomicron* and *Akkermansia muciniphila*.[32–34] Degradation of this protective layer has been associated with ulcerative colitis (UC),[35–37] and understanding how inhibition of proteins responsible for specific steps in mucin *O*-glycan metabolism offers an avenue to more closely examine the processes that underpin mucin degradation by the gut microbiota.

Host and dietary *N*-glycans

Non-barrier glycoproteins, like immunoglobulin A (IgA), are also abundant in the GI tract and are decorated by *N*-glycan oligosaccharides attached to Asn residues (Fig. 1).[38–40] *N*-Glycans are obtained through the diet, including α-mannan from yeast and *N*-glycans from plant-derived dietary fiber. *N*-Glycans usually contain the monosaccharides GlcNAc, fucose, mannose, sialic acid and galactose. Pathogenic microbes can utilize these glycans, providing a fitness advantage and has been implicated as a mechanism for pathogens to evade the immune system.[41,42] Furthermore, IgA coating, or attachment to bacteria, is associated with the ability of certain commensal microbes to invade the colonic mucus layer.[43] Inhibiting GHs involved in *N*-glycan degradation may provide mechanistic insights into how gut microbes use this to their advantage and potentially contribute to dysbiosis.

Xenobiotics

The gut microbiome also plays essential roles in the metabolism of therapeutic xenobiotics.[11] Zimmerman *et al.* analysed the ability of gut bacteria to metabolize 271 drugs, with the majority being chemically modified by at least one bacterial strain.[11] An early well-known characterized example of drug metabolism by the gut microbiota is the chemotherapeutic irinotecan.[8,44,45] The active form of irinotecan is conjugated to a glucuronic acid sugar, inactivating it and marking it for excretion through the GI tract (Fig. 1). GHs produced by gut microbes then remove the glucuronic acid and reactivate the drug, causing severe dose-limiting side effects.[8] Small-molecule GH inhibitors have been shown to eliminate the gut toxicities of irinotecan,[8,44,45] as well as numerous non-steroidal anti-inflammatory drugs and other therapeutics that damage the GI tract *via* a similar mechanism.[46–50]

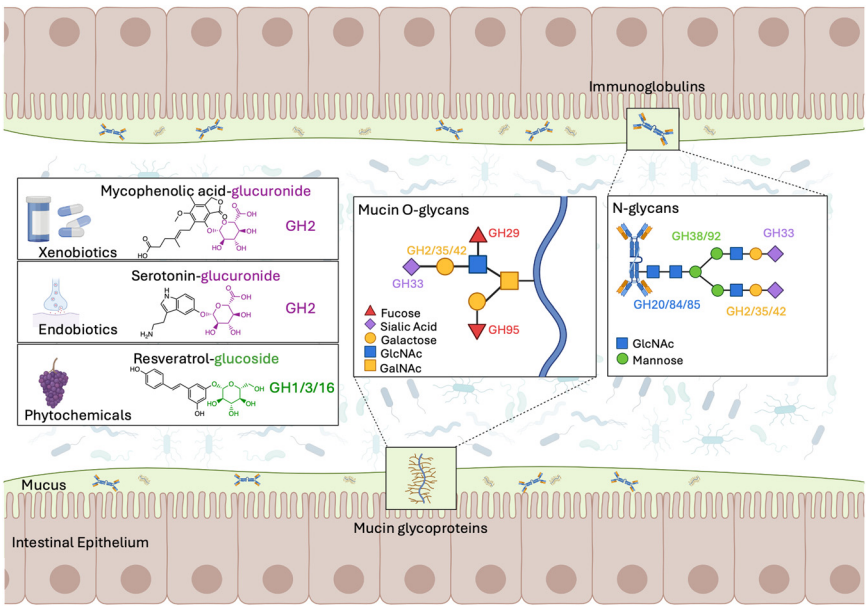

Fig. 1 Substrates for glycoside hydrolases (GH) in the gastrointestinal (GI) tract. The range of carbohydrate substrates present in the GI tract including host-derived glycans (mucins & immunoglobulins), xenobiotic compounds (therapeutics and phytochemicals), and host-generated endobiotic compounds. The GH family responsible for removing the glycans are indicated, showing the diverse enzymes needed to degrade carbohydrate substrates.

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Table 1 Glycoside hydrolases, their substrates and inhibitors

Enzyme	Substrate	Inhibitors[a]	Significance	PDB	Ref.
α-Glucosidase	Carbohydrates, starch	Deoxynojirimycin Acarbose Miglitol Voglibose Cyclophellitol-aziridine	Type II diabetes	3PHA (acarbose) 6C9X (voglibose) 6CA1 (miglitol)	Tan *et al.*,[60] Inouye *et al.*,[61] Niwa *et al.*,[62] Yoon *et al.*[63]
β-Glucosidase	Phytochemicals, plant polysaccharides	Isofagomine Castanospermine Conduritol B epoxide Cyclophellitol-aziridine	Anti-inflammatory, anti-cancer, and antioxidant properties	2J77 (nojirimcyin) 5N6T (cyclophellitol)	Street *et al.*,[64] Saul *et al.*,[65] Braun *et al.*,[66] Gloster *et al.*,[67] Beenakker *et al.*[68]
β-Glucuronidase	Drug metabolites, endobiotic compounds	Saccharolactone Uronic-noeurostegine Inhibitor 1 UNC10201652 UNC4917 UNC10206581 TCH-3562 NCGC00253873 Ceritinib Desloratidine Cyclophellitol-aziridine	Drug toxicity, neurotransmitter and hormone levels	8GEN (UNC10201652) 8GEO (3-OH-desloratidine) 8GEQ (ceritinib) 3LPF (inhibitor1) 6NZG (cyclophellitol)	Wallace *et al.*,[8] Simpson *et al.*,[7] Pellock *et al.*,[69] Jariwala *et al.*,[70] Graboski *et al.*,[71] Challa *et al.*,[72] Roberts *et al.*,[45] Boyland *et al.*,[73] Rasmussen *et al.*,[74] Awolade *et al.*,[75] Pellock *et al.*[76]
β-Galactosidase	*O*-Glycans, *N*-glycans, GOS pre-biotics, plant polysaccharides	PETG Deoxygalactonojirimycin Cyclophellitol-aziridine	Mucin-glycan and immunoglobulin-glycan degradation	5A1A (PETG) 6TSH (nojirimycin)	Bartesaghi *et al.*,[77] Saur *et al.*,[78] Huber *et al.*,[79] De Bruyne *et al.*[80]
α-Fucosidase	Host *O*-glycans and *N*-glycans	DFJ Cyclophellitol-aziridine	Mucin-glycan and immunoglobulin-glycan degradation	2EAC (DFJ) 4WSK (cyclophellitol)	Nagae *et al.*,[81] Jiang *et al.*,[82] Jiang *et al.*[82]
α-Sialidase	*O*-Glycans, *N*-glycans	DANA Zanamivir Oseltamivir Peramivir	HMOs, developmental sialic acid, mucin degradation	8AXI (DAN) 2YA7 (Zanamivir)	Shuoker *et al.*,[83] Gut *et al.*,[84] von Itzstein *et al.*,[85] Kim *et al.*,[86] Babu *et al.*[87]
β-Hexosamindase	*O*-Glycans, *N*-glycans	PUGNAc NAGT	Mucin-glycan and immunoglobulin-glycan degradation	2VVS (PUGNAc)	Beer *et al.*,[88] Knapp *et al.*,[89] Macauley *et al.*[90]
α-Mannosidase	Host *N*-glycans, alpha-mannans yeast cell-wall, plant *N*-glycans	Deoxymannojirimycin Swainsonine Mannoimidazole Kifunensine	Immunoglobulin-glycan degradation	2WW0 (swainsonine) 2WZS (mannoimidazole) 2WVZ (kifunensine)	Thompson *et al.*,[91] Zhu *et al.*,[92] Fuhrmann *et al.*[93]

[a] See relevant enzyme sections for names of inhibitor acronyms.

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Such reagents have helped to define the potential scope of therapeutically targeting the gut microbiota, with the goals of controlling drug toxicity and enhancing drug efficacy.

Dietary glycans

The impact of dietary xenobiotics in the gut microbiota has recently emerged as a major research focus.[10,51] Plant-based dietary compounds often exist as glucose conjugates, such as soy phytoestrogens (*i.e.* daidzin and genistin) and resveratrol (polydatin) (Fig. 1).[10,52] Culp *et al.* demonstrated that these compounds can significantly alter gut microbial composition. Glycoside conjugates are generally less active on host systems than the aglycones created by gut microbial GHs.[10] In plants, such glycosides are cloaked defence mechanisms against pathogens that are activated upon glycoside removal.[10,52]

Recently, Kuziel *et al.* demonstrated the ability of gut microbes to metabolize dietary phenolic glycosides releasing active aglycones that influence host-health.[53] Another class of diet-derived compounds are glucosinolates, which are abundant in cruciferous vegetables, like broccoli and cabbage.[54] A recent paper highlights the ability of a commensal gut microbe, *B. thetaiotaomicron*, to release isothiocyanates conjugated to glucose, utilizing thio-specific GHs.[55] These isothiocyanates have been implicated to possess anti-cancer properties.[56]

Dietary carbohydrates reach the GI tract and are exposed to gut microbes. Examples of dietary carbohydrates include cellulose and starches. Importantly, these molecules reach the GI tract largely intact, where they are then degraded by gut bacteria and fermented to produce short-chain fatty acids (SCFAs). Human milk oligosaccharides, and other dairy products, contain glycans that are not processed by human enzymes but are metabolized by gut microbial GHs. Indeed, the infant gut microbiota consists mostly of Bifidobacteria suggesting the potential in using such oligosaccharides as prebiotic compounds to promote certain species.[57,58] Thus, inhibitors specific to relevant gut microbial GHs would help to define molecular mechanisms involved in diet, host and microbial factors.

Small-molecule CAZyme inhibitors

The complexity of the gut microbiota presents significant challenges for researchers seeking to define how these microbes influence human health. Small-molecule inhibitors provide generally actionable tools for understanding these intricate details and has broadly been reviewed by Woo *et al.*[59] Thorough reviews of GH inhibitors have been presented recently by Asano *et al.*[94] and Kim *et al.*;[95] however, this is not the purpose of this review. Instead, here we focus on the applications of GH inhibitors in the context of the gut microbiota. Recent work has expanded the use of small-molecule inhibitors to investigate GHs in the gut microbiota.[71] Host-targeting GH inhibitors can impact the function of the gut microbiota,[96] and a recent study shows that common therapeutics influence endobiotic glycoconjugate availability by inhibiting gut microbial GHs.[7] To date, most GH inhibitors have been substrate mimics, making selective targeting of microbial GHs difficult (Table 1). Indeed, the most prevalent class of GH inhibitors is derived from nitrogen-containing substrate analogues. Importantly, though, substrate-

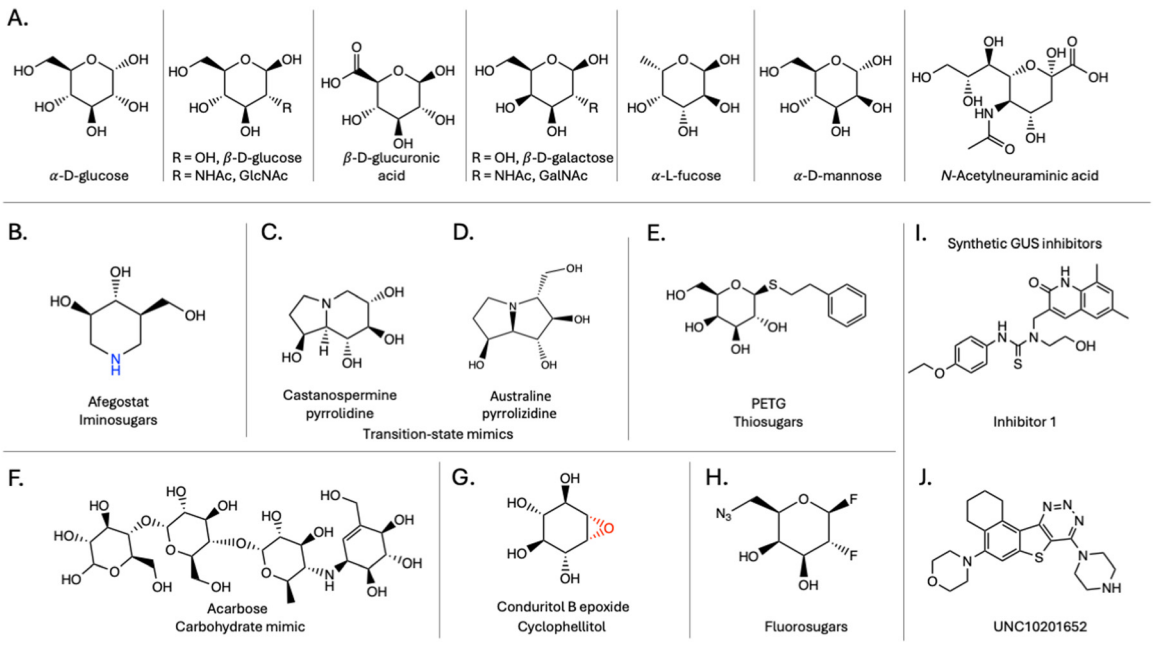

Fig. 2 (A) Chemical structures of monosaccharides, which are the targets for the glycoside hydrolases described in this review. Glycoside hydrolase inhibitor structural classes with representative chemical structures for each class: (B) afegostat, an iminosugar β-glucosidase inhibitor; (C) castanospermine, a pyrrolidine β-glucosidase inhibitor; (D) australine, a pyrrolizidine α-glucosidase inhibitor; (E) PETG, a thiosugar β-galactosidase inhibitor; (F) acarbose, a carbohydrate mimic α-glucosidase inhibitor (G) conduritol B epoxide, a cyclophellitol β-glucosidase inhibitor; (H) 6-azido-2,6-dideoxy-2-fluoro-β-D-galactosyl fluoride a fluorosugar β-galactosidase inhibitor and the synthetic β-glucuronidase inhibitors (I) inhibitor 1 and (J) UNC10201652.

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mimicking chemical probes have been developed and deployed to allow fluorescent labelling of specific GHs.[97] These probes can also be conjugated to biotin to selectively enrich for GHs of interest, using streptavidin beads to increase sensitivity in proteomic studies.[7]

Broadly, GH inhibitors fall into several classes, most prominently including iminosugars (Fig. 2B), thiosugars (Fig. 2E), pyrrolidines (Fig. 2C), pyrrolizidines (Fig. 2D), fluorosugars (Fig. 2H), cyclophellitol-aziridines (Fig. 2G), and synthetic compounds[97,98] (Fig. 2I, J and Table 1). Numerous structural studies have examined the binding modes of these inhibitors, offering a valuable resource for future inhibitor development (Fig. 3). Although a good portion of these compounds have been developed to treat lysosomal storage diseases, this review will discuss their potential in studying gut microbial CAZymes.

Glycoside hydrolases have traditionally been classified according to the two Koshland mechanisms,[99,100] which utilize a catalytic nucleophile and acid/base residue to facilitate hydrolysis. These mechanisms differ in the stereochemistry of the anomeric carbon. Retaining GHs conserve the original configuration while inverting GHs reverse it. In the case of inverting GHs, the active site must accommodate a water nucleophile and therefore has a larger distance between the catalytic residues, providing unique active site architectures. These active site differences likely influence inhibitor binding and suggest mechanism-specific inhibitors could be developed for greater selectivity.

The mechanisms of GHs has been thoroughly reviewed previously by Davies and Henrisatt.[101] Recently, a non-Koshland mechanism was discovered for bacterial GHs, utilizing anionic transition states, in contrast to cationic transition states observed in Koshland mechanisms.[102] Notably the enzymes in this study show broad substrate promiscuity due to the ability for these GHs to accommodate both α- and β-anomers.[102]

These different mechanisms highlight the importance of diverse GH inhibitor classes. Current inhibitors may show preference for GHs of a certain catalytic mechanism over others. Finally, this discovery of non-Koshland GHs, particularly in the context of the gut microbiota, stresses the importance for new classes of GH inhibitors be discovered and to investigate the utility of current GH inhibitors against these non-Koshland GHs.

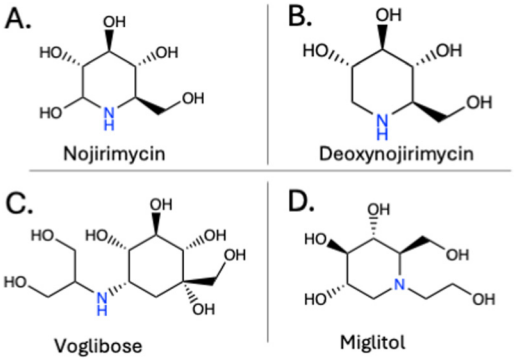

Fig. 4 Chemical structures for representative α-glucosidase inhibitors (A) nojirimycin, (B) deoxynojirimycin, (C) voglibose, and (D) miglitol.

α-Glucosidase inhibitors

Glucose is an abundant monosaccharide in sucrose, maltooligosaccharides and starch. The first known α-glucosidase inhibitor, nojirimycin (Fig. 4A), was discovered in 1966 as an antibiotic produced by Streptomyces[61] and then shown to be an α- and β-glucosidase inhibitor in 1970.[62] A more stable form of this inhibitor, deoxynojirimycin (Fig. 4B), was identified in mulberry leaves and then isolated from *Bacillus subtilis* DSM704.[106,107] Deoxynojirimycin served as a blueprint for subsequent inhibitor development, including those targeting GHs beyond glucosidases. Subsequent α-glucosidase inhibitors include acarbose (Fig. 2F), voglibose (Fig. 4C) and miglitol (Fig. 4D).[108] They were developed to target host α-glucosidases to reduce carbohydrate degradation in the intestines to reduce free glucose levels as a therapeutic intervention for Type II diabetes.[13,14] While originally focused on host enzymes, they also inhibit GHs in the gut microbiota.[63] Tan *et al.* reported that a GH31 α-glucosidase from *Blaubia obeum* is inhibited by acarbose (Fig. 3A), voglibose and miglitol (Table 2).[60]

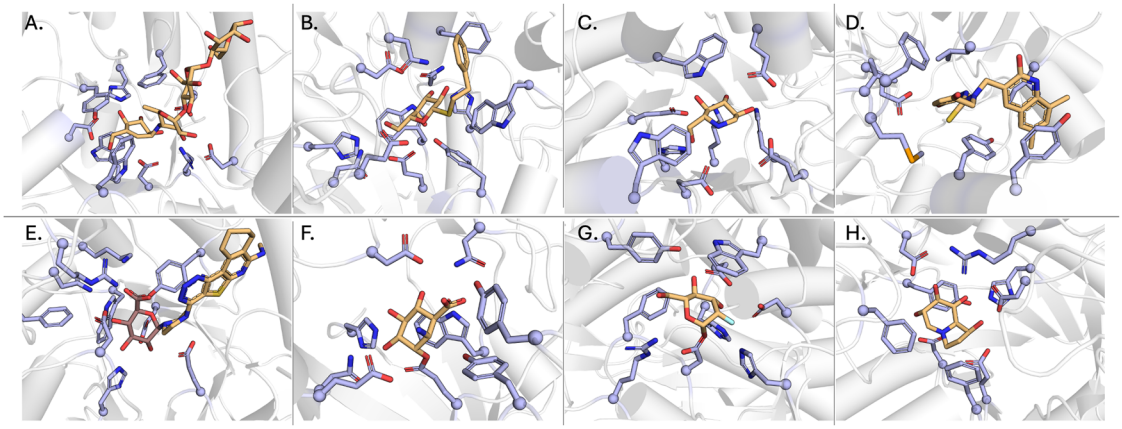

Fig. 3 Binding mode for (A) acarbose in complex with an α-glucosidase (3PHA), (B) PETG in complex with a β-galactosidase (6CVM), (C) deoxynojirimycin bound to an α-glucosidase (2JKE), (D) InhR1 in complex with a β-glucuronidase (5CZK), (E) UNC10206581 in complex with a β-glucuronidase (8UGT), (F) cyclophellitol in complex with a β-glucuronidase (6NZG), (G) fluorosugar in complex with an α-mannosidase (1QX1), (H) castanospermine bound to a β-glucosidase (2PWG). Inhibitors are coloured light orange and active site residues are coloured light blue.

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Table 2 α-Glucosidase inhibitors

Compound	Microbial target	K_i/IC50	Mechanism	Discovery phase	Ref.
Deoxynojirimycin	N/A (not available)	N/A	Competitive	*In vivo* studies	Li *et al.*[103]
Voglibose	Blaubia obeum GH31	8.3 μM[a]	Competitive	*In vivo* studies	Tan *et al.*[60] Bae *et al.*[104]
Miglitol	Blaubia obeum GH31	32 μM[a]	Competitive	FDA approval for type II diabetes	Tan *et al.*[60]
Acarbose	BoSusG	2.2 μM[a]	Competitive	FDA approval for type II diabetes	Brown *et al.*[105]
	SusG	68 μM[a]			
	BoSusA	123 nM[b]			
	SusA	95 nM[b]			
	BoSusB	69 nM[b]			
	SusB	54 nM[b]			
	BoGH97D	134 nM[b]			
	BtGH97H	162 nM[b]			

[a] IC50. [b] K_i.

Notably, voglibose and acarbose have low absorption,[109,110] and therefore remain mostly in the GI tract, where they can interact with microbial α-glucosidases. Furthermore, these inhibitors have been shown to impact the polysaccharide utilization loci employed by microbes in the Bacteroidota phylum, limiting their nutrient scavenging and thus impacting their growth through non-microbicidal means.[105,111,112] Another study found increased short-chain fatty acid production, by-products of carbohydrate fermentation, in patients with Type II diabetes receiving acarbose, indicating that dietary fiber remains intact for fermentation by the gut microbiota.[113]

Recent data have also indicated that the gut microbiota influences the effectiveness of the antidiabetic drug, acarbose.[96,114,115] Work from the Donia Lab showed that gut microbes produce enzymes capable of deactivating acarbose *via* phosphorylation.[96] Furthermore, Tian *et al.* found that increased *Klebsiella grimontii* abundance reduced efficacy in patients treated with acarbose and that acarbose efficacy is reduced in mice treated with *K. grimontii*.[114] *K. grimontii* encodes an acarbose-targeting glucosidase capable of degrading acarbose and reducing its inhibitory effects.[114] Thus, using small molecular inhibitors to target α-glucosidases produced by gut bacteria alters microbial composition and reduces the level of glucose released from complex polysaccharides.

β-Glucosidase inhibitors

Plant phytochemicals are commonly conjugated to a glucose sugar that serves to inactivate the compound until its use is required. Many phytochemicals have antimicrobial properties, acting as a defence mechanism against pathogens, and are thus stored by plants as inactive glucoside conjugates.[52] Plant β-glucosidases activate these reagents when a pathogen is detected. Phytochemicals are obtained by humans through diet and reach the GI tract where they are exposed to microbial β-glucosidases that have the capacity to remove the conjugating glucose, changing the activity of the ingested compound. Well studied phytochemicals include the soy-derived phytoestrogens, daidzein and genistein, which are of particular interest due to their activity on estrogen receptors,[116] potentially interrupting estrogen signalling. Phytoestrogens are commonly found as inactive glucose conjugates, such as daidzin and genistin, making them targets for gut microbial β-glucosidases. The grape-derived phytochemical resveratrol is also commonly found in its glucoside conjugate, polydatin. Phytochemicals are implicated in host health due to their anti-cancer, anti-inflammatory, and antioxidant properties (Fig. 5A).[117] Beyond their roles in activating glucose-conjugated small molecules, gut microbial β-glucosidases are essential proteins for degrading cellulose.[118]

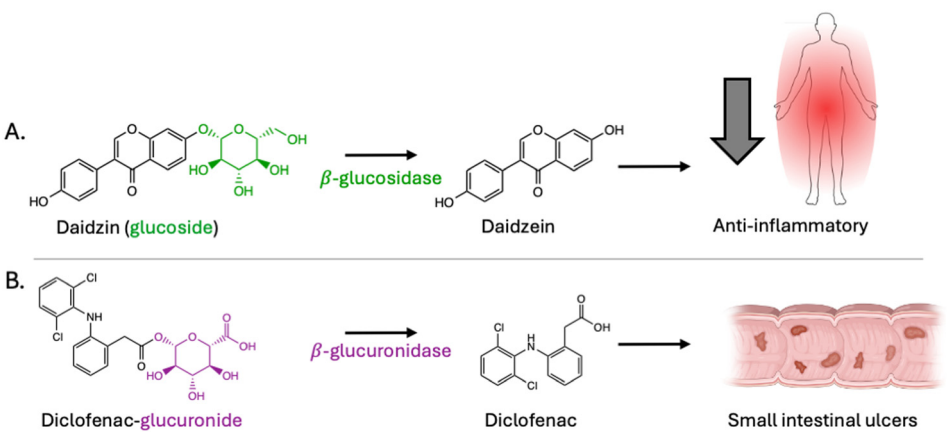

Fig. 5 (A) Daidzin activation pathway by gut microbial β-glucosidases, producing daidzein which has anti-inflammatory properties. (B) Diclofenac reactivation pathway by gut microbial β-glucuronidases resulting in intestinal ulcers.

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There are several β-glucosidase inhibitors: afegostat (isofagomine) (Fig. 2B), an iminosugar designed for treating Gaucher's disease, a lysosomal storage disorder;[64] castanospermine (Fig. 2C), an alkaloid identified from Castanospermum australe seeds,[65] and conduritol B epoxide (Fig. 2G), a mechanism-based covalent inhibitor that served as a prototype for covalent probe development.[66] These inhibitors have seen limited application to the gut microbiota but have been examined as a therapeutic approach for *Clostridium difficile* infections (Table 3). Paparella *et al.* studied the ability of two β-glucosidase inhibitors to block the *Clostridoides difficile* toxins TcdA and TcdB.[119] In this work, the β-glucosidase inhibitors isofagomine and noeuromycin reduced TcdA- and TcdB-mediated cell toxicities by blocking UDP binding to TcdA and TcdB glucosyltransferase domains (GTs) and preventing their activation of the host Rho GTPase.[119] While this work focuses on *C. difficile* infection, future studies are needed to examine the ability of these inhibitors to potentially modulate microbial composition or alter phytochemical levels.

β-Glucuronidase inhibitors

Glucuronidation is a chemical modification that takes place primarily in the liver and gut epithelium and plays a central role in phase II metabolism. Glucuronidation most often inactivates compounds and typically marks them for excretion *via* the urine or bile.[121] However, β-glucuronidases (GUS), produced by gut bacteria, can reverse this process in the GI tract by removing the glucuronide and reactivating the aglycone (Fig. 5B). The first reported GUS inhibitor, saccharolactone (Fig. 6A), acts as a substrate mimic,[73] and other substrate mimics such as uronic-Noeurostegine (Fig. 6B)[74] and iminosugars[75] have also been reported. Because these compounds mimic the native substrate, they broadly inhibit GUS enzymes, targeting both host and bacterial GUS. The lethal lysosomal storage disease Sly Syndrome is caused by inactivating mutations of human GUS (hGUS). Thus, it has been considered important to employ GUS inhibitors that are selective for microbial GUS over hGUS.

Glucuronidation is a common metabolic step for drug compounds, inactivating them and marking them for excretion. A well-examined example is the glucuronidation of SN38, the active metabolite of irinotecan, a chemotherapy drug for colon and pancreatic cancer. Glucuronidated SN38-G is marked for excretion through the bile but when it reaches the gut lumen it is exposed to gut microbial GUS, that remove the glucuronic acid moiety. The reactivated antineoplastic drug SN38 moiety kills intestinal epithelial cells leading to severe, delayed and dose-limiting diarrhoea. The Redinbo Lab showed

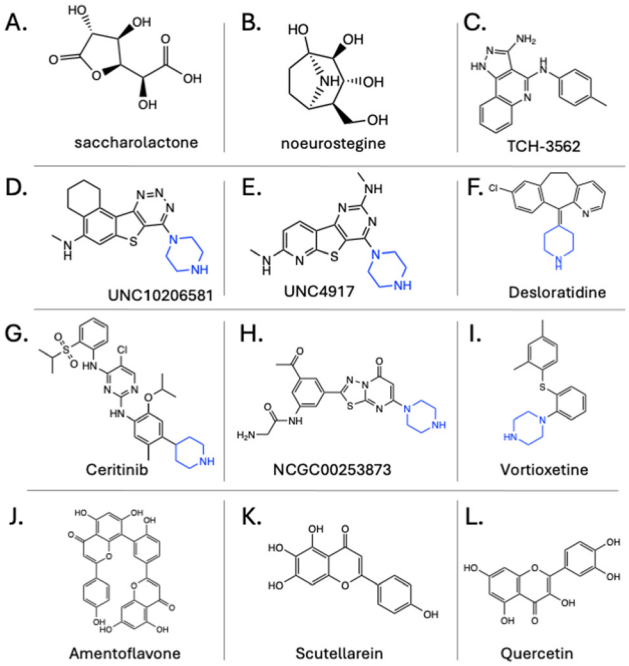

Fig. 6 Chemical structures for representative β-glucuronidase inhibitors (A) saccharolactone, (B) noeurostegine, (C) TCH-3562, (D) UNC10206581, (E) UNC4917, (F) desloratidine, (G) ceritinib, (H) NCGC00253873, (I) vortioxetine, (J) amentoflavone, (K) scutellarein, and (L) quercetin. Reactive piperazine and piperadine moieties are highlighted in blue.

in 2010 that inhibiting gut microbial GUS alleviates GI toxicity caused by SN38 in a rodent model.[8] This initial compound, Inhibitor 1, was identified through a high throughput screen and potently inhibits *E. coli* GUS (Fig. 2I and 3D).

In subsequent work, microbial GUS were found to be a structurally diverse family, with two loop (Loop 1, Loop 2) regions of varying length and some containing a flavin mononucleotide (FMN) binding site.[76,122] These structural differences influence the substrate preferences of microbial GUS. Second generation GUS inhibitors, UNC10201652 (Fig. 2J and 3E) and UNC4917 (Fig. 6E), identified from the same high throughput screen that discovered Inhibitor 1, utilize a piperazine warhead that intercepts the catalytic cycle of the enzyme.[69] The secondary piperazine amine forms a covalent bond with a glucuronic acid in the active site. Structural studies from the Redinbo Lab have identified a key motif that differentiates GH2 GUS from GH2 β-galactosidases.[44] This NxK motif in GUS contacts the carboxyl group on the glucuronic acid and is responsible for its recognition (Fig. 7A). In contrast, GH2

Table 3 β-Glucosidase inhibitors

Compound	Target	K_i/IC50	Mechanism	Discovery phase	Ref.
Afegostat	β-Glucocerebrosidase	5 nM[a]	Competitive	Clinical trials	Street *et al.*,[64] Paparella *et al.*[119]
	TcdB	4.8 μM[b]			
Castanospermine	Almond GH1 β-glucosidase	10 μM[b]	Competitive	*In vivo* studies	Saul *et al.*,[65] Paparella *et al.*[119]
	TcdB	3.4 mM[b]			
Conduritol B epoxide	β-Glucocerebrosidase	4.3 μM[a]	Covalent inhibitor	*In vivo* studies	Kuo *et al.*[120]

[a] IC50. [b] K_i.

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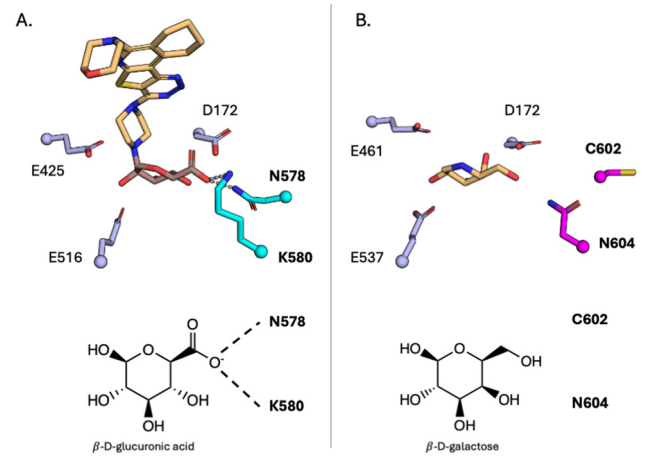

Fig. 7 Active site differences for GH2 β-glucuronidases (GUS) and β-galactosidases (GAL). (A) Active site of a GH2 GUS (PDB 8GEN) bound to UNC10201652-glucuronide, with residues N578 and K580 highlighted in cyan. These residues recognize the carboxyl on glucuronic acid and differentiate enzymes within the GH2 family. (B) Active site of a GH2 GAL bound to deoxygalactonojirimycin (PDB 6TSH) with C602 and N604 highlighted in magenta. These residues differentiate the active sites of these proteins and can therefore be exploited to develop selective inhibitors.

β-galactosidases lack this motif (Fig. 7B). Similarly, other GHs have residues responsible for recognizing specific monosaccharides based on the orientation of hydroxyls. Such features could be exploited in future work to develop inhibitors that are selective for certain enzymes within a GH family. Recent work by Graboski *et al.* optimized the scaffold of UNC10201652 through an SAR campaign creating a potent FMN- and Loop-1 selective GUS inhibitor, UNC10206581.[71] Additionally, the authors illustrate the utility of such inhibitors to target GUS extracted from faecal lysates and in bacterial cell culture. These inhibitors are promising due to their specificity for a structural category of GUS enzymes responsible for liberating small-molecule glucuronide conjugates. Furthermore, these inhibitors can serve as a prototype for developing similar mechanism-based inhibitors of other GHs.

A wide range of human therapeutics reach the gut as inactive glucuronides, and several have been shown to be reactivated by gut microbial GUS including regorafenib,[47] NSAIDs[48–50,123,124] and the immunosuppressant mycophenolate.[46] Modulation of GUS by small molecule inhibitors can alleviate toxicities associated with these therapeutics.[69,73–76,121,122] The ubiquitous consumer product toxin triclosan is also glucuronidated and has been shown to reach the human gut and to cause GI toxicity and carcinogenesis.[125] Blocking gut microbial GUS was recently found to prevent triclosan activation and to prevent colitis in a mouse model.[126] New classes of *E. coli* GUS inhibitors identified through *in silico* screening efforts have recently been reported, providing a diverse collection of GUS targeting small-molecule inhibitors (Fig. 6 and Table 4).[72,127,128] In all cases, the GUS inhibitors being developed are selective for microbial GUS over hGUS.[8,44,45,69,71] Gut microbial GUS inhibitor results have helped to establish that selective compounds may be developed and deployed to modulate other intestinal symbiote GH proteins in future work.

Dietary flavonoids have been identified as microbial GUS inhibitors, highlighting the diverse compounds that inhibit microbial GUS.[129,130] Flavonoid-based formulations have been of significant interest for alleviating chemotherapy-induced toxicity in clinical trials and other animal studies.[131–133] Examples of flavonoids that inhibit microbial GUS are amentoflavone, scutellarein, and quercetin (Fig. 6J–L and Table 4). These compounds are ubiquitous in plants, and they highlight a key mechanism by which diet influences host physiology, potentially leading to avenues for alleviating chemotherapy-induced toxicity. Studies such as these suggest the potential for developing more potent GUS inhibitors based on these natural products.

While drug and xenobiotic inactivation has been a major focus of the gut GUS-mediated reactivation studies outlined above, a wide range of endobiotic hormones, neurotransmitters and other compounds also reach the mammalian GI tract as inactive glucuronides. A recent paper begins to highlight the intricate role of non-host GUS in modulating neurotransmitter and hormone levels.[7] Simpson *et al.* showed that the glucuronides of serotonin, dopamine, estrone, estradiol and thyroxine are all substrates for specific sets of gut microbial GUS.[7] They also demonstrated that commonly used medications at physiologically relevant concentrations can modulate GUS activity, reducing gut and systemic levels of serotonin. The drugs that appear capable of causing these effects contain either a piperazine or piperadine secondary amine that intercept the catalytic cycle of gut microbial GUS using the same mechanism employed by second-generation GUS inhibitors. These results indicate many drugs influence endobiotic homeostasis *via* gut microbial GUS enzymes, and may be capable of impacting other GHs, as well. Finally, several of the medications shown recently to inhibit gut microbial GUS are psychoactive drugs employed for diseases associated with serotonin and dopamine levels, suggesting that their efficacies may in part be due to effects on microbial as well as host factors.[7,9,134,135] These data further support the conclusion that microbial symbiotes play important roles in host immunity, inflammation, neurological, and hormonal changes *via* mechanisms yet to be fully elucidated.

β-Galactosidase inhibitors

Many GH substrates in the GI tract contain galactose, including mucin glycans, galacto-oligosaccharides (GOS), *N*-glycans, and human milk oligosaccharides (HMOs). Several commensal gut microbes produce *endo*- and *exo*-β-galactosidases that are integral to mucin degradation.[140] Potent β-galactosidases inhibitors include 1-deoxy-galactonojirimycin (Fig. 8A), galactosylamine (Fig. 8B),[79] and 2-phenylethyl β-D-thiogalactoside (PETG) (Fig. 2E and 3B).[80] To date, these inhibitors have been studied in the context of lysosomal storage disorders by targeting host enzymes.[141] Interestingly, dietary compounds have been found to influence β-galactosidase activity. For example, caffeine and theophylline mildly inhibit *E. coli* β-galactosidase,[142] suggesting dietary xenobiotics influence galactose liberation by gut microbes.

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Table 4 β-Glucuronidase inhibitors

Compound	Microbial target	IC50	Mechanism	Discovery phase	Ref.
Saccharolactone	*C. perfringens*	6.2 μM	Competitive	*In vitro*	Bai *et al.*[129]
	E. coli	28 μM			
	S. pasteuri	4.7 μM			
TCH-3562	*E. coli*	36 nM	Non-competitive	*In vitro*	Cheng *et al.*[127]
Inhibitor 1	*E. coli*	283 nM	Competitive	*In vivo*	Wallace *et al.*[8]
UNC10201652	*E. coli*	100 nM	Competitive	*In vivo*	Biernat *et al.*,[136]
	E. eligens	410 nM			Simpson *et al.*[7]
	S. agalactiae	133 nM			
	C. perfringens	26 nM			
UNC10206581	*E. coli*	29 nM	Competitive	*In vitro*	Graboski *et al.*[71]
	E. eligens	88 nM			
UNC4917	*E. coli*	80 nM	Competitive	*In vitro*	Pellock *et al.*[69]
Ceritinib	*E. coli*	48 μM	Competitive	FDA approval for ALK–positive metastatic NSCLC	Simpson *et al.*[7]
	S. agalactiae	12 μM			
	C. perfringens	3.4 μM			
	E. eligens	23 μM			
	Gemmiger L1	45 μM			
	Gemmiger FMN	5.0 μM			
	R. hominis 2	11 μM			
	R. inulinvorans	10 μM			
	F. prausnitzii	8.0 μM			
	R. gnavus 3	98 μM			
Desloratidine	*S. agalactiae*	81 μM	Competitive	FDA approved antihistamine	Simpson *et al.*[7]
	C. perfringens	47 μM			
	Gemmiger FMN	38 μM			
	R. hominis	36 μM			
	R. inulinvorans	22 μM			
Vortioxetine	*E. coli*	48 μM	Competitive	FDA approved antidepressant	Simpson *et al.*[7]
	S. agalactiae	6.0 μM			
	C. perfringens	13 μM			
	E. eligens	8.0 μM			
	Gemmiger L1	38 μM			
	Gemmiger FMN	31 μM			
	R. hominis 2	10 μM			
	R. inulinvorans	0.3 μM			
	F. prausnitzii	8.0 μM			
NCGC00253873	*E. coli*	3.8 μM	Competitive	*In vitro*	Challa *et al.*[72]
Scutellarein	*E. coli*	5.8 μM	Competitive	Clinical trials	Weng *et al.*[133] Duan *et al.*[137] Chen *et al.*[138]
Quercetin	*E. coli*	21 μM	Competitive	Clinical trials	Weng *et al.*[133] Ferry *et al.*[139]
Amentoflavone	*C. perfringens*	2.4 μM	Non-competitive	*In vitro*	Bai *et al.*[129]
	S. pasteuri	2.9 μM			
	E. coli	3.4 μM			

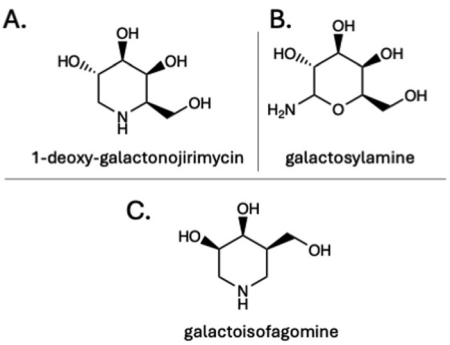

Fig. 8 Chemical structures for representative β-galactosidase inhibitors (A) 1-deoxy-galactonojirimycin, (B) galactosylamine, and (C) galactoisofagomine.

A recent study from Zeng *et al.* demonstrated that a β-galactosidase from *Lactobacillus vaginalis* activates the soy phytoestrogen daidzein, which in turn reduced acetaminophen toxicity by inhibiting farnesyl diphosphate synthase, a ferroptosis pathway enzyme.[143] The researchers show that the beneficial effects from daidzein were eliminated when mice were dosed with the β-galactosidase inhibitor D-Ribono-1,4-lactone, uncovering the importance of β-galactosidases in the availability of beneficial phytochemicals.[143] This also indicates that some β-galactosidases can act on glucose-conjugated small molecules. Additionally, β-galactosidases are essential proteins for the degradation of GOS, and studies show that GOS increases the abundance of lactose degrading microbes β-galactosidase activity.[144] The use of β-galactosidase inhibitors to study gut microbial processes to this point has been limited. However, these reagents offer valuable utility in future studies

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Table 5 β-Galactosidase inhibitors

Compound	Target	K_i	Mechanism	Discovery phase	Ref.
1-Deoxy-galactonojirimycin	*S. pneumoniae* BgaA	34 μM	Competitive	*In vitro*	Singh *et al.*[145]
Galactoisofagomine	*S. pneumoniae* BgaA	25 nM	Competitive	*In vitro*	Singh *et al.*[145]
Galactosylamine	*E. coli* β-galactosidase	59 μM	Competitive	*In vitro*	Huber *et al.*[79]
PETG	*E. coli* β-galactosidase	8 μM	Competitive	*In vitro*	Hadd *et al.*[146]

for uncovering the microbes and microbial factors that are responsible for the degradation key gut substrates (Table 5).

α-Fucosidase inhibitors

Fucose is a common monosaccharide found in host-derived glycans, including mucins and immunoglobulins, both of which are abundant in the gut. Fucose is generally added *via* an α1,2 linkage to galactose or through an α1,3/4/6 linkage to GlcNAc and is commonly found at the termini of glycans. Two main GH families exhibit fucosidase activity: GH29 and GH95. The GH95 family is specific for α1,2 linkages while GH29 is divided into two subfamilies (GH29A and GH29B) based on substrate preference. Fucose is also a prevalent monosaccharide in HMOs, highlighting the importance of microbial α-fucosidases in infant development.[147] The iminosugar fucosidase inhibitor, deoxyfuconojirimycin (DFJ) (Fig. 9A), was originally developed for treating the lysosomal storage disorder, fucosidosis.[148] DFJ is a potent inhibitor for GH29 fucosidases and effectively inhibits the two human fucosidases, FUCA1 and FUCA2.[149,150] More potent inhibitors of fucosidases were developed by modifying DFJ with various aglycone groups at the C1 position, leading to compound **2** (Fig. 9B) with a picomolar binding affinity for *Corynebacterium* sp. Fucosidase (Table 6).[151]

Recently Shuoker *et al.* showed that fucosidases are essential for the growth of *Akkermansia muciniphila* on a mucin substrate and the ability for this taxum to utilize mucin glycans is prevented when DFJ is present (Table 6).[83] These data indicate that for *A. muciniphila* to fully utilize mucin glycans functionally active fucosidases are required to initiate this process.

Additionally, fucosidases are of great interest for HMO degradation, since these oligosaccharides are not metabolized by the infant-produced host factors but fermented in the GI tract by gut microbes.[147] Indeed, the most prevalent HMO is 2′-fucosyllactose,[152] emphasizing the importance of fucosidases in bacterial fermentation during infant development. Thus, fucosidase inhibitors may help to define how bacteria utilize mucin and HMO substrates *via* fucosidase enzymes.

α-Sialidase inhibitors

Sialic acid is a common moiety found at the termini of mucin glycans and HMOs.[20] Like fucose, sialic acid removal facilitates the bulk degradation of the full oligosaccharide. Historically, many inhibitors of sialidases have been developed to combat viral infections.[28] However, these inhibitors offer a promising avenue for unravelling the role of these enzymes in the gut microbiome. One such inhibitor is 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) (Fig. 10A), a transition state analogue of sialic acid.[154] This inhibitor served as the basis for future inhibitor development including, zanamivir (Fig. 10B),[85] oseltamivir (Fig. 10C),[86] peramivir (Fig. 10D)[87] all of which have been developed to target influenza sialidases.[28]

Sialidases have also been studied recently in the vaginal microbiota due to their ability to act on host mucin glycans.[155] Pelayo and colleagues showed that prevalent bacterial sialidases in the vaginal microbiome are effectively inhibited by DANA and zanamivir (Table 7), indicating that these viral sialidase inhibitors can also target bacterial sialidases. Similar studies could be conducted focused on sialidases produced by the gut microbiota to probe their activities against mucin glycans and HMOs. Recently, *A. muciniphila* sialidases have been examined for their importance in mucin utilization, and it was found that that DANA inhibits this taxum's growth on porcine colonic mucus (Table 7).[83] Thus, some bacteria rely on

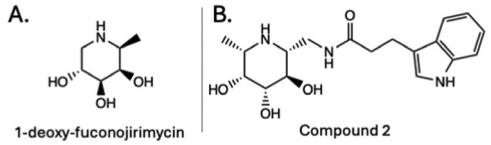

Fig. 9 Chemical structures for representative α-fucosidase inhibitors (A) 1-deoxy-fuconojirimycin, and (B) compound **2**.

Table 6 α-Fucosidase inhibitors

Compound	Microbial target	K_i/IC50	Mechanism	Discovery phase	Ref.
1-Deoxy-fuconojirimycin	*S. roseum* fucosidase	0.22 nM[a]	Competitive	*In vitro*	Bishnoi *et al.*[153] Shuoker *et al.*[83]
	*Am*GH29A	0.60 μM[a]			
	*Am*GH29B	1.5 μM[a]			
	*Am*GH29C	14 μM[a]			
	*Am*GH29D	7.3 μM[a]			
	*Am*GH95A	54 μM[a]			
	*Am*GH95B	24 μM[a]			
Compound **2**	*Corynebacterium* sp. fucosidase	0.46 pM[b]	Competitive	*In vitro*	Chang *et al.*[151]

[a] IC50. [b] K_i.

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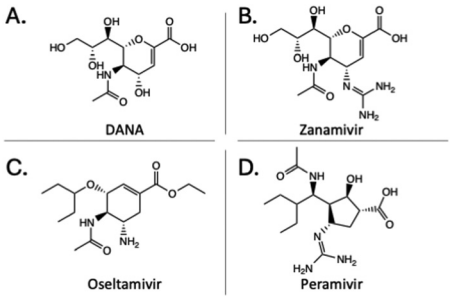

Fig. 10 Chemical structures for representative α-sialidase inhibitors (A) DANA, (B) zanamivir, (C) oseltamivir, and (D) peramivir.

these proteins to access host glycans as a carbon source. Additionally, microbes that encode sialidases can release monosaccharides for other bacteria to utilize. Shuoker *et al.* describe in cross-feeding studies that *A. muciniphila*-released terminal sugars are the utilized by Clostridia bacteria to produce the short-chain fatty acid, butyrate.[83]

β-Hexosaminidase inhibitors

N-Acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) are abundant monosaccharides found in mucins, *N*-glycans, and chitin. GlcNAc is one of the most abundant monosaccharides in mucins, and GalNAc is the first monosaccharide added in all *O*-glycans. β-Hexosamindases are produced by all forms of life and play a key role in the degradation of mucin glycans, immunoglobulin *N*-glycans, and other carbohydrates present in the GI tract. Inhibitors of β-hexosamindases have been developed with a focus on neurodegenerative lysosomal storage diseases, such as Tay-Sachs and Sandhoff diseases. Notable inhibitors include the phenyl carbamate inhibitor, PUGNAc (Fig. 11A),[88] and 1,2-dideoxy-2′-methyl-D-glucopyranoso[2,1-D]-2′-thiazoline (NAGT) (Fig. 11B).[89]

The commensal microbe *Bifidobacterium bifidum* is a key player in HMO and mucin *O*-glycan degradation. A recent report identified an *N*-acetylglucosaminidase (GlcNAcase) specific for sulfated GlcNAc residues.[157] Katoh *et al.* provided evidence for *O*-glycan degradation by this enzyme, along with a thorough structural analysis using X-ray crystallography. Furthermore, they synthesized two sulfo-GlcNACase inhibitors by adding a sulfate to PUGNAc and NAGT (Table 8). The authors then utilize NAGT-6S to inhibit *O*-glycan degradation by *B. bifidum* in culture.[157] GlcNACase inhibitors have been the focus of groups developing novel antimicrobials for drug resistant pathogens like *Staphylococcus aureus*.[158,159] Thus, novel GlcNAcase inhibitors could impact on gut microbiota composition along with advancing our understanding of gut microbial function.

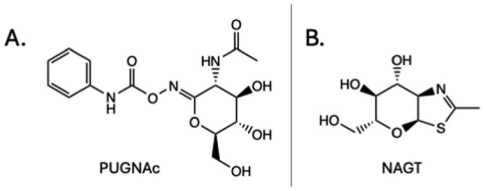

Fig. 11 Chemical structures for representative β-hexosaminidase inhibitors (A) PUGNAc, and (B) NAGT.

α-Mannosidase inhibitors

Mannose is an abundant monosaccharide in *N*-glycans and is commonly found in the glycosylations that decorate immunoglobulins and the glycocalyx. Mannose is also abundant in yeast cell walls obtained through diet. A landmark study illustrated that a prevalent member of the gut microbiota, *B. thetaiotaomicron*, utilizes mannan glycans derived from yeast cell-walls.[160] Additionally, other work has shown that mannosidases from Bacteroides species play key roles in the degradation of plant-derived *N*-glycans, further emphasizing their importance in dietary glycan metabolism.[161] The first reported mannosidase inhibitor was 1-deoxymannojirimycin (Fig. 12A), an iminosugar,[93] and others have subsequently been developed, including swainsonine (Fig. 12B), mannoimidazole (Fig. 12C) and kifunensine (Fig. 12D).

Crystal structures of *B. thetaiotaomicron* mannosidases in complex with swainsonine, mannoimidazole and kifunensine have been reported and reveal conserved interactions.[91,92] In this work from the Davies Lab, 22 mannosidases from *B. thetaiotaomicron* were examined and led to valuable information on substrate preferences and a validated roadmap for using mannosidase inhibitors in the context of the gut microbiome (Table 9).

Table 8 β-Hexosaminidase inhibitors

Compound	Microbial target	K_i	Mechanism	Discovery phase	Ref.
PUGNAc-6S	BbhII	15.4 nM	Competitive	*In vitro*	Katoh *et al.*[157]
NAGT-6S	BbhII	52.3 nM	Competitive	*In vitro*	Katoh *et al.*[157]

Table 7 α-Sialidase inhibitors

Compound	Microbial target	IC50	Mechanism	Discovery phase	Ref.
DANA	*P. timonensis* NanH1	56 μM	Competitive	*In vivo*	Pelayo *et al.*[155] Shuoker *et al.*[83] Karhadikar *et al.*[156]
	P. timonensis NanH2	180 μM			
	P. Bivia NanH	103 μM			
	P. denticola NanH	51 μM			
	G. vaginalis NanH3	29 μM			
	*Am*GH33A	61 μM			
	*Am*GH33B	133 μM			
	*Am*GH181	199 μM			
Zanamivir	*P. timonensis* NanH1	4200 μM	Competitive	FDA approval for influenza	Pelayo *et al.*[155]
	P. timonensis NanH2	34 μM			

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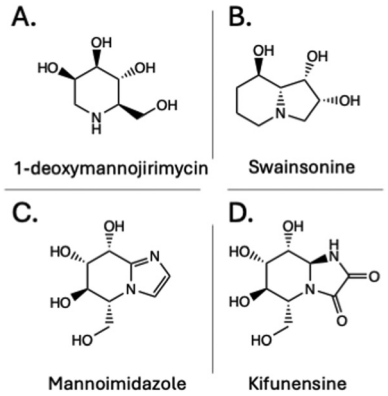

Fig. 12 Chemical structures for representative α-mannosidase inhibitors (A) 1-deoxymannojirimycin, (B) swainsonine, (C) mannoimidazole, and (D) kifunensine.

Glycoside hydrolase activity-based probes

Activity-based probes (ABPs) have gained significant interest in examining gut microbial enzymes in recent years.[163] ABPs are covalent inhibitors, capable of labelling proteins in complex mixtures like cell lysates or proteins extracted from faecal samples. ABPs are specifically designed to target the active site of the protein family of interest and often mimic the native substrate to allow for functional profiling of specific enzymes in a complex mixture. Several groups have used ABPs to fluorescently label proteins or enrich with biotin-conjugated probes for the enzyme of interest (Fig. 3F and 13). Many GH specific ABPs have been developed and have been reviewed recently by Artola *et al.*[97] Here, we focus on uses of ABPs in studying the gut microbiome. A pioneering study was conducted by Bertozzi Lab in 2004.[164] In this report, researchers conducted proteomic analyses of GHs in cell lysates[164] using the fluorosugar, 6-azido-2,6-dideoxy-2-fluoro-β-D-galactosyl fluoride, to label β-galactosidase. Using an azide handle on this probe, researchers were able to label β-galactosidases with a FLAG tag for analysis by western blot.

Another class of GH specific ABPs that has been successful in the past decade are the cyclophellitol-aziridine ABPs (Fig. 3F). This work has largely been accomplished by the Overkleeft Lab, with their initial progress reported in 2010 focused on targeting lysosomal glucocerebrosidase (GBA).[165] Cyclophellitol-aziridine ABPs allowed for specific fluorescent labelling of GBA *in vitro*, in cultured cells and *in vivo*. Several other cyclophellitol derived ABPs have subsequently been created to target a range of GHs including fucosidases,[82] glucosidases,[68,166–169] glucuronidases,[170] mannosidases[171,172] and galactosidases,[120,173] all which have utility in the context of the gut microbiome.

β-Glucuronidase ABPs

The first GH ABPs to be used in a gut microbiota context were the cyclophellitol-aziridine GUS ABPs.[170] These compounds were developed in 2017 to target two hGUS orthologs, GUSB and HPSE, and were found to be effective in fluorescently labelling GUSB extracted from human spleen. They were then used to enrich for GUS in spleen lysates by attaching a biotin and performing a streptavidin pull down prior to proteomic profiling.[170] Inspired by this work Jariwala *et al.* employed these probes to label GUS enzymes extracted from human faecal samples and selectively enriched for them in downstream proteomic analyses.[70] This allowed the researchers to pinpoint a structural subset of GUS enzymes responsible for differential SN38 reactivation in *ex vivo* human samples. ABPs provide a powerful tool for identifying the proteins responsible for drug, toxin and endobiotic reactivation from complex human faecal mixtures. They have been applied to a variety of other substrates relating to GUS enzymes including triclosan,[126] mycophenolate,[46] and endogenous hormones and neurotransmitters.[7] For triclosan, researchers found that a specific subset of the Loop 1 and FMN-binding gut microbial GUS enzymes drove the efficient reactivation of this toxin in the murine GI tract.[126] This was validated by using a GUS inhibitor to block triclosan-induced colitis in a mouse model. Using the same APBB pipeline, researchers were able to pinpoint specific FMN-binding GUS proteins with a unique active site architecture were responsible for the reactivation of the immunosuppressant mycophenolate by human faecal sample extracts.[46] Finally, in terms of endogenous compounds, a range of glucuronidated hormones and neurotransmitters were shown to be processed by specific sub-clades of gut microbial GUS proteins pinpointed from complex human faecal samples. These studies emphasize the influence of the Loop-1 and FMN-binding GUS classes in xenobiotic and endobiotic reactivation.[7] In these examples, the GUS ABP was tethered to a biotin molecule allowing GUS enrichment using streptavidin-coated beads providing a robust signal in downstream mass spectrometry analyses for enzyme identification.

α-Fucosidase ABPs

ABPs have similarly been turned to the study of α-fucosidases produced by gut microbes. The cyclophellitol fucosidase ABP

Table 9 α-Mannosidase inhibitors

Compound	Microbial target	K_i	Mechanism	Discovery phase	Ref.
Mannoimidazole	Bt3130	1.0 μM	Competitive	*In vitro*	Zhu *et al.*[92]
	Bt3965	0.4 μM			
Swainsonine	Bt2199	14 μM	Competitive	*In vivo*	Zhu *et al.*,[92] Tulsiani *et al.*[162]
	Bt3990	5 μM			
Kifunensine	Bt2199	230 μM	Competitive	*In vitro*	Zhu *et al.*[92]
	Bt3990	140 μM			
1-Deoxymannojirimycin	Bt2199	13×10^3 μM	Competitive	*In vitro*	Zhu *et al.*[92]
	Bt3990	12×10^3 μM			

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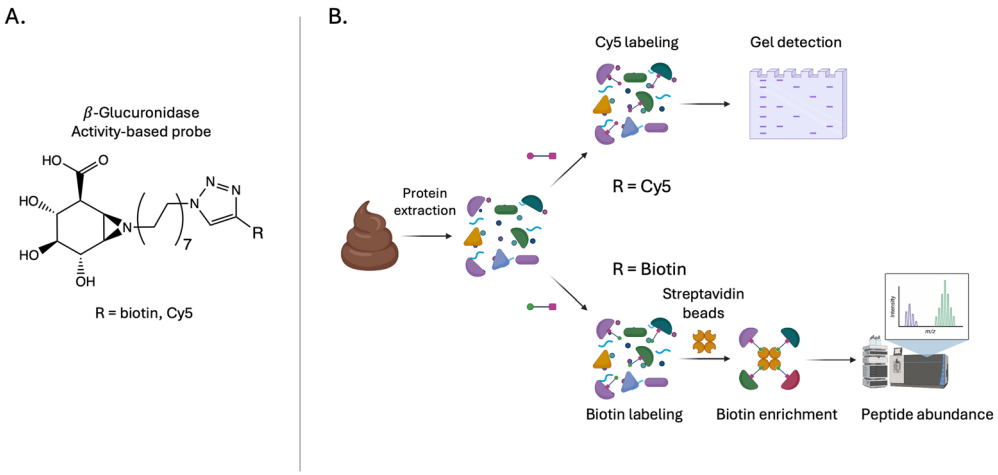

Fig. 13 (A) Chemical structure of a β-glucuronidase activity-based probe. (B) Activity-based protein profiling pipeline, illustrating the ability to label specific GHs in complex biological samples. Probes contain a reactive warhead, mimicking the native substrate, linked to either a fluorophore for in-gel fluorescence detection or biotin for enrichment using streptavidin beads. These probes allow researchers to selectively identify and profile active GHs, enabling the study of GHs in complex biological samples.

labels bacterial fucosidases as well as fucosidases from human spleen, murine spleen, and murine kidney lysates.[82] Furthermore, these probes have been used to identify fucosidase inhibitors through competition assays. Researchers challenged the ability of the ABP JJB256 to label recombinant FUCA1 by incubating with the high affinity fucosidase inhibitor, DFJ, showing that DFJ prevents labelling.[82] In another study this probe was used to label GH29 α-fucosidases secreted by *Bacteroides fragilis* with a fluorophore for in-gel detection.[174] This work highlights the importance of secreted fucosidases in *B. fragilis*-mediated invasion by the microbial pathogen *Campylobacter jejuni*. *C. jejuni* can utilize fucose liberated by *B. fragilis* and fucose utilizing strains of *C. jejuni* have been linked to increased virulence.[175] This probe has also been used to fluorescently label α-fucosidases from mouse faecal extracts.[176] α-Fucosidases were only detected when mice were fed 2′-fucosyllactose, demonstrating that diet influences gut microbial α-fucosidase abundance.[176]

β-Glucosidase ABPs

Conduritol B epoxide was discovered as a covalent inhibitor of β-glucosidases in 1977[66] and cyclophellitol was discovered in 1990,[177] laying the foundation for GH cyclophellitol probes. Breakthrough work in 2010 by the Overkleeft Lab described β-glucosidase-targeting cyclophellitol ABPs, allowing for the labelling of GBA, a retaining β-glucosidase in humans, both *in vitro* and *in vivo*.[165] It was subsequently shown that these cyclophellitol ABPs are potent inhibitors of all 4 retaining β-glucosidases present in humans.[165,166] In 2017, researchers developed a new class of cyclophellitol ABPs, carba-cyclophellitols, by substituting the oxygen for a carbon containing R-group substituents.[68] Resultant ABPs were shown to be active against a GH1 β-glucosidase from *Thermotoga maritima*, indicating one of the utilities of these ABPs in studying bacterial GH1 β-glucosidases.

β-Galactosidase ABPs

Cyclophellitol ABPs for β-galactosidase were developed in 2014 to examine human lysosomal β-galactosidases. More recently, these ABPs were shown to effectively label β-galactosidases in cell lysates and tissue extracts.[120,173] These ABPs offer a strategy to study β-galactosidases produced by gut bacteria and how these enzymes are involved in mucin degradation and GOS catabolism.

α-Mannosidase ABPs

In 2020, researchers reported α-mannosidase cyclophellitol ABPs and demonstrated the ability to label α-mannosidases in HEK293T cells expressing 5 human GH37 α-mannosidases and in murine tissue.[172] These probes could be applied in studying degradation of *N*-glycans present in the GI tract as well as dietary polysaccharides containing mannose.

Future directions

Numerous gut microbiota studies have emphasized the importance of glycan substrates on host-physiology and gut microbial composition.[20,24] Several GH inhibitors have been developed to target host enzymes but can be employed as valuable tools for defining the actions of gut microbial enzymes on a range of host, dietary and microbial substrates. While some studies highlighted here interrogated gut microbial GH-mediated metabolism by using small molecule inhibitors, considerable work remains. Specifically, synthetically derived GH inhibitors, like GUS inhibitors, could allow for microbial-selective GH inhibition.

To date, most GH inhibitors are substrate mimics targeting human proteins, and selectivity for microbial GHs remains elusive for the majority of GH families. Recent advances provide an avenue for overcoming these limitations. The synthetic

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bacterial GUS inhibitors have demonstrated selectivity for bacterial GUS. These GUS inhibitors, identified through high-throughput screening, selectively target bacterial GUS over mammalian GUS, and similar approaches can be applied to other GH families.[8,69,71]

Given these successes, future work should prioritize drug discovery efforts against bacterial GHs. Natural products and iminosugars have dominated the GH inhibitor landscape and expanding into synthetic and polyphenol-derived scaffolds offers the potential for greater selectivity and chemical diversity. Furthermore, many FDA-approved drugs have been found to modulate GH activity, highlighting a resource to guide GH inhibitor development.[7] Specifically, some FDA approved drugs contain 6-member rings with a secondary amine, reminiscent of iminosugar inhibitors, and may be expected to impact GH function. Numerous studies have shown that dietary xenobiotics, like daidzein and genistein, inhibit GHs.[130,133] Such studies implicate the potential for developing polyphenol-derived inhibitors, providing much needed chemical diversity for GH inhibitors.

Groups have successfully identified GUS inhibitors using *in silico* screening, which can readily be implemented for discovering inhibitors of other GH families.[127] Advancements in machine learning and ultra-large-scale virtual screening now makes it feasible to evaluate billions of molecules in silico.[178,179] These technologies could accelerate the discovery of GH inhibitors with greater selectivity. Furthermore, such approaches may enable to discovery of catalytic mechanism selective GH inhibitors, especially of interest due to the recent discovery of non-Koshland bacterial GHs.[102] In addition to inhibitor discovery, there is increasing interest in leveraging microbiota-targeting inhibitors in personalized medicine approaches. Advances in omics technologies could allow for personalized manipulation of the gut microbiota to alleviate GI toxicity from therapeutics or reduce mucin-degrading GH activity associated with inflammation. Thus, future research is expected to consider these and other factors to fully understand the complex interplay between the gut microbiota, glycan metabolism and xenobiotic compounds.

Conflicts of interest

MRR is a founder of Symberix, Inc., and has received research funding from Lilly and Merck.

Data availability

No primary research results, software or code have been included, and no new data were generated or analysed as part of this review.

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