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Structure-based discovery of glycomimetic FmlH ligands as inhibitors of bacterial adhesion during urinary tract infection

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Treatment of bacterial infections is becoming a serious clinical challenge due to the global dissemination of multidrug antibiotic resistance, necessitating the search for alternative treatments to disarm the virulence mechanisms underlying these infections. Uropathogenic Escherichia coli (UPEC) employs multiple chaperone-usher pathway pili tipped with adhesins to colonize various host tissues and habitats. For example, UPEC F9 pili specifically bind galactose or $\beta$-N-acetylgalactosamine epitopes on the kidney and inflamed bladder. Using X-ray structure-guided methods, virtual screening, and multiplex ELISA arrays, we rationally designed aryl galactosides and $\beta$-N-acetylgalactosaminides that inhibit the F9 pilus adhesin FmlH. The lead compound, 29i-NAc, is a biphenyl $\beta$-acetyl-$\beta$-galactosaminoside with a $K_i$ of ~90 nM, representing a major advancement in potency relative to the characteristically weak nature of most carbohydrate-lectin interactions. 29i-NAc binds tightly to FmlH by engaging the residues Y46 through edge-to-face $\pi$-stacking with its A-phenyl ring, R142 in a salt-bridge interaction with its carboxyate group, and K132 through water-mediated hydrogen bonding with its N-acetyl group. Administration of 29i-NAc in a mouse urinary tract infection (UTI) model significantly reduced bladder and kidney bacterial burdens, and coadministration of 29i-NAc and mannose 42269, which targets the type 1 pilus adhesin FimH, resulted in greater elimination of bacteria from the urinary tract than either compound alone. Moreover, FmlH Specifically binds healthy human kidney tissue in a 29i-NAc–inhibitable manner, suggesting a key role for F9 pilin in human kidney colonization. Thus, these glycoside antagonists of FmlH represent a rational antivirulence strategy for UPEC-mediated UTI treatment.

Significance

The emergence of multidrug-resistant bacteria, including uropathogenic Escherichia coli (UPEC), makes the development of targeted antivirulence therapeutics a critical focus of research. During urinary tract infections (UTIs), UPEC uses chaperone-usher pathway pilin tipped with an array of adhesins that recognize distinct receptors with sterochemical specificity to facilitate persistence in various tissues and habitats. We used an interdisciplinary approach driven by structural biology and synthetic glycoside chemistry to design and optimize glycomimetic inhibitors of the UPEC adhesin FmlH. These inhibitors competitively blocked FmlH in vitro, in in vivo mouse UTI models, and in ex vivo healthy human kidney tissue. This work demonstrates the utility of structure-driven drug design in the effort to develop antivirulence therapeutic compounds.
and outcome are determined by complex interactions between host susceptibility and diverse bacterial urovirulence potentials, which can be driven by differences in the expression and regulation of conserved functions. The ability of UPEC to colonize various habitats, such as the gut, kidney, and bladder, depends in large part on the repertoire of adhesins encoded in their genome. The most common mechanism for adhesion utilized by UPEC is mediated through the chaperone–usher pathway (CUP), which generates extracellular fibers termed pili that can confer bacterial adhesion to host and environmental surfaces, facilitate invasion into host tissues, and promote interaction with other bacteria to form biofilms (20). Phylogenetic analysis of Escherichia genomes and plasmids predicts at least 38 distinct CUP pilus types, with single organisms capable of maintaining as many as 16 distinct CUP operons (21). Many of these CUP pilus operons contain two-domain, tip-localized adhesins, each of which likely recognize specific ligands or receptors to mediate colonization of a host and/or environmental niche. For example, the type 1 pilus adhesin FimH binds mannosylated glycopolymers on the surface of the bladder epithelium, which is crucial for the establishment of cystitis (22, 23). The structural basis of mannose (Man) recognition by the N-terminal–receptor binding domain, or lectin domain (LD), of FimH has been leveraged to rationally develop high-affinity aryl mannosides (24–32). In mouse models of UTI, we have previously demonstrated that orally bioavailable mannosides that tightly bind FimH can prevent acute UTI, treat chronic UTI, and potentiate the efficacy of existing antimicrobial treatments like TMP-SMZ, even against antibiotic-resistant Escherichia coli strains (28). Thus, use of mannosides that target the adhesin FimH represents the first successful application of an antivirulence strategy in the treatment of UTI.

A homolog of the type 1 pilus, the F9 pilus, is one of the most common CUP pilus in the E. coli pan genome and an important urovirulence factor employed by UPEC for the maintenance of UTI (21, 33). Our recent work has demonstrated that UPEC upregulates the expression of F9 pilin in response to bladder inflammation and epithelial remodeling induced upon UPEC infection (34). These pilin display the FimH-like adhesin FmlH, which is capable of binding terminal galactose (Gal), β-1,3-N-acetylgalactosamine (GalNAc), or Thomsen-Friedenreich antigen (TF) [Gal[β1-3]GalNAc(6)]. FmlH was shown to bind TF within naïve or infected kidneys and to Thomsen nouvassie antigen (Tn) (GalNAc) within the infected bladder epithelium during chronic, unresolved UTI. Deletion of FmlH in the urosepsis isolate CFT073 resulted in a competitive defect in the ability of this strain to maintain murine UTI in C3H/HeN female mice. Furthermore, vaccination with the LD of FmlH (FmlHLD) as the challenge antigen significantly protected mice from developing UTI. Thus, we have shown that FmlH serves a key role in the UPEC pathogenesis cascade and represents a promising target for antivirulence therapies for UTI in both the bladder and kidney habitats. Herein, we describe the discovery and structure-based optimization of high-affinity aryl galactoside and N-acetylgalactosaminoside FmlH ligands that potently inhibit the function of FmlH. Treatment with these FmlH antagonists significantly reduced bacterial burdens in the kidneys and bladders of infected mice, thereby demonstrating promising translational value in the treatment of UTI in humans. The results of these studies, together with our previous work on FimH mannosides, further support the mechanistic and therapeutic value of antivirulence strategies that leverage structure-function relationships of diverse bacterial adhesins for the rational design of high-affinity glycosides for the treatment of UTI and other bacterial infections.

**Results**

O-nitrophenyl β-Galactoside Identified as Early Lead Inhibitor of the F9 Pilus Adhesin FmlH. We revealed in a previous communication that FmlH binds surface glycan receptors containing terminal Gal, GalNAc, or TF residues (34). Given the role of FmlH in UTI pathogenesis, we aimed to develop high-affinity galactoside antagonists of FmlH through an X-ray structure-guided medicinal chemistry approach. This strategy entailed (i) screening a select library of galactosides through multiplex ELISA arrays for initial lead compound identification; (ii) an iterative process of cocystal structure determination, virtual screening, structure-based ligand design, and in vitro biochemical characterization; and (iii) evaluation of the top lead compound in a mouse model of UTI (Fig. L4). Toward these goals, we first investigated whether Gal, GalNAc, and TF could be adapted to function as soluble, competitive inhibitors of FmlH. To that end, an ELISA-based competition assay was developed to detect binding of FmlHLD to surface-immobilized desialylated bovine submaxillary mucin (ds-BSM) in the presence or absence of soluble compounds (Fig. L4). As expected, Gal, GalNAc, and TF were each capable of inhibiting FmlHLD at a concentration of 1 mM, with GalNAc exerting greater inhibitory potency than TF or Gal. However, neither Man nor glucose (Glc) had any detectable effect on the ability of FmlHLD to bind ds-BSM (Fig. L4). Lactose (Lac), or Gal[β1-4]Glc, was also incapable of inhibiting FmlHLD, demonstrating the high selectivity in which FmlHLD engages Gal-containing glycans (Fig. L4). O-nitrophenyl β-galactoside (ONPG) and isopropyl β-thiogalactoside (IPTG) were also tested for inhibition in this exploratory phase of our search for FmlH inhibitors. While IPTG exerted minor inhibitory activity at 100 μM, ONPG was found to block FmlHLD from interacting with ds-BSM more effectively than Gal, GalNAc, or TF (Fig. L4). The strong inhibitory potency of ONPG suggested that β-galactosides could potentially be rationally designed with higher affinity by specifically targeting residues within and surrounding the sugar binding pocket of FmlH.

Therefore, X-ray crystallography was implemented to elucidate the 3D structures of both apo and ligand-bound FmlHLD (SI Appendix, Table S1). First, a crystal structure of apo FmlHLD was solved at 1.6 Å resolution by molecular replacement using FmlHLD [Protein Data Bank (PDB) ID 3MCY] as the search model. Within this structure, two copies of FmlHLD are found in the asymmetric unit, each of which adopts a canonical β-sandwich fold, with three distinct binding loops (loop 1: residues 10 to 15; loop 2: residues 44 to 53; and loop 3: residues 132 to 142) that form a wide, shallow, solvent-exposed binding pocket (Fig. 1 C and D). Within the binding pocket of both copies resides a sulfate ion, which interacts with residues implicated in Gal binding (Fig. 1D). The putative substrate binding pockets of the two copies of FmlHLD were side by side on one face of the protein, with a second sulfate ion between them. Interactions with ds-BSM were also shown to 2.1 Å and 1.8 Å, respectively. Structural overlay of the apo and ligated crystal structures yields root-mean-square deviation (RMSD) values that fall within 0.6 Å, suggesting that FmlHLD generally adopts the same active or functional conformational state in the absence or presence of ligand (Fig. 1C). This functional conformational state most likely corresponds to a high-affinity conformation of FmlH, as the FmlHLD structures exhibit a higher degree of structural homology to the high-affinity conformation of FmH (RMSD values of 0.8 to 0.9 Å) than to the low-affinity conformation of FimH (RMSD values of 1.7 to 1.9 Å) (34–38).

The cocystal structure of FmlHLD-TF reveals two copies of FmlHLD-TF in the unit cell, in which each TF adopts a distinct ligand conformation (Fig. 1D). In both copies, the terminal Gal in TF occupies the cleft of the binding pocket through direct polar interactions with residues P1, D35, K132, and N140. In contrast, the orientation of the GalNAc in TF differs significantly between the two copies of FmlH. In chain A, the GalNAc sugar points toward loop 3, with the carbonyl group of GalNAc forming a hydrogen bond (H-bond) with the guanidinium group of R142. In chain B, however, the GalNAc packs against and forms a H-bond with the hydroxyl group of Y46. Accordingly, the differences in the orientation of bound ligand across the two copies are accompanied by slight differences in orientation of the side chains of the interacting residues Y46 and R142. The multiple binding modes observed for a single ligand suggests that the
wide, shallow nature of the Gal binding pocket in FmlH would enable galactosides to possibly bind FmlH with diverse interactions and conformations.

The FmlH<sub>LD</sub>-ONPG cocrystal structure also shows two copies of FmlH<sub>LD</sub> in the unit cell, in which a sulfate ion occupies the binding pocket of chain A while ONPG occupies the binding pocket of chain B (Fig. 1D). As expected, the Gal component of ONPG resides in the cleft of the binding pocket, while the solvent-exposed nitrophenyl group mediates a polar or salt-bridge interaction with R142 through an intricate network of H-bonds with water molecules. Furthermore, the positioning of the Gal component of ONPG aligns with that of the Gal residue of TF (Fig. 1C). In all cases, the Gal component of top-scoring galactosides bound to the cleft of the binding pocket, as expected. In addition, most of the high-scoring hits also interacted with specific hot-spot residues near the Gal binding pocket, which we sought to leverage for compound optimization. These hot-spot residues included (i) residue Y46, which caps the top of the binding pocket and can contribute hydrophobic interactions; (ii) residue K132, which lies at the bottom of the sugar binding pocket and can engage polar groups linked to the Gal sugar; and (iii) residue R142, which points toward an empty, solvent-exposed cleft near the binding pocket and can contribute electrostatic interactions (SI Appendix, Fig. S1B). These visual
Design and Synthesis of FmlH-Targeting Galactoside Antagonists. To increase FmlH binding affinity and explore structure-activity relationship (SARs), we constructed a large library of galactoside analogs (Fig. 2). Based on the docking results, we predicted that β-Gal isomers would be preferred over α-Gal and that ortho positioning of functional groups on a phenyl scaffold would best facilitate interactions with specific sites within the binding pocket, namely hot-spot residues Y46 and R142. Accordingly, we synthesized and evaluated small sets of phenyl galactosides with ortho-substituted functional groups (2 to 6; Fig. 2A). We also either purchased or synthesized several other phenyl galactosides, which contained meta or para substituents on the aglycone ring (7 to 11; Fig. 2A), and other aryl and heterocyclic galactosides (12 to 22; Fig. 2B and C). This allowed us to derive meaningful SARs for informing further design and optimization of improved galactosides. In addition, we tested natural-product galactosides isolated from cranberries and other natural sources (23 to 27; Fig. 2D). The promising activity of the simple galactoside ONPG (4β) in the initial screen, coupled with the hot-spot residues identified in virtual screening, prompted us to expand our FmlH-ligand design strategy with a compound series containing biphenyl aglycones (28 to 32; Fig. 2E), such as 2β-Nac, the N-acetyl-β-galactosaminide with an m-carboxylic acid on the B-ring designed to directly interact with the hot-spot residue R142 (SI Appendix, Fig. S1B and C). To confirm the predicted preference for the β-Gal isomers, we also synthesized and tested many corresponding α-Gal isomers. Compounds were synthesized by using one of two general synthetic glycosylation methods involving either a reaction between Gal pentaacetate and phenols promoted by boron trifluoride or a Koenigs–Knorr-type reaction of galactosyl halide with aryl alcohols (SI Appendix, Fig. S2).

Biochemical Characterization of FmlH Antagonists. Selected top-hit glycosides and a few low-scoring analogs from the virtual screen, as well as synthetic galactosides, were tested in the ELISA-based competition assay for their ability to inhibit binding of FmlHLD to ds-BSM. Direct comparison of inhibitory potency among galactosides led to delineation of basic SARs (Fig. 3A–C and SI Appendix, Table S2). When tested at 100 μM, the phenyl β-galactoside 1β (beta isomer of 1; Fig. 2A) exhibited significantly higher binding inhibition (77%) than Gal (8.1%), indicating that the phenyl group significantly enhances binding to FmlHLD (Fig. 3A). Various ortho substituents on the phenyl ring additionally conferred substantial improvements in inhibitory potency, as observed with 2β (87%), 3β (95%), 4β (ONPG; 93%), 5β (97%), and 6β (90%). In contrast, the meta-methoxy groups in compound 7β (76%) did not enhance binding strength compared with 1β. Further, para-substituted functional groups displayed variable inhibitory potencies relative to 1β, with enhancements observed in 8β (86%) and 9β (86%), with no significant effect observed in 11β (78%) or 11β-thio (72%), and with a reduction observed in 10β (65%). Thus, we deduced that the ortho-substituted phenyl β-galactosides generally outperformed other simple phenyl galactosides.

Complex heterocyclic galactosides, such as coumarins 12β (85%) and 14β (89%), which differ only by a methyl group, displayed significant inhibitory potencies against FmlH, while the related galactoside 13β (50%) displayed reduced inhibitory activity, likely because of its fluor substituents (Fig. 3A and SI Appendix, Table S2). Resorufin galactoside 15β (80%) is responsible for augmenting affinity relative to 1β. In contrast, indoles 16β (22%) and 17β (41%) performed poorly as inhibitors of FmlHLD. Napthyl galactosides 18β (46%) and 19β (79%), in addition to isoquinoline 21β (15%), showed no improvement in activity relative to 1β. However, quinoline 20β (95%) displayed significantly higher inhibition than 1β and 18β. This advocates that the electron pair-donating nitrogen atom in 20β is making a specific interaction with FmlH. This observation is consistent with the pattern of SARs, indicating that the ortho position is key to enhancing inhibitory potency against FmlHLD.
We also evaluated naturally occurring galactosides derived from cranberries and other natural sources in this screen (Fig. 3A and SI Appendix, Table S2). These compounds included anthocyanidin (pelargonidin, 23β; cyanidin, 24β; peonidin, 25β) and flavonol (quercetin, 26β; myricetin, 27β) β-galactosides. Generally, these compounds exhibited moderate to weak inhibition of FmIH LD binding, with little enhancement in inhibition relative to Gal (8.1%). The only significant binders were 24β (29%) and 26β (14%). Comparison of the anthocyanidin family indicates that the 3′ or meta-substituted hydroxyl group on the B-ring of 24β is critical for its specific interaction with FmIH. Absence of this meta substituent in 23β (0.7%) or methylation of the hydroxyl group in 25β (3.6%) abrogates potency, suggesting that the hydroxyl group of 24β might participate in a H-bond to a specific residue in the FmIH LD binding pocket. Additional inhibitory screens performed with cranberry-derived compounds
and fractions at 1 mM confirmed the specificity and necessity of the Gal sugar for inhibiting the binding pocket of FmH (SI Appendix, Fig. S3 A and B).

Interestingly, the tested GalNAc-derived compounds possessed significantly higher inhibitory potency compared with their matched-pair Gal-derived counterparts, as exemplified with 4β-NAc (87%) relative to 4β (31%) when tested for inhibition at 10 μM (Fig. 3 B and SI Appendix, Table S2). These results taught us that the N-acetyl group, together with other functional groups, contributes to binding by targeting distinct components of the binding pocket of FmH. In contrast, the galactosides with α-linkages (28β-30α) or disaccharides with aglycone moieties (33 to 35) were generally poor inhibitors of FmH, except for 11α-NAc (82%) (Fig. 3 D and SI Appendix, Table S2).

Consistent with the above-mentioned SARs, the ortho biphenyl galactoside 28β (91%) was more potent than the meta 31β (57%) or para 32β (30%) analogs (Fig. 3 A and SI Appendix, Table S2). Next, we installed a carbonyl group at the meta position on the biphenyl B-ring (29β), intended to target the pocket formed by N140 and R142, and found that 29β exhibited greater inhibition (99%) compared with 28β when tested at 100 μM. This pronounced difference in activity was further highlighted when these compounds were tested for inhibition at 10 μM and 1 μM (Fig. 3 B and C and SI Appendix, Table S2). Importantly, 30β (87%), the methyl ester of 29β, tested at 100 μM resulted in a reduction in binding, suggesting that the negative charge of the carboxylic acid likely mediates a critical electrostatic interaction with R142 of FmHLD. Lastly, we synthesized the GalNAc version of 29β to increase its binding affinity and found that 29β-NAc (93%) had significant improvement in activity over 29β (75%) when tested at 10 μM. Final evaluation of the highest performing galactosides in the ELISA-based competition assay at concentrations of 10 μM and 1 μM allowed for a clearer ranking of compounds, where 29β-NAc clearly stood out as the most potent (Fig. 3 B and C and SI Appendix, Table S2).

**Determination of FmH–Galactoside Binding Affinities.** Bio-layer interferometry (BLI) was pursued to quantitate the binding affinity of the most promising FmH antagonists. First, biotinylated serine-linked TF (Ser-TF) immobilized on streptavidin pins was incubated with varied concentrations of FmHLD, in solution, and steady-state analysis of binding responses revealed a dissociation constant (Kd) of 15.0 ± 0.8 μM (Fig. 3 D). Next, immobilized Ser-TF was incubated in solutions comprising a fixed concentration of FmHLD but varying concentrations of galactosides to determine their inhibitory or dissociation constant (Ki or Kd) values (Fig. 3 E). The BLI-based affinity determinations correlated well with the relative binding strengths measured in the ELISA-based competition assay (Fig. 3 A–C and SI Appendix, Table S2). The two lead compounds, 29β-NAc and 29β, bound tightly to FmHLD, with respective Ki values of ~90 nM and 2.1 μM, which represent a ~7,800-fold and ~330-fold enhancement in binding affinity relative to Gal. Another promising compound, 4β-NAc, bound FmHLD with a Ki value of 2.3 μM. In summary, a combinatorial approach based on virtual screening and structure-guided ligand design led to the discovery of small-molecular weight monomeric glycosides derived from Gal and GalNAc that function as effective antagonists of FmH. Optimization of early hits to high-affinity ortho-biphenyl Gal and GalNAc antagonists was realized via ortho substitution on phenyl aglycones to facilitate interactions that significantly enhanced binding to FmH.

**Structural Basis of Galactoside Inhibition of FmH.** To elucidate the molecular basis for galactoside inhibition of FmH, cocrystal structures of FmHLD bound to 4β, 5β, 20β, and 29β-NAc were determined (Fig. 4 A and B). These galactosides share a common aglycone motif consisting of a phenyl ring with an ortho-substituted functional group. As predicted from computational studies, the sugar portion of all these galactosides resides within the cleft of the binding pocket. The phenyl groups directly attached to the sugar portion of all four compounds lie along the same 3D plane. In this nearly identical conformation, the phenyl ring is oriented perpendicularly to the side chain of residue Y46, revealing edge-to-face π-stacking, which likely contributes to the affinity enhancement observed for all β-galactosides. For 4β, 5β, and 20β, the ortho substituents point toward R142 but are too distant (>7 Å) for direct interaction and, instead, form H-bonds with water molecules that, in turn, interact with residues K132 and R142 (Fig. 4 C and D). Thus, we deduced that the marked affinity enhancement observed for 4β, 5β, and 20β is due to a combination of (i) indirect interactions between the ortho substituent and residues K132 and R142 formed by an intricate network of water-mediated H-bonds and (ii) edge-to-face π-stacking between the phenyl ring and residue Y46.

In contrast to simple phenyl galactosides, the biphenyl scaffold of 29β-NAc presents the carboxylic acid to engage in a direct
charge–charge interaction with the guanidinium side chain of R142 (Fig. 4B). The lower potency of the methyl ester derivative 30β is further evidence that the charge–charge interaction likely drives the observed affinity enhancement (Fig. 4C). The improved affinity of 29β-NAc relative to 29β is also due to additional interactions mediated by the N-acetyl group in H-bonding to a water molecule captured by the biphenyl aglycone and the side chain of residue K132 (Fig. 4B and C).

Altogether, analysis of all X-ray crystal structures of ligand–bound FmlH offers two general mechanisms for the significant enhancement in binding affinity of galactosides relative to Gal: edge-to-face π-stacking with Y46 and polar or electrostatic charge–charge interactions with K132 and R142.

FmlH Antagonist Effectively Treats Murine UTI in Vivo and Prevents Binding to Human Kidney Tissue. We previously reported that FmlH binds to naïve kidney and inflamed bladder tissue and plays a critical role in chronic UTI, as abrogation of its function through genetic deletion or vaccination results in significant attenuation in the ability of UPEC to cause chronic UTI (34). Thus, we hypothesized that galactosides that inhibit the function of FmlH would have efficacy in the treatment and/or prevention of UTI. To assess therapeutic potential, the lead compound 29β-NAc was evaluated for its ability to reduce bacterial burdens in the urinary tracts of C3H/HeN mice during chronic UTI. We previously defined chronic cystitis in C3H/HeN mice as urine titers of >10^6 CFU/mL lasting at least 2 to 4 wk, as well as bladder inflammation and edema at euthanasia (39). Further, C3H/HeN mice are genetically predisposed to vesicoureteral reflux (retrograde flow of urine from the bladder to the kidneys), which can lead to bacterial colonization of the kidneys, renal abscess formation, scarring, and atrophy (40). Accordingly, we observed high levels of kidney colonization by CFT073 in control (vehicle-treated) animals. When delivered intravesically, 29β-NAc significantly reduced bacterial burdens in both the bladder and the kidneys of these mice (Fig. 5A and B). For comparison, mannoside 4Z269, which inhibits the type 1 pilus adhesin FimH, also significantly reduces titers of CFT073 from the bladders and kidneys of infected mice relative to vehicle control (Fig. 5A and B). When administered together, 29β-NAc and 4Z269 eradicated bacteria from the kidney in nearly all mice while also reducing bacterial titers in the bladder, suggesting that FimH mannosides and FmlH galactosides may function synergistically to target distinct bacterial adhesins or communities within the kidney habitat (Fig. 5A and B).

To show relevance to human UTI, we assessed FmlH and FmlH-targeting galactosides through immunofluorescence analysis of FmlHLD binding to human kidney and bladder biopsied tissue determined to be nonmalignant. While FmlHLD does not appear to bind to healthy human bladder tissue, FmlHLD does bind to healthy human kidney tissue, particularly in regions resembling the collecting ducts and distal tubules of the kidney (Fig. 5C and SI Appendix, Figs. S4 and S5). As a negative control, the binding null mutant FmlHLD K132Q, which lacks the ability to bind ds-BSM in vitro (SI Appendix, Fig. S6), was incapable of binding kidney tissue, suggesting that FmlHLD specifically recognizes receptors naturally present in human kidney tissue (Fig. 5C).

These observations are consistent with the previously reported binding phenotypes in mice, in which FmlH can bind naïve mouse kidney tissue, but not naïve mouse bladder tissue, and can bind to receptors in inflamed bladder tissue (34). Moreover, incubation of 29β-NAc with FmlHLD prevented binding to human kidney tissue, suggesting that these results may translate to humans. Importantly, these collective data provide substantial evidence that aryl glycoside–based FmlH antagonists derived from β-Gal or β-GalNAc can serve as an effective therapy for persistent UTIs, including pyelonephritis, for which there is an enormous unmet medical need.

Discussion

UPEC is the causative agent of most UTIs, a common and very costly disease in women, children, and the elderly that is becoming increasingly resistant to antibiotic treatment. By leveraging our expertise in UPEC pathogenesis and structure-based drug design, we developed small-molecule Gal-based FmlH antagonists that show in vivo efficacy in the treatment of chronic UTI in mouse models. Virtual screening combined with rational design led to the identification of several naturally occurring cranberry and synthetic galactosides, the most potent of which binds FmlH with nanomolar affinity. X-ray crystallography revealed that potent galactosides achieve significant enhancements in binding affinity through interactions on opposite sides of the wide Gal binding pocket of FmlH. Appropriately substituted aryl groups, like those found in 4β/4β′-NAc, 5β, and 20β, are seen to mediate edge-to-face π-stacking interactions with Y46 of FmlHLD. Further, the optimized biphenyl aglycone of compound 29β-NAc contains an ideally positioned carboxyl group to mediate electrostatic interactions with R142 in addition to π-stacking interactions with Y46. Evaluation of the lead candidate 29β-NAc in a mouse model of chronic UTI demonstrated significant reductions of bacterial burdens in the mouse kidney and bladder. Combination dosing with mannoside and galactoside resulted in near complete clearance of bacteria from the kidney and significant elimination of bacteria from the bladder. Furthermore, FmlH was shown to bind specifically to...
human kidney tissue, which could be inhibited by 29β-NAc. Additionally, FimH has been shown to be up-regulated in urine samples directly isolated from human patients with UTI compared with expression during in vitro growth in media or normal urine (41), suggesting an important role for FimH in human UTI. Thus, FimH-targeting galactosides represent a rational antivirulence modality for the effective treatment of UPEC-mediated UTI.

Our rational strategy to discover receptor-mimicking galactosides targeting FimH was similar to the strategy we followed for the development of FimH mannosides. However, the design of the galactoside and N-acetylgalactosaminoside antagonists of FimH was met with distinct challenges. The most striking difference between FimH and FimH is the binding affinity for their respective ligands: FimH binds soluble Man with a moderate binding affinity of ~5 to 10 μM, and FimH binds soluble Gal with a weak binding affinity of ~700 μM (34, 42, 43). The weak binding affinity of FimH, which is quite common for most carbohydrate-lectin interactions, rendered the development of high-affinity galactosides much more challenging. This disparity in affinity is a direct consequence of the substantial variance in the shape of the binding pocket. FimH binds Man with high affinity because of the deep, narrow pocket formed by loops 1, 2, and 3, in which loops 2 and 3 mediate specific polar interactions directly to Man and a water molecule and loop 1 serves as an affinity clamp to steric dissociation of Man (53). In contrast, loop 1 in FimH is more distant from loops 2 and 3 than it is in FimH and does not contribute to binding, which results in a widened, solvent-exposed pocket for weak Gal binding (Fig. 1D). In addition, the differences in binding pocket architecture dictate the sterically allowed linkage types. FimH has space at the tip of the LD between its parallel tyrosine gate (residues Y48 and Y137) to accept α-linked moieties, of which bivalent groups confer drastic enhancements in affinity through strong parallel face-to-face π-stacking interactions. In contrast, FimH is capped at the very tip of the pocket with Y46, which biases specificity toward β-linked moieties, of which bivalent groups confer moderate enhancements in affinity through significant edge-to-face π-stacking interactions. Having accounted for these variations, our structure-guided medicinal chemistry approach, coupled with our in vivo work, has clearly demonstrated the future translational impact of galactosides as treatments for UTI.

It is noteworthy that our collective search for high-affinity antagonsists of FimH led to discovery of biphenyl moieties as the preferred aglycone groups for high-affinity galactosides and mannosides, respectively. Pocket geometry dictates the type of biphenyl scaffold that is optimal. Thus, the best FimH-targeting mannosides contain para biphenyls in the alpha stereochemistry, while the best FimH-targeting galactosides contain ortho biphenyls in the beta orientation. However, in both cases, H-bonding donors or acceptors on the B-ring result in significant enhancement in binding affinity through specific interactions outside the sugar binding pocket. Intriguingly, the inhibitory potency conferred by the meta carboxyl on the B-ring of 29β-NAc is also appreciated in the significant inhibitory role of the meta-substituted group on the B-ring in cranberry compounds 240 and 266, which suggests a common pharmacophore between our optimal synthetic compound and natural-product compounds in targeting FimH. This study provides evidence that specific glycosidic compounds in cranberry can specifically bind and inhibit a bacterial adhesin. Furthermore, our work exposes a trend indicating that π-stacking of aromatic aglycones with binding pocket residues in the adhesin is essential in mimicking glycoprotein receptors and for developing tight-binding ligands in each lectin. Mimicking carbohydrates with small molecules is a long-sought-after goal in medicinal chemistry and chemical biology (44–46), and we believe that these results add significantly to this understanding and goal. This information can now be utilized not only in the future optimization of lead compound 29β-NAc as a treatment for UTIs, but also in the rational design of numerous other lectin antagonists for the development of small-molecule glycoside-based drugs aimed at treating infections mediated by E. coli or other micro-organisms (44).

The rapid increase and spread of antibiotic resistance, including multidrug-resistant forms of bacteria, has rendered many antibacterial therapies ineffective and threatens to undermine the biomedical strides made to promote human health and longevity (9). Selection pressures imposed by antibiotics on bacterial pathogens have promoted their proliferation, especially through overuse of antibiotics within the farming industry and inappropriate use or misuse among patients (47–50). Recent reports indicate that patients are now succumbing to bacterial strains which possess broad-spectrum resistance to all last-resort antibiotics, which many fear signals that antibiotic resistance will pave the way for the “next pandemic” (15). Antivirulence strategies that aim to reduce the pathogenicity of bacterial pathogens promise to provide the same therapeutic efficacy as antibiotics without introducing selective pressures that would promote widespread dissemination of resistance (16). Multiple antivirulence efforts will be required to combat the multiple mechanisms by which diverse bacterial pathogens colonize the host, which can include, for example, the targeting of CUP pilus adhesins or the biogenesis machinery responsible for the assembly of CUP pili (51). As highlighted in this work, UPEC employs an armament of diverse CUP pilus to colonize and persist within changing local environmental niches. Similarly, CUP pilus targeting is gratefully demonstrated that targeting more than one CUP adhesin may indeed be a more effective strategy for combating UTIs. Herein, we have highlighted the overwhelming value of applying a deep mechanistic understanding of structure-function-virulence relationships of bacterial adhesins to the rational design of high-affinity carbohydrate glycomimetics for the treatment of UTI. This demonstration serves as a general model for the rational approach necessary to target virulence factors and disrupt their role in bacterial infections.

Materials and Methods

Ethics Statement. All animal experiments were conducted according to the National Institutes of Health (NIH) guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after pertinent review and approval by the Institutional Animal Care and Use Committee at Washington University School of Medicine (protocol 20150226). Deidentified human tissue was obtained from the Tissue Procurement Core at Washington University School of Medicine.

Protein Expression and Purification. FimH residues 1 to 160 from UPEC strain UTI89 was fused to c-terminus six-histidine tag (i.e., FimH6His) were cloned into the IPTG-inducible plasmid pTrc99A. This construct was then transformed and expressed in E. coli strain C600. Periplasm were isolated as previously described and dialyzed four times against PBS plus 250 mM NaCl (34). FimH6His was purified from this periplasmic fraction by cobalt affinity chromatography through elution with 150 mM imidazole. FimH6His was exchanged into 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.5) and 50 mM NaCl, concentrated to 6 mg/mL, and stored stably at 4 °C for use in biochemical and biophysical assays.

In Silico Docking and Virtual Screening. A library of approximately 1 million members comprising 85% naturally occurring sugars and 15% mannosides were used in docking. Virtual screening of this library, which comprised galactosides ranging from 150 to 900 Da in molecular mass, yielded a mean docking score of

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4.18 kJ), with a standard deviation of 0.73 kcal/mol and a range of 4 to 9 kcal/mol (SI Appendix, Fig. S1A). To prioritize hits, we abstained from directly comparing raw binding scores, as large, lipophilic molecules tend to have artificially high predicted binding interactions due to their contribution to hydrophobic interactions as calculated by the empirical scoring function of AutoDock Vina (52). Instead, the results of the virtual screen were evaluated per group efficiency (GE), which, in this context, measures the contribution of the aglycone group within each galactoside (indicated as X in the following equation) to the docking score (DS) with respect to the number of heavy atoms (HA) present in the aglycone group [GE = (DSX−DSα/(HAX−HASSOC))]. Top hits were defined as galactosides with a GE value greater than 1.25 times the SD (σ = 0.0016 kcal/mmol) per HA) above the library mean (μ = 0.0011 kcal/mol per HA), which constituted the top ~10% of highest scoring galactosides (SI Appendix, Fig. S1A).

Synthesis of Galactosides and N-acetyl Aminagalactosides. Galactosides and N-acetyl aminagalactosides were synthesized by standard glycosylation chemistry, including boron trifluoride-mediated glycosidation and the Koenigs-Knorr reaction, respectively (SI Appendix, Fig. S2). In method A, boron trifluoride-promoted glycosylation of phenols with Gal pentaacetate yielded corresponding acetylated aryl galactosides, which were treated with sodium methoxide in methanol to provide the corresponding aryl galactosides (1β to 3β, 5β to 7β, 18β to 20β, 24α to 26α, and 28β to 30β). In method B, GalNaC and Gal analogs (20β-NAc, 21β, 28β-NAc, and 29β-NAc) were synthesized from galactosyl halide and aryl alcohols via a Koenigs-Knorr-type reaction, which yielded aryl galactosides that were then deacetylated by treatment with methanethiol in ethanol.

ELISA. Immulon 4HBX 96-well plates were coated overnight with 5 μg of bovine submaxillary mucin (Sigma). Coated wells were then treated with 100 μL of Arthrobacter ureafaciens sialidase (10 μIU/mL) diluted in PBS for 1 h at 37 °C. Thereafter, wells were incubated with 200 μL of blocking buffer (PBS plus 1% BSA) for 2 h at 23 °C, followed by incubation with 100 μL of biotinylated FmlHαβ blocked in blocking buffer to 20 μg/mL in the presence or absence of galactoside compounds for 1 h at 23 °C. After washing three times with PBS plus 0.05% Tween-20, 100 μL of streptavidin-HRP conjugate (Bangs Laboratories) was added to each well for 1 h at 23 °C. After a final round of washing, plates were developed with 100 μL of tetramethylbenzidine (BD Biosciences) substrate and quantified within 1 to 2 min with 50 μL of 1 M H2SO4, and absorbance was measured at 450 nm. This assay was used to determine percent inhibition values and inhibitory constant (IC50) values where indicated.

BLL. Streptavidin pins were first dipped in a baseline in PBS (pH 7.4) for 120 s, followed by loading of 5 to 10 μg/mL biotinylated Ser-TF (Toronto Research Chemicals) in PBS for 300 s, quenching by 10 μg/mL biotinylin PBS for 240 s, and another baseline step in PBS for 120 s. Thereafter, pins were dipped in PBS for 120 s and transferred to protein samples (varying concentration of FmlHαβ or fixed concentration of FmlHαβ with varying concentration of galactoside compounds) for association to 300 to 600 s. Equilibrium binding response values were used to determine the affinity of interaction between each FmlHαβ and immobilized Ser-TF under a 1:1 binding model or between FmlHαβ and galactosides in solution under a competitive one-site binding model.

Protein Crystallization and Structure Determination. Crystals of apo FmlHαβ in 10 mM Hepes (pH 7.5) and 50 mM NaCl were grown by mixing 2 μL of protein (6 mg/mL) and 2% PEG8000 and equilibrated against 1 mL of mother liquor in the reservoir. These crystals were transferred into cryoprotectant (1 M LiSO4, 10% PEG8000, and 25% glycerol). Diffraction data for TF, 4β (in space group C 12 1), and 29β-NAc structures were collected at 100 K at an in-house facility equipped with a rotating anode Rigaku MicroMax 007 generator, a Rayonix Marmux X-ray source, and a Mar345 image plate detector. Diffraction data for apo, 4β, 5β, and 20β structures were collected at 100 K at the ALS Beamline 4.2.2. Data were indexed and integrated in iMosflm (55), XDS (56), or HKL2000 and scaled by Scala (57). The phase problem was solved by molecular replacement using Phaser-MR in PHENIX (58) with FmlHαβ from PDB ID 3MCY. Several rounds of refinements were performed in PHENIX to improve the final models.

Mouse Infections. Seven- to 8-week-old female C3H/HeN mice were obtained fromEnvigo. Mice were anesthetized and inoculated via transurethral catheterization with 50 μL of CFT073 bacterial suspension (~1 × 10^8 to 2 × 10^9 CFU in total) in PBS. Mice experiencing high titers of bacteriuria (~10^9 CFU/mL and edematous and inflamed bladders when killed after 2 wk, or chronic cystitis (39), were then transurethrally inoculated either with 50 mg/kg compound or vehicle control (10% DMSO). Mice were killed 6 h posttreatment, and bacteria colonizing the bladder or kidney were plated for quantification.

Immunofluorescence. Frozen, deidentified human bladder and kidney sections were obtained from the Tissue Procurement Core and stored stably at ~80 °C. These tissue section slides were removed from the freezer and allowed to thaw at room temperature for 10 to 20 min. After applying a hydrophobic barrier around the tissue, slides were rehydrated in 200 μL of PBS plus 1% BSA and 0.2% Triton X-100 in PBS for 10 min. Buffer was gently aspirated and slides were blocked for 1 h at room temperature with 200 μL of buffer. Thereafter, buffer was gently aspirated and slides were incubated with 200 μL of sample overnight at 4 °C. Samples diluted in buffer included 50 μg/mL FmlHαβ, wild-type (WT), 50 μg/mL FmlHαβ and 50 μg/mL FmlHβ WT incubated with 100 μM 29β-NAc. Samples were gently aspirated and slides were washed three times in buffer for 5 min each. Next, slides were incubated with our mouse antihemolytic polyclonal antibody (1,500 dilution in buffer) for 1 h at room temperature. Slides were washed again three times in buffer and then incubated in the dark with donkey anti-mouse IgG Alexa Fluor 594 and Wheat Germ Agglutinin Alexa Fluor 633 (each 1:500 dilution in buffer) for 1 h at room temperature. Slides were washed once with buffer and then incubated in the dark with DAPI (1:1,000 dilution in buffer) for 5 min at room temperature. After washing twice with buffer, coverslips were mounted using 80 μL of mounting media. Slides were loaded onto a Zeiss LSM 880 Confocal Laser Scanning Microscope (Carl Zeiss, Inc.) equipped with a diode 405 to 430 laser, a HeNe 543 laser, and a HeNe 633 laser. Images were acquired with a 20×, 0.8 numerical aperture Zeiss Plan Achromat objective using ZEN 2 imaging software.

Statistics. Mouse data are compiled from two (42269 plus 29β-NAc) or three (all other treatments) independent experiments, with four or five mice per group per experiment. These data were analyzed using the uncorrected two-tailed Mann-Whitney U test in GraphPad Prism v.5. ELISA data were reported as box-and-whisker plots indicating the mean, 2.5th, 25th, 75th, and 97.5th percentiles of at least two independent experiments, with three technical replicates per experiment.

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