Screening Small Molecule Glycosylation Inhibitors and Assessing Their Impact on Glycoprotein Biosynthesis and Bacterial Fitness

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Abstract: *Helicobacter pylori* is a gram-negative, spiral-shaped pathogenic bacterium that colonizes the human stomach. Once infection is established, gastric cancer and peptic ulcers can occur, among other complications. Antibiotics have been a powerful tool in the treatment of pathogenic bacteria. The current treatment of *H. pylori* is "triple therapy," and involves using at least two different antibiotics. The third medication is usually proton pump inhibitors (PPIs) to help the stomach heal. However, this has not been effective. Excess use of broad-spectrum antibiotics leads to antibiotic resistant mechanisms in bacterial pathogens (Quintana, 2022). Broad spectrum antibiotics also often disrupt the normal gut microbiome (Quintana, 2022). Alternative methods are needed to minimize negative side effects and selectively treat antibiotic resistant "priority pathogens" of concern. Bacterial glycans are compelling therapeutic targets due to their distinctive monosaccharides and role in bacterial pathogenicity. Prior studies have shown that disruption in bacterial glycosylation leads to reduced adhesion to host cells, biofilm formation, motility, and thus, host colonization (Quintana, 2022). Thus, the research of the Dube lab has focused on targeting glycans that coat the surface of bacterial cells. Recent work by Isabella Quintana previously demonstrated S-glycoside inhibitors based on rare bacterial monosaccharides to be effective in disrupting glycan biosynthesis and bacterial fitness. I replicated some experiments to confirm these findings. I also explored complementary angles using carbohydrate-binding proteins to confirm that S-glycoside inhibitors effectively impeded glycan biosynthesis in a concentration-dependent manner. Overall, small molecule inhibitors have shown to selectively target antibiotic resistant pathogens, thus increasing our tools to treat infection.

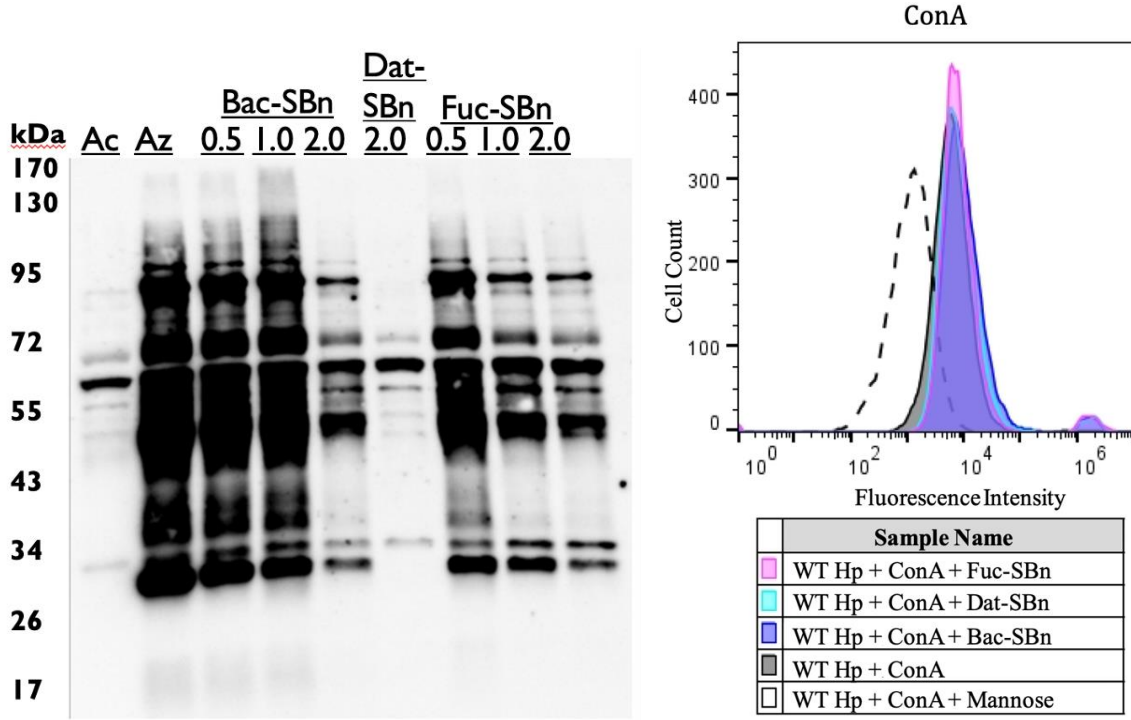
Project Objectives: This research aims to assess the effectiveness of S-glycoside inhibitors based on rare bacterial monosaccharides in disrupting glycan biosynthesis by measuring glycoprotein biosynthesis and changes in cell surface glycan architecture.

Methodology Used: Small molecule inhibitors - benzylS-glycosides on bacterial monosaccharides, Bac, DATDG, and FucNAc were used as substrate decoys of bacterial glycosyltransferase. These bacterial monosaccharides were chosen due to their quick synthesis of scaffolds and their utilization in priority pathogens (Williams, 2020). The goal was to assess the effectiveness of S-glycosides as metabolic inhibitors to impede bacterial glycosylation. This was done by 1) confirming inhibition of glycoprotein biosynthesis and 2) confirming changes in cell surface glycan architecture.

To confirm inhibition, glycoprotein biosynthesis was measured using Metabolic Oligosaccharide Engineering (MOE) and a gel-based analysis through Western Blot. For MOE, *H. pylori* were inoculated into 3.5 mL of liquid culture, with an optical density measured at 600nm (OD600) between 0.4-0.5 (Quintana, 2023b). Samples were treated with a negative, azide-free control (0.5 mM Ac4GlcNAc), or were metabolically labeled with 0.5 mM Ac4GlcNAz. The positive control contained no inhibitor. All other samples were supplemented with 0.5, 1.0, or 2.0 mM of BacSBn, DATSBn, or FucSBn S-glycoside inhibitors. There were 11 samples in total. *H. pylori* cultures were incubated for 4 days under microaerophilic conditions (14% CO2, 37 °C). Following metabolic labeling, bacterial cells were probed for glycosylation inhibition via Western blot. Cells were lysed and resultant protein lysates were standardized (BioRad's DC protein concentration assay) to a protein concentration of ∼2.5 mg mL–1 prior to reaction with 250 μM Phos-FLAG overnight at room temperature (Quintana, 2023a**)**. Reacted lysates were loaded onto a 12% Tris–HCl SDS-PAGE gel, separated by electrophoresis, and transferred to nitrocellulose paper (Quintana, 2023a**)**. Anti-FLAG-HRP was employed to visualize FLAG-tagged proteins via chemiluminescence (Quintana, 2023a**)**.

To explore a complementary lens, fluorescent, carbohydrate-binding proteins were used to confirm changes in cell surface glycan architecture in inhibitor treated samples. This was accomplished using a lectin binding assay. *H. pylori* were treated with 2.0 mM BacSBn, DATSBn, or FucSBn or left untreated (5 samples) for 3 days and then were probed with Alexa Fluor 488-conjugated Concanavalin A (ConA) (Quintana, 2023a**)**. As a negative control, ConA was preincubated with 400 mM mannose (carbo-block) prior to binding to untreated *H. pylori* (Quintana, 2023a). Cells were analyzed by flow cytometry on a BD Accuri C6+ (BD Biosciences) instrument, with 10,000 live cells gated for each replicate experiment (Quintana, 2023a**)**. Data were analyzed by using FlowJo software (Ashland, OR) (Quintana, 2023a**)**.

Results Obtained:



Significance and Interpretation of Results: The results of the western blot (left) confirmed that S-glycoside inhibitors impeded *H. pylor*i glycosylation in a concentration-dependent manner. The first lane of the western blot (Ac) was the negative control. It did not contain the azide handle necessary for signal production and thus had no signal. The next lane (Az) was grown in the presence of azide and thus had the greatest signal present. The signal represented a read out of normal, fully elaborated glycans. The following lanes from experimental samples were compared to the positive control. Experimental samples with a similar signal to the positive control (Az) were considered unaffected by the inhibitor (Quintana, 2023b). Samples with a reduction in signal were considered affected by the inhibitor. In samples treated with Bac-SBn and Fuc-SBn inhibitors, there was a slight reduction of signal at the lower concentration (0.5 mM). Because the signals were relatively unchanged in comparison to the positive Az control, we concluded that the 0.5mM concentration did not affect glycan biosynthesis. When Bac-SBn, DAT-SBn, and Fuc-SBn inhibitor concentrations were raised to 2.0 mM, there was a significant reduction in azide signal relative to the Az lane, and thus we concluded they impeded glycan biosynthesis (Quintana, 2023b). DAT-SBn only has one lane representing the highest concentration due to a procedural error. However, all the inhibitors showed a significant reduction of signal at the highest concentration (2.0mM). From these results, we concluded that the S-glycoside inhibitors inhibited *H. pylori* glycosylation in a concentration-dependent manner.

The results of the fluorescent carbohydrate-binding lectin assay (right) acted as a complementary means to assess the effect of S-glycoside inhibitors on impeding glycan biosynthesis. The lectin *Concanavalin A* (Con A) was used to bind to untreated *H. pylori* versus *H. pylori* treated with 2.0 mM thioglycosides BacSBn, DATSBn, or FucSBn. Results (Figure 1) showed that ConA bound to untreated *H. pylori* at modest levels, and ConA binding was diminished by pretreatment of ConA with high concentrations of its monosaccharide ligand mannose (Quintana, 2023a). Binding untreated *H. pylori* with the carboblock mannose confirmed that the lectins were functional. This sample ensured that the monosaccharide ligand mannose did appreciably bind to ConA. Relative to untreated *H. pylori*, cells that were treated with BacSBn, DATSBn, and FucSBn displayed an increase in ConA binding via increased fluorescence (Figure 1). The data confirm a change in cell surface glycan architecture, and it provides bioorthogonal complementary evidence of glycan inhibition. This result is in line with previous reports of small molecules or genetic disruption of glycoprotein biosynthesis causing increased lectin binding (Quintana, 2023a**)**. The research in the Dube Lab has the potential to contribute to the discovery of a new set of antibiotic alternatives, revolutionizing the treatment and outcomes for patients infected with drug resistant bacterial infections.

Figures/ Charts:

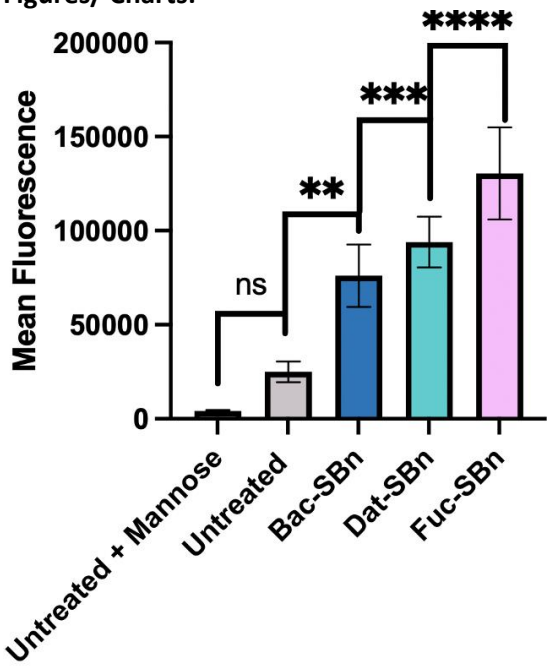


Figure 1. Wildtype *H. Pylori* treated with inhibitors exhibited increased ConA lectin binding compared to untreated wildtype *H. pylori***.**

Acknowledgments and References:

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