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. Infection can cause peptic ulcers and gastric cancer. While antibiotics have once been a powerful tool in the treatment of pathogenic bacteria, excess use of broad-spectrum antibiotics has led to an increase in antibiotic-resistant mechanisms. Broad-spectrum antibiotics also often disrupt the normal gut microbiome. Alternative methods are needed to mitigate negative side effects and selectively treat antibiotic resistant "priority pathogens" of concern, as categorized by the World Health Organization (WHO). The Dube lab focuses on utilizing bacterial glycans as therapeutic targets due to their distinctive monosaccharides and role in bacterial pathogenicity. Prior studies from the Dube Lab have shown substrate decoys O-glycosides and S-glycosides based on rare bacterial monosaccharides to have inhibitory effects on *H. pylori* glycan biosynthesis and bacterial fitness (Williams, 2023; Quintana, 2023a). This summer, to strengthen our toolbox and discover agents with enhanced potency, I conducted parallel experiments on two classes of novel small molecule inhibitors of *H. pylori* glycoprotein biosynthesis. The first inhibitor is protected Glc-1-C-phosphonate, termed C-glycoside 1, DT13 (Figure 1A). It is a glucose analog that contains a non-hydrolyzable carbon connection, effectively acting as a glycosyltransferase (GT) inhibitor. The second class of inhibitor includes novel inhibitors 3F-FucNHAc-OBn and 3F-FucNHAc-SBn, termed SSK-10 and SSK-11 respectively (Figure 1B, 1C). The SSK inhibitors were created to determine whether the precedented O-glycoside and S-glycoside substrate decoy inhibitors could be made more effective with the addition of a chain terminator (F-sugar). All inhibitors were found to inhibit *H. pylori* glycoprotein biosynthesis, and C-glycoside 1, DT13 was found to elicit negative effects on bacterial fitness, thus impacting *H. pylori* pathogenicity. My research in the Dube Lab aims to contribute to the development of necessary, novel therapeutics that target bacterial glycans in the face of increasing antibiotic resistance.

Project Objectives: This research aims to assess the effectiveness of SSK-10, SSK-11, and C-glycoside 1,DT13 inhibitors in disrupting glycan biosynthesis in *H. pylori* by measuring glycoprotein biosynthesis and changes in cell surface glycan architecture. Bacterial fitness assays were also utilized to determine the effects of inhibited glycoprotein biosynthesis from C-glycoside 1, DT13 on bacterial growth, motility, and biofilm formation.

Methodology Used: To assess the effectiveness of 1, DT13, SSK-10, and SSK-11 as metabolic inhibitors in impeding *H. pylori* glycosylation, experiments were run to measure glycoprotein biosynthesis via molecular weight using Metabolic Oligosaccharide Engineering (MOE) and Western Blot. Most of the experimental protocols used have been adapted from previous Dube lab members due to similarity in workflow. 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. Samples were treated with a negative, azide-free control (0.5mM Ac4GlcNAc), or were metabolically labeled with 0.5mM Ac4GlcNAz (Quintana, 2023b). The positive control contained no inhibitor. All other samples were supplemented with either 0.025mM or 0.1mM of C-glycoside 1, DT13, or 0.5mM, 1.0mM or 2.0mM of SSK-10 or SSK-11. *H. pylori* cultures were incubated for 4 days under microaerophilic conditions (14% CO2, 37 °C).

Following metabolic labeling, bacterial cells were probed for *H. pylori* glycosylation inhibition via Western blot. Cells were lysed, and the protein lysates were standardized to a concentration of ~2.5 mg/mL prior to reaction with 20μM Phos-FLAG overnight at room temperature. 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).

Biorthogonal, complementary assays were then conducted to confirm glycoprotein inhibition by utilizing fluorescent carbohydrate-binding protein Alexa Fluor 488 Concanavalin A (ConA) to confirm changes in cell surface glycan architecture in inhibitor-treated samples. Each experiment contained an untreated sample. All remaining samples included *H. pylori* treated with either 0.025mM or 0.1mM of C-glycoside 1, DT13, or 0.5mM, 1.0mM, or 2.0mM of SSK-10 or SSK-11. The samples were left for 3 days at 37 °C with gentle shaking under microaerophilic conditions. They were then probed with ConA. As a negative control, ConA was preincubated with 400mM mannose (carbo-block) prior to binding to untreated *H. pylor*i to ensure the use of functional lectins (Quintana, 2023a). Cells were analyzed by flow cytometry on a BD Accuri C6+ instrument, with 10,000 live cells gated for each replicate experiment (Quintana, 2023a). Data were analyzed by using FlowJo software.

After confirming glycoprotein inhibition, bacterial fitness assays were conducted to determine the impacts of inhibited glycan biosynthesis from C-glycoside 1, DT13 on *H. pylori* fitness, specifically growth, biofilm formation, and motility. Bacterial growth was monitored during log phase until bacteria reached stationary phase - over the course of 8 days. Cells were initially inoculated into liquid media at a starting OD600 of ~0.1 and cultured in the absence of inhibitors or the presence of either 0.025mM or 0.1mM of C-glycoside 1, DT13, or 0.5mM, 1.0mM, or 2.0mM of SSK-10 or SSK-11 at 37 °C with gentle shaking under microaerophilic conditions. The OD600 was measured by using a SPECTROStar Nano 96-well plate reader (Quintana, 2023a).

Motility was recorded by measuring the ability of *H. pylori* to swarm on soft agar over the course of 18 days. *H. pylori* cultures were standardized to an OD600 between 0.4 and 0.5 in rich media and then incubated with no inhibitor or either 0.025 or 0.1mM of C-glycoside 1, DT13, or 0.5mM, 1.0mM, or 2.0mM of SSK-10 or SSK-11 under microaerophilic conditions. Cells from each culture were pelleted and resuspended. 10 µL of the concentrated culture was plated onto soft agar plates supplemented with 4% agar and 10% fetal bovine serum (Quintana, 2023a). Plates were incubated at 37 °C and 14% CO2, and the colony diameter was measured and imaged for 18 days.

Finally, the ability of *H. pyl*ori to form a biofilm in the absence or presence of C-glycosides was assessed by standardizing *H. pylori* to an OD600 of 0.4 to 0.5 in rich liquid media in the absence of inhibitor or with C-glycoside 1, DT13 at 0.025 or 0.1mM. Samples were added in triplicate to the sidewalls of a 96-well plate (Quintana, 2023a). The bacteria were incubated for 5 days at 37 °C and 14% CO2. After incubation, the medium was carefully removed, and the biofilm was stained with 0.15% crystal violet (Quintana, 2023a). After staining the triplicate wells to visualize biofilm production, side-view images were taken. The stained wells were then solubilized in 30% acetic acid in water, and the absorbance of the solution was quantified at 550 nm using a SPECTROstar Nano plate reader (Quintana, 2023a).

Results: The results of the western blots confirmed that both C-glycoside and SSK inhibitors impede *H. pylori* glycosylation (Figure 2A). The first lane of each 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. This 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. Conversely, samples with a reduction in signal were considered affected by the inhibitor. The results from the western blot (Figure 2A, left) exhibited strong inhibition in glycoprotein biosynthesis from compounds SSK-10 and SSK-11 at 1.0mM and 0.5mM, respectively. The western blot of C-glycoside 1, DT13 (Figure 2A, right) exhibited little glycoprotein biosynthesis inhibition at 0.025mM, but strong inhibition at 0.1mM.

The results of the fluorescent carbohydrate-binding lectin assay acted as a complementary means to assess the effect of the inhibitors on impeding glycan biosynthesis. The fluorescent lectin Con A was used to bind to untreated *H. pylori* versus *H. pylor*i treated with varying concentrations of inhibitor. Results (Figure 2B) demonstrated that ConA binding was reduced by pretreatment of ConA with high concentrations of its monosaccharide ligand mannose (Quintana, 2023a). This confirmed that the lectins were functional as the monosaccharide ligand mannose did appreciably bind to ConA. Relative to untreated *H. pylori*, cells that were treated with SSK-10 (Figure 2B, left) at 0.5mM, 1.0mM, and 2.0mM displayed an increase in ConA binding via increased fluorescence. The data not shown for SSK-11 contained nearly identical results. Similarly, relative to untreated *H. pylori*, cells that were treated with C-glycoside 1, DT13 (Figure 2B, right) at 0.025mM and 0.1mM displayed an increase in ConA binding via increased fluorescence. The data confirm a change in cell surface glycan architecture, and it provides biorthogonal complementary evidence of glycoprotein biosynthesis inhibition from all compounds. This result is in line with previous reports of small molecules or genetic disruption of glycoprotein biosynthesis causing increased lectin binding (Quintana, 2023a).

Due to timing, bacterial fitness assays were run only on C-glycoside 1, DT13. Inhibition of glycan biosynthesis in *H. pylori* at 0.1mM concentrations negatively impacted motility, growth, and biofilm formation at statistically significant levels (Figure 3A).

Significance and Interpretation of Results: The Glc-1-C-phosphonate glycosyltransferase inhibitor demonstrates promising results. The inhibitor impedes *H. pylori* glycoprotein biosynthesis at concentrations as low as 0.1mM and elicits negative fitness effects in the same concentrations. Further studies could be used to inform the selectivity of the inhibitor, as well as toxicity towards mammalian cells. Bacterial fitness assays could also be conducted on both SSK-10 and SSK-11 to determine effects of the inhibitors on *H. pylori* pathogenicity. My 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

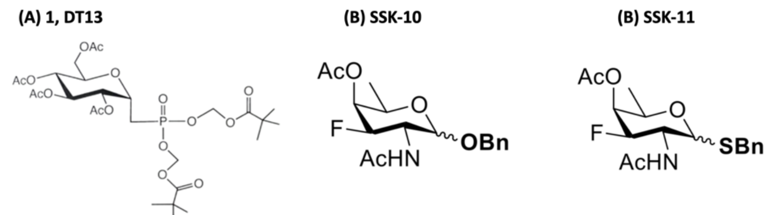
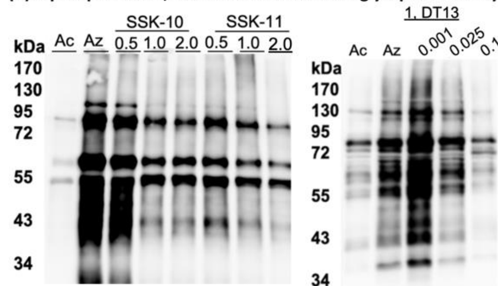


Figure 1. Novel small molecule inhibitors. A) Chemical structure of Glc-1-C-phosphonate, termed C-glycoside 1, DT13. This compound is a glycosyltransferase inhibitor and is the protected, most relevant version of the C-glycoside compounds. B) Chemical structure of 3F-FucNHAc-OBn, termed SSK-10. This compound is a combination chain terminator (F-sugar) and substrate decoy (O-glycoside based on a rare bacterial monosaccharide FucNHAc). C) Chemical structure of 3F-FucNHAc-SBn, termed SSK-11. This compound is also a combination chain terminator (F-sugar) and substrate decoy (S-glycoside based on a rare bacterial monosaccharide FucNHAc).

Figure 2

(A) C-phosphonate 1, DT13 cause inhibition of glycoprotein biosynthesis in *H.pylori*



(B) C-glycoside 1, DT13, SSK-10, and SSK-11 cause perturbed cell-surface glycan architecture in *H. pylori*

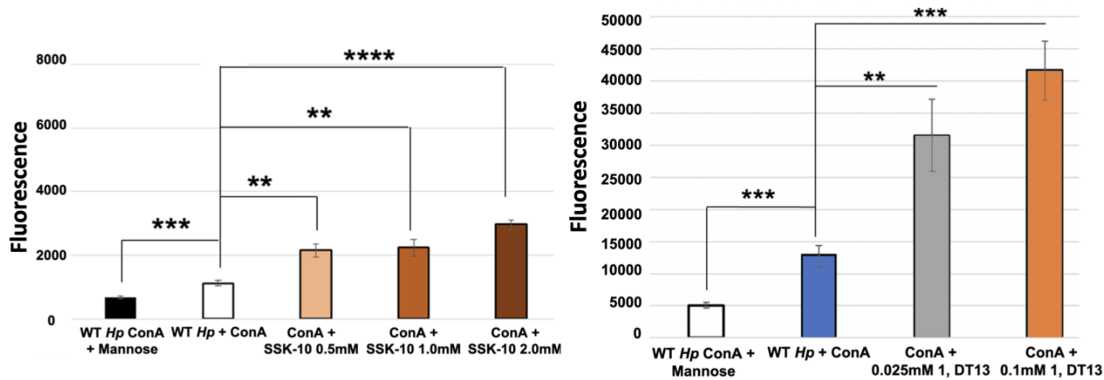


Figure 2. Novel compounds inhibit glycoprotein biosynthesis in *Helicobacter pylori*. A) Western blot analysis reveals significantly diminished glycoprotein biosynthesis in *H. pylori* upon treatment with SSK-10 at 1.0mM, and with SSK-11 at 0.5mM (left). *H. pylori* upon treatment with 1, DT13 at 0.025mM reveals little to no inhibition, while 1, DT13 at 0.1mM demonstrates strong inhibition. B) Flow cytometry analysis reveals increased binding of fluorescent ConA lectin in *H. pylori* treated with SSK-10 (left) and 1, DT13 (right), consistent with perturbed cell surface glycan architecture. By contrast, pretreatment of ConA with 400 mM mannose (carbo-block) prior to probing untreated *H. pylori* led to decreased binding, signifying functional lectins. SSK-11 data not shown were replicable with SSK-10 data. The western blot data shown are representative of replicate experiments (n = 2).

Figure 3

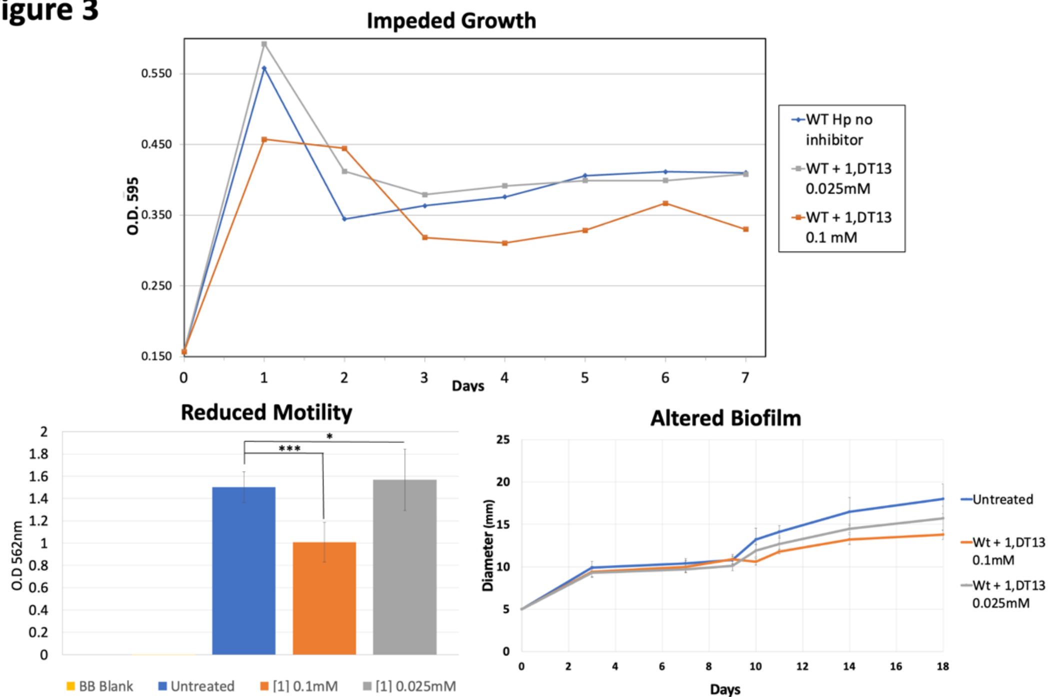


Figure 3. Novel C-glycosides decrease fitness in *Helicobacter pylori*. Measurement of growth, motility, and biofilm formation demonstrates significant fitness defects in *H. pylori* treated with C-glycoside 1, DT13 at 0.1mM. The data shown are representative of replicate experiments (n = 2).

Acknowledgements and References:
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