High-Throughput Screening Identifies Novel Inhibitors of the Acetyltransferase Activity of *Escherichia coli* GlmU[†]

Mark P. Pereira,¹ Jan E. Blanchard,² Cecilia Murphy,² Steven L. Roderick,³ and Eric D. Brown^{1,2*}

Department of Biochemistry and Biomedical Sciences and Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario L8N 3Z5, Canada¹; McMaster High Throughput Screening Laboratory, Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada²; and Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461³

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The bifunctional GlmU protein catalyzes the formation of UDP-*N*-acetylglucosamine in a two-step reaction using the substrates glucosamine-1-phosphate, acetyl coenzyme A, and UTP. This metabolite is a common precursor to the synthesis of bacterial cell surface carbohydrate polymers, such as peptidoglycan, lipopolysaccharide, and wall teichoic acid that are involved in the maintenance of cell shape, permeability, and virulence. The C-terminal acetyltransferase domain of GlmU exhibits structural and mechanistic features unique to bacterial UDP-*N*-acetylglucosamine synthases, making it an excellent target for antibacterial design. In the work described here, we have developed an absorbance-based assay to screen diverse chemical libraries in high throughput for inhibitors to the acetyltransferase reaction of *Escherichia coli* GlmU. The primary screen of 50,000 drug-like small molecules identified 63 hits, 37 of which were specific to acetyltransferase activity of GlmU. Secondary screening and mode-of-inhibition studies identified potent inhibitors where compound binding within the acetyltransferase active site was requisite on the presence of glucosamine-1-phosphate and were competitive with the substrate acetyl coenzyme A. These molecules may represent novel chemical scaffolds for future antimicrobial drug discovery. In addition, this work outlines the utility of catalytic variants in targeting specific activities of bifunctional enzymes in high-throughput screens.

The acetylated amino-sugar *N*-acetylglucosamine (GlcNAc) is a metabolite of central importance to the biosynthesis of the bacterial cell envelope. The activated form of GlcNAc, UDP-GlcNAc, is at a branchpoint between the synthesis of three key bacterial biosynthetic pathways: peptidoglycan and lipid A and lipopolysaccharide in gram-negative bacteria, and teichoic acid in gram-positive bacteria. This activated precursor is necessary for the assembly of these molecules on the intracellular face of the cytoplasmic membrane, and perturbing UDP-GlcNAc synthesis can have grave effects on the fitness of the bacteria. Both peptidoglycan and lipid A are necessary for cell viability (13, 24), while lipopolysaccharide and teichoic acid contribute to the virulence of bacterial pathogens (23, 28). Hence, the discrete inhibition of UDP-GlcNAc biosynthesis would simultaneously target multiple essential and virulence-determining pathways.

Although UDP-GlcNAc is an essential metabolite in both eukaryotes and prokaryotes, distinct differences in biosynthesis observed between UDP-GlcNAc synthases from members of each kingdom can be exploited for selective inhibition of prokaryotic UDP-GlcNAc synthesis. In bacteria, UDP-GlcNAc is efficiently synthesized from the glycolysis intermediate fructose-6-phosphate in four enzymatic steps beginning with amidation to glucosamine-6-phosphate followed by isomerization

to glucosamine-1-phosphate (GlcN-1-P) by the GlmS and GlmM enzymes (2). The 456-amino-acid bifunctional enzyme GlmU then catalyzes the two final steps of UDP-GlcNAc synthesis: the acetylation of GlcN-1-P using acetyl coenzyme A (AcCoA), followed by nucleotidyl activation with UTP (10). In eukaryotes, however, the biosynthesis of UDP-GlcNAc is markedly different at the acetylation step of the phosphorylated amino-pyranose. The substrate of the eukaryotic analogue for the acetyltransferase reaction (GNA1 [glucosamine-6-phosphate *N*-acetyltransferase 1] [*Candida albicans*]) is GlcN-6-P, which is subsequently isomerized by AGM1 (*N*-acetylglucosamine-phosphate mutase 1) (*Candida albicans*) to GlcNAc-1-P for nucleotidyl activation with UTP by the enzyme UAP1 (UDP-*N*-acetylglucosamine pyrophosphorylase 1) (*C. albicans*) (16). The differing substrate specificity of the GlmU enzyme's acetyltransferase activity from the eukaryotic analogue highlights this activity as having promise for specific perturbation of bacterial UDP-GlcNAc synthesis. In addition to differences in substrate specificity, the acetyltransferase domain of GlmU and the eukaryotic analogue GNA1 are structurally distinct. Although the N terminus of GlmU enzyme encodes an uridylyltransferase orthologous to the eukaryotic enzyme UAP1, the C-terminal acetyltransferase domain of GlmU is characterized by a hexapeptide repeat sequence that leads the enzyme to adopt a left-handed beta helix fold (18), a characteristic of select bacterial acyltransferases. The eukaryotic enzyme GNA1 that catalyzes the acetylation of GlcN-6-P does not adopt this fold but a structurally conserved fold of the GNAT (Gcn5-related *N*-acetyltransferase) family of acetyltransferases (26). These key structural and mechanistic differ-

* Corresponding author. Mailing address: Department of Biochemistry and Biomedical Sciences and Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada. Phone: (905) 525-9140, ext. 22454. Fax: (905) 522-9033. E-mail: ebrown@mcmaster.ca.

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ences have highlighted the acetyltransferase activity of GlmU as an excellent target for antimicrobial design.

Although the structure of the acetyltransferase domain of GlmU has been studied meticulously, small-molecule inhibitors of this activity that can serve as chemical probes for careful mechanistic studies or scaffolds for antibacterial drug design are still not available. To date, only electrophilic thiol-reactive molecules, such as maleimides and iodoacetamide, have been listed as inhibitors of the acetyltransferase domain (7, 21). These compounds, however, are nonspecific inhibitors that react with an active site thiol and hence do not show promise for the development of chemical probes of mechanism or for drug discovery. Here we present a high-throughput screen of the *Escherichia coli* enzyme GlmU against 50,000 drug-like small molecules from Maybridge (Cornwall, United Kingdom) to evaluate their effect on the acetyltransferase activity of this enzyme. The challenge that was afforded in the selective screening of this reaction is sensitivity of the acetyltransferase domain to reducing conditions (21). The presence of a cysteine in the acetyltransferase active site positioned near the acetyl group of AcCoA necessitates reducing agents, such as dithiothreitol (DTT), to maintain the enzyme in a catalytically active state, preventing the use of typical CoA assay reagents, such as dithionitrobenzene. Through the use of a combination of wild-type (WT) GlmU and a variant lacking acetyltransferase activity (GlmU_{H363N}), we report here a robust coupled assay to screen for inhibitors specific to the acetyltransferase reaction of the GlmU enzyme. Using this assay, a total of 63 molecules, 37 specific to the acetyltransferase activity of GlmU, were selected for further study. The potency and specificity of three identified small-molecule inhibitors highlight them as excellent candidates for further study.

MATERIALS AND METHODS

Materials. 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), DTT, magnesium chloride (MgCl₂), and AcCoA were purchased from BioShop Canada Inc. (Burlington, Ontario, Canada). Library compounds were purchased from Maybridge (Cornwall, United Kingdom). All other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Protein expression and purification. Native *E. coli* GlmU was purified as previously described (19). Briefly, a pET3a expression vector harboring the *glmU* gene was transformed into *E. coli* BL21(DE3) which massively expressed the enzyme in the absence of induction. After streptomycin sulfate precipitation of DNA and centrifugation, the supernatant was applied to a Poros HQ50 anion-exchange column and eluted with an NaCl gradient (0 to 0.75 M). The fractions were analyzed by polyacrylamide gel electrophoresis, pooled, and concentrated for separation using Superdex 200 prep grade gel filtration chromatography. The apparent purity of the enzyme was >98% as judged by Coomassie blue-stained polyacrylamide gel electrophoresis. The GlmU_{H363N} mutant enzyme was overexpressed and purified using the same protocol.

Coupled acetyltransferase assay. The acetyltransferase activity of GlmU was monitored in a coupled assay with the end product inorganic phosphate detected using malachite green (4) as described here. The formation of GlcNAc-1-P (*N*-acetylglucosamine-1-phosphate) was coupled with the uridylyltransferase reaction of GlmU and pyrophosphate hydrolysis by the addition of excess GlmU_{H363N} (acetyltransferase-negative mutant of GlmU) and excess inorganic pyrophosphatase. The components of the reaction mixture included 25 mM HEPES (pH 7.6), 1 mM DTT, 5 mM MgCl₂, 0.01% Triton X-100 (vol/vol), 2% dimethyl sulfoxide (DMSO) (vol/vol), 20 mM inorganic pyrophosphatase, 100 μM UTP, 100 μM GlcN-1-P, 100 μM AcCoA, 0.25 nM WT GlmU, and 25 nM GlmU_{H363N} in a 50-μl volume. The reaction was started with the addition of substrates AcCoA and GlcN-1-P (glucosamine-1-phosphate) and quenched after a 4-min incubation with 100 μl of malachite green detection reagent (0.034% malachite [wt/vol], 1.05% ammonium molybdate [wt/vol] in 1 M HCl).

Primary screening methodology. A screen of the acetyltransferase activity of GlmU against 50,000 small molecules from the Maybridge library (Cornwall, England) was performed in 96-well plates (catalog no. 3370; Corning, Corning, NY) in duplicate using either a fully automated Beckman/Coulter Sagian core system or a stand-alone Biomek FX workstation (Beckman Coulter Inc., Fullerton, CA) for setup. Assay absorbance was read in a Spectramax Plus³⁸⁴ plate reader (Molecular Devices, Sunnyvale, CA). Compounds dissolved in DMSO were added to reaction mixtures to a final concentration of 20 μM and preincubated with enzyme prior to the start of the reaction with AcCoA and GlcN-1-P. Neat DMSO was added to both high controls (with enzyme) and low controls (without enzyme). Calculation of Z' , a measure of the quality of the high-throughput screen (30), was performed using equation 1, where σ_h , σ_l , μ_h , and μ_l are the standard deviations (σ) and the averages (μ) of the high (h) and low (l) controls:

$$Z' = 1 - \left(\frac{3\sigma_h + 3\sigma_l}{|\mu_h - \mu_l|} \right) \quad (1)$$

Secondary screening methodology. Primary screening hits were tested for acetyltransferase-specific inhibition by assaying the conversion of AcCoA to CoA directly. AcCoA and CoA were separated by paired-ion high-performance liquid chromatography (PIC-HPLC) and detected by UV absorbance spectroscopy at 260 nm. The PIC-HPLC gradient used included a 10 mM tetrabutylammonium hydrogen sulfate and 50 mM potassium phosphate (pH 5.5) mobile phase with a 6-minute gradient from 24% to 30% acetonitrile on a C₁₈ Novapak reverse-phase column (Waters, Mississauga, Ontario, Canada). Absorbance peaks were integrated to determine reaction velocities.

A dose-response assessment (50% inhibitory concentration [IC₅₀]) was determined for each "hit" compound obtained from the primary screen using the PIC-HPLC assay. The reaction buffer consisted of 25 mM HEPES (pH 7.6), 0.01% Triton X-100, 1 mM DTT, 5 mM MgCl₂, 2% DMSO, and 0.25 nM WT GlmU. AcCoA and GlcN-1-P concentrations were fixed at 100 μM, and inhibitors were added at half-logarithmic dilutions from 0.1 μM to 100 μM. Data were fit by nonlinear least-squares regression using Sigmaplot 8.0 (SPSS Inc., Chicago, IL) and equation 2, solving for IC₅₀, where %*A* is percent activity, *L* is minimum %*A*, *H* is maximum %*A*, *I* is the concentration of inhibitor, and *s* is the slope factor:

$$\%A = L + \frac{H - L}{1 + 10^{(\log IC_{50} - I)s}} \quad (2)$$

Each primary screen hit was tested for nonspecific inhibition by the addition of 0.01 mg/ml bovine serum albumin (BSA) to the primary screen reaction (14). Compounds (at 10 μM) were tested for inhibition of the acetyltransferase activity in the presence of the BSA protein.

Mode-of-inhibition studies. Mode-of-inhibition and *K_i* values were determined by simultaneously changing the inhibitor concentration and either the AcCoA or GlcN-1-P concentration (12.5 μM to 200 μM). The resulting curves were fit by nonlinear least-squares regression to models describing competitive, uncompetitive, or mixed inhibition (25) using the Enzyme Kinetics Module 1.1 of Sigmaplot 8.0 (SPSS Inc., Chicago, IL).

RESULTS

Suitability of the developed assay to high-throughput screening. To predispose the developed assay to inhibitors of the acetyltransferase domain, the acetyltransferase reaction was maintained as rate limiting. With the addition of 100-fold excess of GlmU_{H363N} (25 nM) over WT GlmU (0.25 nM), the rate of the reaction was increased 3.5-fold, consistent with the rate differences between the two enzymatic reactions (21 s⁻¹ for uridylyltransferase and 77 s⁻¹ for acetyltransferase [10]). The ratio of mutant enzyme to WT enzyme was saturating for uridylyltransferase activity, while maintaining GlmU_{H363N} at a low concentration to prevent appreciable binding of the tested compounds to the variant. An excess of inorganic pyrophosphatase ensured efficient cleavage of pyrophosphate to maintain the acetyltransferase activity as rate limiting. The developed assay was found to be linear with both time and the

FIG. 1. (A) Scheme of the developed coupled acetyltransferase assay for *E. coli* GlmU. The addition of excess GlmU_{H363N} and inorganic pyrophosphatase (PPase) have rendered the acetyltransferase activity rate limiting. The 2 units of inorganic phosphate produced by each acetyltransferase turnover are detected by absorbance of 660 nm after the addition of malachite green assay reagents. (B) Evaluation of GlmU acetyltransferase screen. The high controls (100% residual activity; circles) and the low controls (0% residual activity; squares) are plotted. The assay shows a large hit window between the high and low controls with a calculated Z' factor of 0.71.

concentration of WT enzyme. A schematic outlining the reaction is displayed in Fig. 1A.

The statistical parameter Z' described by equation 1 defines a measure of the quality of the high-throughput screen (30). It describes the window between high and low controls to which we can determine our lead "hits"; a well-defined hit window is indicated by a value greater than 0.5 (30). The described screening methodology produced a Z' value of 0.71 (Fig. 1B).

Primary screen of GlmU acetyltransferase activity identifies 63 "hits." A total of 50,000 molecules were tested in duplicate for inhibition of GlmU as seen in Fig. 2. A hit cutoff of 75% residual activity was originally determined by calculating 3 standard deviations of the high controls below 100% activity (hit cutoff = $100\% - 3\sigma_h$). This cutoff yielded 215 hits, some of which appeared to be clustered with test compound molecules centered at 100% activity in Fig. 2 and were likely false-positive results. Further, a 75% cutoff produced a relatively large number of hits that would have been prohibitive to the use of an HPLC assay for secondary screening. (The latter was highly sensitive and specific to the GlmU acetyltransferase reaction.) On the other hand, using test compound data instead of control data, a statistical cutoff of 45% residual activity

was determined to yield 39 hits from for the entire 50,000-molecule data set; this cutoff excluded many active molecules and raised concern that false-negative results might affect data quality. A compromise hit threshold was selected by using the midpoint between the two values. A cutoff value of 60% residual activity resulted in the identification of 63 hits, with a hit rate of 0.13%. From visual inspection of the inhibition data in Fig. 2, this threshold appears to be the most appropriate cutoff value.

Specificity of inhibitors to GlmU's acetyltransferase domain. Each "hit" molecule from the primary screen was tested for activity against the acetyltransferase domain of GlmU using a reverse-phase PIC-HPLC-based separation, monitoring the conversion of AcCoA to CoA. Thirty-seven molecules were able to inhibit the acetyltransferase reaction, leaving the remaining molecules as either inhibitors of the uridylyltransferase and pyrophosphatase reactions or as false-positive results from the primary screen.

Each of the thirty-seven molecules was tested for their dose-response relationship with GlmU. Molecules that produced inhibition in a nonidealistic regression to equation 2, that is, molecules that did not inhibit close to 100% of enzyme activity at high inhibitor concentrations, had slope values greater than 1.5, or had R^2 values less than 0.95 for the fit, were filtered out. Seventeen of the 37 molecules had a nonidealistic regression to equation 2, and the dose-response assessment of the remaining 20 compounds yielded IC_{50} s in the range of 0.09 to 2 μ M. Additionally, to filter out any promiscuous inhibitors, the activity of these compounds (at 10 μ M) was tested in the presence of 0.01 mg/ml BSA using the coupled primary screening assay to observe whether inhibition of GlmU could be abrogated by the presence of this protein. This test can detect inhibitors that are active through nonspecific interference with the target enzyme and distinguishes those that form specific inhibitor-enzyme complexes (5, 14). In this assay, maximal inhibition was observed in the presence of BSA for 7 of the 20 compounds. These data were used to prioritize the compounds for further study. Three molecules where inhibition was not perturbed in the presence of BSA showed potent inhibition, were confirmed by mass spectrometry, and were available for reorder from Maybridge. These three molecules were selected for further study.

FIG. 2. Replicate plot of the screen of 50,000 small molecules against *E. coli* GlmU acetyltransferase reaction. The percent residual activity (%RA) of duplicates is plotted on opposite axes. Data points that fall on a slope of 1 are considered to be in agreement. Molecules within the outlined area below 60% residual activity for each replicate reproducibly showed inhibition below the determined cutoff value and were selected for further study.

FIG. 3. Inhibition studies with MAC0021939. Initial rate data of different amounts of inhibitor and either AcCoA (A) or GlcN-1-P (B). The fixed substrate was maintained at 100 μ M. MAC0021939 was used at concentrations of 0 nM ($\bullet$), 50 nM ($\circ$), 100 nM ($\blacktriangledown$), 200 nM (∇), and 300 nM ($\blacksquare$). (A) The inhibitor is competitive with regard to AcCoA with a K_{ic} of 17.2 ± 2.30 nM (B) and uncompetitive with GlcN-1-P with a K_{iu} of 189 ± 17.7 nM.

Mode-of-inhibition studies for MAC0021939, MAC0008028, and MAC0029665. The modes of inhibition for three molecules with the acetyltransferase activity of GlmU, MAC0021939, MAC0008028, and MAC0029665, were tested to determine the form of the enzyme that binds to the inhibitor. In the case of both MAC0021939 and MAC0008028, uncompetitive inhibition was observed with GlcN-1-P having uncompetitive inhibition constant (K_{iu}) values of 189 ± 17.7 nM and $1.11 \pm 0.08 \times 10^3$ nM, respectively, and competitive inhibition was observed with AcCoA having competitive inhibition constant (K_{ic}) values of 17.2 ± 2.30 nM and 404 ± 59.1 nM, respectively (Fig. 3 and 4). MAC0029665 also showed uncompetitive inhibition with respect to GlcN-1-P ($K_{iu} = 28.2 \pm 1.83$ nM); however, it showed mixed inhibition with AcCoA with a fourfold-lower K_{ic} (22.8 ± 2.7 nM) than K_{iu} (100.9 ± 28.4 nM) (Fig. 5).

DISCUSSION

The potential of developing antimicrobials that target UDP-GlcNAc incorporation into extracellular polysaccharides has been validated biochemically. The facts that the flux of metab-

FIG. 4. Inhibition studies with MAC0008028. Initial rate data of different amounts of inhibitor and either AcCoA (A) or GlcN-1-P (B). The fixed substrate was maintained at 100 μ M. MAC0008028 was used at concentrations of 0 nM ($\bullet$), 1,000 nM ($\circ$), 2,000 nM ($\blacktriangledown$), and 3,000 nM ($\blacksquare$). (A) The inhibitor is competitive with regard to AcCoA with a K_{ic} of 404 ± 59.1 nM (B) and uncompetitive with GlcN-1-P with a K_{iu} of $1.11 \pm 0.08 \times 10^3$ nM.

FIG. 5. Inhibition studies with MAC0029665. Initial rate data of different amounts of inhibitor and either AcCoA (A) or GlcN-1-P (B). The fixed substrate was maintained at 100 μ M. MAC0029665 was used at concentrations of 0 nM ($\bullet$), 20 nM ($\circ$), 40 nM ($\blacktriangledown$), 60 nM (∇), and 80 nM ($\blacksquare$). (A) The inhibitor shows mixed inhibition with regard to AcCoA with a K_{ic} of 22.8 ± 2.70 nM and K_{iu} of 100.9 ± 28.4 nM. (B) Uncompetitive inhibition with GlcN-1-P is observed with a K_{iu} of 28.2 ± 1.83 nM.

olites through the UDP-GlcNAc synthesis pathway is tightly controlled by the GlmS riboswitch (3) and that expression of the GlmU polypeptide has been shown to be essential to the viability of *E. coli* (15) highlight the importance of UDP-GlcNAc synthesis to cellular viability. Furthermore, the clinically used antibiotic phosphomycin targets the addition of enoyl-pyruvate to UDP-GlcNAc by MurA in peptidoglycan synthesis (27), and Williams et al. describe a peptide with antimicrobial properties that inhibits a R-3-hydroxyacylchain transfer to UDP-GlcNAc in the first committed step of lipid A biosynthesis (29). Similarly, the antibacterial tunicamycin inhibits the UDP-*N*-acetylglucosamine:poly(isoprenyl) phosphate GlcNAc-1-P transferase reaction involved in numerous pathways (6, 12, 22), including the first step in teichoic acid biosynthesis (11). As these antimicrobials target pathways utilizing UDP-GlcNAc, it stands to reason that targeting the biosynthesis of UDP-GlcNAc would be as profitable.

Although the acetyltransferase activity of GlmU is an ideal reaction to design specific chemical probes to affect the synthesis of bacterial cell envelope polymers due to an absence of a eukaryotic ortholog, specific inhibitors for this activity are not available. To date, the pursuit of small-molecule inhibitors for GlmU has focused only on the uridylyltransferase reaction (17), highlighting a need for high-throughput screening campaigns targeting the acetylation of GlcN-1-P. In order to specifically screen for inhibitors of the acetyltransferase activity of GlmU against a library of compounds, we sought to develop a robust assay amenable to an automated system. Here we report the development of a screening system incorporating a reducing environment and a non-thiol-modifying reporter system, two factors necessary for maintaining *E. coli* GlmU activity. The developed assay system uses a coupled assay that maintains the enzyme in a reducing environment while targeting the non-rate-limiting acetyltransferase activity.

Coupled reactions have shown utility where an enzyme activity is unsuitable for high-throughput assay development (1, 9). The second reaction of GlmU was an opportune coupling reaction, as one of the final products, inorganic pyrophosphate, is readily cleaved by inorganic pyrophosphatase for detection by malachite green. The fact that the uridylyltransferase reaction

was rate limiting, with a 3.7-fold-lower turnover of the uridyltransferase domain than that of the acetyltransferase domain (21 s^{-1} and 77 s^{-1} , respectively) (10) was problematic, as the coupled assay would be predisposed to select for inhibitors of the uridyltransferase activity. To circumvent this problem, an excess of the variant GlmU_{H363N} that lacks acetyltransferase activity was used as a coupling enzyme. The H363N variant is a catalytic change that prevents the activation of GlcN-1-P prior to acetyltransfer (20) but does not impact the uridyltransferase domain. The fact that we observed that 59% of the primary screen hits target the acetyltransferase reaction suggests that the developed assay was successful at selecting for the activity of interest, the non-rate-limiting reaction of a bifunctional enzyme. It is important to note that although the assay was developed to limit primary screen hits to only that of the acetyltransferase domain, it is difficult to completely exclude inhibitors of the coupling enzymes.

Both the primary and secondary screening methodologies were targeted at identification of small-molecule inhibitors that bound directly to the acetyltransferase domain of GlmU. The primary screening campaign was performed at the concentration of $100 \text{ }\mu\text{M}$ for all substrates. This concentration is subsaturating for AcCoA and GlcN-1-P and saturating for UTP (10). Holding AcCoA and GlcN-1-P at concentrations below saturating conditions made the screen especially sensitive to competitive inhibitors with respect to AcCoA and GlcN-1-P. The high concentrations of UTP were intended to select against competitive inhibitors of this substrate of the uridyltransferase reaction. We reasoned that competitive inhibitors that bind to the enzyme active site would have the most utility as specific perturbants of the left-hand beta helix fold of the acetyltransferase domain of GlmU. The secondary screening methodologies employed were selected to discern small-molecule inhibitors with an interaction specifically for the GlmU acetyltransferase domain. From mode-of-inhibition studies, it is clear that the lead molecules distinguished by this line of experimentation fit that criteria.

The mode-of-inhibition studies for the three molecules with the acetyltransferase activity of GlmU, MAC0021939, MAC008028, and MAC0029665, determined that these small molecules are inhibiting enzyme activity through specific interactions with the GlmU enzyme. Additionally, the modes of inhibition for these small molecules have aided our understanding of the mechanism of the acetyltransferase reaction. Uncompetitive inhibition was observed in the case of all three inhibitors with the substrate GlcN-1-P, while competitive inhibition (MAC0021939 and MAC008028) and mixed inhibition (MAC0029665) was observed with AcCoA. An inhibitor that is competitive with a substrate suggests a similar site of binding for both molecules and that the same form of the enzyme is bound (apo or in complex with another substrate) (25). The inhibition patterns observed suggest that MAC0021939 and MAC008028 occupy the same binding site as AcCoA does. Since the binding site is known for competitive inhibitors, these molecules are excellent starting points for cocrystallization and structure-based drug design. An uncompetitive inhibitor binds after the varied substrate, and the enzyme-substrate-inhibitor complex is strengthened with increased substrate concentration to produce a dead-end complex to prevent catalysis (25). A mixed inhibitor incorporates both competitive and uncom-

petitive properties with respective strengths dictated by the K_{ic} and K_{iu} constants. In ordered bisubstrate catalysis, an uncompetitive inhibitor of the first binding substrate is competitive with the second binding substrate (25). The facts that uncompetitive inhibition is observed with GlcN-1-P and competitive inhibition is observed with AcCoA for both MAC0021939 and MAC008028 suggest that these inhibitors bind in an ordered manner where GlcN-1-P binds first, followed by the tested inhibitor. These results suggest an ordered Bi Bi kinetic mechanism with GlcN-1-P binding preceding that of AcCoA for GlmU's acetyltransferase reaction. This model, however, is interesting in light of the electron density for the bound substrate AcCoA in the crystal structure of GlmU (20), which suggests that AcCoA can bind Apo enzyme. The presence of a sulfate in the active site, however, may be mimicking the phosphate of GlcN-1-P, allowing for AcCoA binding. These results highlight the use of these inhibitors as probes of mechanism for left-handed beta helix acyltransferases.

To date, only thiol-reactive molecules have been reported to affect the acetyltransferase activity of GlmU (7, 21). Burton et al. showed that application of these molecules, which include iodoacetamide and *N*-ethylmaleimide, to catheters showed a decrease in urinary tract infections postapplication and attributed their antimicrobial activity to the inhibition of GlmU (7). These molecules are electrophilic in nature and modify a large number of cysteine-containing proteins (8) and will not be useful for mechanistic studies of GlmU or for development of a broad-range antimicrobial therapeutic. In this communication, we developed an assay to isolate nonelectrophilic inhibitors specific to the acetyltransferase reaction of GlmU. The work highlights the use of catalytic variants to assay specific activities of bifunctional enzymes. Using our developed coupled assay in a high-throughput inhibitor screen, we discovered three potent inhibitors specific to the acetyltransferase activity of GlmU. Although these molecules were not found to inhibit the growth of *E. coli* MC1061 in culture, further screening campaigns with larger chemical collections using the described screening strategy may be successful in identifying molecules that specifically target the GlmU enzyme and have antibacterial activity. From the work presented here, the identified inhibitors of the acetyltransferase activity are good lead candidates for optimization of biological activity by structure-activity relationship studies and for cocrystallization with the GlmU enzyme for potential drug design.

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