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Identification of Active-Site Inhibitors of MurG Using a Generalizable, High-Throughput Glycosyltransferase Screen

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Peptidoglycan is a cross-linked carbohydrate polymer that forms layers around bacterial cell membranes.[1] One of its primary functions is to protect bacterial cells from lysis due to fluctuations in internal osmotic pressure. The machinery for peptidoglycan biosynthesis is highly conserved in both Gram-negative and Gram-positive bacteria, and each of the enzymes involved in the pathway is a potential target for antibiotic chemotherapy.[2] In recent years, inhibitors have been identified for all of the peptidoglycan-synthesizing enzymes except MurG,[3] the enzyme that catalyzes the final intracellular step in the biosynthetic pathway (Figure 1).[4] MurG is a member of a superfamily of nucleotide-sugar glycosyltransferases (NDP-Gtases), enzymes that catalyze the transfer of sugars from nucleotides such as UDP/TDP or GDP to a variety of acceptors.[5] Like kinases, nucleotide-glycosyltransferases are ubiquitous and play roles in a wide range of biological processes, some of which are pathogenic. Despite considerable effort, it has generally been difficult to design good inhibitors of Gtases.[6] Furthermore, the most potent inhibitors typically contain negatively charged diphosphates or mimics thereof, and thus have limited potential as leads.

High-throughput screening (HTS) has been used successfully to identify neutral inhibitors of protein kinases and phosphatases that compete with the negatively charged substrates of these enzymes.[8] Therefore, we thought it might be possible, by screening large numbers of compounds, to identify inhibitors containing structural elements that mimic the functions of the diphosphoryl group of the nucleotide-sugar donor used by MurG. These functions include positioning the uridine and hexose substituents and contributing favorably to binding. Here, we describe a fluorescence-based substrate displacement assay that was used to screen about 50,000 compounds against MurG.[9] The assay, which has allowed us to identify a promising family of MurG inhibitors, can be readily adapted to screen other glycosyltransferases for potential inhibitors.

Our goal was to identify compounds that compete with UDP-GlcNAc for binding to the active site of MurG in the hope that some of these compounds would contain structural elements that mimic the functions of the diphosphate. We decided that the best way to enrich screening hits for competitors of UDP-GlcNAc would be to use a high-throughput assay based on displacement of the hexose donor from the active site. The X-ray structure of a co-complex of MurG containing UDP-GlcNAc shows that the C2 *N*-acetyl group on the donor is solvent exposed and the protein makes no contacts to the methyl group (Figure 2a).[7] Therefore, we anticipated that donor analogues containing *N*-acyl modifications would bind to MurG. Accordingly, we synthesized the fluoresceinated UDP-GlcNAc analogue **1** (Figure 2b) and evaluated it for use in a displacement assay. The anisotropy of **1** increased significantly in the presence of MurG. From the change in anisotropy as a function of MurG concentration, we calculated a dissociation constant of $1.7 \pm 0.2\ \mu$M for **1**. Adding unlabeled UDP-GlcNAc, UDP, or UMP to a preequilibrated mixture of MurG and

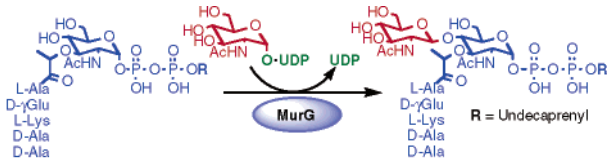

Figure 1. Reaction catalyzed by MurG.

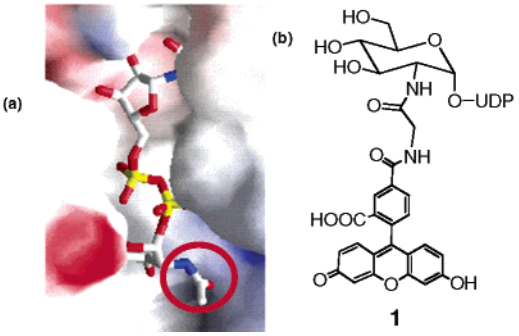

Figure 2. (a) Electrostatic surface representation of the MurG binding cleft showing the exposed C_2 *N*-acyl group of UDP-GlcNAc.[7] The uracil ring is buried. (b) Structure of the labeled UDP-GlcNAc probe.

1 caused a decrease in anisotropy, showing that all three of these molecules displace **1** from the active site, albeit at different concentrations. Dissociation constants for the three ligands were calculated from the concentration dependence of the anisotropy change.[10] The K_D of UDP-GlcNAc was found to be identical to that of **1**, implying that the fluorophore does not affect binding. The K_D of UDP ($2.3\ \mu$M) was found to be much better than that of UMP ($89\ \mu$M), consistent with the IC_{50} values measured previously.[11] These experiments suggested that it would be possible to distinguish weaker binders from better binders on the basis of changes in anisotropy at fixed concentrations of **1**, MurG, and the putative binder.

We developed a miniaturized fluorescence polarization assay and used it to screen 48,877 compounds in duplicate over 5 days at the Institute for Chemistry and Cell Biology, a collaborative screening facility located at Harvard Medical School. Compounds were screened in 384-well plates. Each plate contained two control wells, one with MurG and **1** alone and the other with MurG, **1**, and 25 μM UDP. Under the assay conditions, the well containing UDP gave a polarization reading that was approximately 50% that of the wells containing only MurG and **1**. Test compounds were added in 100 nL of DMSO to the sample wells to give a final concentration of 25 μg/mL in a final volume of 20 μL. Because our aim was to identify ligands that approached or exceeded UDP in binding to MurG, we scored as positive only those wells in which the fluorescence signal reproducibly dropped by more than 50%. There were 277 compounds that scored positive by this criterion, for a hit rate of 0.6%. The true hit rate is undoubtedly lower than this because a significant number of the hit compounds were fluorescent.

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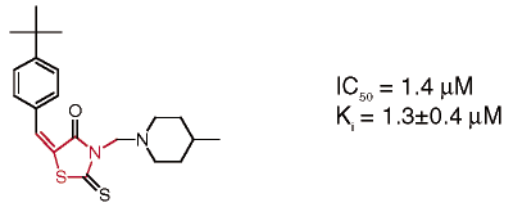

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Figure 3. Representative compound from the family of MurG inhibitors with the common core highlighted.

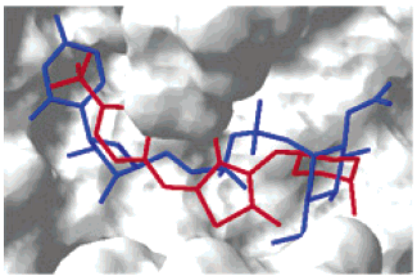

Figure 4. Overlay of **2** (red) with UDP-GlcNAc (blue) bound to the MurG crystal structure. Some residues have been cut away to expose the uracil ring.

We selected 44 compounds, representing several different structural classes, for secondary screening using a kinetic assay in which the production of radiolabeled product was measured.[12] Compounds were tested in duplicate at a concentration of ~5 μM. At this concentration and under the same assay conditions, UDP inhibited the enzymatic reaction by 50%. As before, we wanted compounds that were similar or better inhibitors than UDP, and so we selected only those that reproducibly inhibited the enzyme by more than 50% under the assay conditions. Eleven of the 44 compounds met this criterion. Seven of these 11 compounds have a five-membered, nitrogen-containing heterocyclic core with an alkyl or aryl substituent at N-1 and an arylidene substituent at the 3 position. A representative of the family, and the most potent of the seven related inhibitors, is shown in Figure 3. Compound **2** is a competitive inhibitor of MurG with respect to the UDP-GlcNAc substrate (Supporting Information).

The high percentage of inhibitors with a similar core is striking and suggests that these compounds share a common binding mode. Manual docking of the inhibitors into the UDP-GlcNAc binding pocket of MurG reveals that the compounds are best accommodated when the five-membered ring is located in the vicinity of the diphosphate binding site with the N-1 substituent oriented toward the GlcNAc binding site and the arylidene substituent oriented toward the uridine binding site (Figure 4).[13] Thus, by using a NDP-sugar displacement assay, we have identified a family of MurG inhibitors with a neutral core that may mimic the diphosphate moiety of UDP-GlcNAc with respect to the display of substituent groups. We are currently trying to obtain crystals of MurG with some of these inhibitors to evaluate the proposed binding mode and to provide information to guide the design of better inhibitors.

The displacement assay used here for high-throughput screening is simple to implement and can be readily adapted to screen any glycosyltransferase in which at least one modifiable group on the nucleotide-sugar is solvent exposed. Crystal structures of existing glycosyltransferases can serve as starting points for the design of suitable fluorescent substrate analogues, and structural similarities between glycosyltransferases imply that fluorescent analogues that work for one GTase will also work for related GTases.[5] It may be possible to identify families of scaffolds that mimic diphosphates in different conformations. Such a set of scaffolds would be invaluable for the diversity-oriented synthesis of libraries to be screened for glycosyltransferase inhibition.

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Supporting Information Available: Synthetic scheme and characterization of **1**; anisotropy curves; experimental details for secondary screening; assay conditions and inhibition pattern for compound **2** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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