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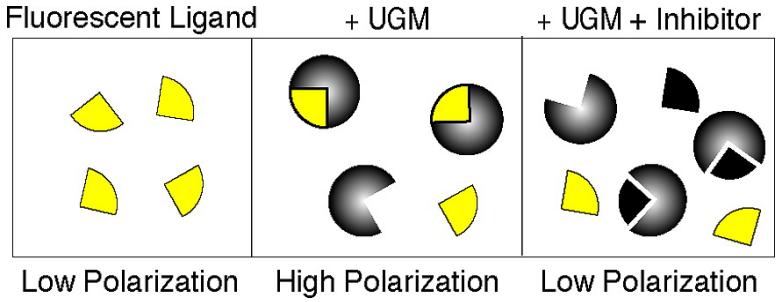

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Identification of Inhibitors for UDP-Galactopyranose Mutase

Michelle Soltero-Higgin, Erin E. Carlson, John H. Phillips, and Laura L. Kiessling*

Departments of Biochemistry and Chemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received April 6, 2004; E-mail: kiessling@chem.wisc.edu

Galactofuranose (Gal*f*) residues play vital roles in the growth of many pathogenic prokaryotes and lower eukaryotes. For example, they are essential components in the cell wall of the bacterium *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB).[1,2] TB kills approximately 2 million people annually. Gal*f* residues are also found in the lipophosphoglycan (LPG) of *Leishmania* spp., and these parasites cause the fatal visceral leishmaniasis.[3] The Gal*f* residues in the polysaccharides of Gram negative bacteria such as *Klebsiella pneumoniae* and *Escherichia coli* influence virulence.[4,5] Gal*f* residues, however, are not found in mammals. Thus, blocking their incorporation into polysaccharides essential for pathogen viability or virulence may lead to novel therapeutics.[6] A key enzyme involved in Gal*f* metabolism is uridine 5′-diphosphate (UDP)-galactopyranose (Gal*p*) mutase (herein referred to as UGM but also known as Glf). UGM facilitates the isomerization of UDP-Gal*p* and UDP-Gal*f* (Figure 1). Thus, UGM facilitates the production of the biosynthetic glycosyl donor UDP-Gal*f*, which is used by galactofuranosyl transferases. Here, we report the identification of UGM inhibitors using a high-throughput assay.

Few blueprints for generating UGM inhibitors exist. The structure of the flavoenzyme was determined using X-ray crystallography,[7] but the structure of a substrate−enzyme complex is not known. Inhibitors based on uridine have been identified; however, the lack of cell permeability of many of these compounds is problematic.[8] We wished to examine a more structurally diverse set of compounds with favorable cell permeability properties; therefore, we sought to screen a large diverse library to identify candidates for UGM inhibition. Accordingly, we turned our attention to the development of a high-throughput assay.

A medium throughput assay to monitor UGM activity was developed by Scherman et al.[8] This approach relies on the differences in the products of periodate cleavage of the pyranose and furanose forms. Thus, the production of radioactive formaldehyde generated from reaction of the furanose form is monitored. Because UDP-Gal*f* is generated in low yield (7%) from UDP-Gal*p*, this assay lacks the sensitivity needed to screen large libraries of compounds.

We sought to implement a high-throughput assay using fluorescence polarization (FP) to identify inhibitors of UGM.[9] Recently, FP has been applied to identify inhibitors of another enzyme, MurG, that acts on UDP-sugars,[9] and we have used FP previously to monitor carbohydrate binding to a lectin.[10] In FP, the emission of a fluorescent compound excited with plane-polarized light is measured. Differences in polarization will occur that depend on whether the fluorescent probe is bound to UGM (tumbling slowly) or released into solution by a competitive inhibitor (tumbling rapidly). FP offers high sensitivity with minimal sample manipulation; therefore, it is well-suited for high-throughput screening. Here, we present the assay and results from its implementation using the *K. pneumoniae* UGM.

To identify UGM inhibitors using FP, a fluorescent probe that binds the active site of UGM is required. In designing the necessary probe, we reasoned that the UDP moiety of the substrates would contribute the majority of the substrate-binding energy.[11−13] In contrast, the sugar-binding region of the active site must accommodate the structurally different saccharides (Gal*p* vs Gal*f*). Accordingly, we anticipated that the attachment of a fluorescent reporter through the β-phosphate of UDP would afford a probe with the requisite properties. A linker was included in the design to minimize steric clashes of the fluorophore with binding site residues. Compound **1** was generated by coupling uridine-5′-diphosphohexanolamine to fluorescein isothiocyanate.

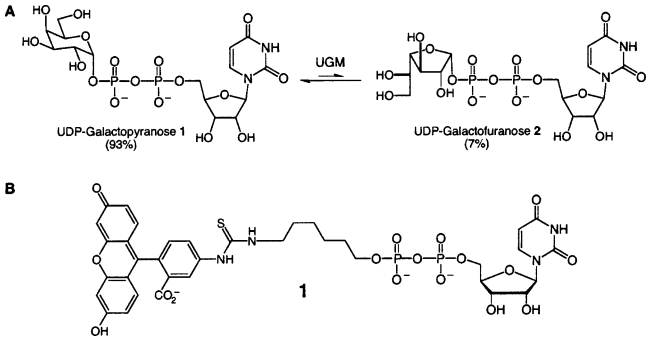

Figure 1. (A) The reaction catalyzed by UGM. (B) The structure of the fluorescent probe **1**.

Because it is not known how UGM interacts with its substrate, it was critical to determine whether compound **1** binds to the enzyme. When UGM was titrated into a solution of **1**, an increase and then saturation in fluorescence polarization was observed, confirming that compound **1** is a ligand for UGM.[14] To ascertain whether compound **1** binds specifically in the active site, we tested it in a competition assay with an inhibitor of the enzyme, UDP.[15,16] As expected, a decrease in fluorescence polarization was observed upon UDP addition. Next, we determined whether compound **1** could block the enzymatic activity of UGM. We monitored the production of UDP-Gal*p* from UDP-Gal*f* in the presence of UGM and compound **1** using an HPLC assay.[14] The IC_{50} value for inhibition of UGM activity is 1.3 ± 0.1 μM. We further characterized the interaction of **1** and UGM using isothermal titration calorimetry (ITC). Fluorescent **1** binds UGM with a K_d of 0.25 ± 0.06 μM, and it interacts with 1:1 stoichiometry. Compound **1** binds 40-fold more tightly to UGM than does UDP.[15] Notably, the fluorescein substituent is stabilizing. Indeed, compound **1** is one of the most potent ligands for UGM reported to date. These results indicate that UGM has an extended binding site that inhibitors can exploit.

Using compound **1**, we adapted our FP assay to a 384 well microtiter plate format. This configuration provided the means to screen a library of 16 000 diverse small molecules (less than 500 Da) designed to be cell permeable (Chembridge DIVERSet). We screened all the compounds at a final concentration of 330 μM against a solution of compound **1** and UGM. Each plate contained the UGM inhibitor UDP, as we sought to identify compounds that

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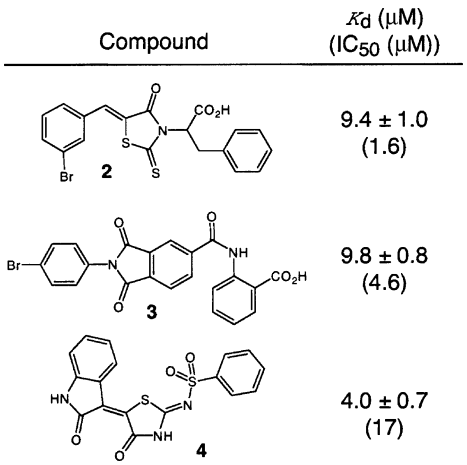

Figure 2. Compounds identified from the high-throughput FP screen that inhibit the catalytic activity of UGM. The IC_{50} values refer to the concentration at which 50% of the enzymatic activity of UGM was inhibited.

interact with comparable or superior affinity. In addition, samples of **1** with and without UGM were included on each plate to calibrate FP values. To eliminate false positives, the intrinsic fluorescence of each library member was determined and those compounds (<1%) whose signal exceeded the detection limit of the plate reader were eliminated. Approximately 0.4% of the compounds were identified as hits in this assay (FP values less than or equal to those obtained in the presence of UDP).

Compounds identified in the screen were tested in the FP assay at 10-fold lower concentration, and dissociation constants for the most active compounds were determined. Seven library members had K_d values below 10 μM, and these were tested for their ability to inhibit the catalytic activity of UGM. Inhibition constants were established by measuring UGM activity in the presence of a range of inhibitor concentrations using the HPLC assay. Examples are presented in Figure 2. The data indicate that the FP assay can be used to identify effective inhibitors of UGM.

Examination of the identified inhibitors reveals that they possess common features (Figure 2). Each is composed of a five-membered ring heterocycle with two carbonyl or carbonyl-related (e.g., thiocarbonyl or imino) hydrogen bonding acceptors. This arrangement of functionality may serve as a diphosphate mimic, as has been suggested.[17,18] Moreover, compounds **2** and **4** have substituents appended in a 1,3 arrangement to the five-membered ring heterocycle. Interestingly, heterocycles with substituents in this orientation have also been found to inhibit two enzymes involved in bacterial peptidoglycan synthesis: the glycosyltransferase MurG[9] and the NADPH-dependent enolypyruvyl reductase MurB.[18] Both of these enzymes act on UDP-containing substrates.

As mentioned above, compounds identified in other screens as inhibitors of enzymes that act on UDP-sugar substrates are similar to those we found. Specifically, a common feature is an aromatic substituent appended to the heterocyclic core.[9] In the case of UGM, we hypothesize that π-stacking interactions may occur between an aromatic group and the highly conserved active site tryptophan ($W156_{E.coli}$) side chain. This residue is believed to interact with uracil ring in the natural substrate in a similar fashion.[7]

The UGM inhibitors identified here, however, possess two aromatic substituents; many inhibitors of other UDP-sugar-utilizing enzymes contain a single aromatic group. To determine whether UGM inhibitor **2** is selective, we tested its ability to block an α-1,3-galactosyltransferase,[19] which uses UDP-Gal as a substrate. When the inhibitor was added at 30 μM, no inhibition was observed. Thus, the ligands identified here exploit unique features of the UGM binding site. We postulate that large polarizable flavin isoalloxazine ring system may provide a site for extensive hydrophobic and aromatic interactions.[20] The increase in binding affinity for compound **1** over UDP is consistent with such a model.

The high-throughput FP assay described here provides a general strategy to identify inhibitors of the many pathogens possessing Gal*f* residues. Preliminary experiments indicate that the fluorescent probe **1** binds tightly to the UGM from *M. tuberculosis*, and progress on identifying inhibitors of this enzyme is underway. Finally, given the site of fluorophore attachment, we note that **1** could be used to probe other enzymes that act on UDP- or TDP-sugars.

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Supporting Information Available: Detailed experimental procedures, including example binding curves and compound synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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