Chemistry & Biology, Vol. 11, 703–711, May, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.02.024

Identification of Selective Inhibitors for the Glycosyltransferase MurG via High-Throughput Screening

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Summary

Nucleotide-glycosyltransferases (NDP-Gtfs) play key roles in a wide range of biological processes. It is difficult to probe the roles of individual glycosyltransferases or their products because, with few exceptions, selective glycosyltransferase inhibitors do not exist. Here, we investigate a high-throughput approach to identify glycosyltransferase inhibitors based on a fluorescent donor displacement assay. We have applied the screen to *E. coli* MurG, an enzyme that is both a potential antibiotic target and a paradigm for a large family of glycosyltransferases. We show that the compounds identified in the donor-displacement screen of MurG are selective for MurG over other enzymes that use similar or identical substrates, including structurally related enzymes. The donor displacement assay described here should be adaptable to many other NDP-Gtfs and represents a new strategy to identify selective NDP-Gtf inhibitors.

Introduction

MurG is a nucleoside diphospho-glycosyltransferase (NDP-Gtf) involved in murein biosynthesis. It catalyzes the transfer of N-acetyl glucosamine (GlcNAc) from UDP-GlcNAc to Lipid I, an N-acetyl muramic acid (MurNAc) derivative that is anchored to the cytoplasmic surface of the bacterial cell membrane [1, 2] (Figure 1). The GlcNAc-MurNAc product of the MurG reaction is the minimal subunit of the peptidoglycan polymer that surrounds and protects bacterial cell membranes. Because peptidoglycan is required for the survival of bacterial cells, all of the enzymes involved in its biosynthesis are targets for new antibiotics [3]. We have recently become interested in strategies to identify inhibitors of MurG for two reasons [4–8]. First, biologically active MurG inhibitors would represent a novel class of antibiotics with which to combat resistant bacterial strains. Second, MurG is a prototypical member of a large but poorly understood class of enzymes, the NDP-Gtfs, which play key roles in eukaryotic and prokaryotic cell biology [9–14]. Although the utility of specific inhibitors of individual Gtfs as tools with which to probe glycosylation pathways is widely recognized, the best strategy to develop such inhibitors is unclear.

Most approaches to the design of NDP-Gtf inhibitors have typically fallen into two major categories [15–22]. One approach involves making compounds in which the nucleoside or nucleotide portion of the NDP-sugar donor is elaborated with other groups, either singly or in a combinatorial fashion [15, 18, 23, 24]. Since the NDP portion of the glycosyl donor typically plays a significant role in donor binding, it is anticipated that the nucleotide scaffold will favor Gtf binding, while the other elements will provide selectivity for the specific Gtf. Many bisubstrate analogs fall into this category of potential inhibitors, and some low micromolar (IC_{50}) inhibitors have been identified following this approach. The most potent compounds typically contain an intact NDP group because the nucleosides alone usually do not bind very well [18, 23, 24]. A drawback of this approach is that NDP groups do not have the appropriate characteristics for cell penetration, and it is unlikely that NDP-based Gtf inhibitors will be useful for blocking Gtfs inside cells.

The second major approach toward Gtf inhibitors uses iminocyclitols or related glycosidase inhibitors as scaffolds [17–19, 25]. Glycosidic bond hydrolysis involves the formation of an oxonium ion-like transition state [26], which is stabilized by carboxylate side chains in the enzyme active site. Iminocyclitols are believed to mimic this transition state. Since the transition state for glycosyl transfer is also believed to involve a species with oxonium ion-like character [10], iminocyclitols have also been investigated as Gtf inhibitors. Unfortunately, iminocyclitols and other glycosidase inhibitors have proven to be poor inhibitors of Gtfs [18], perhaps because the oxonium-ion-like species in Gtf reactions forms an ion pair with the nucleotide leaving group instead of with a carboxylate group. Consistent with this hypothesis, it has been found that iminocyclitols that are poor NDP-Gtf inhibitors on their own show synergistic inhibition with nucleotides [27]. In any event, glycosidase inhibitors have not proven to be good starting points for Gtf inhibitor design.

An alternative approach to identifying Gtf inhibitors is to screen a purified enzyme against large combinatorial libraries to generate leads that can be optimized. Although Gtfs have been screened against small biased libraries, high-throughput screening of Gtfs is still in its infancy [28, 29]. Recently, we reported a strategy for high-throughput screening of *E. coli* MurG that involves displacement of a fluorescently labeled UDP-GlcNAc derivative from the glycosyl donor binding site [30]. We have suggested that this screening strategy can be readily adapted to screen many other Gtfs. Herein, we describe the rationale underlying this strategy, and we address a key issue: whether a screen based upon displacement of an ubiquitous cellular substrate such as UDP-GlcNAc can possibly yield selective inhibitors. Our initial results suggest that it will be possible to discover selective Gtf inhibitors using this strategy. The implications of our findings are discussed.

Results

The challenge in any high-throughput screen is to develop a simple, reliable assay that can be adapted to

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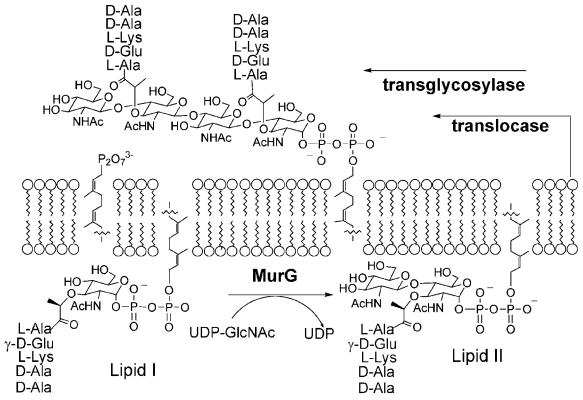


Figure 1. MurG Plays an Essential Role in Peptidoglycan Biosynthesis

MurG, a membrane-associated glycosyltransferase, converts Lipid I to Lipid II, which is translocated across the membrane and then polymerized by the transglycosylases.

screen large numbers of compounds. We envisioned a screen that would be straightforward, technically simple, and inexpensive. An assay based on displacement of a fluorescently labeled glycosyl donor fulfilled these criteria. *E. coli* MurG is an ideal model system to use in developing a donor displacement assay because there is an X-ray structure of the enzyme containing an intact glycosyl donor molecule [8]. From this crystal structure, we can predict how the glycosyl donor can be modified without disrupting enzyme binding. Our structure shows that most of the glycosyl donor is buried in the active site cleft with numerous contacts to the enzyme (Figure 2A). However, the methyl group of the N-acetyl moiety at C2 protrudes from the active site and does not contact the enzyme. The structure suggested that it would be possible to modify the N-acyl substituent with a fluorophore without affecting the interactions of the rest of the glycosyl donor with the enzyme. Based on our structural analysis, we prepared the fluorescently labeled N-acetyl glucosamino derivative **F1** (Figure 2B) and evaluated its ability to function as a glycosyl donor for MurG. Although it reacts more slowly than natural UDP-GlcNAc, **F1** was found to be a substrate for MurG, showing that the N-acyl group is a suitable position to modify.

We next evaluated the ability of the fluorescent glycosyl donor to bind to MurG by titrating a solution of **F1** with the enzyme while monitoring fluorescence polarization. The polarization increases as MurG is added, indicating that **F1** binds. The data fit well to an equation for 1:1 binding, and we calculated a dissociation constant of 1.4 ± 0.2 μM for the interaction. Addition of UDP-GlcNAc to the MurG:**F1** solution causes the polarization to drop, consistent with competitive displacement of the fluorescent donor from the active site [30]. We then tested the assay on a series of compounds that are known to inhibit MurG. The binding constant for each inhibitor was determined from the concentrations required to displace the fluorescent glycosyl donor; the results correlate with the IC_{50}s determined previously using a kinetic assay [5] (Table 1). These initial experiments validated the displacement assay and suggested that it would be possible to discriminate between strong and weak binders in a high-throughput assay by monitoring changes in polarization at a single concentration of compound.

Screening

We established a set of conditions (2.6 μM MurG, 0.33 μM fluorescent ligand concentrations) at which 25 μM UDP caused a 50% drop in fluorescence polarization. UDP was used to standardize conditions because it binds to MurG with a dissociation constant of 2.6 ± 0.2 μM, and we wanted to select for compounds with comparable or better affinity. The conditions were adapted to a 20 μl scale, and the screening was carried out in 384-well microplates. We then screened approximately 64,000 molecules in duplicate at a concentration of 25 μg/ml of each inhibitor (molecular weights of the compounds in the library typically fell into the range of 400–600 g/mol). The molecules screened came from a variety of different compound libraries, including commercial and diversity-oriented synthesis (DOS) libraries (see Experimental Procedures), to ensure that a wide range of structural diversity was represented in the sam-

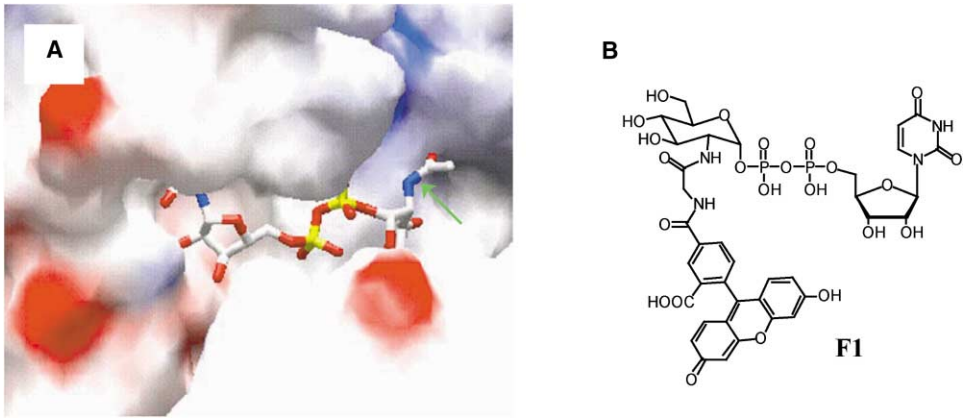


Figure 2. Design of the Fluorescent Ligand Used for High-Throughput Screening

(A) Crystal structure of the binding pocket of the MurG:UDP-GlcNAc complex.

(B) The probe used in high-throughput screening is shown. For details on the preparation of **F1**, see Helm et al. [30].

Table 1. Dissociation Constants of Donor Sugar UDP-GlcNAc and Its Analogs

Compound	F1	UDP-GlcNAc	UDP-GalNAc	UDP	UMP	ADP
Dissociation constant (μM)[a]	1.8 $\pm$ 0.2	1.4 $\pm$ 0.2	73.1 $\pm$ 14.1	2.6 $\pm$ 0.2	99.6 $\pm$ 12.2	88.2 $\pm$ 26.9
Signal change at 25 μM	—	60%	10%	50%	<10%	<10%

[a] Dissociation constants were measured using the fluorescence polarization displacement assay.

ple set. Compounds that reproducibly caused a 50% or greater drop in polarization were scored as hits. Using this cutoff, we identified 456 hits (0.6% hit rate). A number of the compounds in the libraries fluoresce, thereby causing a change in the polarization unrelated to the displacement of **F1** from the binding site. Therefore, the actual hit rate is lower than 0.6%.

We selected 220 compounds from the 456 hits to evaluate for MurG inhibition in a kinetic assay that monitors the incorporation of radioactivity from radiolabeled UDP-GlcNAc into Lipid II. UDP was again used to standardize the secondary assay. Reaction conditions were chosen wherein a 5 μM concentration of UDP caused a 50% drop in product formation. Under these reaction conditions, both the UDP-GlcNAc and Lipid I concentrations are within 2-fold of their K_m values. Since we wanted to identify compounds with comparable or better potency than UDP, we evaluated each test compound in duplicate at a concentration of 5 μg/ml and scored as positives all those that inhibited MurG by at least 40%. Fifty-five out of 220 compounds (25%) met or exceeded this cutoff, and we measured the IC_{50} values for these compounds using the same radiometric assay. In general, the IC_{50} values of the compounds fell between 1 and 10 μM. For some compounds, the IC_{50} curves revealed that complete inhibition could not be achieved: at some critical concentration inhibition decreased significantly. We interpreted this behavior as indicating that some compounds aggregate at higher concentrations. The aggregation clearly interferes with the ability of these compounds to inhibit MurG activity. However, these same compounds were identified as hits in the ligand-displacement screen at concentrations above the critical concentration for aggregation. It has previously been observed that compound aggregation can lead to false positives in kinetic assays [31], but these results suggest that the phenomenon can also lead to false negatives. The displacement assay is apparently less sensitive to aggregation-induced artifacts.

An analysis of the screening results revealed that many of the compounds identified in the secondary screen contain a 1,3-disubstituted heterocyclic core. In fact, 31 out of the 55 compounds (56%) were found to contain one of the cores shown in Figure 3 [32]. We selected a subset of the hits (compounds **1**, **5**, and **7**) for further kinetic analysis and found that they were competitive inhibitors with respect to UDP-GlcNAc (Figure 4). Although the compounds contain a potentially reactive α,β-unsaturated carbonyl motif, these results show that inhibition does not involve covalent modification of the enzyme. The structural similarities between the inhibitors evidently reflect the fact that they bind to the same region of the enzyme. The limited structural diversity of the hits should simplify the analysis of structure-activity relationships.

It is worth noting that some of the inhibitors identified in our displacement screen bear intriguing structural similarities to inhibitors that are proposed to compete with the NDP-sugar substrates of a variety of other enzymes [33, 34]. In particular, similar heterocyclic cores are found in inhibitors of other NDP-sugar processing enzymes. We and others have proposed that certain scaffolds mimic the diphosphate linkages found in these NDP-sugar substrates with respect to orienting the attached substituents toward the nucleoside and hexose binding sites [30, 35]. We are attempting to obtain cocomplexes of MurG with some of the inhibitors bound so that we can evaluate the role of the heterocyclic cores in binding. If the heterocylic cores do prove to function as mimetics of the diphosphate linkage, then it may be possible to make focused Gtf inhibitor libraries by diversifying around a small number of cores.

Selectivity

An enormous number of enzymes in both eukaryotic and prokaryotic cells utilize UDP-GlcNAc or related nucleotide sugars as substrates. Our screen is based on displacement of an UDP-GlcNAc derivative, prompting concerns that the hits obtained will bind to many UDP-hexose processing enzymes. To assess the utility of the donor displacement assay, we needed to determine whether the inhibitors obtained were selective for MurG over other enzymes that use similar or identical substrates.

MurG is an inverting glycosyltransferase that catalyzes the formation of a β glycosidic linkage from an α-linked UDP-sugar donor [8]. Therefore, we chose to test the hits against two other inverting glycosyltransferases that also use UDP donors. One of these Gtfs, O-linked GlcNAc transferase (OGT), is a eukaryotic glycosyltransferase that uses UDP-GlcNAc as a glycosyl donor [36]. Sequence-based computational studies have suggested that OGT belongs to the same structural superfamily as MurG [37]. The other Gtf we tested, GtfB, is a prokaryotic Gtf involved in the biosynthesis of glycopeptide antibiotics [38]. Its natural donor is UDP-glucose, and an X-ray structure shows that its structure is remarkably similar to that of MurG [39]. In fact, the C-terminal domains of MurG and GtfB, which contain the glycosyl donor binding sites, have an rmsd of 0.6 Å over all C-terminal C-α atoms. In addition to these Gtfs, we also evaluated MurA, an enolpyruvoyl transferase that converts UDP-GlcNAc to the corresponding C3 enolpyruvate derivative, and PBP1b, a prokaryotic enzyme that forms the glycan chains of peptidoglycan [40]. The substrate for PBP1b is the undecaprenyl-diphosphoryl-disaccharide donor, Lipid II. Thus, MurA uses UDP-GlcNAc but is not a Gtf, whereas PBP1b is a Gtf that uses a diphospholipid rather than a diphosphonucleoside as a leaving group.

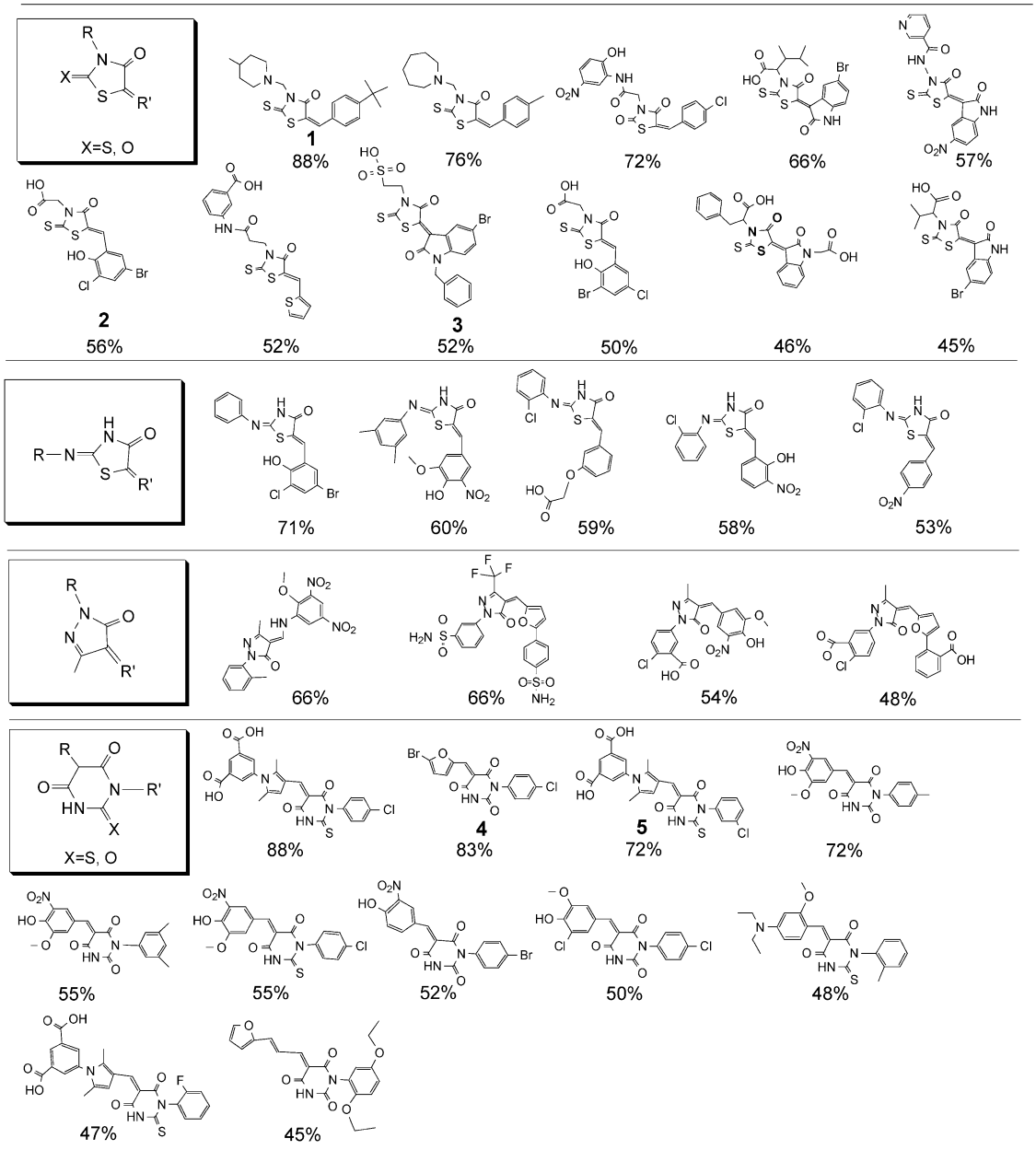


Figure 3. Selected MurG Inhibitors

Structures of selected compounds that caused >40% MurG inhibition at a concentration of 2.5 μg/ml (~5 μM). Four conserved core structures are indicated. The C-C double bond geometries have not been experimentally established. All compounds were from commercially available ChemDiv libraries. Assay conditions are provided in Experimental Procedures.

Each of the compounds shown in Table 2 was screened for its ability to inhibit the panel of enzymes. The compounds were tested in duplicate or in triplicate at three different concentrations, ranging from 5 μM to 100 μM. For solubility reasons, higher concentrations were not evaluated. Up to the highest concentrations tested, none of the compounds inhibited OGT, MurA, or PBP1b. Since the first two of these enzymes utilize UDP-GlcNAc substrates, these results show that it is possible to screen for displacement of UDP-GlcNAc from one enzyme without selecting for compounds that bind to all UDP-GlcNAc processing enzymes. An examination of the structures in the PDB database shows that the conformations of UDP-hexose substrates bound to enzymes can vary significantly (Figure 5A). The variations occur largely around the diphosphate linkage, which has shallow rotational barriers and the ability to adopt a large number of isoenergetic conformations. Thus, the nucleoside and hexose portions of the substrate can be presented in very different orientations depending on the enzyme, reflecting large differences in the substrate binding pockets. Inhibitors that are relatively rigid, as are most of our hit compounds, would not be expected to bind equivalently to enzymes with such differently

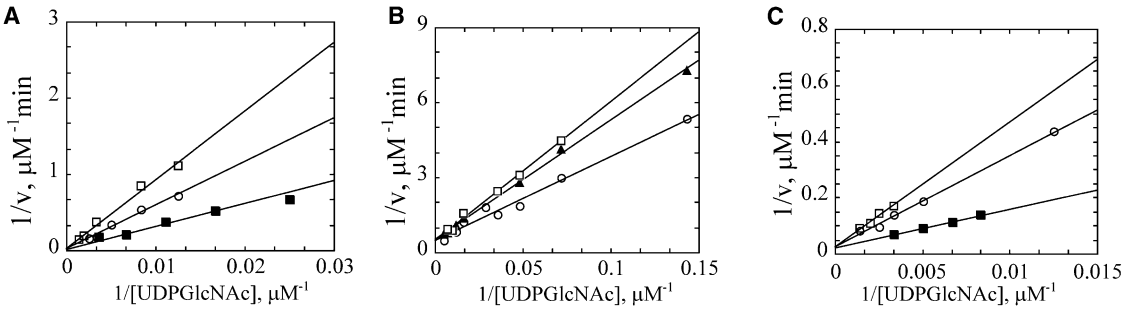


Figure 4. Kinetic Characterization of Selected MurG Inhibitors

Double reciprocal plots for the MurG reaction with UDP-GlcNAc as the variable substrate in the presence of inhibitors **1** (A), **5** (B), and **7** (C). Conditions for the colorimetric (A and C) and radiometric (B) assays are described in Experimental Procedures.

(A) 0 (■), 1 (○), and 2 μM (□) compound **1** at a fixed concentration of C_{20} Lipid I (60 μM) with the concentration of UDP-GlcNAc varied from 40–700 μM.

(B) 0 (○), 1 (▲), and 2 μM (□) compound **5** at a fixed concentration of C_{10} Lipid I (20 μM) with the concentration of UDP-GlcNAc varied from 7–210 μM.

(C) 0 (■), 3 (○), and 4.5 μM (□) compound **7** at a fixed concentration of C_{20} Lipid I (60 μM) with the concentration of UDP-GlcNAc varied from 40–700 μM.

shaped active sites even if they utilize the same substrate.

We did find that some of the MurG inhibitors also inhibit GtfB, a glycosyltransferase that is known to have a donor binding site very similar to that of MurG (Figure 5B). Given the similarities in the glycosyl donor binding domains, it may not be surprising that some of the inhibitors inhibit GtfB. However, it is still possible to achieve remarkable selectivity. For example, compound **5**, a competitive inhibitor of MurG with respect to UDP-GlcNAc, is selective for MurG by a factor of at least 30. Although MurG and GtfB have very similar folds, their amino acid compositions are different, and there are many differences in the details of the donor binding sites. Based both on our experimental results and structural considerations, we think that glycosyl donor displacement screens show great promise for the identification of selective Gtf inhibitors. Furthermore, we think that identifying and optimizing the features that provide for discrimination will substantially improve the selectivity of our initial hits.

Discussion

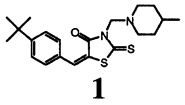
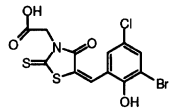
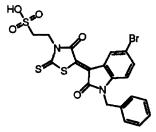
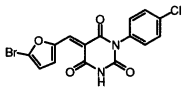
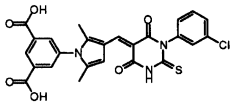
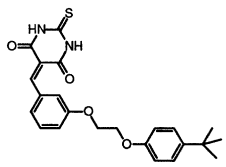
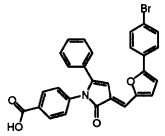
Glycosyltransferases play many roles in cells, most of which are poorly understood. For example, NDP-Gtfs in the endoplasmic reticulum and the Golgi apparatus are known to be involved in the assembly of the oligosaccharides that are presented on glycoproteins and glycolipids [41–43]. These glycoproteins and glycolipids mediate a variety of cell-cell recognition events, and it is known from mutational studies and knockout experiments that alterations in the patterns of glycosylation can be pathogenic. However, it has been difficult to probe the roles of individual glycosyltransferases or their products because, with few exceptions, selective glycosyltransferase inhibitors do not exist. Because such compounds would be useful for a variety of purposes, there is great interest in strategies to make Gtf inhibitors.

We began to explore strategies for the high-throughput screening of Gtfs in order to identify good starting points for the design of selective Gtf inhibitors. We initially considered using a kinetic assay to screen MurG for compounds that inhibit enzymatic activity. Although we have devised both radiometric and colorimetric assays to monitor MurG activity [5, 7], we decided against using a kinetic assay screening for several reasons. One reason is that we obtain the Lipid I substrate analogs used in the kinetic assays by chemical synthesis [4, 5], and we did not relish the idea of using large amounts of this compound in a random screen. In addition, kinetic assays also require precise timing and can be technically demanding, particularly if they involve the use of secondary enzymes to detect product, as does our colorimetric assay [7]. Assays involving secondary enzymes are also subject to false positives related to inhibition of the read-out enzymes. Finally, many of the compounds found in libraries are relatively nonpolar and form micellar aggregates at low concentrations, and kinetic assays are highly sensitive to inhibition by these aggregates [31].

We felt that a donor displacement assay offered several potential advantages over a kinetic assay, including technical simplicity and reduced sensitivity to artifacts related to compound aggregation. In addition, we hoped that a majority of hits would be competitive inhibitors with respect to UDP-GlcNAc and would thus fall into a limited number of structural classes. In contrast, a kinetic screen of a two-substrate enzyme such as MurG could yield inhibitors that operate by several mechanisms. False positives related to compound aggregation may further increase the numbers of different types of inhibitors identified in a kinetic screen, making it difficult to analyze the hits to design better compounds. As we have reported above, the hits identified in the donor displacement assay fall into a small number of categories, and this should simplify structural analysis.

Although we perceived several advantages of a donor-based displacement assay for screening, the single most important reason for exploring the strategy is that it can potentially be extended to other NDP-Gtfs. A handful of crystal structures of Gtfs are now available,

Table 2. IC_{50} of Inhibitors against Selected Enzymes

Compounds	IC_{50} (μM) MurG	OGT	MurA	PBP1b	GtfB
 1	1.4	>100[a]	>100[a]	>100[a]	20
 2	3.4	>100	>100	>100	4
 3	3.5	>100	>100	>100	15
 4	6.4	>100	>100	>100	11
 5	1.4	>100	>100	>100	50
 6	4.0	>100	>100	>100	100
 7	5.4	>100	>100	>100	50

[a] No inhibition was observed, but this compound aggregates at high concentrations.

and sequence analysis reveals that thousands of other Gtfs are structurally related to these enzymes [14]. Therefore, it is possible to predict, using a combination of structural data and sequence information, where a glycosyl donor might be labeled with a fluorescent tag to enable a displacement assay. Whether it is worth screening other Gtfs using such a strategy, however, depends on whether results of the MurG screen appear promising. The donor substrate used by MurG, UDP-GlcNAc, is a substrate for many enzymes in both prokaryotic and eukaryotic cells. Furthermore, a majority of the 7000 known and putative Gtfs use UDP or TDP sugar donors. Therefore, it was essential to assess whether compounds identified via high-throughput screening of MurG could inhibit other enzymes that use similar or identical substrates. We examined four other enzymes, two of which are Gtfs that are either known (GtfB) or proposed (OGT) to be structurally related to MurG. Like MurG, these enzymes are inverting Gtfs that form β-glycosides from α-linked UDP-GlcNAc (OGT) or UDP-glucose (GtfB) donors. The results show that the compounds identified using the donor displacement assay are indeed selective for MurG over these other NDP-Gtfs as well as other enzymes that utilize UDP-GlcNAc as a substrate (MurA). These findings bode well for the utility of donor-based displacement assays for high-throughput screening of Gtfs.

We are attempting to obtain cocomplexes of MurG with various inhibitors bound in order to understand the mode of binding in detail. Combined with the screening data on the hit compounds, this information should make it possible to prepare focused libraries of com-

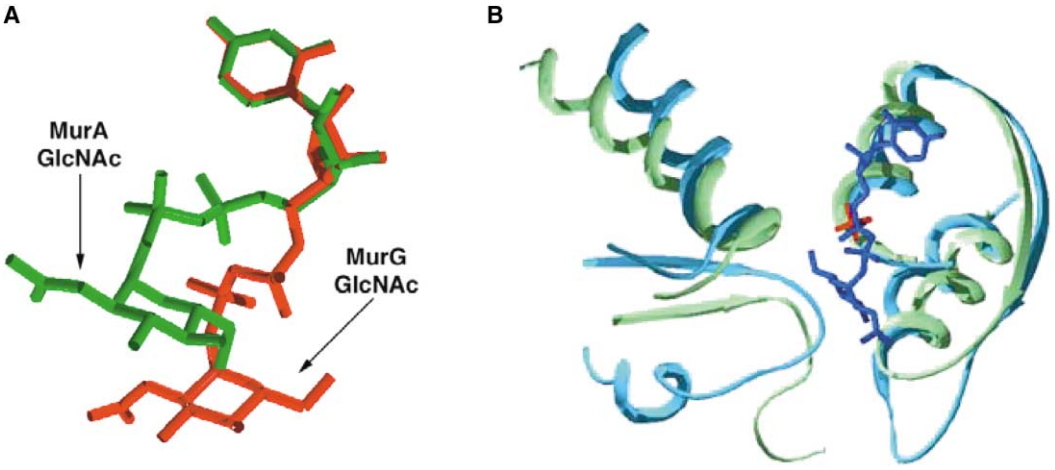


Figure 5. Substrate and Active-Site Geometries for Different UDP-GlcNAc Processing Enzymes

(A) Overlay of UDP-GlcNAc from the MurG:UPD-GlcNAc complex (red) and from the MurA:UDP-GlcNAc complex (green) showing different conformations.

(B) Overlay of the donor binding site of MurG (light blue) with the corresponding binding site of GtfB (green). The sulfate ion (red) in GtfB is superimposed with the α-phosphate of UDP-GlcNAc (dark blue) in MurG.

pounds to identify better inhibitors. Additionally, we are exploring the utility of the donor-displacement assay described here to screen other UDP-sugar transferases.

Significance

Glycosyltransferases play key roles in all organisms. Understanding these roles requires the ability to manipulate individual glycosyltransferases. Selective glycosyltransferase inhibitors would be valuable tools for probing the functions of glycosyltransferases and their products. With few exceptions, however, selective glycosyltransferase inhibitors do not exist. Furthermore, it is not yet clear how one might proceed in developing such inhibitors. We are exploring the utility of a high-throughput screen based upon displacement of a fluorescent glycosyl donor to discover inhibitors for a prototypical glycosyltransferase, *E. coli* MurG. This donor displacement assay enables us to screen large numbers of compounds rapidly, and we have shown here that several compounds identified from a donor displacement screen of MurG are selective for MurG over closely related enzymes that use similar or identical substrates. The high-throughput screening strategy described here can be adapted to screen other Gtfs and represents a general strategy to identify selective small molecule inhibitors of Gtfs that can be used to probe glycosylation pathways.

Experimental Procedures

High-Throughput Screening

The high-throughput screens were carried in 384-well plates. A solution containing 50 mM HEPES (pH 8.0), 0.33 μM compound **1**, and 2.6 μM MurG was added to the wells. 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. About 64,000 compounds were screened in duplicate at the Institute of Chemistry and Cell Biology, a collaborative screening facility located at Harvard Medical School. The compounds screened were from a variety of different libraries, including Bionet, Maybridge, Peakdale, ChemDiv Combilab and International collections, the NCI Structural Diversity Set, NINDS Custom Collection, and the ICCB Discretes Collections 2, 3, and 4 (a series of compounds synthesized by ICCB chemists), the dihydropyrancarboxamide diversity oriented syntesis (DOS) set, and the ICCB DOS set 1 (a set of compounds from a variety of DOS libraries).

Kinetic Assay Used for Secondary Screening of MurG Inhibitors

Assays were carried out by incubating inhibitors at a final concentration of 2.5 μg/ml ($\sim$5 μM) with 14 μM UDP-^{14}C-GlcNAc and 15 μM C_{20} Lipid I analog [44] in 10 μl MurG reaction buffer (50 mM HEPES [pH 7.9], 5 mM $MgCl_2$). Reactions were started by adding 0.5 μl of 0.01 mg/ml MurG stock (in 20 mM Tris, [pH 7.9], 150 mM NaCl, and 50 mM EDTA) to the substrate solution and were quenched after 2 min by adding 10 μl 0.1% SDS and chilling on ice. Products and starting material were separated by cellulose chromatography (3MM Whatman chromatography paper) in isobutyric acid: 1N NH_4OH = 5:3 and quantitated by scintillation counting.

IC_{50} Measurements

IC_{50} measurements for MurG were carried out using the same assay conditions as above, except that inhibitor concentration was varied from 0.5 μM to 20 μM. Data were fit using Prism to the equation

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{(X - \log IC_{50}) * h}},$$

where X is the logarithm of the inhibitor concentration, Y is the reaction rate, and h is the Hill slope.

Inhibition Kinetics

Compounds **1** and **7** were subjected to more detailed kinetic analysis using a fluorescence-based assay described previously [7]. In this assay, the formation of UDP is coupled enzymatically to the oxidation of NADH. Briefly, reactions were carried out in 384-well microplates, and the decrease in NADH fluorescence was monitored at 465 nm using a Perkin-Elmer HTS 700 Plus Bio-Assay Plate Reader. Each reaction contained MurG reaction buffer (50 mM HEPES [pH 7.9], 5 mM $MgCl_2$), 0.5 mM phospho(enol)pyruvate (PEP),

0.2 U/μl lactic dehydrogenase, 3 U/μl pyruvate kinase (added as 10 U/μl stock solution in 100 mM K_2HPO_4 [pH 7.6]), 0.25 mM NADH, 60 μM C_{20} Lipid I analog, an appropriate amount of UDP-GlcNAc, ranging from 40–700 μM, and 1 μl enzyme 100-fold diluted from a 10 mg/ml stock (in 20 mM Tris [pH 7.9], 150 mM NaCl, and 50 mM EDTA). Reaction volumes were 30 μl. All of the components except for the MurG substrates and MurG were premixed in a reservoir and dispensed into each well. The substrates were then added and the reaction mixtures were incubated for 5 min until a stable background rate was achieved. MurG was then added and the fluorescence was monitored for 5–10 min. A time course for each reaction was obtained. The initial rates were determined by calculating the slopes (linear fitting) of the initial linear portion of the reaction time course curves using KaleidaGraph (Synergy Software). Inhibition studies were carried out in duplicate at two different concentrations of the inhibitors (**1**, **7**). The Ki was calculated from the replots of the double reciprocal curves using the following equation for competitive inhibition:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \bullet \left(1 + \frac{I}{K_{is}}\right) \bullet \frac{1}{S}.$$

Compound **5** interferes with the NADH fluorescence and cannot be studied using this assay. The inhibition pattern of this compound was determined using the radiometric kinetic assay decribed above.

Assay for MurA

MurA was purified followed the procedure descried by McCoy et al. [45]. Cells were grown at 37°C to OD_{600} (60 ml LB) and induced with IPTG for another 6 hr. Cells were harvested and resuspended in 1.5 ml of 50 mM Tris (pH 7.5), 2 mM dithiothreitol (DTT), and sonicated. Cell debris was removed from the samples by centrifugation at 39,000 rpm for 30 min, and the sample supernatant was desalted using a Pharmacia NAP-10 column equilibrated with 50 mM Tris (pH 7.5)/2 mM DTT. The purity of the protein is higher than 90%. The assay mixtures (final volume 50 μl) contained 50 mM Tris (pH 7.5), 2 mM DTT, 10 mM UDP-GlcNAc, 20 μg MurA, 2% DMSO, and 0, 5, 50, 100 μM inhibitors. The mixtures were preincubated for 15 min at 37°C, and the reaction was started by the addition of 2.5 μl 100 mM phospenolpyruvate (PEP). After 30 min of incubation, 800 μl of color reagent [45] was added to stop the reaction. After another 1 min, 1 μl citrate solution was added, the mixture was incubated for 30 min at room temperature, and the OD_{660} was measured using a sample without UDP-GlcNAc as reference.

Assay for Transglycosylase PBP1b

The protein was purified as previously described [46]. Assays were carried out as reported [46] by separately incubating varying amounts of ^{14}C-GlcNAc-labeled C_{35} Lipid II (specific activity, 273 cpm/pmol) and inhibitors (5, 50, and 100 μM) in eppendorf tubes containing 9 μl of buffer (50 mM HEPES at pH 7.5, 10 mM $CaCl_2$, 1000 U/ml penicillin G, 0.2 mM decyl PEG, and 11% DMSO) and 1 μl PBP1b (from a solution freshly prepared by diluting the 50% glycerol stock 20-fold into 5 mM Tris buffer [pH 8.0], containing 8 mM decyl PEG) for 10 min. Reactions were started by adding 1 μl of the above PBP1b mixture to the substrate solution and were typically stopped after 15 min by adding 10 μl ice-cold 10 mM Tris (pH 8.0) containing 0.2% Triton X-100. Reactions were left on ice until spotted on cellulose chromatography paper (3MM Whatman chromatography paper). Products and starting material were separated following the methods of Anderson et al. (isobutyric acid: 1N NH_4OH = 5:3) and quantitated by scintillation counting [47].

Assay for GtfB

GtfB was overexpressed and purified as previously described [38]. The assay for GtfB activity was adapted from a reported procedure [38]. Inhibitors, dissolved in DMSO, were added to 20 μl reactions containing 50 nM GtfB, 2 mM UDP-glucose, 75 mM Tricine (pH 9), 4 mM TCEP, and 600 μM vancomycin aglycone; the final concentrations of inhibitors are from 5 to 100 μM. For consistency, all reactions contained 5% DMSO. Reactions were incubated at 37°C for 1.5 hr, then quenched with 20 μl cold methanol and centrifuged at 13,000 rpm for 10 min. Reaction mixtures were resolved by analytical HPLC, using a gradient of 0%–45% acetonitrile in water/0.1% TFA over 15 min, at a flow rate of 1 ml per minute. Product formation was monitored by UV absorbance at 285 nM. Estimated IC_{50} values for GtfB were determined based on the inhibition observed at 0 μM, 5 μM, 15 μM, and 100 μM (see Supplemental Data).

Assay for OGT

The mitochondrial splice variant of OGT, minus the first 50 amino acids, was cloned into pET 32b and expressed from Novagen BL21 (DE3) cells essentially as described [48]. The cell pellet was lysed with B-PER (Pierce) and rLysozyme (Novagen), and the soluble fraction was purified using His-Bind Resin (Novagen) [48]. Reactions were performed in buffer containing 10 mM Tris (pH 7.4) with 100 μg /ml BSA. The peptide substrate (KKKYPGGSTPVSSANMM) was used at 1 mM concentration. ^{14}C UDP-GlcNAc (300 mCi/mmole specific activity) was added to a final concentration of 11 μM. Inhibitors tested were added in 0.5 μl of DMSO (at final concentrations of 15 and 100 μM), and the final reaction volume was 20 μl. The reactions were quenched after 50 min at room temperature with 10 μl of formic acid and spotted onto phosphocellulose (Whatman P81) paper disks. The disks were washed for 3 × 5 min with 1% phosphoric acid, 1 min with acetone, and dried before counting. Product formation was measured by liquid scintillation counting.

Supplemental Data

Supplemental data are available at http://www.chembiol.com/cgi/content/full/11/5/703/DC1.

Received: December 31, 2003
Revised: February 17, 2004
Accepted: February 24, 2004
Published: May 21, 2004

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