Drug Targeting *Mycobacterium tuberculosis* Cell Wall Synthesis: Development of a Microtiter Plate-Based Screen for UDP-Galactopyranose Mutase and Identification of an Inhibitor from a Uridine-Based Library

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A microtiter plate assay for UDP-galactopyranose mutase, an essential cell wall biosynthetic enzyme of *Mycobacterium tuberculosis*, was developed. The assay is based on the release of tritiated formaldehyde from UDP-galactofuranose but not UDP-galactopyranose by periodate and was used to identify a uridine-based enzyme inhibitor from a chemical library.

The enzyme UDP-galactopyranose mutase (Glf), which catalyzes the formation of UDP-galactofuranose (UDP-galf) from UDP-galactopyranose (UDP-galp), plays a key role in the biosynthesis of the galactofuran component of the cell wall of *Mycobacterium tuberculosis*, as shown in Fig. 1. Many attributes of Glf suggest it as a drug target. Glf has been shown to be essential for mycobacterial growth (5). No analogous enzymatic reaction takes place in humans. UDP-galactopyranose mutase from *Escherichia coli* has been crystallized, its structure has been determined (3, 6), and the structure of the protein from *M. tuberculosis* has been just been determined to 2.5 Å (Jim Naismith, personal communication). However, a convenient assay appropriate for inhibitor searches has been lacking.

Previously, Glf has been assayed via high-performance liquid chromatographic separation of UDP-galf and UDP-galp (1). In an earlier attempt to provide a simpler assay, UDP-galf was used as the substrate (in the reverse direction) and the UDP-galp product was converted with two helper enzymes to UDP-glucuronic acid with concomitant reduction of NAD to NADH. However, this assay is not suitable for screening for inhibitors of the enzyme because of the difficulty of obtaining large amounts of UDP-galf, concerns about looking for inhibitors when running the reaction in the reverse direction, and the drawback that helper enzymes are required. Thus, we designed a microtiter plate-based assay to screen for potential inhibitors of this enzyme.

The *M. tuberculosis* Glf enzyme was expressed in *E. coli* (8). Purification of Glf from the *E. coli* extract resulted in an en-

zyme preparation that was less reliable in activity; this is believed to be due to irreversible loss of the flavin adenine dinucleotide cofactor (4). The *E. coli* strain (BL21) used to express *M. tuberculosis* Glf is a *galE* mutant and also does not have the *E. coli* *glf* gene found in *E. coli* K-12 (2, 7). Consequently, *E. coli* proteins do not act on the UDP-Galp substrate. In the assay described below, the amount of enzyme was adjusted so that about 5% of the UDP-galp present was converted to UDP-galf. This was a compromise in that enough radioactivity was produced for reproducibility but equilibrium had not been reached at 7% UDP-Galf (4).

The theory behind the new assay for Glf is presented in Fig. 2. Thus, tritiated formaldehyde is released from the product (UDP-[6-³H]Galf) but not the substrate (UDP-[6-³H]Galp) after treatment with periodate. Therefore, to assay the uridine-based library (9), 23 μ l of a cocktail consisting of 25 mM HEPES (pH 7.2), 25 nmol of MgCl₂, 25 nmol of freshly prepared NADH, and 2.5 nmol (~0.08 μ Ci) of UDP-[6-³H]galactopyranose (American Radiolabeled Chemical, St. Louis, Mo.) was added to each well of a microtiter plate. Each of the ~1,300 potential inhibitors (final concentration, 20 μ M) was added in 2 μ l of dimethyl sulfoxide to a single well (2 μ l of dimethyl sulfoxide was added to control wells also). The reaction was started with the addition of 2 μ l (500 ng) of a crude *M. tuberculosis* Glf enzyme preparation (8) (diluted 12-fold to a concentration of 500 ng/2 μ l beforehand in a 25 mM solution of HEPES [pH 7.2] containing 10 mM NADH to reduce the flavin adenine dinucleotide coenzyme). The reaction mixtures were incubated at room temperature for 20 min; this was followed by the addition of 5 μ l of 250 mM sodium acetate (pH 4.4) containing 200 mM sodium *meta*-periodate (NaIO₄) to each well and a 15-min incubation in the dark at room temperature. The NaIO₄ was then neutralized by the addition of 3 μ l of ethylene glycol (undiluted) and 5 min of incubation at room temperature. After the addition of 100 μ l of water to each well, the periodate-treated mixture was transferred to a

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FIG. 1. (A) Conversion of UDP-galactopyranose to UDP-galactofuranose as catalyzed by UDP-galactopyranose mutase (Glif, Rv3809c). (B) Structure of *M. tuberculosis* arabinogalactan demonstrating the key structural role of galactofuranosyl residues.

FIG. 2. Assay for UDP-galactopyranose mutase. UDP-galf, but not UDP-galp, forms neutral tritiated formaldehyde when treated with sodium periodate.

300- μl column of MTO-Dowex 1×8 (200 to 400 mesh) in the acetate form (Supelco, Bellefonte, Pa.) in 96 wells (7 mm [inside diameter] by 3 cm [height]) with a filter (Empore; 3M, St. Paul, Minn.). The samples were processed through the Dowex column by using a vacuum, and the columns were washed with an additional 100 μl of water. One-third of each processed sample (60 μl) was transferred to a 96-well scintillation plate (Perkin-Elmer Life Sciences, Boston, Mass.); this was followed by the addition of 180 μl of Optiphase Supermix (Perkin-Elmer Life Sciences) scintillation fluid and counting on a Perkin-Elmer Life Sciences Microbeta Trilux scintillation system.

A single active component, 320KAW73 (Fig. 3), was detected in the library. The component was resynthesized and purified and shown to have a 50% inhibitory concentration of approximately 6 μM (Fig. 3). The starting materials of the synthesis were also assayed for activity; the aldehyde (compound C in Fig. 3) showed a small amount of activity at 100 μM but no significant activity at lower concentrations. Other, similar compounds in the library that did not show detectable activity are shown in Fig. 4.

Development of a microtiter-based assay for Glf required overcoming the major hurdle of a convenient method by which to detect the product UDP-galf in a fashion consistent with microtiter plate techniques. The recognition that C-6 and its attendant hydrogens are released from UDP-galf but not from

UDP-galp with periodate treatment, along with the commercial availability of UDP-[6- ^3H]galactopyranose, led to the solution of this problem (Fig. 2). A second problem, only partially overcome, is that the equilibrium value of the reaction lies strongly on the side of the substrate, such that only about 7% UDP-galf is present at equilibrium (4, 8). Thus, ideally, the assay should be done in the linear range of UDP-galf production, perhaps up to about 1% conversion, but this is not practical because of the amounts of radioactivity needed and the fact that a certain background production of neutral radioactivity (corresponding to $\sim 1\%$ conversion to UDP-Galf) occurs in the absence of enzyme. Hence, the compromise value of 4 to 6% conversion results in an assay less sensitive to inhibitors and more sensitive to random noise than ideal. We routinely include UDP at 0.1 to 1 mM as a positive control, as under the assay conditions used, UDP inhibits Glf by about 50% at 200 μM . In continuing to screen additional libraries, we did obtain false-positive assay results but these were readily identified by retesting.

Beyond the development of an assay for UDP-galactopyranose mutase, a uridine analog inhibitor of the enzyme has been uncovered. Its structure is rather similar to some in the library that were inactive (Fig. 4). Of special note is the fact that the phenylsulfonyl diphosphate analog was needed as the two other spacers designed to mimic diphosphate were inactive even with the same *ortho*-hydroxyl di-*meta* nitro substituents

FIG. 3. Activity of 320KAW73 (A) and its precursors (B and C). The data shown are averages of values.

FIG. 4. Comparison of 320KAW73 with related compounds in the screening library that were inactive.

(Fig. 4). Unfortunately, this inhibitor was not active against whole *M. tuberculosis*, presumably because of entry problems. However, the inhibitor may be very valuable for forming co-crystals with the enzyme. Further screening with a commercial library of drug-like compounds (obtained from Nanosyn, Menlo Park, Calif.) is in progress.

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