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Development of an Activity Assay for Discovery of Inhibitors of Lipopolysaccharide Transport

Luisa S. Gronenberg and Daniel Kahne*

Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 01238, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received December 8, 2009; E-mail: kahne@chemistry.harvard.edu

Antibiotic-resistant Gram-negative infections are a serious health threat. Unlike Gram-positive bacteria, Gram-negative organisms have an outer membrane (OM) that restricts access to many antibiotics that might otherwise be useful. This membrane contains an outer leaflet composed of lipopolysaccharide (LPS), which creates a hydrophilic barrier that prevents easy passage of small hydrophobic molecules (Figure 1).[1] Because improper synthesis, transport, or assembly creates a defective (leaky) OM, it has been suggested that inhibitors of LPS biogenesis could be useful antibiotics, either alone or in combination with known antibiotics. Indeed, inhibitors of LpxC, the enzyme that catalyzes the first committed step in LPS biosynthesis, have shown promise as antibacterial agents.[2]

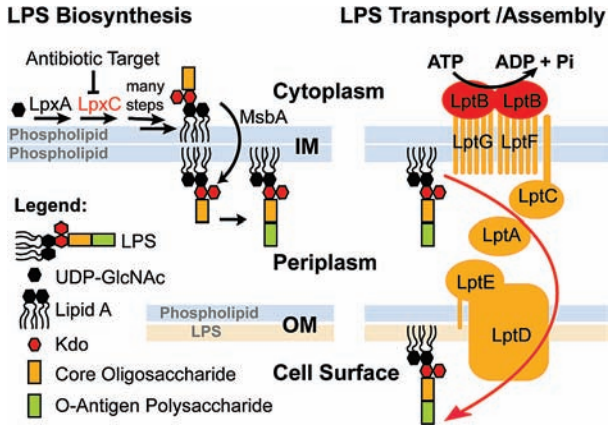

Figure 1. LPS biosynthesis, transport, and assembly.

LPS is partially synthesized in the cytoplasm and then flipped from the inner to the outer leaflet of the inner membrane (IM),[3a] where the biosynthesis is completed by the ligation of O-antigen to the core oligosaccharide of LPS (Figure 1, left).[3b,c] The LPS is then transported to the outer leaflet of the OM by seven recently discovered Lpt proteins, which are essential in most Gram-negative bacteria (Figure 1, right).[4a−e] Energy is required to initiate extraction from the IM of the LPS molecule, which contains five myristoyl (C_{14}) chains and one lauroyl (C_{12}) chain. The periplasmic space contains no ATP to drive membrane extraction; instead, an ATP-binding cassette (ABC) transporter containing a cytoplasmic ATPase, LptB, as well as two transmembrane components, LptF and G, is proposed to perform this function. Although ABC transporters have received considerable attention as targets for cancer chemotherapy, bacterial ABC transporters have been largely overlooked as possible antibacterial targets. The purified LptBFG transporter was recently shown to exhibit ATPase activity, but this membrane protein complex is not suitable for high-throughput screening.[4f] Here we have reconstituted the activity of the soluble ATPase component LptB and developed a high-throughput assay for activity. By screening a kinase inhibitor library, we identified several ATP-competitive inhibitors. Targeting ATPase components of essential ABC transporters in bacteria may be a good strategy for antibiotic discovery.

LptB was overexpressed as a C-terminal eight-histidine fusion protein in *Escherichia coli* and purified over Ni-NTA resin (yield ~20 mg/L of culture) (Figure S1 in the Supporting Information). To monitor activity, we adapted a continuous assay that couples ATP hydrolysis to NADH oxidation (Figure S2).[5] Reactions were monitored by measuring the decrease in fluorescence intensity of NADH (λ_{ex} = 340 nm, λ_{em} = 465 nm). ATPase activity was observed in the presence of Mg^{2+} or Mn^{2+}; no reaction was observed with Zn^{2+}, Ni^{2+}, or Ca^{2+} (Figure S3). Rates were 4- to 5-fold higher for ATP hydrolysis than for hydrolysis of GTP, CTP, or UTP (Figure 2A). To ensure that the ATPase activity was due to LptB and not to a copurifying contaminant, we expressed a mutant protein in which the predicted catalytic glutamate was changed to glutamine.[6] The mutant protein, LptB-E163Q, had no activity in the assay (Figure S4).

To analyze the kinetic parameters of LptB, we measured the reaction rate over a range of ATP concentrations using optimized buffer conditions. Nonlinear regression (GraphPad Prism) showed that the data fit an allosteric sigmoidal model better than a Michaelis−Menten model. The value of k_{cat} was found to be 6.2 ±

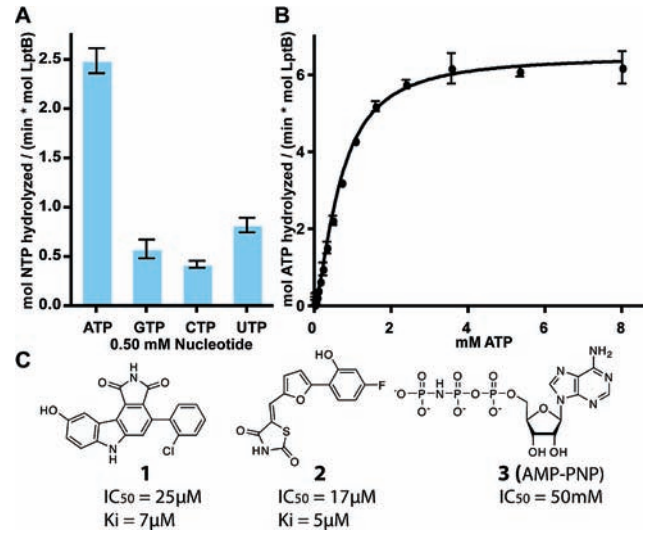

Figure 2. Development of kinetic assay for LptB and its use in discovering inhibitors. (A) Analysis of nucleotide selectivity for LptB. (B) Rate of ATP hydrolysis with respect to ATP concentration. (C) Two validated LptB inhibitors, **1** and **2**, discovered in this screen (Figure S4). For comparison, IC_{50} values are shown for AMP-PNP (**3**). The K_i calculated for **3** on the basis of the IC_{50} value is 25 mM (Figures S6 and S7).[8]

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0.1 min^{-1}, which is comparable to those for several other ABC transporter ATPases.[6b] The apparent K_m (K') was found to be 0.56 $\pm$ 0.04 mM, with a Hill coefficient of 1.5 $\pm$ 0.1. These data indicate that ATP binding to LptB is cooperative (Figure 2B) and suggest that LptB functions as a dimer, consistent with the reported LptBFG complex stoichiometry[4f] and the known mechanisms of all other characterized ABC transporter ATPases.[6]

To screen for small-molecule inhibitors, we adapted the assay to a 384-well plate format. Reactions were initiated by the addition of ATP, and the fluorescence was read after 60 min. Inactive LptB-E163Q was used as a control for full inhibition (positive control). DMSO-treated LptB was used as a control for no inhibition (negative control). To eliminate false positives due to fluorescent library compounds, the plates were read after library transfer but before the reaction was initiated by addition of ATP. The signal window of the assay, determined by calculating the Z' factor for a plate containing only positive and negative controls, was 0.83 (Figure S5).[7]

We screened 244 compounds from two commercially available kinase inhibitor libraries, composed mostly of ATP-competitive inhibitors. Compounds belonging to two different structural classes were selected for further analysis (**1** and **2**; Figure 2C). Both compounds were found to be competitive with respect to ATP, as expected on the basis of the composition of the libraries. The inhibitor binding constants (K_i) were found to be in the micromolar range. The compounds had no activity against a wild-type strain of *E. coli*; however, they had minimum inhibitory concentrations consistent with their K_i values against a strain of *E. coli* with a leaky OM (Table S1). It is likely that the lack of activity against the wild-type strain is related to poor OM penetration. Whether the observed antibiotic activity is due to inhibition of LptB is not yet known; there are other essential ATPases in *E. coli* that could be in vivo targets of these compounds.

A key motivation for identifying small-molecule inhibitors of ABC transporter ATPases is that better tools are needed for biochemical studies, as nonhydrolyzable ATP analogues are poor inhibitors.[6c] In fact, a commercially available, nonhydrolyzable ATP analogue, AMP-PNP (**3**) (Figure 1C), was 3 orders of magnitude less potent than inhibitors **1** and **2** reported here. We are currently working to identify inhibitors with improved potency that are based on the scaffolds identified. Crystals of LptB containing bound inhibitors would also facilitate structure-based design for improved specificity.

In eukaryotic systems, inhibition of ABC transporter-dependent drug efflux pumps (e.g., P-glycoprotein) has been validated as an approach for cancer chemotherapy.[9] In bacteria, drug efflux pumps have been considered as targets, but the clinically relevant pumps are not ABC transporters.[10a] However, there are numerous ABC transporters that play important roles in cell envelope biogenesis.[10b,c] Inhibiting these transporters has enormous potential as a new antibiotic strategy.[11] We have identified the first compounds that inhibit the ATPase component of a bacterial ABC transporter, one that is essential to viability. The approach of screening for inhibitors of important bacterial ATPases in ABC transporters could leverage a vast body of work for small-molecule leads,[12] some of which have already been tested in humans.[13]

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Supporting Information Available: LptB expression, purification, and kinetic characterization; detailed assay conditions and screening procedures; inhibitor characterization; and complete ref 13a. This material is available free of charge via the Internet at http://pubs.acs.org.

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