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

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Abstract

The outer membrane of Gram-negative bacteria contains an outer leaflet composed of lipopolysaccharide (LPS) that is transported to this location by a pathway that is essential for viability. It has been suggested that inhibitors of this pathway could be useful antibiotics. Herein we reconstitute the activity of the ATPase component (LptB) of the ABC transporter that initiates LPS transport and assembly. We developed a high throughput assay and screened a library of kinase inhibitors against LptB. We identified two classes of ATP-competitive inhibitors. These are the first inhibitors of the ATPase component of any bacterial ABC transporter. The small molecule inhibitors will be very useful tools for further biochemical studies of the proteins involved in lipopolysaccharide transport and assembly.

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

LPS is partially synthesized in the cytoplasm and is then flipped from the inner to the outer leaflet of the inner membrane (IM),^{3a} where 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 outer membrane 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 (C₁₄) and one lauroyl (C₁₂) chains. 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

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Supporting Information Available: LptB expression, purification and kinetic characterization, detailed assay condition and screening procedures, inhibitor characterization. The complete citation for Ref. ^{13a} is also included. This material is available free of charge via the Internet at <http://pubs.acs.org>

activity, but this membrane protein complex is not suitable for high throughput screening.^{4f} Here we reconstitute the activity of the soluble ATPase component, LptB, and develop 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 8-histidine fusion protein in *Escherichia coli* and purified over Ni-NTA resin (yield ~20 mg/L of culture) (Figure S1). 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 ($\lambda_{\text{ex}} = 340 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$). 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 four- to five-fold higher for ATP hydrolysis compared with hydrolysis of GTP, CTP, or UTP (Figure 2A). To ensure that ATPase activity was due to LptB and not to a co-purifying 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. Non-linear regression (GraphPad Prism) showed that the data fit an allosteric sigmoidal model better than a Michaelis-Menten model. k_{cat} was found to be $6.2 \pm 0.1 \text{ min}^{-1}$, which is comparable to several other ABC transporter ATPases.^{6b} The apparent K_{m} (K') was found to be $0.56 \pm 0.04 \text{ mM}$, with a Hill coefficient of 1.5 ± 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 with 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 fluorescence was read after 60 minutes. 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 of mostly ATP-competitive inhibitors. Compounds belonging to two different structural classes were selected for further analysis (Figure 2C, 1 and 2). Both compounds were found to be competitive with respect to ATP, as expected based on the composition of the libraries. The inhibitor binding constants (K_i) are in the μM range. The compounds have no activity against a wild-type strain of *E. coli*; however, they have MICs consistent with their K_i values against a strain of *E. coli* with a leaky OM (Table S1). Likely, the lack of activity in 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*, which 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; non-hydrolyzable ATP analogs are poor inhibitors.^{6c} In fact, a commercially available, non-hydrolyzable ATP analog, AMP-PNP (Figure 1C, 3), was three orders of magnitude less potent than the inhibitors (**1**, **2**) reported here. We are currently working to identify inhibitors with improved potency 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 that have already been tested in man.¹³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LPS Biosynthesis

LPS Transport /Assembly

Figure 1.
LPS biosynthesis, transport and assembly.

Figure 2. Development of kinetic assay for LptB and its use to discover 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 IC₅₀ values are shown for **3**, AMP-PNP. The calculated K_i for **3**, based on the IC₅₀, is 25mM.⁸ (Figures S6,7)