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The antibiotic novobiocin binds and activates the ATPase that powers lipopolysaccharide transport

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Abstract

Novobiocin is an orally active antibiotic that inhibits DNA gyrase by binding the ATP-binding site in the ATPase subunit. Although effective against Gram-positive pathogens, novobiocin has limited activity against Gram-negative organisms due to the presence of the lipopolysaccharide-containing outer membrane, which acts as a permeability barrier. Using a novobiocin-sensitive *Escherichia coli* strain with a leaky outer membrane, we identified a mutant with increased resistance to novobiocin. Unexpectedly, the mutation that increases novobiocin resistance was not found to alter gyrase, but the ATPase that powers lipopolysaccharide (LPS) transport. Co-crystal structures, biochemical, and genetic evidence show novobiocin directly binds this ATPase. Novobiocin does not bind the ATP binding site but rather the interface between the ATPase subunits and the transmembrane subunits of the LPS transporter. This interaction increases the activity of the LPS transporter, which in turn alters the permeability of the outer membrane. We propose that novobiocin will be a useful tool for understanding how ATP hydrolysis is coupled to LPS transport.

Graphical abstract

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Supporting Information: Supplemental figures and tables, experimental procedures, compound analysis, protein purification and crystallization protocols. This material is available free of charge on the ACS Publications website.

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Gram-negative bacteria are naturally resistant to many antibiotics due to the presence of the outer membrane, a unique asymmetric bilayer with phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet.¹ LPS is a large glycolipid containing multiple fatty acyl chains that comprise the hydrophobic region of the outer leaflet.² Phosphate groups attached to the core sugars bind divalent metal cations to form a well-ordered polyelectrolyte barrier (Figure S1).^{1a} Many toxic molecules are unable to penetrate this barrier, and many of those that somehow cross the barrier are immediately exported by multi-drug efflux pumps.³ In order to develop strategies to breach the outer membrane and enable entry of a wider range of antibiotics, we have been working to elucidate how the cell assembles the outer membrane. We frequently generate mutants in which the machines that build the outer membrane have been altered and then probe the barrier function of their outer membranes with antibiotics.⁴ Here, we describe how this approach led to the discovery that the DNA gyrase inhibitor novobiocin binds to and increases the activity of the transport machine responsible for assembling LPS in the outer membrane in *Escherichia coli*. Novobiocin binds the conserved ATPase that powers LPS transport, and structures we report here may guide the design of other small molecules that bind to ATP-binding cassette (ABC) transporters to alter their function.

The synthesis of LPS is completed at the inner membrane and from there LPS must be transported to the cell surface.⁵ This transport requires a machine because it is highly unfavorable to extract a molecule containing as many as six long hydrocarbon chains from a membrane. *E. coli* contains seven essential LPS transport (Lpt) proteins, LptABCDEFG, which are proposed to form a trans-envelope complex (Figure 1A).⁷ LptB₂FG comprise an ABC system (Figure 1A) with a homodimer of cytoplasmic LptB ATPases complexed to a transmembrane LptFG heterodimer.^{6c,6d,6f,8} ATP hydrolysis by LptB is required for LPS extraction from the inner membrane and transport through the periplasm.^{8c,9} LptC receives LPS from an inner membrane component of the ABC system and passes it to LptA for transit across the periplasm to the outer membrane translocon, LptDE.¹⁰

To better understand how LptB₂FG functions, we made *E. coli* strains that have defects in the LPS assembly machinery that make cells permeable to antibiotics. One such strain carries the *lptB1* allele, which produces an LptB variant with a C-terminal defect (see SI for description) that causes sensitivity to a panel of antibiotics that do not kill wild-type *E. coli* (Figure 1B). We raised mutants in the *lptB1* background that could restore the impermeability to these antibiotics to wild-type levels. We obtained two classes of suppressor alleles encoding amino acid changes in *lptB1*. One class, exemplified by *lptB1*(G33C), increased resistance to all antibiotics tested to wild-type levels, presumably by correcting the defect in *lptB1* function (Figure 1B bottom left, Table S1). The other class of

suppressors, exemplified by *lptB1(R144H)*, dramatically increased resistance to novobiocin (Figure 1B bottom right, Table S1), a known DNA gyrase inhibitor,¹¹ while moderately increasing resistance to erythromycin¹² and not increasing resistance to rifampicin, bacitracin or another gyrase inhibitor,¹³ nalidixic acid (Figure 1B bottom right, Table S1). Therefore, we speculated that novobiocin might interact directly with LptB1(R144H) and affect its function, leading to a restoration of the impermeability of the outer membrane. Indeed, we found that, in a dose-dependent manner, novobiocin increases the resistance of *lptB1(R144H)* cells to other antibiotics (Figure S2). Furthermore, wild-type and *lptB1(R144H)* strains exhibit similar sensitivity to novobiocin (Table 1), suggesting that high levels of novobiocin still inhibit gyrase in the mutant. Together, these data indicate that sub-inhibitory concentrations of novobiocin increase the impermeability of the outer membrane of the *lptB1(R144H)* mutant likely by improving LPS transport.

To determine if and how novobiocin binds to LptB, we soaked novobiocin into LptB-ADP crystals and obtained a 2.0-Å structure of a co-complex (Figure 2A). ADP is still present in the co-complex, and novobiocin binds adjacent to the groove in LptB that accommodates the coupling helices from LptFG^{8a,8c,14} (Figure 2B, Figure S3). Coupling helices are a conserved motif in ABC systems that connect the transmembrane domains (here, LptFG) to the nucleotide-binding domains (here, LptB).¹⁴ Notably, novobiocin contacts LptB residue F90 (Figure 2C), which interacts with LptFG.¹⁴ This residue is invariant in LptB, and non-conservative substitutions at this position are lethal because they disrupt proper formation of the LptB₂FG complex.^{8c} Novobiocin thus binds a critical position at the LptB-LptFG interface.

We wondered whether novobiocin binds wild-type LptB in cells, as it clearly shows dose-dependent suppressor effects in *lptB1(R144H)* cells and co-crystallizes with LptB. We did not expect to observe activation of LptB by novobiocin in cells with wild-type-LptB₂FG machines because they already maintain an impermeable outer membrane. Instead, based on the site where novobiocin binds in LptB, we decided to use a different mutant that produces wild-type LptB but has a defect in the coupling helices of LptFG that interact with LptB. The coupling helices in LptFG each contain a strictly-conserved glutamate, and changing both glutamates to alanine (*lptFG(ch)*) causes a lethal loss of function (Figure 3A, LB panel).¹⁴

We previously found that the *lptFG(ch)* mutant can be rescued by replacing the charged arginine side chain at position 91 in LptB to an uncharged serine (Figure 3A, compare *lptFG(ch)* and *lptFG(ch)-lptB(R91S)*).¹⁴ Because novobiocin contacts R91 (Figure 2C), we speculated that it might rescue growth of *lptFG(ch)* by masking the charge. Indeed, we found that novobiocin enables the *lptFG(ch)* mutant to grow (Figure 3A). We conclude that novobiocin suppresses Lpt defects *in vivo* by interacting with wild-type LptB at R91; therefore, novobiocin interacts with LptB in cells in a similar manner to that observed in the crystal structure.

We wondered whether it would be possible to separate novobiocin's ability to suppress the *lptFG(ch)* defects *in vivo* from its activity against DNA gyrase. To test this, we synthesized a carboxamide derivative, novobiocin-adamantyl (Figure 3B), containing a bulky group that

we predicted would impair binding to DNA gyrase, but hoped would not affect binding to LptB. Compared to novobiocin, novobiocin-adamantyl showed reduced activity against DNA gyrase *in vitro* (Figure S4) and was ~100× less potent *in vivo* against the leaky *lptB1* strain and an efflux pump-deficient *tolC* strain (Table 1, Figure S5). Nevertheless, novobiocin-adamantyl suppressed the lethality of *lptFG(ch)* (Figure 3A, compare *lptFG(ch)* between panels, and Figure S6). In addition, the *lptB1(R144H)* strain, unlike its parent *lptB1* strain, was as resistant to novobiocin-adamantyl as the wild-type (Table 1). We therefore concluded that novobiocin-adamantyl, like novobiocin, interacts directly with LptB. In support of this hypothesis, we obtained a 1.95-Å complex of LptB-ADP-novobiocin-adamantyl that shows that novobiocin-adamantyl binds the same site as novobiocin (Figure 3C). These data imply that we can decouple the gyrase and LptB activities of novobiocin.

To estimate the affinity of novobiocin for LptB, we synthesized an otherwise identical analogue in which a phenolic hydroxyl was substituted with a ¹⁹F-label (fluorobiocin, Figure 3B). Solution NMR binding experiments indicate that fluorobiocin is a low micromolar binder (Figure S7). This measured affinity is consistent with the concentrations required for novobiocin to bind wild-type LptB and rescue *lptFG(ch)* *in vivo*.

To investigate how *lptB(R91S)* and novobiocin or novobiocin-adamantyl suppress impaired growth of the *lptFG(ch)* strain, we used a previously established assay to evaluate time-dependent LPS release from inner membrane vesicles to an LptA variant (LptA*) containing the UV-crosslinkable unnatural amino acid *para*-benzoyl-phenylalanine (*pBPA*).⁹ The assay detects via immunoblot LPS that becomes crosslinked to LptA* in the presence of UV light. Right-side out vesicles were made from *E. coli* cells overexpressing either wild-type or substituted LptB₂FGC complexes, soluble LptA* was added, and reactions were incubated with a vehicle control, novobiocin, or novobiocin-adamantyl before being UV irradiated at different time points (Figure 4A). Protein levels across vesicle preparations were assayed via immunoblot and found to be comparable (Figure S9). Vesicles containing wild-type complexes of LptB₂FGC showed a pronounced time-dependent increase in LPS-LptA* adducts (Figure 4B, lanes 1-4). By comparison, vesicles containing LptB₂FG(ch)C complexes released less LPS over time (Figure 4B, lanes 5-8). These experiments provide direct evidence that the amino acid changes in LptFG(ch), which were known to affect complex formation with LptB, impair LPS release. We found that wild-type levels of LPS release could be restored by preparing vesicles from cells expressing LptB(R91S) rather than LptB with LptFG(ch)C (lanes 9-12).

We also found that novobiocin and novobiocin-adamantyl stimulate LPS release from the LptB₂FG(ch)C vesicles in a concentration dependent manner (Figure 4C). Importantly, these compounds also increase LPS release from vesicles containing fully wild-type LptB₂FGC complexes (Figure 4D) at micromolar concentrations similar to those observed in the *in vitro* binding assay with fluorobiocin. Therefore, we conclude that novobiocin and novobiocin-adamantyl rescue *lptFG(ch)* *in vivo* by binding wild-type LptB and activating LPS release by the inner membrane Lpt components.

Here we report that the natural product antibiotic novobiocin binds LptB₂FG, the LPS ATP-dependent transporter, and increases its activity. We found this effect because certain *lpt*

mutants compromise LPS transport, creating a permeable outer membrane. This allows novobiocin to enter the cell and activate LPS transport, which restores the impermeability of the outer membrane and thereby increases their resistance to antibiotics including novobiocin. Moreover, we observe *in vitro* that novobiocin stimulates LPS release by wild-type LptBFGC from membrane vesicles. The mechanism by which novobiocin affects coupling between LptB and LptFG, and therefore LPS release, remains under investigation. We presume that binding facilitates one or more steps in the catalytic cycle involving ATP binding, hydrolysis, and release as this binding site is critical for coordinating nucleotide binding with LPS release.^{8a,8c,14}

We believe that novobiocin will be a useful tool for understanding how the transporter couples ATP hydrolysis to LPS release. This is the first structure of a non-substrate small molecule bound to the nucleotide-binding domain of any ABC transporter. It may be possible to use the synthetic tools and structural information described here to make derivatives of novobiocin that inhibit rather than activate the transporter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

An amino acid change in LptB1 selectively confers resistance to novobiocin. a) Schematic of the LPS transport system. LptB₂FG (blue) provides the energy for LPS transport from the inner membrane (IM) to the outer membrane (OM). b) Lawns of bacteria were grown in the presence of disks containing novobiocin (nov), bacitracin (bac), erythromycin (erm), rifampicin (rif), and nalidixic acid (NA). The diameter of the zone of total (clear) or partial (hazy) growth inhibition around the disks was measured as an indicator of antibiotic susceptibility (see Table S1 for quantification). The *lptB1* allele encodes a defective LptB ATPase that renders the strain generally susceptible to antibiotics when compared to the wild type. Intragenic suppressor mutations in *lptB1* can decrease sensitivity to all antibiotics [*lptB1(G33C)*] or to novobiocin [*lptB1(R144H)*]. Growth around erythromycin disk is partially restored in *lptB1(R144H)* cells. See Figure S2 for details.

Figure 2.

The co-complex of novobiocin bound to LptB. a) Surface representation of the structure of an LptB monomer bound to one molecule of novobiocin and one molecule of ADP. b) Schematic showing the orientation of the novobiocin-binding site relative to the groove in LptB that accommodates the coupling helices from LptFG. c) Novobiocin contacts LptB at residues F90 and R91, previously shown to interact with LptFG. The F_O-F_C omit map is contoured at 3σ .

Figure 3.

Novobiocin and a derivative bind to LptB *in vivo*. a) Comparison of fitness on solid media for *lptFG(wt)*, *lptB(R91S)*, coupling helix mutant *lptFG(ch)*, and *lptFG(ch)-lptB(R91S)*. Overnight cultures grown in minimal medium were serially diluted tenfold and spotted onto LB agar plates with and without compounds. Novobiocin and novobiocin-adamantyl rescue growth defects of the coupling helix mutant *lptFG(ch)* similarly to the genetic suppressor *lptFG(ch)-lptB(R91S)*. b) Scheme showing the synthesis of fluorobiocin (fbn) and novobiocin-adamantyl (adn). Detailed experimental procedures and characterization (^1H , ^{13}C , and HRMS) provided in supplementary material. c) Co-crystallization of LptB with novobiocin-adamantyl shows that it binds LptB in the novobiocin-binding site (LptB-ADP-ADN). The F_O-F_C omit map is contoured at 3σ .

Figure 4.

Novobiocin and novobiocin-adamantyl restore the ability of complexes containing LptFG(ch) to extract LPS from membrane vesicles *in vitro*. a) Schematic showing the assay to monitor LPS release from right-side-out vesicles to LptA* (LptA(I36pBPA)). Vesicles containing overexpressed wild-type or mutant LptB₂FGC complexes were incubated with LptA* and ATP at 30°C to initiate LPS extraction and transport from the vesicles to LptA*. Samples were taken at different time points and UV-irradiated to crosslink LPS to LptA*, and accumulation of LPS-LptA* adducts was detected using LPS immunoblots. b) LPS was released from vesicles containing wild-type complexes of LptB₂FGC in a time-dependent manner. Complexes containing LptFG(ch) and wild-type LptB released less LPS than fully wild-type complexes; this defect of LptFG(ch) in LPS release was suppressed by LptB(R91S) in place of wild-type LptB. c) The defect in LPS release seen with complexes containing LptFG(ch) and wild-type LptB was also suppressed by novobiocin or novobiocin-adamantyl. Addition of either agent restored LPS release from vesicles to LptA* in a dose-dependent manner. d) Novobiocin and novobiocin-adamantyl increase LPS release from vesicles containing over-expressed wild-type LptB₂FGC, but not LptB(E163Q)₂FGC, a catalytically dead variant.

Table 1

Novobiocin-adamantyl is less potent than novobiocin against wild type, *lptB1*, *lptB1(R144H)*, and efflux pump-deficient *tolC* strains. Minimum inhibitory concentrations measured after a 15-hr incubation period at 37 °C.

	Minimum inhibitory concentration (μM)	
	Novobiocin	Novobiocin-adamantyl
wild type	62.5-125	>500
<i>lptB1</i>	<0.5	125
<i>lptB1(R144H)</i>	62.5-125	500
<i>tolC</i>	2	125-250