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Novobiocin enhances polymyxin activity by stimulating lipopolysaccharide transport

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Abstract

Gram-negative bacteria are challenging to kill with antibiotics due to their impenetrable outer membrane containing lipopolysaccharide (LPS). The polymyxins, including colistin, are the drugs of last resort for treating Gram-negative infections. These drugs bind LPS and disrupt the outer membrane; however, their toxicity limits their usefulness. Polymyxin has been shown to synergize with many antibiotics including novobiocin, which inhibits DNA gyrase, by facilitating transport of these antibiotics across the outer membrane. Recently, we have shown that novobiocin not only inhibits DNA gyrase, but also binds and stimulates LptB, the ATPase that powers LPS transport. Here, we report the synthesis of novobiocin derivatives that separate these two activities. One analog retains LptB-stimulatory activity but is unable to inhibit DNA gyrase. This analog, which is not toxic on its own, nevertheless enhances the lethality of polymyxin by binding LptB and stimulating LPS transport. Therefore, LPS transport agonism contributes substantially to novobiocin-polymyxin synergy. We also report other novobiocin analogs that inhibit DNA gyrase better than or equal to novobiocin, but bind better to LptB and therefore have even greater LptB stimulatory activity. These compounds are more potent than novobiocin when used in combination with polymyxin. Novobiocin analogs optimized for both gyrase inhibition and LPS transport agonism may allow the use of lower doses of polymyxin, increasing its efficacy and safety.

Graphical abstract

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Author Contributions

These authors contributed equally to the work.

The increase in antibiotic-resistant bacterial infections poses a major public health threat.¹ Gram-negative bacteria are particularly difficult to treat because they have an outer membrane that prevents the entry of many antibiotics that kill Gram-positive organisms.² Due to the spread of antibiotic resistance and a lack of antibiotics effective against Gram-negative organisms,³ colistin and polymyxin B, members of the polymyxin class of antimicrobial peptides, have seen increased clinical use as drugs of last resort.⁴ Although they are efficacious against nearly all Gram-negative bacteria, they can cause irreversible kidney damage at therapeutic doses.⁵ Therefore, strategies to lower the dosage of polymyxin required to clear resistant Gram-negative infections are being actively sought.

Polymyxins disrupt the outer membrane by binding lipopolysaccharide (LPS),⁶ which is largely responsible for barrier function (Figure 1A, 1B). Consequently, polymyxin has been investigated in combination therapy to increase the penetrance of antibiotics that normally cannot cross the outer membrane.^{4b, 7} Novobiocin (Figure 1C), which inhibits DNA gyrase but generally exhibits poor activity against Gram-negative organisms,⁸ is one antibiotic shown to synergize with polymyxin (Figure S1, Table S1).⁹ It has been proposed that polymyxin-mediated disruption of the outer membrane barrier allows more novobiocin to penetrate into the cell and inhibit DNA gyrase.¹⁰ However, the basis of the bactericidal mechanism behind this combination is poorly understood.^{9b} We recently reported that novobiocin binds LptB,¹¹ the ATPase that powers the seven-protein LPS transport (Lpt) machine that delivers LPS to the cell surface (Figure 1A).¹²

We wondered whether the ability of novobiocin to stimulate LPS transport plays a role in its synergy with polymyxin. As novobiocin itself does have gyrase activity, synergy between **1** and polymyxin may reflect that activity as well as LPS agonism. We needed to separate the two activities of novobiocin to address whether LPS transport agonism is crucial for synergy with polymyxin.

We prepared a novobiocin analog, **2** (Figure 1C) lacking the L-noviose sugar of **1**, which is known to make important contacts with GyrB.¹⁵ An *in vitro* gyrase supercoiling assay revealed that the activity of **2** against *Acinetobacter baumannii* gyrase was at least 260-fold lower than that of **1** (Figure 2A, S2). We chose *A. baumannii* as our model strain because it is considered the highest priority Gram-negative pathogen to counteract according to the

World Health Organization.¹⁶ Consistent with a loss of gyrase activity, **2** does not have significant antibiotic activity even against Gram-positive organisms that are susceptible to novobiocin (Table S2).

After decoupling gyrase activity from **2**, we wondered whether **2** could bind the LPS transporter. We developed a ¹⁹F NMR binding assay to measure the affinity of **1** and **2** to the purified five-protein complex, LptB₂FGC,^{12a, 12b, 17} that extracts LPS from the inner membrane (Figure 1A). We prepared fluorinated derivatives of **1** and **2** (Figure S3) and we monitored changes in the ¹⁹F linewidths of these derivatives in response to increasing concentrations of the LptB₂FGC (Figure 2B). Both compounds bind LptB₂FGC with single-digit micromolar K_D values. Therefore, **2** lacks gyrase activity but maintains affinity for its second target in the inner membrane complex.

We have shown using a photocrosslinking assay^{12a} that novobiocin **1** stimulates LPS transport^{11a} by accelerating extraction of LPS from the inner membrane and transfer to LptA,¹⁸ the protein that forms the bridge to the outer membrane translocon.^{12c, 19} We used this same assay (Figure 2C) to test if **2**, like **1**, stimulates LPS transport. Compared to the DMSO control, **2** showed increased photocrosslinking to LptA containing a UV-crosslinkable amino acid (Figure 2D). Taken together, these results show that this novobiocin derivative lacks gyrase activity but maintains LPS transport activity.

We next sought to test whether **2** would show synergy with polymyxin B even though it does not inhibit DNA gyrase and has no antibiotic activity. We carried out a checkerboard assay using *A. baumannii* as a test strain. At concentrations of **2** that clearly stimulate LPS release (Figure 2D), the minimal inhibitory concentration of polymyxin B is reduced by at least five-fold despite the compound having no antibiotic activity (Figure 2E). This effect is independent of gyrase inhibition because compound **2** showed comparable synergy against an *A. baumannii* strain containing a mutation in gyrase that makes it insensitive to novobiocin (Figure S1). As **2** has no activity on its own even against permeabilized strains²⁰ (Figure S4), these results suggest that **2** potentiates the lethal effects of polymyxin B by acting as an agonist of LPS transport. That is, **2** potentiates the antimicrobial effects of polymyxin rather than vice-versa.

If the LPS transport stimulation activity of novobiocin plays a role in the synergy observed with polymyxin B, we reasoned that novobiocin analogs with improved LPS transport stimulation activity should also improve the synergy with polymyxin B. We screened other aminocoumarins for stimulation of LPS release and discovered that the natural product clorobiocin was exceptionally active (Figure S5). We wondered whether the 5-methylpyrrolylcarbonyl group²¹ is the key moiety in clorobiocin responsible for increased stimulation.

Obtaining the pyrrole-modified noviose **3** required selectively performing an acylation reaction on one of four hydroxyl groups, a challenge others have faced with this scaffold.²¹⁻²² We found that Yamamoto's Sc(OTf)₃-catalyzed acylation method²³ converted descarbamyl novobiocin to **3** with high regioselectivity for the equatorial L-noviose alcohol and no detectable acylation of the phenolic hydroxyls (Figure 1C, ESI). **3** showed both

improved activity against gyrase and increased LPS transport stimulation activity (Figure 3A, C). Consistent with the latter finding, the fluorinated NMR probe **3F** bound the LPS transporter with higher affinity than **1F** (Figure 3B). We found that **3** is superior to **1** in its ability to reduce the lethal concentration of polymyxin B (Figure 3D). Notably, **3** showed synergy with polymyxin B against gyrase-resistant as well as gyrase-sensitive strains, whereas **1** only showed synergy against the gyrase-sensitive strain at the concentrations tested. This finding suggests that the LPS stimulatory activity plays an important role in the synergy.

To further probe whether LPS transport stimulation activity plays a critical role in the observed synergy, we examined the crystal structure of LptB in complex with novobiocin to identify positions likely to affect LptB₂FGC binding. Replacing the benzamide in **1** with a cyclopropylamide (**4**) (Figure 1C, S6) was predicted to perturb the interaction with LptB residue R91,^{11a, 17} which is known to play a role in transducing information from the ATPase to the transmembrane proteins LptF and LptG.^{11b, 17, 24} Although the activities of **4** and **1** against gyrase were similar, the fluorinated probe **4F** did not bind LptB₂FGC (Figure 3B), and **4** did not stimulate LPS release (Figure 3C). Therefore, the LPS stimulatory activity of novobiocin can be separated from gyrase inhibitory activity by changing structural features of the benzamide. Compound **4** did not synergize with polymyxin B against either the sensitive or resistant strains, but interpreting this result was complicated because, unlike **1** and **3**, the compound showed no activity against *A. baumannii* on its own.

We synthesized additional compounds, focusing on improving the LPS stimulatory activity without changing gyrase activity. This effort led to compound **5**, which contains an aryl substituent on the noviose carbamate and a rigidified benzamide substituent. Compound **5** showed no change in gyrase activity compared with **1** (Figure 4A), but a 10- to 100-fold increase in LPS release (Figure 4B). Like **2** and **4**, compound **5** also had no antibiotic activity against *A. baumannii*, but unlike **4**, it showed synergy with polymyxin B against both gyrase-sensitive and gyrase-resistant strains (Figure 4, ESI). These results provide additional evidence that LPS transport stimulation contributes to the synergy of novobiocin and some of its derivatives with polymyxin B.

We have demonstrated that it is possible to improve the LPS release agonism of novobiocin by making structural changes to the noviose sugar and replacing the benzamide with a suitable group. We have also shown that novobiocin derivatives that maintain gyrase activity but are better LPS release agonists show greatly improved synergy with polymyxin. For example, at 0.016 $\mu\text{g/mL}$ ($\sim 0.1\times$ MIC), **3** reduces the amount of polymyxin B needed to kill *A. baumannii* by 30-fold (Figure 3D). Why increasing LPS transport enhances the antibacterial activity of polymyxin remains unclear, but studies to address this question are underway. We now have a rational framework for the design of novobiocin analogs with both improved gyrase inhibition and LPS transport stimulation activity. Co-administration of polymyxin with novobiocin analogs optimized for both activities could represent an effective strategy to reduce polymyxin toxicity in the treatment of recalcitrant Gram-negative infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Proposed mechanisms for polymyxin and novobiocin

A) The polymyxins bind lipopolysaccharide (LPS), a molecule that is translocated from the inner membrane to the outer membrane by the seven-membered Lpt complex. Novobiocin binds gyrase and inhibits DNA supercoiling. It also stimulates LPS transport by binding LptB. B) Structures of polymyxin B and colistin. C) Structure of novobiocin and derivatives used in this study. Conditions: a) 5 M NaOH/H₂O, 80 °C, 45 min. b) 1 M NaOH/H₂O, 50 °C, 45 min. c) 5-methyl-1*H*-pyrrole-2-carboxylic anhydride (1.5 eq.), 10 mol % Sc(OTf)₃, MeCN, 23 °C, 3 h. d) NRRL-B-3652 heavy cell suspension¹³, water, N₂ atmosphere, 16 h. e) cyclopropanecarbonyl chloride (0.8 eq.), pyridine, −40 °C, 16 h. f) 3-(naphthalen-2-yl)benzoyl chloride (0.8 eq.), pyridine, −40 °C, 16 h. g) PhNCO (1.3 eq.), 2 mol % MoO₂Cl₂, DCM, 23 °C, 12 h.¹⁴ See supporting information for full experimental details.

Figure 2. Polymyxin B-mediated killing by novobiocin does not depend on its gyrase activity

A) **2** displays no inhibitory activity against gyrase. Compounds were incubated with purified *A. baumannii* gyrase, buffer, and relaxed pBR322 plasmid for 30 minutes at 37 °C. The supercoiling reaction was then quenched and the DNA topoisomers were separated by gel electrophoresis and stained. At higher than 80 μ g/mL, **1** and **2** precipitate in the gyrase assay buffer. B) **1F** and **2F** both display similar binding affinities to LptB₂FGC. Error bars represent the mean $\pm$ S.E.M (n = 3). C) Schematic of LPS transport assay with right-side-out (RSO) vesicles overexpressing LptB₂FGC and purified LptA* = LptA(I36pBPA). D) **2** retains activity on LptB₂FGC. Compounds, ATP, and LptA* were incubated with the vesicles for 1 hour at 30 °C prior to UV irradiation at 365 nm for 5 minutes. LPS $\times$ LptA* crosslinks were detected by LPS immunoblots. E) **2** synergizes with polymyxin B in a gyrase-independent fashion. Checkerboard broth microdilution assay against wildtype and gyrase-resistant *A. baumannii*. The black squares denote concentrations of **2** that reduce the polymyxin B concentration by at least five-fold. The data are representative of a minimum of two biological replicates.

Figure 3. A novobiocin derivative with improved LPS transport stimulating activity reduces the lethal concentration of polymyxin B

A) **1** and **4** inhibit gyrase supercoiling at 0.63 $\mu\text{g/mL}$; **3** inhibits at 0.16 $\mu\text{g/mL}$. B) **1F** and **3F** bind to LptB₂FGC, while **4F** does not. NMR binding experiments were performed as in Figure 2. N/D (not determined). C) Substitution of the carbamate in **1** for 5-methyl-1H-pyrrole-2-carboxylate in **3** increases LPS transport activity relative to **1**. **4** does not stimulate LPS release. D) **3** potentiates polymyxin B activity at least eightfold better than **1** does.