Combating gram-negative resistance: targeting the cell envelope

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Chapter 1:

Introduction

Background

Antimicrobial resistance (AMR) is a threat to global health. In 2016, it was projected that in 2050 approximately 10 million annual deaths would be attributed to AMR[1]. More recently, a systematic analysis of data from 1990 to 2021 corroborated that projection of approximately 10 million annual deaths attributable to or associated with AMR by 2050, equating to a 70 % increase between 2022 and 2050. However, it is also projected that with improved care and access to antibiotics, and especially the development of new treatments for gram-negative infections, a total of over 100 million deaths could be prevented between 2025 and 2050[2].

The collection of bacteria that cause the highest burden through infection and development of resistance are commonly referred to as the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.[3]. *Escherichia coli* was not part of the original ESKAPE pathogens, but multidrug resistant *E. coli* infections have emerged as a significant concern[4]. *E. coli, K. pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp. are gram-negative bacteria, possessing an outer membrane which forms an additional barrier preventing antibiotics from entering the cell and contributing to inherent resistance to many antibiotics. In addition, efflux pumps actively expel many antibiotics from the cell[5].

The World Health Organization (WHO) reported a widespread increase in antimicrobial resistance between 2016 and 2020[6]. Carbapenem- and third-generation cephalosporin-resistant *Enterobacteriaceae* together with carbapenem-resistant *Acinetobacter* are classified as 'critical priority pathogens'[7]. Optimizing therapeutic strategies against these bacteria, including existing, novel, and combination antibiotic regimens, is a key priority identified by the WHO for mitigating the burden of AMR on human health[8]. The development and effects of AMR are not exclusive to humans; they are interconnected with (animal-) agriculture and the environment. Therefore, addressing AMR requires a 'One Health' approach, considering all aspects of human, animals, environmental health [9,10].

Combating AMR requires a multi-faceted strategy including the development of adjuvants that can (re)sensitize resistant bacteria to antibiotics, the optimization and broadening of the spectrum of existing antibiotics, and the discovery and development of antibiotics with novel bacterial targets. This chapter introduces antibiotic targets in the gram-negative cell envelope, along with the sources and discovery methods of novel antibiotics.

Envelope targets for antibiotic development

Novel antibiotic targets, especially in gram-negative bacteria, are essential for combating antimicrobial resistance and have been extensively reviewed[11–15]. Most of the recently approved antibiotics and those in late-stage clinical trials are β-lactam/β-lactamase inhibitor (BL/BLI) combinations[13]. Although effective, these combinations alone are insufficient for combating multidrug-resistant (MDR) infections, highlighting the need for antibiotics with novel targets. An ideal antibiotic target is essential for bacterial survival, highly conserved, easily accessible, with low potential for resistance development, and without human orthologs. Targeting the bacterial cell envelope is advantageous, as the outer membrane and periplasm are more accessible than the cytoplasm. Additionally, molecules directly targeting components of the outer membrane are typically not subject to efflux pump activity, as they do not need to traverse the inner membrane[16].

Many potential antibiotics in early or preclinical development stages target components of the cell envelope of gram-negative bacteria. These targets can be broadly categorized into two groups: those targeting the lipopolysaccharide (LPS) biosynthesis and transport, and those targeting the transport and assembly of membrane-associated proteins[12]. Figure 1 illustrates the most important established and novel targets for antibiotics in the outer leaflet of gram-negative bacteria.

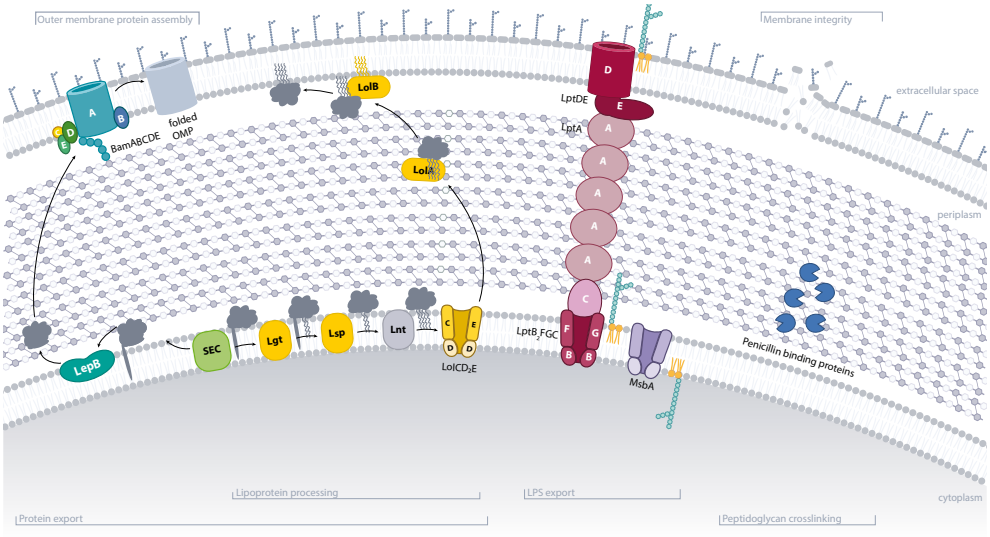

Figure 1: Targets for antibiotics in gram-negative bacteria (Adapted from [11,17]). Inner-membrane protein translocase SEC (light green), lipoprotein processing enzymes Lgt and Lsp, localization of lipoprotein (Lol) pathway (yellow), signal protease LepB (turquoise), β-barrel assembly complex (Bam) (blue/green), Lipopolysaccharide (LPS) transport machinery (Lpt), LPS-flippase MsbA, Penicillin binding proteins and general membrane integrity.

Lipopolysaccharide biosynthesis and transport

Within LPS biosynthesis, two known targets involved in lipid A synthesis are LpxH and LpxC; inhibitors of these enzymes exhibit antimicrobial activity[18,19]. After synthesis, MsbA flips lipid A and LPS across the inner membrane[20] and this protein is the target of multiple antimicrobial molecules[21–24]. Several proteins of the lipopolysaccharide transport machinery (Lpt) can be targeted as well. LptA bridges the periplasmic space between the inner and outer membrane by interacting with LptC[25]. Molecules inhibiting the interaction between the two proteins show antibacterial activity[26,27]. Inhibitors of LptB, which is part of the transporter complex LptBFGC in the inner membrane, can enhance polymyxin activity[28], or exhibit direct antibacterial activity[29]. Murepavidin, an inhibitor of the outer membrane protein LptD, shows promising antibacterial activity[30,31], however, Phase III clinical trials were terminated due to toxicity issues[32].

Envelope protein transport and assembly

Unfolded periplasmic and outer membrane proteins, as well as virulence factors and toxins, are transported across the inner membrane via the Sec translocase system consisting of the channel subunit SecYEG and the ATPase SecA[33,34]. While SecYEG is needed for efficient transport, SecA alone can facilitate the transport of proteins without a signal peptide[35]. There are several inhibitors of the SecA channel with activity against gram-negative bacteria (reviewed in[36]).

After translocation across the inner membrane lipoproteins undergo further processing before being transported to the outer membrane. Specifically, lipoprotein diacylglyceryl transferase (Lgt) lipidates the protein, while signal peptidase II (LspA) cleaves the signal peptide, before the proteins are further processed and transported to the outer membrane by the localization of lipoproteins (Lol) system[37]. LspA is essential in gram-negative bacteria and is highly conserved. It is the target of two known natural products with antibacterial activity: globomycin and myxovirescin[38,39]. While Lgt is not essential, one inhibitor has been shown to inhibit growth[40]. LolCDE is a protein complex that releases lipoproteins from the inner membrane prior to transport to the outer membrane[41]. The complex is highly conserved in pathogenic bacteria, while LolCDE in commensal gram-negative bacteria differs, making the known inhibitors selective for pathogens[42,43]. Lipoprotein processing and LspA inhibitors are further discussed in **Chapter 4**.

Signal peptides of non-lipoprotein outer membrane proteins are cleaved by signal peptidase I (LepB). LepB inhibitors exhibit antibacterial activity[44], and compound RG6436 (Genentech) is currently undergoing Phase I clinical trials[45]. Outer membrane proteins (OMPs) are folded into the outer membrane by the βbarrel assembly machinery (Bam) complex which consists of the OMP BamA and the lipoproteins BamB, BamC, BamD, BamE. BamA acts as a chaperone facilitating the folding of other OMPs[46]. The BamA inhibitor darobactin was discovered in 2019[47]. Its discovery led to the subsequent identification of multiple derivatives called bamabactins[48–50]. Recent screening efforts by Merck and Genentech have also identified BamA-binding cyclic peptides. However,

these compounds exhibit relatively high minimum inhibitory concentrations (MICs) or only exhibit activity at low BamA expression levels[51,52]. The Bam mechanism and its inhibitors are further discussed in **Chapter 2**.

Other targets

Other envelope-associated targets include proteins in the bacterial fatty acid biosynthesis machinery[53], among which FabI, a reductase, is considered to be a particularly promising target. The FabI inhibitor afabicin currently is in Phase II clinical trials for the treatment of staphylococcal infections[54,55]; its analogue, fabimycin, is active against gram-negative bacteria[56].

The target of the first discovered class of antibiotics, β-lactams, is penicillin-binding-proteins (PBPs), which crosslink the peptidoglycan layer. Bacteria become resistant to β-lactams by the expression of βlactamases[57]. One class of β-lactamases, the extended-spectrum β-lactamases (ESBLs), is further discussed in Chapter 5.

The outer membrane itself is the target of polymyxins, which permeabilize the cell, leading to cell death[58]. Polymyxin B nonapeptide (PMBN), consisting of the cyclic heptapeptide and two linear amino acids of polymyxin B, as well as numerous PMBN analogues, effectively disrupt the outer membrane without causing cell death. They can act as synergists with antibiotics targeting cytoplasmic processes to overcome intrinsic resistance of gram-negative bacteria and have been extensively reviewed[59,60]. Polymyxins and PMBN are further discussed in **Chapter 3**.

Another way of overcoming intrinsic resistance facilitated by the outer membrane is by exploiting active transport mechanisms into the cell. Siderophores are molecules that sequester iron from the environment and are actively imported. Conjugating antibiotics to siderophores can greatly increase their activity[61,62]. The cephalosporin-siderophore conjugate cefiderocol has been approved by the FDA and EMA in 2019 and 2020[63].

The inhibition of efflux pumps could help overcome multi drug resistance (MDR) caused by their overexpression[64,65]. Lastly, inhibiting the transport of capsule components has been shown to facilitate killing of bacteria by the complement system[66].

Strategies for Discovery and Characterization

Following the 'golden-age' of antibiotic discovery from the 1940s-60s, a 'discovery void' emerged, characterized by a decades-long absence of new antibiotic classes[67]. While advancements in discovery and screening methods have recently led to the discovery of a few new classes of antibiotics, the rapidly evolving nature of antimicrobial resistance maintains an urgent need for new antimicrobial agents[68].

Sources of novel antimicrobials

Novel antimicrobial molecules originate from various sources, predominantly through the screening of libraries containing natural extracts or synthetic molecules in antibiotic discovery programs. Natural products are the basis of the majority of the recently discovered anti-gram-negative compounds that have progressed to late-stage preclinical development and have been recently reviewed[69]. While easily accessible antibacterial natural products are thought to be largely exhausted, the vast majority of all bacteria are "uncultured", representing a potentially untapped source of novel antibiotics[69].

Another valuable method of discovery of novel antibiotics is the analysis, cloning, and heterologous expression of biosynthetic gene clusters (BGCs). Through genome mining, where genomes of organisms are analyzed based on homology to known BGCs, products of 'silent' BGCs or those from difficult-to-culture organisms can be characterized. Another approach is the direct cloning of environmental DNA into heterologous hosts[70]. In addition, with advancements in machine learning and artificial intelligence, *in silico* methods such as virtual screenings and computer-based drug design are likely to become increasingly relevant in drug development, including in antibiotic discovery[71].

De novo peptide selection approaches, using techniques such as phage- or mRNA display, allow for the screening of vast libraries of linear or cyclic peptides. Hits from these screens are usually generally based on a peptide's high binding affinity for a chosen molecular target, which does not guarantee inhibitory or antimicrobial activity[72]. Phage display has been used to screen libraries of peptides and antibodies against various bacterial targets as well as whole cells (reviewed in[73]). Several known and established antibiotics

are macrocyclic peptides, thus using peptide display techniques represents a promising approach[74]. The recently published BamA-binding macrocyclic peptides with antibacterial activity[51,52] illustrate the potential of the display techniques. While the activity of the BamA-binding peptides was limited, it can be anticipated that with different targets and further optimization, highly effective antimicrobial peptides may be identified.

Screening methods in antibiotic development

High-Throughput Screening (HTS) methods for the discovery of antimicrobial molecules have been reviewed recently[75]. The traditional strategy for the discovery of antimicrobials is whole-cell screening (WCS), where bacteria are cultured with libraries of natural extracts or synthetic molecules. Such phenotypic screens led to the discovery of multiple classes of antibiotics in the 1940s, 50s and 60s[76]. More advanced cellular target-based WCS employ engineered bacteria that either possess an altered expression level of a given target or express a reporter substrate under the control of pathway-specific promoters. This approach has also led to the discovery of several antimicrobial molecules (reviewed in[77]).

Peptides targeting the outer membrane can be displayed on the surface of bacteria using Surface Localized Antimicrobial display (SLAY), where active peptides cause cell death and can be identified by DNA sequencing[78]. This approach has been used to identify macrocyclic, βhairpin peptides with antimicrobial activity[79,80].

Phenotypic screening of whole cells is agnostic to the mechanism of action of the molecules and is therefore highly valuable in the discovery of novel antibiotic targets. However, whole-cell approaches are limited by the requirements that active compounds must not be significantly affected by efflux and must be able to easily reach their target, either through sufficient cell permeability or by targeting exposed cell surface components. Biochemical assays employing purified molecular targets overcome such limitations and enable the discovery of specific inhibitors, whose antibiotic-like properties can be subsequently optimized[81,82].

Challenges in the discovery of antimicrobials targeting the gram-negative envelope

While targeting the gram-negative cell envelope has advantages, it also presents several challenges. Many targets are embedded or associated with hydrophobic environments, favoring hydrophobic compounds in HTS. This, however, can lead to challenges with solubility, stability, bioavailability, and pharmacokinetics[14]. Generally, target-based screenings focus on a single target, which often result in a hit with a single mode of action. However, more complex mechanisms of action are advantageous, as they reduce the likelihood of resistance development through a simple target modification[76].

Using natural products in drug discovery presents several challenges. These challenges include the initial collection and cultivation of organisms and the subsequent preparation of extracts. The active molecule must be present at sufficient concentrations in the extract to register as a hit in the initial screen. Additionally, other molecules in the extract might interfere with the assay by causing false negatives/positives through signal interference or acting synergistically. Especially with phenotypic WCS, the risk of finding compounds with general toxicity or rediscovering known antibiotics is substantial[75].

In addition to overcoming scientific obstacles, antibiotic development is also fraught with economic challenges. Most major pharmaceutical companies have discontinued their antibiotic research and development programs over the years[83]. This is primarily attributed to the substantial effort required for developing new antibiotics coupled with their relatively low profitability, primarily due to typically short-term use and the rapid emergence of resistance[84,85]. Push incentives like the Global Antibiotics Research and Development Partnership (GARDP) and Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) have been established to stimulate and support antibiotic development efforts[83].

Thesis Outline

This thesis explores both mechanisms of antibiotic resistance and novel strategies for antimicrobial discovery, with a focus on membrane-associated bacterial targets and resistance mechanisms.

Chapter 1 provides a general introduction to this thesis. It reviews the current literature on antibiotic targets in the gram-negative cell envelope and offers insight into strategies for the discovery and development of novel antimicrobials.

Chapter 2 explores the use of mRNA display to discover antimicrobial peptides. We identified peptides that bind BamA-enriched outer membrane vesicles (OMVs) and characterized their activity, demonstrating the feasibility of this technique for targeting the outer membrane, albeit with room for optimization.

Chapter 3 focuses on the design and evaluation of nanobody-drug conjugates. In doing so, we conjugated a BamA-targeting nanobody (nanoE6) to the membrane disrupting polymyxin B nonapeptide (PMBN) and assessed their antimicrobial activity against wild-type and lipopolysaccharide-deficient *E. coli*..

Chapter 4 evaluates the use of globomycin-containing natural product extracts in an *in vitro* FRET assay measuring LspA activity. We show that such extracts can be effectively screened in this system, paving the way for future high-throughput screening efforts.

Chapter 5 involves the characterization of various β-lactamases from gram-negative bacteria found in Dutch dairy cattle. We evaluate the ability of these β-lactamases to hydrolyze various β-lactams focusing on cloxacillin, a narrow-spectrum antibiotic widely used in dairy cattle, and demonstrate that this can contribute to the selection of ESBL-producing *E coli.*

Chapter 6 summarizes and contextualizes the findings of the previous chapters, highlighting the importance of researching and developing novel antibiotics to combat emerging resistance.

Literature

1. O'Neill, J. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. https://apo.org.au/node/63983 (2016).
2. Naghavi, M. *et al.* Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. *The Lancet* **404**, 1199–1226 (2024).
3. Rice, L. B. Federal Funding for the Study of Antimicrobial Resistance in Nosocomial Pathogens: No ESKAPE. *J. Infect. Dis.* **197**, 1079–1081 (2008).
4. Poirel, L. *et al.* Antimicrobial Resistance in Escherichia coli. *Microbiol. Spectr.* **6**, 10.1128/microbiolspec.arba-0026–2017 (2018).
5. Impey, R. E., Hawkins, D. A., Sutton, J. M. & Soares da Costa, T. P. Overcoming Intrinsic and Acquired Resistance Mechanisms Associated with the Cell Wall of Gram-Negative Bacteria. *Antibiotics* **9**, 623 (2020).
6. *Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022*. (2022).
7. *WHO Bacterial Priority Pathogens List, 2024: Bacterial Pathogens of Public Health Importance to Guide Research, Development and Strategies to Prevent and Control Antimicrobial Resistance*. https://www.who.int/publications/i/item/9789240093461.
8. *Global Research Agenda for Antimicrobial Resistance in Human Health*. https://www.who.int/publications/i/item/9789240102309.
9. Action against antimicrobial resistance requires a One Health approach. https://www.who.int/europe/publications/i/item/WHO-EURO-2024-9510-49282-73655.
10. *COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS Pathway to a Healthy Planet for All EU Action Plan: 'Towards Zero Pollution for Air, Water and Soil'*. (2021).
11. Theuretzbacher, U., Blasco, B., Duffey, M. & Piddock, L. J. V. Unrealized targets in the discovery of antibiotics for Gram-negative bacterial infections. *Nat. Rev. Drug Discov.* **22**, 957–975 (2023).
12. Theuretzbacher, U., Jumde, R. P., Hennessy, A., Cohn, J. & Piddock, L. J. V. Global health perspectives on antibacterial drug discovery and the preclinical pipeline. *Nat. Rev. Microbiol.* 1–17 (2025) doi:10.1038/s41579-025-01167-w.
13. Theuretzbacher, U. Evaluating the innovative potential of the global antibacterial pipeline. *Clin. Microbiol. Infect.* **31**, 903–909 (2025).

14. Romano, K. P. & Hung, D. T. Targeting LPS biosynthesis and transport in gram-negative bacteria in the era of multi-drug resistance. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1870**, 119407 (2023).

15. Storek, K. M., Sun, D. & Rutherford, S. T. Inhibitors targeting BamA in gram-negative bacteria. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1871**, 119609 (2024).

16. Silver, L. L. A Gestalt approach to Gram-negative entry. *Bioorg. Med. Chem.* **24**, 6379–6389 (2016).

17. Konovalova, A., Kahne, D. E. & Silhavy, T. J. Outer Membrane Biogenesis. *Annu. Rev. Microbiol.* **71**, 539–556 (2017).

18. Karthikeyan, D., Kumar, S. & Jayaprakash, N. S. A comprehensive review of recent developments in the gram-negative bacterial UDP-2,3-diacylglucosamine hydrolase (LpxH) enzyme. *Int. J. Biol. Macromol.* **267**, 131327 (2024).

19. Zhou, P. & Hong, J. Structure- and Ligand-Dynamics-Based Design of Novel Antibiotics Targeting Lipid A Enzymes LpxC and LpxH in Gram-Negative Bacteria. *Acc. Chem. Res.* **54**, 1623–1634 (2021).

20. Padayatti, P. S. *et al.* Structural Insights into the Lipid A Transport Pathway in MsbA. *Structure* **27**, 1114-1123.e3 (2019).

21. Bonifer, C. & Glaubitz, C. MsbA: an ABC transporter paradigm. *Biochem. Soc. Trans.* **49**, 2917–2927 (2021).

22. Alexander, M. K. *et al.* Disrupting Gram-Negative Bacterial Outer Membrane Biosynthesis through Inhibition of the Lipopolysaccharide Transporter MsbA. *Antimicrob. Agents Chemother.* **62**, 10.1128/aac.01142-18 (2018).

23. Wang, H. *et al.* Cerastecins inhibit membrane lipooligosaccharide transport in drug-resistant Acinetobacter baumannii. *Nat. Microbiol.* **9**, 1244–1255 (2024).

24. Zhang, G. *et al.* Cell-based screen for discovering lipopolysaccharide biogenesis inhibitors. *Proc. Natl. Acad. Sci.* **115**, 6834–6839 (2018).

25. Schultz, K. M. *et al.* Binding and transport of LPS occurs through the coordinated combination of an array of sites across the entire Escherichia coli LPS transport protein LptA. *Protein Sci.* **32**, e4724 (2023).

26. Zhang, X. *et al.* Identification of an anti-Gram-negative bacteria agent disrupting the interaction between lipopolysaccharide transporters LptA and LptC. *Int. J. Antimicrob. Agents* **53**, 442–448 (2019).

27. Moura, E. C. C. M. *et al.* Thanatin Impairs Lipopolysaccharide Transport Complex Assembly by Targeting LptC–LptA Interaction and Decreasing LptA Stability. *Front. Microbiol.* **11**, (2020).

28. Mandler, M. D. *et al.* Novobiocin Enhances Polymyxin Activity by Stimulating Lipopolysaccharide Transport. *J. Am. Chem. Soc.* **140**, 6749–6753 (2018).

29. Zampaloni, C. *et al.* A novel antibiotic class targeting the lipopolysaccharide transporter. *Nature* **625**, 566–571 (2024).

30. Sader, H. S., Dale, G. E., Rhomberg, P. R. & Flamm, R. K. Antimicrobial Activity of Murepavadin Tested against Clinical Isolates of Pseudomonas aeruginosa from the United States, Europe, and China. *Antimicrob. Agents Chemother.* **62**, 10.1128/aac.00311-18 (2018).

31. Srinivas, N. *et al.* Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in Pseudomonas aeruginosa. *Science* **327**, 1010–1013 (2010).

32. Polyphor Ltd. *A Phase II, Open-Label, Multicenter Study to Assess the Tolerance, Safety, Efficacy and Pharmacokinetics/Pharmacodynamics (PK/PD) of POL7080 in the Treatment of Patients With Acute Exacerbation of Non-Cystic Fibrosis Bronchiectasis Due to Pseudomonas Aeruginosa Infection Requiring Intravenous Treatment*. https://clinicaltrials.gov/study/NCT02096315 (2017).

33. Xie, K. & Dalbey, R. E. Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. *Nat. Rev. Microbiol.* **6**, 234–244 (2008).

34. Dalbey, R. E. & Kuhn, A. Protein Traffic in Gram-negative bacteria – how exported and secreted proteins find their way. *FEMS Microbiol. Rev.* **36**, 1023–1045 (2012).

35. Hsieh, Y. *et al.* SecA Alone Can Promote Protein Translocation and Ion Channel Activity: SecYEG INCREASES EFFICIENCY AND SIGNAL PEPTIDE SPECIFICITY *. *J. Biol. Chem.* **286**, 44702–44709 (2011).

36. Jin, J. *et al.* SecA inhibitors as potential antimicrobial agents: differential actions on SecA-only and SecA-SecYEG protein-conducting channels. *FEMS Microbiol. Lett.* **365**, fny145 (2018).

37. Buddelmeijer, N. The molecular mechanism of bacterial lipoprotein modification—How, when and why? *FEMS Microbiol. Rev.* **39**, 246–261 (2015).

38. Dev, I. K., Harvey, R. J. & Ray, P. H. Inhibition of prolipoprotein signal peptidase by globomycin. *J. Biol. Chem.* **260**, 5891–5894 (1985).

39. Xiao, Y., Gerth, K., Müller, R. & Wall, D. Myxobacterium-Produced Antibiotic TA (Myxovirescin) Inhibits Type II Signal Peptidase. *Antimicrob. Agents Chemother.* **56**, 2014–2021 (2012).

40. Diao, J. *et al.* Inhibition of Escherichia coli Lipoprotein Diacylglyceryl Transferase Is Insensitive to Resistance Caused by Deletion of Braun's Lipoprotein. *J. Bacteriol.* **203**, 10.1128/jb.00149-21 (2021).

41. Narita, S. & Tokuda, H. Bacterial lipoproteins; biogenesis, sorting and quality control. *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids* **1862**, 1414–1423 (2017).

42. Muñoz, K. A. *et al.* A Gram-negative-selective antibiotic that spares the gut microbiome. *Nature* **630**, 429–436 (2024).

43. Breidenstein, E. B. M. *et al.* SMT-738: a novel small-molecule inhibitor of bacterial lipoprotein transport targeting Enterobacteriaceae. *Antimicrob. Agents Chemother.* **68**, e00695-23 (2023).

44. Szałaj, N. *et al.* Bacterial type I signal peptidase inhibitors - Optimized hits from nature. *Eur. J. Med. Chem.* **238**, 114490 (2022).

45. Genentech: Our Pipeline - RG6436. https://www.gene.com/medical-professionals/pipeline/rg6436.

46. Gu, Y. *et al.* Structural basis of outer membrane protein insertion by the BAM complex. *Nature* **531**, 64–69 (2016).

47. Imai, Y. *et al.* A new antibiotic selectively kills Gram-negative pathogens. *Nature* **576**, 459–464 (2019).

48. Miller, R. D. *et al.* Computational identification of a systemic antibiotic for gram-negative bacteria. *Nat. Microbiol. 2022* 1–12 (2022) doi:10.1038/s41564-022-01227-4.

49. Groß, S. *et al.* Improved broad-spectrum antibiotics against Gram-negative pathogens via darobactin biosynthetic pathway engineering †. (2021) doi:10.1039/d1sc02725e.

50. Modaresi, S. M. *et al.* Antibiotics that Kill Gram-negative Bacteria by Restructuring the Outer Membrane Protein BamA. 2024.12.16.628070 Preprint at https://doi.org/10.1101/2024.12.16.628070 (2024).

51. Walker, M. E. *et al.* Antibacterial macrocyclic peptides reveal a distinct mode of BamA inhibition. *Nat. Commun.* **16**, 3395 (2025).

52. Sun, D. *et al.* The discovery and structural basis of two distinct state-dependent inhibitors of BamA. *Nat. Commun.* **15**, (2024).

53. Zhang, Y.-M., White, S. W. & Rock, C. O. Inhibiting Bacterial Fatty Acid Synthesis *. *J. Biol. Chem.* **281**, 17541–17544 (2006).

54. Debiopharm International SA. *Randomized Open-Label Active-Controlled Study to Assess the Safety, Tolerability, and Efficacy of Afabicin IV/Oral in the Treatment of Patients With Bone or Joint Infection Due to Staphylococcus*. https://clinicaltrials.gov/study/NCT03723551 (2025).

55. Wittke, F. *et al.* Afabicin, a First-in-Class Antistaphylococcal Antibiotic, in the Treatment of Acute Bacterial Skin and Skin Structure Infections: Clinical Noninferiority to Vancomycin/Linezolid. *Antimicrob. Agents Chemother.* **64**, 10.1128/aac.00250-20 (2020).

56. Parker, E. N. *et al.* An Iterative Approach Guides Discovery of the FabI Inhibitor Fabimycin, a Late-Stage Antibiotic Candidate with In Vivo Efficacy against Drug-Resistant Gram-Negative Infections. *ACS Cent. Sci.* **8**, 1145–1158 (2022).

57. Mora-Ochomogo, M. & Lohans, C. T. β-Lactam antibiotic targets and resistance mechanisms: from covalent inhibitors to substrates. *RSC Med. Chem.* **12**, 1623–1639 (2021).

58. Mohapatra, S. S., Dwibedy, S. K. & Padhy, I. Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions. *J. Biosci.* **46**, 85 (2021).

59. Klobucar, K. & Brown, E. D. New potentiators of ineffective antibiotics: Targeting the Gram-negative outer membrane to overcome intrinsic resistance. *Curr. Opin. Chem. Biol.* **66**, 102099 (2022).

60. Wesseling, C. M. J. & Martin, N. I. Synergy by Perturbing the Gram-Negative Outer Membrane: Opening the Door for Gram-Positive Specific Antibiotics. *ACS Infect. Dis.* **8**, 1731–1757 (2022).

61. Negash, K. H., Norris, J. K. S. & Hodgkinson, J. T. Siderophore–Antibiotic Conjugate Design: New Drugs for Bad Bugs? *Molecules* **24**, 3314 (2019).

62. Page, M. G. P. Siderophore conjugates. *Ann. N. Y. Acad. Sci.* **1277**, 115–126 (2013).

63. Syed, Y. Y. Cefiderocol: A Review in Serious Gram-Negative Bacterial Infections. *Drugs* **81**, 1559–1571 (2021).

64. Nishino, K., Yamasaki, S., Nakashima, R., Zwama, M. & Hayashi-Nishino, M. Function and Inhibitory Mechanisms of Multidrug Efflux Pumps. *Front. Microbiol.* **12**, (2021).

65. Nakashima, R. *et al.* Structural basis for the inhibition of bacterial multidrug exporters. *Nature* **500**, 102–106 (2013).

66. Kong, L. *et al.* Single-molecule interrogation of a bacterial sugar transporter allows the discovery of an extracellular inhibitor. *Nat. Chem.* **5**, 651–659 (2013).

67. Silver, L. L. Challenges of Antibacterial Discovery. *Clin. Microbiol. Rev.* **24**, 71–109 (2011).

68. Hutchings, M. I., Truman, A. W. & Wilkinson, B. Antibiotics: past, present and future. *Curr. Opin. Microbiol.* **51**, 72–80 (2019).

69. Lewis, K. *et al.* Sophisticated natural products as antibiotics. *Nature* **632**, 39–49 (2024).

70. Libis, V. *et al.* Multiplexed mobilization and expression of biosynthetic gene clusters. *Nat. Commun.* **13**, 5256 (2022).

71. da Silva, T. H., Hachigian, T. Z., Lee, J. & King, M. D. Using computers to ESKAPE the antibiotic resistance crisis. *Drug Discov. Today* **27**, 456–470 (2022).

72. Randall, J. R., Wang, X., Groover, K. E., O'Donnell, A. C. & Davies, B. W. Using display technologies to identify macrocyclic peptide antibiotics. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1870**, 119473 (2023).

73. Huang, J. X., Bishop-Hurley, S. L. & Cooper, M. A. Development of Anti-Infectives Using Phage Display: Biological Agents against Bacteria, Viruses, and Parasites. *Antimicrob. Agents Chemother.* **56**, 4569–4582 (2012).

74. Luther, A., Bisang, C. & Obrecht, D. Advances in macrocyclic peptide-based antibiotics. *Bioorg. Med. Chem.* **26**, 2850–2858 (2018).

75. Ayon, N. J. High-Throughput Screening of Natural Product and Synthetic Molecule Libraries for Antibacterial Drug Discovery. *Metabolites* **13**, 625 (2023).

76. Brötz-Oesterhelt, H. & and Sass, P. Postgenomic Strategies in Antibacterial Drug Discovery. *Future Microbiol.* **5**, 1553–1579 (2010).

77. Landeta, C. & Mejia-Santana, A. Union Is Strength: Target-Based and Whole-Cell High-Throughput Screens in Antibacterial Discovery. *J. Bacteriol.* **204**, e00477-21 (2022).

78. Tucker, A. T. *et al.* Discovery of Next-Generation Antimicrobials through Bacterial Self-Screening of Surface-Displayed Peptide Libraries. *Cell* **172**, 618-628.e13 (2018).

79. Randall, J. R. *et al.* Designing and identifying β-hairpin peptide macrocycles with antibiotic potential. *Sci. Adv.* **9**, eade0008 (2023).

80. Randall, J. R. *et al.* Adapting antibacterial display to identify serum-active macrocyclic peptide antibiotics. *PNAS Nexus* **2**, pgad270 (2023).

81. Muñoz, K. A. & Hergenrother, P. J. Facilitating Compound Entry as a Means to Discover Antibiotics for Gram-Negative Bacteria. *Acc. Chem. Res.* **54**, 1322–1333 (2021).

82. Richter, M. F. & Hergenrother, P. J. The challenge of converting Gram-positive-only compounds into broad-spectrum antibiotics. *Ann. N. Y. Acad. Sci.* **1435**, 18–38 (2019).

83. Blaskovich, M. A. T. & Cooper, M. A. Antibiotics re-booted—time to kick back against drug resistance. *Npj Antimicrob. Resist.* **3**, 1–20 (2025).

84. Bradley, J. S. *et al.* Anti-infective research and development—problems, challenges, and solutions. *Lancet Infect. Dis.* **7**, 68–78 (2007).

85. Skender, B. The demise of the antibiotic pipeline: the Bayer case. *Humanit. Soc. Sci. Commun.* **11**, 1–10 (2024).