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β-lactam combinations against multidrug-resistant *Enterobacterales*

Exploring combination effects and resistance development

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Abstract
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The β-lactam antibiotics are a cornerstone in treating bacterial infections, but the increasing prevalence of antibiotic resistance worldwide threatens their effectiveness. The main driver of β-lactam resistance is the production of β-lactamases, which are bacterial enzymes that inactivate the antibiotic. Moreover, resistance to multiple antibiotic classes is common in β-lactamase producing bacteria, further limiting treatment options. At the same time, few novel antibacterial agents are reaching the market. To address this challenge, antibiotic combination therapy is employed to enhance the effects of existing drugs against multidrug-resistant bacteria. Yet, there is a lack of knowledge regarding which antibiotics to combine to achieve the best effect. The investigations in this thesis evaluate the potential and limitations of combinations involving β-lactams, β-lactamase inhibitors and colistin against multidrug-resistant *Enterobacterales in vitro*.

In the first paper, we investigated resistance mechanisms to three commonly used β-lactam/β-lactamase inhibitor combinations (BLBLIs) in an *Escherichia coli* strain encoding multiple β-lactamases. We found that β-lactamase gene amplifications were a key driver of resistance, with variations in the amplification pattern depending on the BLBLI combination. Clinical resistance could be reached by gene amplifications for ampicillin-sulbactam and piperacillin-tazobactam, whereas ceftazidime-avibactam resistance required multiple genetic changes.

In the second paper, we evaluated the efficacy of double-carbapenem combinations against *E. coli* and *Klebsiella pneumoniae* producing carbapenemases (KPC-2, OXA-48, NDM-1, and NDM-5). Synergistic effects were most commonly observed against OXA-48-producing strains, whereas the efficacy was low against KPC-2 and negligible against NDM producers.

In the third and fourth papers, we evaluated the antibacterial activity of colistin in combination with BLBLIs. Considering that reduced membrane permeability is associated with decreased susceptibility towards BLBLIs, adding colistin may be beneficial since its membrane-disrupting effect may increase the entry of other drugs. In paper three, we showed synergistic effects with colistin and ceftazidime-avibactam against a KPC-2-producing *K. pneumoniae* strain with porin deficiencies. However, when systematically assessing the impact of porin loss on the synergistic potential of colistin in combination with BLBLIs in paper four, we did not find any clear association between porin loss and synergy.

These studies provide insight into the therapeutic potential and limitations of combinations, including β-lactam antibiotics against strains with different setups of resistance genes. More research is required to understand how to best use the newly introduced BLBLI combinations to preserve their activity and enhance the value of the available antibiotics for future generations.

Keywords: Gram-negative bacteria, antibiotic combination therapy, antibiotic resistance, beta-lactams, beta-lactamase inhibitors

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Rajer, F., **Allander**, **L**., Karlsson, PA., Sandegren, L. (2022) Evolutionary trajectories toward high-level β-lactam/β-lactamase inhibitor resistance in the presence of multiple β-lactamases. *Antimicrob Agents Chemother.,*66(6):e00290-22.

II. **Allander**, **L**., Vickberg, K., Lagerbäck, P., Sandegren, L., Tängdén, T. (2022) Evaluation of *in vitro* activity of double-carbapenem combinations against KPC-2-, OXA-48- and NDM-producing *Escherichia coli* and *Klebsiella pneumoniae*. *Antibiotics*, 11(11):1646.

III. **Allander**, **L**., Vikdahl, E., Chatzopoulou, M., O'Jeanson, A., Lagerbäck, P., Tängdén, T. Evaluation of ceftazidime-avibactam in combination with colistin against KPC-2-producing *Klebsiella pneumoniae* with porin deficiency in static and dynamic time-kill experiments. *Manuscript*

IV. **Allander**, **L**., Vickberg, K., Fermér, E., Söderhäll, T., Sandegren, L., Lagerbäck, P., Tängdén, T. Impact of porin deficiency on the synergistic potential of colistin in combination with β-lactam/β-lactamase inhibitors against *Klebsiella pneumoniae*. *Manuscript*

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Other papers by the author not included in this thesis:

Olsson, A., **Allander**, **L**., Shams, A., Al-Farsi, H., Lagerbäck, P., Tängdén, T. (2023) Activity of polymyxin B combinations against genetically well-characterised *Klebsiella pneumoniae* producing NDM-1 and OXA-48-like carbapenemases. *Int J Antimicrob Agents*, 106967.

Örmälä-Tiznado, A., **Allander**, **L**., Maatallah, M., Kabir, M., Brisse, S., Sandegren, L., Coorens, M., Giske, C. G. (2023). Molecular characteristics, fitness and virulence of high-risk and non-high-risk clones of carbapenemase-producing *Klebsiella pneumoniae*. *Microbiology Spectrum*. *Accepted manuscript*

Palica, K., Vorácová, M., Skagseth, S., Andersson Rasmussen, A., **Allander L**., Hubert, M., Sandegren, L., Schrøder Leiros, HK., Andersson, H., Erdélyi, M. (2022). Metallo-β-lactamase inhibitor phosphonamidate monoesters. *ACS Omega.*, 7(5):4550-4562

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Abbreviations

BLBLI	β-lactam/β-lactamase inhibitors
CRE	Carbapenem-resistant *Enterobacterales*
ESBL	Extended spectrum β-lactamase
FICI	Fractional inhibitory concentration index
HGT	Horizontal gene transfer
LPS	Lipopolysaccharides
MBL	Metallo-β-lactamase
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentrations
MLST	Multilocus sequence typing
OMP	Outer membrane proteins
PBP	Penicillin-binding proteins
WHO	The World Health Organization

Introduction

Several decades have passed since the introduction of antibiotics into clinical practice in the 1940s, a medical breakthrough that remarkably impacted health care worldwide. Antibiotics are used not only to treat acute bacterial infections but have also paved the way for many other medical advances where patients are at increased risk of bacterial infections, such as immunosuppressant treatment for cancer patients, invasive surgeries, and neonatal care. Antibiotics are, therefore, used routinely within the medical field, yet antibiotics are drugs that become ineffective the more we use them. Following the introduction of an antibiotic, the effectiveness of the drug is continuously reduced because of antibiotic resistance emerging in bacteria. Resistance development towards antibiotics is a natural evolutionary process allowing bacteria to adapt and survive. However, we have accelerated this process because of overuse and misuse of antibiotics (1). This has contributed to alarming rates of resistance emergence in pathogenic bacteria. Together with the lack of development of new antibiotics, we find ourselves running out of effective antibiotic treatments to combat common infections (2). A comprehensive assessment of the global burden of bacterial resistance revealed that 4.95 million deaths were associated with resistance in 2019 alone, including 1.27 million deaths directly attributable to bacterial resistance (3).

In 2017, the WHO presented a priority list of antibiotic-resistant bacteria (4). The list was intended to advise and promote research and development of new antibiotics against the pathogenic bacteria that constitute the greatest threat to public health. The list highlights, in particular, the challenge of Gram-negative bacteria that are resistant to multiple antibiotics. Multidrug resistance (MDR) is typically defined as the lack of susceptibility to at least one agent in three or more antimicrobial categories (5). In the first group on the WHO priority list, we find *Enterobacteriaceae*, a large family of Gram-negative bacteria within the order *Enterobacterales*, which includes many clinically important bacteria. This thesis work addresses the two leading bacterial species within this family with regard to morbidity and mortality: *Escherichia coli* and *Klebsiella pneumoniae* (4).

Gram-negative bacteria

In 1884, Hans Christian Gram developed a staining technique that classified most bacteria into two large groups based on the cell wall structure that encases the bacterial cytoplasm (6). In Gram-negative bacteria, the cell wall consists of an inner and an outer membrane separated by the periplasmic space in which a thin peptidoglycan layer resides. The inner membrane is a phospholipid bilayer, while the outer membrane contains phospholipids in the inner leaflet and lipopolysaccharides (LPS) in its outer leaflet (Figure 1). The outer membrane in Gram-negative bacteria serves as a protective barrier yet allows selective passage of nutrients into the cell and harmful products out of the cell (7). Gram-positive bacteria lack the outer membrane but have a much thicker multi-layered peptidoglycan than Gram-negative bacteria.

The bacterial cell wall is the drug target for many clinically significant antibiotics. The cell wall is a prime target because its structural integrity is essential for bacterial survival, its composition is unique to bacteria, and it allows for broad-spectrum activity as several features of cell walls are conserved across various pathogens (8).

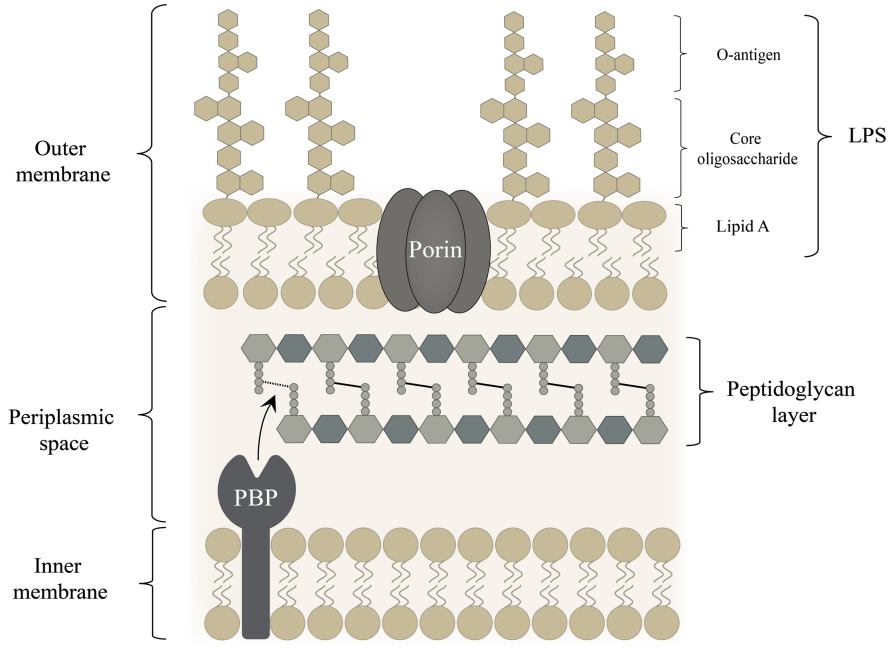


Figure 1. Structure of the cell wall in Gram-negative bacteria. Major structural components are labelled. Abbreviations: PBP; penicillin-binding protein.

Components of the bacterial cell wall

Lipopolysaccharides (LPS)

The LPS structure plays a significant role in structural integrity, barrier function, and bacteria-host interplay. The LPS molecule comprises three structural components with distinct properties (Figure 1). Lipid A is the hydrophobic anchor of the LPS structure that constitutes the outer leaflet of the outer membrane. Lipid A is a potent endotoxin whose release upon cell lysis can overstimulate the host immune system, resulting in life-threatening septic shock in the host. Attached to lipid A is the hydrophilic core oligosaccharide. Electrostatic interactions of negatively charged groups in lipid A and the core region with cations (Ca^{2+} and Mg^{2+}) help link the LPS molecules together and stabilise the LPS leaflet. The outermost domain of the LPS molecule is the O-antigen, which consists of hydrophilic repeating oligosaccharides. The O-antigen is the most diverse component of the LPS molecule, and its composition can vary between strains of the same species. Hence, modification of the O-antigen contributes to evasion of the host immune system. The barrier properties of the LPS (outer leaflet) arise from its amphipathic composition. The hydrophobic portion of lipid A restricts the passage of hydrophilic compounds, whereas the core oligosaccharide and O-antigen also provide hydrophilic character to the LPS, making the outer membrane resist the passage of hydrophobic compounds as well (9).

Porins

Porins are small pore-forming proteins that span across the outer membrane and allow for the uptake of water-soluble nutrients and other hydrophilic compounds, including hydrophilic antibiotics (10). The porins are trimeric structures made of β-barrels embedded in the outer membrane, which create an open water-filled channel allowing for the passive diffusion of small hydrophilic molecules. The selective permeability of porins is determined by their size, shape, and charge, which restrict the entry of larger or hydrophobic molecules. The porins are generally abundant in the outer membrane of Gram-negative bacteria, and several different types of porins exist. The expression of the various porins is controlled by two-component signalling systems that respond to various environmental cues (10,11). The major porins in *E. coli* (OmpC and OmpF) are the most well-studied porin channels. The OmpF pore is slightly larger than OmpC, which generally makes it easier for substrates to pass through the OmpF pore. In *K. pneumoniae*, two major porins, OmpK35 and OmpK36, are homologous to OmpF and OmpC, respectively (11).

Peptidoglycan layer

The peptidoglycan layer is a strong and dynamic polymer structure that determines the shape of the bacteria and protects the cell from bursting due to intracellular osmotic pressure. It is a mesh-like structure composed of long polymers of alternating units of N-acetylmuramic acid (MurNac) and N-acetylglucosamine (GlcNac) disaccharides crosslinked via short peptide chains. The synthesis of the peptidoglycan layer is vital for cell division and elongation (12). The disruption of this process has been a successful target strategy of some of the most efficient and widely used antibiotics (8,13).

The peptidoglycan biosynthesis begins in the cytoplasm, where a series of Mur enzymes (MurA-MurF) catalyse the formation of the MurNac and GlcNac precursors and subsequently add a peptide chain to the MurNac unit (12). In most Gram-negative bacteria, the peptide side chain consists of five amino acid residues: L-alanine (L-ala), γ-d-glutamate (D-glu), meso-diaminopimelic acid (mDAP), and D-alanine (D-ala)-D-ala. The composition of the peptide sequence may vary among bacterial species, but the D-ala-D-ala terminus is generally conserved (14). The MurNac-pentapeptide is then linked with a GlcNac unit, generating the disaccharide building block. The building blocks are then transported across the inner membrane, entering the periplasmic space, where they can be incorporated into the growing peptidoglycan chain (12,15).

Once in the periplasm, the penicillin-binding proteins (PBP) catalyse the polymerization of the glycan chains (transglycosylation) and the crosslinking of the neighbouring peptide side chains (transpeptidation) to form the solid mesh-like structure (Figure 2). The PBP transpeptidases recognise the D-ala-D-ala terminus in the peptide chain and facilitate the formation of a peptide bond between the D-ala residue at the fourth position and the amino acid residue in the third position (mDAP) of an adjacent chain. The terminal D-ala residue is released from the target chain during this process. Some PBPs can limit the further extension of the peptidoglycan by removing the terminal D-ala from the stem pentapeptides (DD-carboxypeptidation) or hydrolyse the peptide bond connecting two glycan strands (endopeptidation). This process controls the amount of pentapeptide substrates available and is vital for maintaining cell shape (12,16).

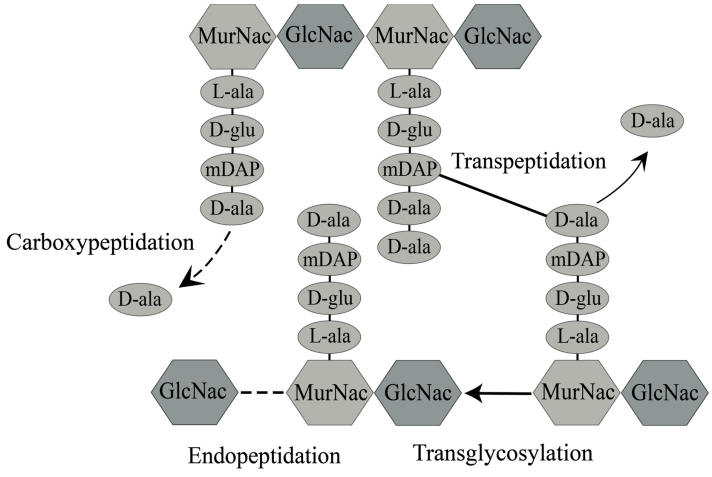


Figure 2. Synthesis and remodelling of the peptidoglycan layer facilitated by Penicillin-binding proteins (PBP). Abbreviations: MurNac, N-acetylmuramic acid; GlcNac, N-acetyl- glucosamine; L-ala, L-alanine; D-glu, γ-d-glutamate; mDAP, meso-diaminopimelic acid; D-ala, D-alanine.

Escherichia coli

Escherichia coli (*E. coli*) is one of the most studied microorganisms worldwide and a bacterial species that encompasses great diversity. *E. coli* is a commensal member of the gastrointestinal tract in humans and other animals, yet a very versatile and common pathogen. *E. coli* causes a variety of diseases, including intra-abdominal, urinary tract, respiratory tract, central nervous system, and bloodstream infections (17). *E. coli* is the leading cause of both community- and hospital-acquired urinary tract infections (UTIs) and is one of the most common pathogens in bloodstream infections (2,18,19). The severity of the disease depends on the site of infection, host factors, and the bacterial characteristics. Pathogenic *E. coli* possesses specific virulence attributes, which confers an increased ability to adapt to new environmental niches and allows them to cause a broad spectrum of diseases. These virulence factors include adhesins, toxins, and polysaccharide capsule. The genes for these virulence factors are commonly encoded on mobile genetic elements and can be transferred between strains (17).

Genome sequencing has enabled mapping of the population genetics of pathogenic *E. coli* and revealed that specific clonal lineages are responsible for most infections associated with virulence, antibiotic resistance, and clinical outbreaks worldwide. Multilocus sequence typing, or MLST, is one typing method that has been widely applied for the characterisation of population structures. This method identifies an allelic profile based on a fixed set of conserved genes (housekeeping genes) for a given isolate and correlates the given allele profile to a sequence type (ST). The use of whole-genome sequencing

has allowed for the expansion of the traditional MLST to a larger number of genes with core genome multilocus sequence typing (cgMLST) and whole genome multilocus sequence typing (wgMLST). ST131 is one of the most common sequence types of *E. coli* isolated clinically and an important example of a multidrug-resistant extraintestinal pathogenic lineage disseminated worldwide (20).

Moreover, *E. coli* is also undisputedly one of the most used model organisms in microbiological research. The laboratory strain *E. coli* K-12 and its derivatives have been vital in advancing genetics, biochemistry, and physiology. In 1997, the *E. coli* K-12 derivate MG1655 was the first *E. coli* strain to have its complete genome sequence published (21). The availability of the whole sequence promoted extensive use of MG1655 worldwide. *E. coli* K-12 was initially isolated from a diphtheria patient in 1922, but after decades of being cultured and passaged in the laboratory, it has adapted to the laboratory environment. However, due to the enormous amount of information collected over such a long time, *E. coli* MG1655 remains widely used as a model organism for studying genetics and the evolution of clinically relevant characteristics (22,23).

Klebsiella pneumoniae

Klebsiella pneumoniae (*K. pneumoniae*) was first described when it was isolated in 1882 from the lungs of a patient who died of pneumonia (24). It later became evident that *K. pneumoniae* can cause a variety of infections in addition to pneumonia, including urinary tract infections, bloodstream infections, and sepsis. *K. pneumoniae* can be found in the environment, including water and soil, and can contaminate medical devices. Furthermore, *K. pneumoniae* commonly colonises human mucosal surfaces, including the gastrointestinal tract and oropharynx (25). Compared to *E. coli*, *K. pneumoniae* is less commonly associated with community-acquired infections. Instead, infections with *K. pneumoniae* are typically observed in vulnerable patients with underlying diseases and risk factors for infection in the hospital setting, e.g., neonates, the elderly, and immunocompromised individuals (26). Studies show that *K. pneumoniae* strains isolated from infection sites are often identical to those found during screening of the patients' own microbiota (27–29). This opportunistic pathogen is a common cause of hospital-acquired infections, causing mostly ventilator-associated pneumonia but also catheter-associated UTIs, post-surgical wound infections and bloodstream infections (2,26,30).

However, there is an increased emergence of hypervirulent *K. pneumoniae* lineages, which are not considered opportunistic and can cause invasive infections in otherwise healthy people (31). The virulent phenotype is derived from acquiring large virulence plasmids encoding, for example, siderophore systems and increased capsule production. Virulence and multidrug resistance have historically been associated with separate populations of *K. pneumoniae*.

Multidrug-resistant strains were more commonly associated with healthcare-associated infections (e.g., ST258, ST11, ST14, and ST15) and the emergence of severe community-acquired infections caused by hypervirulent strains occurred in parallel (e.g., ST23, ST26 ST57, and ST163). However, as both antibiotic resistance and virulence factors are associated with mobile genetic elements, we are now observing the convergence of resistance and virulence, resulting in the evolution of multidrug-resistant hypervirulent strains (32).

Antibiotics

Antibiotics are antimicrobial substances with various structures and properties capable of killing or inhibiting the growth of bacteria. Prior to antibiotics, even common infections could become life-threatening, and surgical procedures carried a much higher risk of post-surgical infections (33). The "golden era" of antibiotic discovery refers to a time period typically spanning from the 1940s to the 1960s when most of the antibiotic classes we use today were discovered and introduced to the market. During this period, scientists systematically explored soil microorganisms for their antibiotic-producing capabilities, leading to the identification of the chemical scaffolds of most clinically used antibiotics. Initially characterised by rapid discoveries and introductions of new antibiotic classes, this era gave hope in overcoming antibiotic resistance through continuous innovation. However, the rapid emergence of antibiotic resistance, depletion of natural resources, complexity of discovery, and lack of economic incentives led pharmaceutical industries to gradually withdraw from antibiotic discovery, leading to what is often described as a "discovery void" (34).

Below is a summary of the most commonly used antibiotics to treat Gram-negative infections that are within the scope of the thesis. However, it is worth mentioning that the choice of antibiotics varies over time and by region based on factors such as antibiotic resistance patterns and local clinical guidelines.

β-lactam antibiotics

Alexander Fleming's ground-breaking discovery of the first β-lactam antibiotic (penicillin) in 1928 paved the way for the antibiotic age (35). Since then, many different antibiotic classes have been discovered and developed. Yet the β-lactam antibiotics continue to be the most important class of antibiotics used to treat Gram-negative as well as Gram-positive bacterial infections. The β-lactams are well tolerated, effective, and are the most frequently prescribed group of antibiotics worldwide (36,37).

Mechanism of action

The β-lactam antibiotics exert their bactericidal activity by inhibitory binding to penicillin-binding proteins (PBPs), resulting in disruption of the peptidoglycan synthesis. The PBPs were discovered and named for their affinity to bind the β-lactam antibiotic penicillin. There are several different types of PBPs present in most bacteria. The PBPs can be categorised into high-molecular-mass (HMM) and low-molecular-mass (LMM) PBPs. In many Gram-negative bacteria such as *E. coli*, HMM PBPs 1-3 are essential enzymes encompassing transpeptidases and transglycosylases, whereas LMM PBPs 4-6 are D-carboxypeptidases and are generally non-essential (16). Various PBPs are present in varying quantities per cell and exhibit different affinities for β-lactam antibiotics. The high similarity in biochemical structure between the β-lactam antibiotic and the terminal D-ala–D-ala moiety of the peptide side chain of the peptidoglycan facilitates binding to the active site of PBPs. After binding, a reaction between the β-lactam ring and the catalytic serine in the active site of the PBP forms a covalent bond, resulting in the inactivation of the PBPs. The irreversible inhibition of the PBPs destabilises and weakens the peptidoglycan structure, ultimately resulting in loss of viability and cell lysis (38,39).

Groups of β-lactam antibiotics

Since the discovery of penicillin, several groups of β-lactam antibiotics have been found and further developed to improve characteristics such as potency, spectrum of activity and to counter the emergence of resistance. Currently, four major classes of β-lactam antibiotics are in clinical use: penicillins, cephalosporins, carbapenems, and monobactams. All β-lactam antibiotics contain the characteristic β-lactam ring in their chemical structure (amide group and carboxylate group or sulfonic acid) but differ in side groups attached to the β-lactam scaffold (Figure 3) (39).

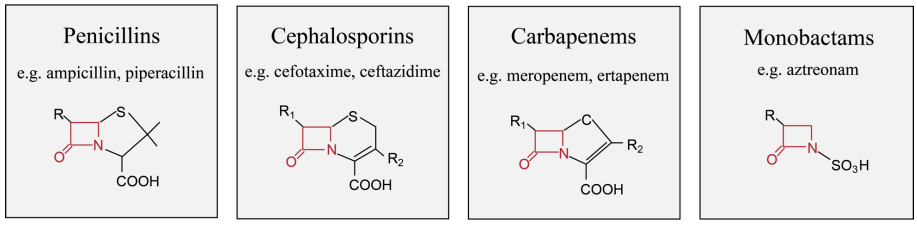


Figure 3. Different groups of β-lactam antibiotics, with their common β-lactam ring structure highlighted in red.

Penicillins

The first penicillin introduced into clinical practice was benzylpenicillin in the 1940s. It was used to treat infections caused by Gram-positive staphylococci and streptococci (40). Modifications of penicillin led to the development of semisynthetic penicillin derivates that are effective against a broad range of bacteria. This included, for example, aminopenicillins (e.g., ampicillin and amoxicillin) that broadened the antibacterial activity of penicillin to include most Gram-negative bacteria and extended-spectrum penicillins (e.g. piperacillin) which also have activity against *Pseudomonas* species (39). The extensive use of penicillins following their introduction into clinical practice quickly led to the development of resistance and prompted the search for new β-lactam antibiotics (38).

Cephalosporins

Cephalosporins were initially discovered in the 1940s, and the first cephalosporins were made available for clinical use in the 1960s (41–43). The cephalosporins have continuously been improved with the development of new "generations" of these drugs (e.g., ceftriaxone, cefotaxime and ceftazidime) with different properties in terms of, for example, antibacterial spectrum and stability against resistance mechanisms (39). Cephalosporins are a primary treatment choice for Gram-negative bacterial infections, and the availability of multiple generations of cephalosporin antibiotics provides a range of treatment options. However, resistance is a major concern and resistance towards third-generation cephalosporins in *E. coli* and *K. pneumoniae* are highlighted as major contributors to the global burden of antibiotic resistance (3,4).

In 2019, a new cephalosporin derivate named cefiderocol was first approved for clinical use. Cefiderocol is a synthetic compound consisting of a cephalosporin component attached to a siderophore. This siderophore binds to iron and facilitates bacterial cell entry through active iron transporters (44). Cefiderocol is currently a last-resort treatment option against Gram-negative bacteria, such as *Enterobacterales* and *P. aeruginosa,* that are resistant to other β-lactam antibiotics (45).

Carbapenems

The carbapenems were discovered in the 1970s and possess a broad spectrum of activity and great potency (38,46). Imipenem was the first clinically available carbapenem and has been widely used; however, it exhibits poor chemical stability. Other carbapenems, including meropenem, ertapenem and doripenem, have similar potent spectrums but better chemical stability (47). A key factor in the efficacy of carbapenems is their ability to efficiently inhibit multiple essential PBPs (PBP1a, 1b, 2 and 3) (38,47). Carbapenems are often referred to as last-line antibiotics for treatment against severe infections with multidrug-resistant Gram-negative bacteria (38). The increasing prevalence of

carbapenem resistance is therefore a serious concern (3,4). While carbapenem resistance remained quite rare in invasive *E. coli* isolates for most countries in Europe, 33% of the countries reported resistance percentages of 25% or above in *K. pneumoniae* isolates in 2021 (48).

Monobactams

Monobactams are a monocyclic β-lactam antibiotics. The only monobactam in clinical use is aztreonam, which was approved in 1984. Aztreonam exhibits activity against Gram-negative aerobic bacteria while lacking activity against Gram-positive bacteria or anaerobes. Aztreonam binds tightly to PBP3 in Gram-negatives, with weaker binding to other PBPs, leading to filamentation followed by cell lysis (39,49).

β-lactam/β-lactamase inhibitors

The widespread use of β-lactam antibiotics has accelerated the emergence of resistance in pathogenic bacteria. Resistance to β-lactam antibiotics can occur through multiple mechanisms, which will be expanded upon in subsequent sections. However, for the purpose of this section, it is fitting to introduce the main mechanism of β-lactam resistance in Gram-negative bacteria: the β-lactamases. The β-lactamases are bacterial enzymes that cleave the β-lactam ring and thereby inactivate the antibiotic. One of the primary classification systems in use for β-lactamases is the Ambler system, which groups β-lactamases into separate molecular classes (A, B, C, and D) based on their amino acid sequences. Structural class A, C and D comprises serine β-lactamases that use a reactive serine residue in their active site to cleave the β-lactam ring. Class B enzymes are metallo-β-lactamases (MBL) that use catalytically functional metal ions (Zn^{2+}) in their active site to cleave the β-lactam ring (50).

Given that these enzymes challenge the therapeutic use of β-lactam antibiotics, the idea arose to develop combinations comprising a β-lactamase inhibitor along with a β-lactam antibiotic. The β-lactamase inhibitors inactivate the β-lactamase enzyme and prevent it from acting on the β-lactam antibiotic. In the mid-1970s, pharmaceutical companies launched considerable research efforts to search for a potent inhibitor of the TEM-1 enzyme, the main β-lactamase of interest at the time (51). Screening of natural products resulted in the discovery and development of clavulanic acid, which, together with amoxicillin, became the first β-lactam/β-lactamase inhibitor (BLBLI) pair in clinical use during the 1980s (51,52). Further investigations of the inhibition mechanism of clavulanic acid led to the development of the synthetic inhibitor sulbactam, which was partnered with ampicillin, and later, the more potent inhibitor tazobactam, which was partnered with piperacillin. These are so-called suicide inhibitors and bind irreversibly to the β-lactamase enzyme, resulting in the inactivation of the inhibitor. These inhibitors are structurally similar to β-lactam antibiotics but typically have no or low antibacterial activity on their

own (38). Clavulanic acid, sulbactam and tazobactam inhibit many class A β-lactamases but have reduced effectiveness against strains that produce multiple β-lactamases and are ineffective against class A β-lactamases capable of inactivating carbapenem antibiotics. Additionally, these inhibitors typically have insufficient activity against class B, C and D β-lactamases (51). Nevertheless, they still have an important role to play. Oral amoxicillin-clavulanic acid and intravenous piperacillin-tazobactam are frequently used for common infections and are recommended for the treatment of low-risk, non-severe infections caused by third-generation cephalosporin-resistant *Enterobacterales* (45).

The search for β-lactamase inhibitors with a broader inhibitory spectrum resulted in, for example, avibactam, relebactam, and vaborbactam. In contrast to the previous inhibitors, these are so-called non-β-lactam inhibitors, which are typically not suicide inactivators but inactivate most β-lactamase enzymes in a reversible manner. This interaction results in the regeneration of active enzyme and intact inhibitor (51). Avibactam, relebactam and vaborbactam are not active against the MBL enzymes (class B) but effectively inhibit clinically important class A and class C β-lactamases. Moreover, avibactam can inhibit class D enzymes that inactivate carbapenems. Avibactam is currently available in combination with ceftazidime, a third-generation cephalosporin, while relebactam and vaborbactam are partnered with carbapenems, imipenem and meropenem respectively (53). Ceftazidime-avibactam is recommended as a first-line option against severe infections with carbapenem-resistant *Enterobacterales* (CRE), with the exception of MBL-producing strains. Meropenem-vaborbactam and, to a somewhat lesser extent, imipenem-relebactam are also recommended as treatment options against CRE, mainly against strains producing class A enzymes that inactivate carbapenems (KPC variants) (45,54,55).

There are currently no clinically available inhibitors against class B β-lactamases. The commercially available serine β-lactamase inhibitors are ineffective because of the structural and mechanistic differences of the MBL enzymes in class B. The development of MBL inhibitors has proven to be a challenging task. For example, the MBLs exhibit large structural diversity and belong to a larger superfamily of metalloproteins found in various domains of life. Achieving an effective broad-spectrum MBL inhibition while maintaining selectivity is therefore challenging (56).

β-lactamase inhibitor	β-lactam partner	Spectrum of inhibition by β-lactamase inhibitor									
		Ambler classification	A				B			C	D	
		Examples of β-lactamases	TEM	SHV	CTX-M	KPC	NDM	VIM	IMP	AmpC	OXA	OXA-48
Clavulanic acid	Amoxicillin		✓	✓	✓							
Sulbactam	Ampicillin		✓	✓	✓							
Tazobactam	Piperacillin			✓	✓					*	*	
Avibactam	Ceftazidime		✓	✓	✓	✓				✓	*	✓
Vaborbactam	Meropenem		✓	✓	✓	✓				✓		
Relebactam	Imipenem		✓	✓	✓	✓				✓	*	

✓ Useful inhibitory activity.

* Variable inhibitory activity. Tazobactam has generally low but measurable activity against class C enzymes (AmpC) and can inhibit only certain OXA enzymes (e.g., OXA-2 and OXA-32). Avibactam and relebactam are typically not a potent inhibitors of most OXA-enzymes but can inhibit some variants (e.g., OXA -23 and OXA-24), in addition to OXA-48.

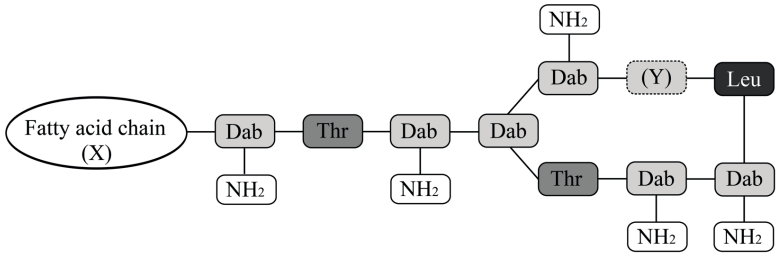
Figure 4. Spectrum of enzymatic inhibition by commercially available β-lactam/β-lactamase inhibitor combinations.

Commercially available BLBLI combinations typically come in a fixed-dose ratio, except amoxicillin-clavulanic acid, which is available in different ratios. The selection of these fixed dose pairings is influenced by *in vitro* activity and *in vivo* efficacy. For example, the commercial 4:1 ratio of ceftazidime-avibactam was supported by survival studies in infected mice (53). The standard method for *in vitro* susceptibility testing for most BLBLI combinations is broth microdilution with a fixed concentration of inhibitor alongside a range of β-lactam concentrations (57,58). According to this approach, it is expected that once the threshold concentration of the inhibitor is exceeded, the inhibitor's impact on the minimum inhibitory concentration (MIC) is minimal (53,59). Moreover, some studies suggest that this approach more clearly separates susceptible and resistant bacterial isolates while minimizing categorical errors (60,61). However, if concentrations *in vivo* surpassing this threshold value leads to a further reduction in susceptibility, the fixed concentration approach may not reflect the true contribution of the inhibitor and, consequently, *in vivo* efficacy (53).

Polymyxin antibiotics

Polymyxins are lipopeptides with antibacterial activity primarily against common Gram-negative bacteria. Polymyxins were discovered in the late 1940s (62,63). However, they became excluded from treatment regimens shortly after their approval in the late 1950s due to their high risk of toxicity and the arrival of other antibiotic classes that were considered to be associated with fewer side effects (64). Nevertheless, the polymyxins have resurfaced as last-resort antibiotics following the increased prevalence of multidrug-resistant Gram-negative bacteria. The two polymyxins available for clinical use are polymyxin B and colistin (polymyxin E) (65). Polymyxin B and colistin exhibit a comparable spectrum of activity and mechanism of action because of their structural similarity. The polymyxins have a complex structure

composed of a hydrophilic cyclic heptapeptide with a tripeptide side chain linked to a hydrophobic fatty acid tail. Positive-charged amino groups are attached to the cyclic peptide (Figure 5) (66).



(X) Fatty acid chain residues: 6-methyloctanoic acid for colistin A and polymyxin B1, and 6-methylheptanoic acid for colistin B, and polymyxin B2.

(Y) Amino acid differeing between colistin and polymyxin B: Leu for colistin and Phe for polymyxin B.

NH2 Differ for colistimethate (CMS):-NH-CH_2-SO_3H.

Figure 5. Structure of polymyxin antibiotics (polymyxin B and colistin). Abbreviations Dab, diaminobutyric acid; Thr, Threonine; Leu, Leucine; Phe, Phenylalanine.

The primary target for polymyxins is the outer membrane of Gram-negative bacteria. The positively charged, hydrophilic cyclic peptide structure of polymyxins binds to the negatively charged lipid A component of the LPS structure. This electrostatic interaction causes displacement of the cations (Ca^{2+} and Mg^{2+}) that link the LPS molecules together, destabilising the LPS leaflet. This, in turn, allows the hydrophobic fatty acid chain to be inserted into the outer membrane. This insertion destabilises the outer membrane's structure, increasing its permeability. Polymyxins can then traverse the outer membrane and reach the inner membrane, causing further disruption and destabilisation. The loss of membrane integrity results in leakage of essential cellular content and, ultimately cell lysis (64).

Polymyxins are mainly administered intravenously to treat severe infections but may also be used topically as cream or drops to treat, for example, otitis externa (swimmers' ear). Polymyxin B is administrated to patients in its active form, whereas colistin is administered as an inactive prodrug, colistimethate (also known as colistin methanesulfonate, CMS). The hydrolysis of CMS in the human body results in the release of the active colistin. The pharmacokinetic properties therefore differ between CMS and polymyxin B. The therapeutic window for polymyxins is narrow, and following intravenous administration, polymyxins exhibit reversible nephrotoxicity in up to 50% of the patients but also, less frequently, neurotoxicity. It is worth acknowledging that there are considerable variations in plasma exposure in studies investigating the population pharmacokinetics of polymyxins (64,65).

Other antibiotics classes

Treatment options are often very limited in carbapenem-resistant strains due to co-resistance to multiple antibiotic classes in these strains. In addition to the polymyxins, aminoglycosides, fosfomycin, and tigecycline quite often remain active *in vitro* and may be considered.

Aminoglycosides are broad-spectrum antibiotics that act through inhibitory binding to the 30S subunit of the ribosome, resulting in disruption of protein synthesis (67). Streptomycin was the first aminoglycoside and was introduced in 1944 (68). Several aminoglycosides were developed in the years that followed and became widely used. However, aminoglycosides are associated with toxicity (nephrotoxicity and ototoxicity), which in the 1980s resulted in a transition away from their use in favour of less toxic alternatives like third-generation cephalosporins, carbapenems and fluoroquinolones (67). However, due to the increased emergence of multidrug-resistant Gram-negative bacteria, there is a renewed interest in aminoglycosides as an alternative treatment when first-line drugs are ineffective or unavailable. Currently, clinically relevant aminoglycosides include gentamicin, amikacin, and tobramycin (45,54). Aminoglycosides have proven clinical efficacy as monotherapy for UTIs but should always be combined with a second active antibiotic for other systemic infections and for patients in septic shock (45).

Fosfomycin is a broad-spectrum antibiotic discovered in 1969 (69). Fosfomycin targets cell-wall synthesis by inhibiting UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), which is a key enzyme in the early stage of peptidoglycan biosynthesis in the cytoplasm. Consequently, the precursors of the peptidoglycan building blocks cannot be formed, resulting in disruption of cell wall integrity, cell lysis and ultimately cell death. Resistance to fosfomycin develops rapidly *in vitro* but less frequently *in vivo*. Yet, it remains a concern for the sustainable use of fosfomycin in clinical practice, and consequently, combination therapy is always recommended also for fosfomycin except for UTIs (45,70). Orally administered fosfomycin is used for uncomplicated UTI located in the urinary bladder but results in inadequate systemic concentrations for other indications. Intravenous fosfomycin is increasingly used to treat infections caused by multidrug-resistant bacteria and was recently reintroduced on the Swedish market (45,54,55).

Tigecycline is a structural derivative of tetracycline antibiotics and was approved for clinical use in 2005. It is a broad-spectrum protein synthesis inhibitor that binds to the 30S ribosomal subunit and prevents binding aminoacyl-tRNA molecules to the A site of the ribosome. Since carbapenem-resistant *Enterobacterales* often display *in vitro* susceptibility to tigecycline, it has emerged as an alternative treatment option (71). However, there is limited evidence for the effectiveness of tigecycline compared to other antibiotics, and its effect is uncertain in severe infections. Tigecycline has been associated with higher mortality compared to other antibiotics (mainly carbapenems) in

critically ill patients, especially when used for respiratory tract infections. Therefore, it is only recommended when other suitable treatments are lacking (45).

Antibiotic resistance mechanisms

While the therapeutic use of antibiotics has undeniably accelerated the emergence of resistance, resistance in itself is an ancient phenomenon and has existed long before we employed antibiotics for medical purposes. Most antibiotics that we currently rely on, like the β-lactams, are natural products originating from various environmental microorganisms that produce these compounds to compete and dominate an environmental niche. Consequently, microbes have long encountered antibiotics and have evolved mechanisms to withstand them as means of survival (72,73).

Antibiotic resistance can arise through several mechanisms by which bacteria escape or are protected against the actions of antibiotics. Resistance can be intrinsic, whereby bacteria possess an innate ability to withstand antibiotic exposure. Bacteria can also acquire resistance to antibiotics via mutations in chromosomal genes and by acquisition of new genetic material through horizontal gene transfer (74). Moreover, some bacteria may also temporarily increase their ability to tolerate antibiotic exposure by changes in gene and/or protein expression triggered by environmental factors such as stress and nutrient availability (75).

Gram-negative bacteria are intrinsically resistant to certain antibiotics due to the impermeable nature of the bacterial outer membrane, a feature stemming from its asymmetrical structure. The outer membrane also poses a major challenge to the development of novel antibiotics for Gram-negative bacteria as it requires the ability of the molecules to cross the membrane to reach their targets inside the cell. For example, the commonly used glycopeptide antibiotic vancomycin efficiently inhibits peptidoglycan crosslinking in Gram-positive bacteria but is ineffective against Gram-negative bacteria due to its inability to cross the outer membrane. Furthermore, the porins embedded in the outer membrane allow for the passive diffusion of small hydrophilic compounds, such as β-lactam antibiotics, but exclude larger compounds (11).

The ways by which bacteria can acquire resistance are typically divided into three groups: (i) degradation or modification of the antibiotic, (ii) regulation of influx/efflux of antibiotics in and out of the cell, and (iii) modification or replacement of the target (76). The interplay between multiple resistance mechanisms in the same bacterium can result in high-level resistance towards antibiotics. One example of this phenomenon in Gram-negative bacteria is the development of resistance towards β-lactam antibiotics.

Resistance to β-lactam antibiotics

The world of β-lactamases

The main mechanism of β-lactam resistance in Gram-negative bacteria is the production of β-lactamases. The β-lactamases cleave the β-lactam ring open through hydrolysis, thereby inactivating the antibacterial properties of the β-lactam antibiotic. The β-lactamases are ancient enzymes whose origin has been traced back long before the clinical introduction of β-lactams (77). For example, phylogenetic analyses have estimated that the class A serine β-lactamases have been in existence for over 2 billion years, and metagenomic studies of ancient permafrost sediments revealed amino acid sequences with similarities to the currently ubiquitous TEM β-lactamases (73,78). The PBPs are believed to be the ancestors of the serine β-lactamases because of their structural similarities with an active site serine residue that facilitates the formation of an acyl-enzyme intermediate with the β-lactam ring (Figure 6). The β-lactamases rapidly hydrolyse the resulting acyl-enzyme and inactivate the β-lactam. In contrast, the hydrolysis reaction by PBPs is slow, and the formation of the PBP-β-lactam complex is essentially considered irreversible within the lifetime of a bacterial cell. Given the functional and structural differences between the serine β-lactamases and the MBLs, it is believed that MBLs evolved from proteins other than the PBPs (77).

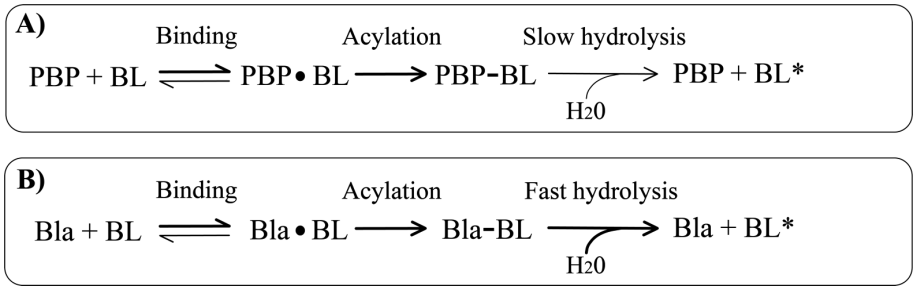


Figure 6. Reaction pathway for binding of a β-lactam substrate to PBP (**A**) or to a serine β-lactamase (**B**). Abbreviations: PBP, penicillin-binding protein; BL, β-lactam; BL*, hydrolysed β-lactam; Bla, β-lactamase.

The β-lactamases are a diverse group of enzymes where only one amino acid change can greatly alter the functionality of the enzyme. To date, thousands of unique β-lactamase genes have been identified, encoding enzymes with various hydrolytic activity against different β-lactams (77). The β-lactamases can be grouped in several ways according to structure and function. The structural Ambler classification system (class A to D) was introduced earlier in the section on BLBLI combinations and is expanded on below. Of note, however, functional classification schemes based on, for example, substrate profiles have also been proposed (79). Other functional names are commonly used to describe groups of enzymes that span more than one class or group. The most

prominent examples are two clinically important groups of β-lactamases, the extended-spectrum β-lactamases (ESBL) and carbapenemases. The ESBLs may belong to structural class A, C or D and hydrolyses penicillins and cephalosporins. Carbapenemases encompass enzymes from class A, B or D and are capable of hydrolysing most β-lactams, including carbapenems, to various degrees (80).

Class A serine β-lactamases

Class A is a large group and comprises enzymes with a wide range of activities. Commonly encountered class A β-lactamases found in *Enterobacterales* include TEM, SHV, CTX-M and KPC enzymes (80). TEM-1 was first described in the 1960s when it was discovered in an *E. coli* strain isolated from a Greek patient named Temoneira, hence the name TEM (81). TEM-1 can hydrolyse penicillins and early cephalosporins. However, through the accumulation of mutations, over 200 TEM variants have been described with varying hydrolytic activity. The first ESBLs were derivatives of TEM-1 and SHV-1 enzymes with amino acid substitutions that broadened their substrate profile to include the extended-spectrum cephalosporins (82).

In the past, the most prevalent ESBL-families in Gram-negative bacteria were variants of TEM and SHV. However, since the early 2000s, CTX-M-type ESBLs, particularly the CTX-M-15 variant, have spread worldwide and become the most common type of ESBL (80). The dissemination of CTX-M-15 has been mainly associated with the spread of *E. coli* ST131 (80). CTX-M was first recognized in the late 1980s and received its name after its hydrolytic activity against cefotaxime (CTX) and Munich (-M), the city in Germany where the enzyme was found in an *E. coli* isolate from a child with otitis media (82,83). The CTX-M enzymes are today frequently found in clinical isolates of *E. coli* and *K. pneumoniae* but appear to originate from a chromosomal β-lactamase in environmental *Kluyvera* species (80,82). In general, the CTX-M enzymes tend to exhibit more efficient hydrolysis of cefotaxime and ceftriaxone compared to ceftazidime, although the extent of this spectrum varies between CTX-M variants (82). For example, CTX-M-15 confers resistance to ceftazidime because of an amino acid change (Asp140Gly) that enables access to the more bulky ceftazidime molecule (84,85). The CTX-M enzymes are typically inhibited by the commercially available β-lactamase inhibitors, e.g. clavulanic acid and tazobactam, to various degrees (51).

During the late 1990s, a family of β-lactamases known as *K. pneumoniae* carbapenemases (KPC) started appearing along the east coast of the United States. KPC carbapenemases have a large and shallow active site, enabling interactions with a wide range of β-lactam antibiotics from all classes (86). Generally, KPC enzymes efficiently hydrolyse penicillins, cephalosporins, monobactams (aztreonam) and carbapenems but show weak hydrolytic activity against ceftazidime. KPC enzymes are typically not inhibited by the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam but by several

of the newer enzyme inhibitors such as avibactam, vaborbactam and relebactam (51). As the name suggests, the KPC carbapenemases are most commonly detected in *K. pneumoniae* but are also found in other species of *Enterobacterales*. The widespread dissemination of KPC has been primarily attributed to the expansion of *K. pneumoniae* isolates belonging to the highly successful clonal complex 258 (CC258) or ST258 (32,80). The most common KPCs are currently KPC-2 and KPC-3 (80). The prevalence of KPC varies depending on geographical region; however, KPC enzymes are becoming established in an increasing number of countries and have been responsible for multiple outbreaks worldwide. Some high-prevalence countries include the United States, Greece, Israel, Colombia, and Brazil (80,87).

Class B metallo-β-lactamases

The class B metallo-β-lactamases (MBLs) are structurally and mechanistically distinct from the serine β-lactamases of class A, C and D. The MBLs efficiently hydrolyse most β-lactams, including carbapenems, but lack the ability to hydrolyse monobactams and display poor hydrolytic activity against cefiderocol (44,88). Among the carbapenemases, the MBLs are generally the most efficient at hydrolysing carbapenems. Moreover, they are not inhibited by any commercially available β-lactamase inhibitor (88). However, the β-lactamase inhibitor avibactam is currently being developed in combination with aztreonam (monobactam) to target *Enterobacterales* carrying both serine β-lactamases and MBLs. Aztreonam is not hydrolysed by MBLs. However, since most MBL-producing strains co-produce other enzymes that hydrolyse aztreonam (e.g., AmpC, ESBL), avibactam can be given as a partner drug to protect aztreonam from hydrolysis. The aztreonam-avibactam combination is at the end of phase III trials (89). Treatment recommendations for severe infections caused by MBL-producing *Enterobacterales* include cefiderocol or ceftazidime-avibactam in combination with aztreonam while awaiting the arrival of aztreonam-avibactam on the market (45,54).

The most common MBLs detected in clinical isolates are the imipenemase (IMP), Verona imipenemase (VIM), and New Delhi metallo-β-lactamase (NDM) families (80). NDM-1 was initially reported in 2009 in a *K. pneumoniae* isolate obtained from a patient in Sweden returning from New Delhi, India (90). Within a few years, NDM had disseminated worldwide and became endemic in some countries in South Asia and the Middle East. Today, NDM has become the most prevalent MBL-type among *Enterobacterales* in many parts of the world (88,91).

Class C serine β-lactamases

The class C enzymes, also known as AmpC-type β-lactamases, are cephalosporinases that primarily hydrolyses cephalosporins, including expanded-spectrum cephalosporins, but also penicillins and some monobactams to a lesser extent. Avibactam and other newer β-lactamase inhibitors, such as relebactam and vaborbactam, are active against AmpC enzymes (51). Most AmpC-type β-lactamases are chromosomally encoded, and in some Gram-negative bacteria, the expression of the enzyme can be induced following exposure to some β-lactams. AmpC β-lactamases can also be constitutively expressed at high levels on plasmids. CMY-2 is the most common plasmid-encoded AmpC-type and can be detected worldwide in Gram-negative bacteria, including *E. coli* and *K. pneumoniae* (92).

Class D serine β-lactamases

Class D encompasses several families of β-lactamases, but most class D enzymes belong to the OXA family. The OXA-type enzymes were initially classified based on their oxacillin-hydrolysing characteristics, hence the name oxacillinases (OXA). However, they are now regarded as a diverse group of enzymes that exhibit variations in their substrate profiles and amino acid sequences (93). OXA-1 was one of the first OXA types described in the 1960s (94). OXA-1 is considered a narrow-spectrum variant but can hydrolyse some cephalosporins, including cefepime, a fourth-generation cephalosporin (93). Interestingly, OXA-1 remains frequent and is prevalent among strains producing CTX-M-15 (80). There are several OXA-types associated with an ESBL phenotype, and most are derivatives of OXA-2 and OXA-10 (82). Within the OXA family, there are also variants with a generally weak but significant hydrolytic activity against carbapenems. In addition, the OXA carbapenemases typically have activity against penicillins and narrow-spectrum cephalosporins but do not significantly hydrolyse the extend-spectrum cephalosporins (e.g., ceftazidime).

The most globally prevalent OXA carbapenemase in *Enterobacterales* is OXA-48, followed by its derivatives, OXA-48-like carbapenemase (95). OXA-48 was first identified in Turkey in 2001 when it was isolated from a carbapenem-resistant *K. pneumoniae* isolate (96). *Enterobacterales* with OXA-48 are considered endemic in various parts of the world, with higher prevalence observed in specific regions (e.g., parts of the Middle East, North Africa, and the Mediterranean), but hospital outbreaks or case reports are documented worldwide. Since the carbapenemase activity of OXA-48 is low, these isolates often display a modest increase in carbapenem MICs and are often classified as carbapenem-susceptible with standard *in vitro* susceptibility testing. Resistance to carbapenems in OXA-48-producing strains typically requires the co-production of other β-lactamases (e.g., ESBLs) or porin mutations (95). However, poor *in vivo* and clinical outcomes have been reported

following treatment with carbapenems against OXA-48-producing *Enterobacterales* despite these bacteria often displaying *in vitro* susceptibility (97). The OXA enzymes are generally not inactivated to the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam. However, there are exceptions, such as OXA-2 and OXA-32, that are inhibited by tazobactam. Avibactam inhibits some of the class D β-lactamases, most importantly it is the only available inhibitor with effective inhibition of OXA-48 (51,93)

Dissemination of β-lactamases: the role of plasmids and mobile genetic elements

The mobilization of β-lactamases among bacteria has enabled the acquisition and rapid dissemination of β-lactam resistance in Gram-negative pathogens (77,98,99). This is achieved through the actions of mobile genetic elements, which together facilitate the transfer of β-lactamase genes within and between bacterial cells. Bacteria can transfer genetic material (including resistance genes) between each other through the processes of horizontal gene transfer (HGT). There are three mechanisms of horizontal transfer: (i) transformation: uptake of extracellular DNA that is then incorporated in the genome of the recipient, (ii) transduction: transfer of bacterial genetic material by bacteriophages, and (iii) conjugation: genetic transfer of plasmids or integrative conjugative elements through cell-to-cell contact (100).

Clinically relevant β-lactamases are often associated with mobile genetic elements and are commonly encoded on transferable plasmids. Dissemination of plasmids and other mobile genetic elements across a range of Gram-negative pathogens by the action of HGT has been a key factor in the successful spread of these enzymes (80,98). Plasmids are self-replicating extrachromosomal DNA molecules that often encode proteins for their own horizontal transfer from one bacterial cell to another (101). Another important player in the mobilization of resistance genes is transposons. Transposons are mobile genetic elements able to move themselves (and associated resistance genes) from one genetic location to another. This activity is mediated by the recognition of inverted repeats on the transposon sequence, and the genetic material in between the repeats is then randomly inserted in any part of the genome by site-directed recombination. Transposons can have varying sizes, the smallest being designated insertion sequence (IS) elements that only encode the transposase gene flanked by the inverted repeats. Resistance genes can occasionally get trapped between two IS elements. This cluster of genes can then move together and become part of the bacterial chromosome or a plasmid. Furthermore, integrons are genetic elements that contain a site-specific recombination system that allows for the incorporation and expression of genes, including resistance genes, contained within so-called gene cassettes. Integrons can be found on mobile genetic elements such as plasmids or transposons (98).

TEM-1 was the first plasmid-encoded β-lactamases described in literature. Being transposon and plasmid-mediated enabled the widespread

dissemination of the TEM-1 β-lactamase. Within a few years after its first isolation, TEM-1 spread worldwide and is now found in many Gram-negative pathogens (82). The mobilization of β-lactamases has resulted in Gram-negative bacteria frequently co-producing multiple β-lactamases with different hydrolytic spectra. This phenomenon is especially noticeable in carbapenemase-producing bacteria (80). For example, approximately 90% of the 51 clinical carbapenemase-producing *E. coli* and K. *pneumoniae* isolates studied in **paper II** co-produced other β-lactamases (mainly CTX-M).

Reduced membrane permeability and efflux

Even though the production and dissemination of β-lactamases are the main drivers for high-level β-lactam resistance in Gram-negative bacteria, alterations in the permeability of the bacterial membrane also play an important role (102–104). Reduced permeability and increased efflux of antibiotics are commonly found in isolates showing resistance to several antibiotics. In order for the β-lactams to exert their activity, they need to cross the outer membrane in Gram-negatives to reach their PBP targets. Even though porins embedded in the outer membrane restrict the uptake of several compounds due to size and charge constrictions, they provide an entry route for many hydrophilic antibiotics, including the β-lactam antibiotics (11). In *E. coli,* the porins OmpC and OmpF facilitate entry of β-lactams, whereas *K. pneumoniae* express their homologues OmpK36 and OmpK35 (11,105,106). It has been reported that OmpK35 and OmpK36 allow more efficient diffusion of β-lactams than OmpF and OmpC, emphasising the vital role the porins play in antibiotic activity in *K. pneumoniae* (105).

Consequently, porin alterations can result in reduced susceptibility to β-lactam antibiotics (11,104,105,107). For example, a specific duplication of a glycine-aspartate pair in loop 3 of OmpK36 has been shown to cause pore restriction and thus reduced susceptibility to cephalosporins and carbapenems (108). Loss of porins does typically not entirely prevent the entry of β-lactams but reduces the periplasmic drug concentration to a level where low-level hydrolytic activities by β-lactamases may result in significant reductions in antibiotic susceptibility. Production of ESBLs that alone possess negligible hydrolytic activity against carbapenem antibiotics may cause resistance to ertapenem in the presence of porin loss (109–111). In addition to inactivating genetic changes within the porin-encoding genes, alterations in regulatory genes are also commonly described. For example, mutations in *ompR* (outer membrane porin response regulator), which encodes a regulator of OmpC and OmpF expression, have been associated with decreased antibiotic susceptibility (107).

Antibiotics that have successfully entered the cell can also be actively transported out by efflux pumps embedded in the bacterial membrane, resulting in decreased intracellular drug concentrations. Many efflux pumps can transport a variety of structurally diverse compounds and are known as

multidrug resistance efflux pumps. The AcrB-TolC efflux pump is a prominent multidrug efflux system found in Gram-negative bacteria, including *E. coli* and *K. pneumoniae*. Antibiotics that act as substrates of the AcrB-TolC efflux pump include, for example, β-lactams, chloramphenicol, fluoroquinolones and tetracyclines. Active efflux of β-lactams generally has a small effect on susceptibility when the β-lactams can diffuse rapidly through the porins. However, when porin permeability is reduced, the impact of efflux may become more pronounced. Moreover, mutations in the regulatory systems (AcrR, MarR, Sox, Rob, and Ram) can increase the expression of AcrB-TolC. In most cases, active efflux mainly serves as a stepping stone to resistance and interacts with other mechanisms to increase resistance levels (74,103)

Modification of target

Modification of drug targets that reduce the affinity to the antibiotic is a common resistance mechanism against several antibiotic classes (74). The alteration of PBPs as a resistance mechanism towards β-lactams has mainly been described in Gram-positive bacteria (112). However, there are reports where specific PBP mutations have been associated with a substantial decrease in susceptibility towards β-lactams in Gram-negative bacteria such as *E. coli* (113).

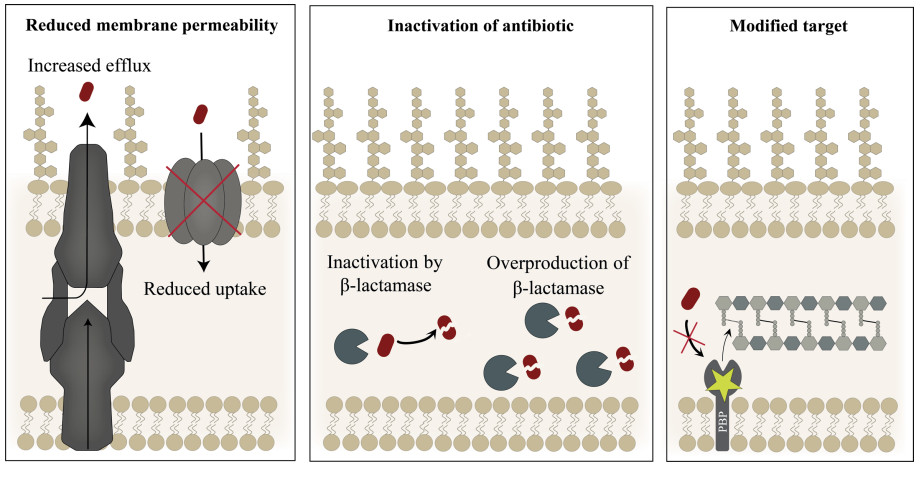


Figure 7. Overview of mechanisms of resistance that bacteria employ against β-lactam antibiotics, including reduced membrane permeability, efflux, enzymatic inactivation, and modification of target. Abbreviations: PBP, penicillin-binding protein.

Resistance to β-lactam/β-lactamase inhibitor combinations

As with other antibiotic agents, resistance towards BLBLI combinations emerged following their introduction into clinical practice (51). The mechanisms leading to resistance against BLBLI closely resemble those for β-lactam antibiotics alone. Resistance is associated with several mechanisms, including porin deficiencies, increased β-lactamase production and/or β-lactamase mutations (114).

Amino acid substitutions at critical sites of β-lactamases

Amino acid substitutions at critical sites of β-lactamases are a main driver for resistance towards BLBLI (51,115). Many inhibitor-resistant β-lactamases were initially variants of the TEM-1 enzyme. The inhibitor-resistant TEM resisted inhibition by clavulanic acid and sulbactam but typically remained susceptible to inhibition by tazobactam and avibactam (51,116). For newer BLBLIs, there are several reports of ceftazidime-avibactam resistance due to single amino-acid substitutions in the KPC carbapenemase (117–124). Moreover, substitutions in ESBLs, like CTX-M-15, have also been shown to contribute to lowered susceptibility to ceftazidime-avibactam (120,125). Interestingly, these mutations have at times been associated with an increased susceptibility towards β-lactams alone (124–126).

Overproduction of β-lactamases

The effect of β-lactamases can be further enhanced by increasing the abundance of β-lactamases produced by a single cell, resulting in that bacteria can cope with higher drug concentrations. Overproduction of β-lactamases can occur through elevated gene expression or increased gene copy number due to gene amplifications and increased plasmid copy number (98,127–129). The level of gene expression can be altered through mutations or the insertion of mobile genetic elements into regulatory regions. This has, for example, been described for resistance towards penicillin/β-lactamase inhibitor combinations by TEM enzymes (130,131). Moreover, gene duplication and amplifications by which a genetic region gets repeated, is a phenomenon commonly reported to increase the level of resistance (127,132). Gene amplifications are typically unstable resistance mechanisms that are frequently lost in the absence of antibiotic selection (127,132).

It has long been recognised that increased gene copy number of β-lactamases confer resistance (127,133). A recent study reported that amplification of OXA-1 and CTX-M-15 coupled with porin disruption was associated with carbapenem resistance in *Enterobacterale*s in a clinical setting (134). Furthermore, mechanisms of elevated copy number have become increasingly important in clinical settings because of their role in resistance towards BLBLI combinations (114,135–137). We show in **paper I** that amplifications of different β-lactamases decrease the susceptibility to specific BLBLI

combinations. Importantly, we found that amplifications in combination with mutations in CTX-M-15 and mutations that reduced drug influx rendered the ESBL-producing strain clinically resistant towards ceftazidime-avibactam. Several other studies report that an increased copy number of KPC has been associated with resistance towards novel BLBLI combinations, including ceftazidime-avibactam, meropenem-vaborbactam and imipenem-relebactam (53,122,138,139).

Reduced membrane permeability

As indicated above, porin modifications contribute to the emergence of resistance against novel BLBLIs when combined with other resistance mechanisms (122,134,137,140,141). In **paper IV,** we observed that porin loss (knockouts of *ompK35* and *ompK36*) resulted in a considerable decrease in susceptibility to β-lactams in combination with avibactam. Furthermore, a significantly higher concentration of avibactam was required to achieve maximum inhibition in strains with porin loss compared to strains with intact porins, suggesting that these porins play a role in the entry of avibactam. However, avibactam has also been shown to diffuse through other porin channels, e.g., OmpK37 and PhoE (142,143). While many β-lactamase inhibitors share some structural similarities with β-lactam antibiotics, they are generally not considered major substrates for efflux pumps such as AcrB-TolC. However, if the β-lactam partner is more efficiently expelled from the cell, efflux may subsequently contribute to reduced susceptibility to BLBLI combinations. For example, efflux and changes in permeability are important drivers of resistance to meropenem-vaborbactam and imipenem-relebactam, whereas efflux seems to play a small role in emergence of resistance towards ceftazidime-avibactam (114,143).

Modification of PBP

Most β-lactamase inhibitors have low affinity for PBPs and limited antibacterial activity alone, with some exceptions (51). Yet mutations in PBP can change its affinity for the β-lactam antibiotic and may, therefore, be associated with reduced susceptibility to BLBLI combinations. A specific insertion in PBP3 has been associated with reduced susceptibility towards ceftazidime-avibactam in *E. coli*; however, this is attributed to the fact that PBP3 is a primary target for ceftazidime (113,144). Interestingly, multiple mutations in different PBPs emerged during short-term evolution experiments with ceftazidime-avibactam, as outlined in **paper I**. Further investigation is required to comprehend the impact of these mutations on resistance.

Multidrug resistance in β-lactamase producers

β-lactamase-producing *Enterobacterales* typically display resistance towards several antibiotic classes, thereby limiting treatment options (80). Through the action of mobile genetic elements can wide arrays of resistance genes be captured, accumulated, and disseminated among bacteria, resulting in the emergence of multidrug-resistant pathogens. Plasmids found in clinical settings often harbour numerous resistance genes (145,146). This phenomenon can be exemplified by the pUUH239.2 plasmid included in **paper I**. This multidrug resistance plasmid was isolated from a nosocomial outbreak of a *K. pneumoniae* clone at the Uppsala University Hospital (UUH), Uppsala, Sweden, in May 2005 (147). The pUUH239.2 plasmid encodes genes conferring resistance to macrolides [*mphR(A)*, *mrx* and *mph(A)*], trimethoprim (*dhfrXII*), β-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$), sulphonamides (*sul1*), aminoglycosides (*aadA2*), aminoglycosides/fluoroquinolones (low level, *aac(6')-1b-cr*) and tetracycline (*tetA* and *tetR*) (148).

Furthermore, 82% of the 51 clinical carbapenemase-producing *E. coli* and *K. pneumoniae* isolates studied in **paper II** carried acquired resistance genes towards three or more other antibiotic classes. Carbapenem-resistant strains typically carry resistance mechanisms to standard antibiotics such as fluoroquinolones and trimethoprim-sulfamethoxazole but typically retain *in vitro* susceptibility to older drugs such as polymyxin antibiotics (55). However, polymyxin resistance is also emerging, resulting in clinical failure (149,150). Colistin resistance among carbapenem-resistant isolates has developed rapidly in hospitals and countries with high use of colistin (151). This has raised concerns regarding the emergence of polymyxin resistance when used as monotherapy (65). Moreover, resistance is frequently reported *in vitro* (152–155). In **paper III,** we observed resistance emergence in KPC-2-producing *K. pneumoniae* at 16 hours following single-drug exposure to colistin at 1 mg/L (corresponding to free concentration in plasma at steady state). Further investigation is required to elucidate the mechanism of resistance in our study. The most common resistance mechanism in *Enterobacterales* is modification of the LPS structure, resulting in impaired polymyxin binding. Adding cationic groups to lipid A increases the charge of the LPS, thereby reducing the affinity of polymyxins for the outer membrane. Specific mutations in the regulatory components that result in constitutive activation and upregulation of LPS modification have been reported to cause polymyxin resistance in *K. pneumoniae* and *E. coli* (66). The identification of transferable plasmid-mediated colistin resistance genes (*mcr*) has further increased the risk of colistin resistance dissemination among bacteria (66,156).

Antibiotic combination therapy

Antibiotic combination therapies are employed in clinical practice to enhance the activity of existing antibiotics. Of note, BLBLI combinations are considered monotherapy since they are only available for treatment as one substance. The purpose of using antibiotic combination therapy can be to broaden the antibacterial spectrum, to achieve additive or synergistic effects, and to lower the risk of emergence of resistance during treatment (157,158). Clinical studies have shown better clinical outcomes with combination therapy as compared to monotherapy in severe infections caused by carbapenem-resistant *Enterobacterales* (159–162). Nevertheless, recommendations for particular drug combinations are typically unavailable due to a lack of clinical data (45,55). Also, the quality of evidence is low as it is based almost exclusively on observational studies with small sample sizes and unadjusted analyses. Moreover, the definition of "combination therapy" differs among studies, including the number of antibiotics used, treatment regimens, dosing and durations, and the phenotypic and genotypic characterisation of causative pathogens is usually limited or absent (45,55).

Drugs that have been avoided in the past are being reintroduced as part of combination regimens due to their *in vitro* activity against multidrug-resistant pathogens. Considering that the older antibiotics such as polymyxins, aminoglycosides, fosfomycin and tigecycline have uncertain efficacy in severe CRE infections, it is often recommended that they be used in combination with another drug that exhibits *in vitro* activity. This approach is generally advised when CRE strains are susceptible *in vitro* solely to polymyxins, aminoglycosides, tigecycline, or fosfomycin or when BLBLIs are unavailable. However, specific recommendations for particular drug combinations are typically not available (45,55).

Combination therapy including β-lactam antibiotics

Combination therapy that includes β-lactam antibiotics, either as a double combination or in combination with other antibiotics, has been suggested even though their activity may be affected by enzymatic activity (163–165). An advantage of this approach is that the β-lactam antibiotics are safe and well-tolerated compared to other treatment options such as aminoglycosides and polymyxins. Antibiotic combination treatments explored in this thesis are described below.

Double-carbapenem therapy

Double-carbapenem combination therapy involves using two different carbapenem antibiotics to enhance treatment efficacy (166). While some observational studies have shown promise with double-carbapenem therapy, demonstrating improved outcomes for patients with severe infections, these

studies have important limitations with regard to size and bias (45,165,166). In contrast, a recent observational study reported that double-carbapenem therapy was not superior to single-carbapenem regimens with regard to mortality (167). Moreover, *in vitro* data on the synergistic effects of double-carbapenem combinations are conflicting (168–171). The variable results between the studies may partly be due to strain-dependent factors. Clinical carbapenemase-producing isolates typically carry several resistance mechanisms, which may influence susceptibility and the ability of a combination to act synergistically. Most previous studies on double-carbapenem treatment have focused primarily on KPC-producing *K. pneumoniae*, whereas data on *K. pneumoniae* producing other carbapenemases and *E. coli* are scarce.

Double-carbapenem combination treatment most often includes ertapenem alongside either meropenem or doripenem (166). The rationale behind the double-carbapenem approach is based on a higher affinity of ertapenem for carbapenemase enzymes and the hypothesis that ertapenem would serve as a "suicide drug", allowing the second carbapenem to exert its antibacterial effects (168,172). However, our findings in **paper II** did not support this notion as we did not observe better overall *in vitro* effects with ertapenem-containing combinations compared to meropenem with doripenem. In fact, since the hydrolysis of ertapenem is fast and the enzyme is not permanently inhibited (173–175), it is arguable whether an ertapenem-containing combination would be superior to increasing the exposure of a more stable drug, such as meropenem. In **paper II,** we found that the *in vitro* antibacterial effect with two-drug carbapenem combinations was often similar to the effect observed when doubling the concentration of either of the single drugs

β-lactam/β-lactamase inhibitors in combination with other drugs

Monotherapy is recommended for BLBLI combinations like ceftazidime-avibactam for CRE (45,54). Yet, ceftazidime-avibactam is often prescribed alongside other antibiotics, such as polymyxins, aminoglycosides or tigecycline (45,54,157,176). Moreover, the emergence of resistance during treatment with ceftazidime-avibactam has been reported (177–180). Resistance during treatment ranged between approximately 2 and 10%. To date, there is limited data on the use of combination therapy with ceftazidime-avibactam to improve treatment effectiveness and limit the emergence of resistance during treatment (176,181). Moreover, *in vitro* studies show conflicting results (182–185) regarding synergistic potential and prevention of resistance, possibly due to differences in susceptibility and resistance mechanisms in the tested strains. Polymyxins (polymyxin B and colistin) are widely applied in combination therapy against severe CRE infections (45,54). Polymyxin antibiotics do have a bactericidal effect on their own by disrupting the outer membrane of Gram-negative bacteria, but they may also potentiate the activity of other drugs by increasing their entry into the cell when given in combination (65).

Considering that reduced membrane permeability is associated with decreased susceptibility towards BLBLI combinations (as described earlier), adding a polymyxin antibiotic could be beneficial to increase the periplasmic concentration of BLBLIs in strains with major porin alterations. In **paper III,** we showed an improved *in vitro* effect with adding colistin to ceftazidime-avibactam against a clinical KPC-2-producing *K. pneumoniae* strain with porin deficiency. However, when systematically assessing the impact of porin loss on the synergistic potential of colistin in combination with BLBLIs in **paper IV**, we did not find any clear association between porin loss and synergy. Further investigations are required to elucidate other factors that may come into play in clinical isolates where synergy is observed.

In vitro methods to study antibiotic combination effects

Drug combination effects are typically categorized as synergistic, additive, or antagonistic, according to whether the combined effect is stronger than, equal to or weaker than the sum of the individual activities (186). However, it is important to acknowledge that the use and interpretation of this terminology are dependent on the context and require clear definitions (158,187,188). At the time of writing, *in vitro* susceptibility testing of antibiotic combinations is not part of routine diagnostics in clinical microbiology laboratories. Hence, there is no standardized evaluation of antibiotic combination effects in relation to clinical breakpoints. Nevertheless, there are some well-established and widely used *in vitro* methods aiming to assess whether a combination exhibits an enhanced effect and improved bacterial killing compared to each drug alone (188). The most commonly used *in vitro* methods include the checkerboard assay and time-kill experiments (158,188,189). Despite the widespread use of these methods, systematic assessments of the correlation between *in vitro* data on combination effects and clinical outcomes remain scarce. The existing clinical studies show conflicting results and are not easily compared due to their observation design, small sample sizes and variability in patient populations, infection site, treatments and pathogens (189–191). Randomised controlled trials are warranted but are very difficult to perform. In the absence of clinical evidence*, in vitro* data can be useful to guide future *in vivo* studies. *In vitro* methods relevant to the scope of the thesis are summarised below.

Checkerboard assay

The checkerboard assay builds on the conventional MIC determination with broth microdilution, whereby bacterial growth is read by eye in liquid culture against a series of dilutions (generally 2-fold) of antibiotics. By varying the concentration of both drugs in a checkerboard pattern in the microtiter plate, the susceptibility (MIC) to each drug alone and in combination can be assessed (188,192). The combination effects can be categorized by using The Fractional Inhibitory Concentration Index (FICI). The FICI is determined as

follows: FICI= (MIC of drug A in combination / MIC of drug A alone) + (MIC of drug B in combination / MIC of drug B alone). Because of the recognized limitations of the accuracy of the broth dilution method of a 2-fold dilution above and below the MIC, an FICI of ≤ 0.5 is considered synergistic, an FICI ranging from > 0.5 to ≤ 4 is considered indifferent, and an FICI value of > 4.0 is considered antagonistic (192). The advantage of the checkerboard and the FICI is the simplicity. Nevertheless, it is a quite crude measure limited by visual interpretations of MIC at a single time point (24 hours) (193).

Automated time-lapse microscopy

Our research group have previously evaluated the use of automated time-lapse microscopy (oCelloScope, Philips BioCell A/S, Allerød, Denmark) to detect bacterial growth and to screen for antibiotic combinations against multidrug-resistant Gram-negative bacteria (194,195). The instrument generates a series of images of the wells in a 96-well plate at a predetermined time interval. These images are then processed and translated into bacterial growth curves using growth kinetics analysis (GKA) algorithms. The GKA algorithms are based on two parameters: Background Corrected Absorption (BCA), which measures the intensity of dark objects and Segmentation and Extraction of Surface Area (SESA), which measures contrast against the background. A BCA value > 8 and a maximum SESA value ($SESA_{max}$) > 5.8 are used as cut-off values to indicate a bacterial density of $>10^6$ colony-forming units per millilitre (CFU/mL). If BCA and $SESA_{max}$ were below these cut-offs with a combination but not with any of the constituent single antibiotics at the same concentration, the combination is considered to exhibit an enhanced effect (194–197). Based on the information provided by the screening, we can move forward with interesting combinations in time-kill experiments, which provide more precise data on bacterial density and reductions in CFU/mL.

Static time-kill

Time-kill experiments are generally considered the standard method for testing antibiotic combination effects *in vitro* (188). In 1999, the National Committee on Clinical Laboratory Standards (NCCLS), now known as CLSI (Clinical and Laboratory Standards Institute), provided a standardized protocol for time-kill assays (189). In short, the activity of the drugs is tested alone or in combination against a standardized bacterial inoculum (~ 6 $\log_{10}$ CFU/mL) in liquid by multiple sampling during, usually, a 24-hour time period. The viable cell count is determined at each time point and plotted against time. Synergy is then defined as ≥ 2 $\log_{10}$ decrease in CFU/mL with the combination compared to the most effective single drug. A bactericidal effect is defined as a ≥ 3 $\log_{10}$ reduction in CFU/mL (99.9% killing) compared to the starting inoculum. The advantages of the time-kill method are that it allows for the observation of changes in bacterial concentration over time following antibiotic exposure and has a low detection limit (1 $\log_{10}$ CFU/mL). However, time-kill

experiments are labour-intensive and time-consuming, limiting the number of concentrations that can be tested simultaneously (189,198).

Dynamic time-kill

In static time-kill experiments, antibiotic activity is assessed using just one initial dose, after which the antibiotic concentrations continuously decrease throughout the course of the experiment. Dynamic *in vitro* models can be used to conduct time-kill experiments with antibiotic concentrations mimicking patient pharmacokinetics. In this model, bacteria are exposed to dynamic drug concentrations generated by a continuous flow of growth medium through the model. By modulating the dosing times and the flow of medium through the system, the dynamic drug concentrations are tailored to reflect the dosing regimens given to patients. Bacterial concentrations are determined through continuous sampling and determination of viable cell count (199). The same definitions for synergy and bactericidal effect as in static time-kill experiments are typically used in dynamic time-kill studies when assessing the efficacy of antibiotic combinations (200). This approach, while more time-consuming and costly, brings us closer to the clinical situation due to the use of dynamic drug concentrations. It also enables prolonged experiments spanning several days, providing detailed information on bacterial killing, regrowth, and delayed emergence of resistance (199,201).

An in-house dynamic *in vitro* model has been set up and evaluated for studying antibiotic combination therapy against Gram-negative bacteria (202). This model is based on an *in vitro* kinetic model developed by Löwdin et al. (203). In short, a peristaltic pump is used to withdraw media from the bacterial compartment and incoming media is drawn into the compartment by negative pressure. Filters retain the bacteria in the compartment. The calculation of flow rates in the model is based on the compound with the shortest half-life (compensations can be made for the other drugs in reservoir media).

Current investigations

In the projects included here, we explore combination treatments aiming to enhance the activity of existing β-lactam antibiotics. In **paper I,** we investigate the resistance mechanisms against three different BLBLIs against bacteria with several different β-lactamases. In **paper II**, we evaluate the antibacterial activity of double-carbapenem combinations against carbapenemase-producing *E. coli* and *K. pneumoniae*. In **papers III** and **IV,** we evaluate the antibacterial activity of colistin in combination with BLBLIs against *K. pneumoniae* and the impact of porin loss on the synergistic potential with this combination. The main objectives are to assess how genetic determinants for β-lactam resistance affect the activity of combinations and subsequently assess which resistance profiles can be circumvented with different combinations of drugs.

Paper I

Evolutionary trajectories towards high-level β-lactam/β-lactamase inhibitor resistance in the presence of multiple β-lactamases

Several studies have investigated the mechanisms behind resistance towards different β-lactam/β-lactamase inhibitor (BLBLI) combinations (115,135,136) but the major focus often lies with just one type of β-lactamase. Clinical isolates often encode multiple β-lactamases (80,204) and therefore, we wanted to study the emergence of resistance to different BLBLI combinations in a strain encoding multiple β-lactamases.

The multidrug-resistance plasmid, named pUUH239.2, was isolated from an outbreak clone of *K. pneumoniae* at the Uppsala University Hospital in Sweden (147,148). The pUUH239.2 plasmid (encoding $bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{CTX-M-15}}$) was conjugated to the well-studied *E. coli* MG1655 (148). In this study, we utilised *E. coli* MG1655 pUUH239.2 to investigate how the evolutionary trajectories of resistance development towards three commonly used BLBLI combinations, ampicillin-sulbactam (SAM), piperacillin-tazobactam (TZP) and ceftazidime-avibactam (CZA), were affected by the simultaneous presence of three different β-lactamases; TEM-1, OXA-1 and CTX-M-15.

The BLBLI combinations are administered to patients in ratios of 2:1 for SAM, 8:1 for TZP and 4:1 for CZA (57). To reflect the antibiotic-inhibitor selection during treatment, the clinical ratios were used to perform *in vitro* selection of mutants with reduced susceptibility to combinations of β-lactam antibiotics and β-lactamase inhibitors.

Gene amplifications of the β-lactamase genes were the main driver for resistance to all three BLBLI combinations in the selection of one-step mutants. This is in accordance with previous studies on resistance development towards BLBLI combinations (116,135,136). The amplification pattern generally differed depending on which antibiotic-inhibitor combination that was used for selection. SAM mutants displayed high-level gene amplifications of $bla_{\text{TEM-1}}$, whereas TZP mutants displayed amplifications of $bla_{\text{OXA-1}}$. In contrast, CZA mutants displayed a mix of gene amplifications of β-lactamases together with mutations in porin-related genes and a single amino acid change in $bla_{\text{CTX-M-15}}$ (G238D). Interestingly, there were instances of collateral sensitivity. For example, one CZA mutant with an 8-fold decrease in susceptibility to CZA (ratio 4:1) exhibited increased susceptibility to piperacillin, SAM and TZP.

Short-term liquid evolutions were performed with increasing concentrations of the different antibiotic-inhibitor combinations. Similarly, as observed for one-step selection, gene amplifications of β-lactamases were identified in mutants evolved in SAM and TZP. The TZP mutants mainly contained amplification of a plasmid region involving $bla_{\text{OXA-1}}$ together with mutations in genes involved in the respiratory chain. The SAM mutants had amplifications of the region including $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$, rather than $bla_{\text{TEM-1}}$ as observed in the one-step mutants. Mutants evolved with CZA displayed a more complex picture. There were amplifications of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-1}}$ together with a high number of mutations in genes associated with the cell wall/membrane, metabolism, replication, and translation. Similarly, as with one-step selection, amino acid substitutions in CTX-M-15 arose during the evolution experiments (N132K, P167T and G238D).

In conclusion, this study demonstrates the impact of different gene amplification patterns, involving one or more β-lactamase genes, on resistance towards specific BLBLI combinations. Clinical resistance can be reached by gene amplifications for SAM and TZP, whereas clinical resistance towards CZA requires several genetic changes combined, such as amplifications, reduced drug influx and/or mutations in CTX-M-15. Our findings illustrate how the presence of multiple β-lactamases shapes the evolutionary trajectories towards resistance to different BLBLI combinations.

Paper II

Evaluation of *in vitro* activity of double-carbapenem combinations against KPC-2-, OXA-48- and NDM-producing *Escherichia coli* and *Klebsiella pneumoniae*

Double-carbapenem combinations have shown synergistic potential against carbapenemase-producing *Enterobacterales* and have been suggested for clinical use. In this study, we evaluated the activity of double-carbapenem combinations against 51 clinical KPC-2-, OXA-48-, NDM-1, and NDM-5-producing *E. coli* and *K. pneumoniae* isolates. Many isolates encoded additional β-lactamases and had inactivating mutations in porin-encoding genes (*ompC*/*ompK36*, *ompF/ompK35*). We also included strains constructed from *E. coli* (ATCC 25922) in which we systematically varied the genetic setup of carbapenemases in an otherwise isogenic background. The *E. coli* constructs are valuable tools to account for the individual contributions of the different carbapenemases. The carbapenemase genes $bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ were inserted into the cryptic chromosomal *bgl* operon using λ-red recombineering. The *bgl* operon is silent during *in vitro* conditions and has previously been deemed an appropriate insertion site (205). The activity of two-drug combinations of ertapenem (ETP), meropenem (MEM), and doripenem (DOR) was evaluated in 24 h time-lapse microscopy screening experiments complemented with cell viability testing (spot assay), and in static time-kill experiments.

Synergistic effects with at least one of the double-carbapenem combinations were most commonly observed for OXA-48-producing strains, whereas the efficacy of the combinations was low against KPC-2 and negligible against NDM producers. This may be attributed to the different hydrolytic capabilities of the different carbapenemases. NDM exhibits highly efficient hydrolytic activity against carbapenems, while OXA-48 exhibits poor hydrolytic activity against carbapenems compared to KPC-2 and especially NDM (206,207). The OXA-48 producers, therefore, generally display higher carbapenem susceptibility. This aligns with the general observation that synergy was more common in carbapenem-susceptible isolates. Synergy rates were higher with all three combinations in clinical *E. coli* isolates susceptible to meropenem and doripenem compared to the resistant isolates ($P \leq 0.0131$). For clinical *K. pneumoniae* isolates, the synergy rate with ertapenem and meropenem was higher in isolates susceptible to meropenem and doripenem ($P \leq 0.0003$). However, no significant association was detected for the other combinations, probably owing to the lower susceptibility rates in *K. pneumoniae*. Because we limited drug concentrations to not exceed the maximum free drug concentration in plasma, the probability of synergy is expected to be lower against clinical isolates with lower susceptibility.

In 24 h static time-kill experiments with constructed *E. coli* strains, synergy was observed at early time points against the KPC-2- (ETP+DOR and MEM+DOR) and OXA-48-producing strains (ETP+MEM and ETP+DOR) at concentrations of 1x MIC. However, bacterial killing was often similar to that of either of the single drugs at 2x MIC. No synergistic activity was observed against the NDM-1-producing construct.

We could not conclude that one double-carbapenem combination was generally superior to another in our data set. Hence, our data does not support the notion that ertapenem is a generally preferred carbapenem in combination treatment due to its high affinity for the carbapenemase enzymes.

In conclusion, our findings suggest that the benefit of double-carbapenem combinations against carbapenemase-producing *E. coli and K. pneumoniae* is limited, especially against isolates resistant to the constituent antibiotics and those that produce NDM.

Paper III

Evaluation of ceftazidime-avibactam in combination with colistin against KPC-2-producing *Klebsiella pneumoniae* with porin deficiency in static and dynamic time-kill experiments

Ceftazidime-avibactam is recommended as a first-line treatment against severe infections caused by carbapenem-resistant *Enterobacterales,* with the exception of MBL-producing strains (45). To date, there is limited data on the use of ceftazidime-avibactam in combination with another antibiotic (176,181). In this study, we evaluated the activity of ceftazidime-avibactam (CAZ-AVI) in combination with colistin (COL) against a clinical KPC-2-producing *K. pneumoniae* strain in static and dynamic time-kill experiments.

The studied strain belonged to ST258 and, in addition to KPC-2, encoded several β-lactamases, including CTX-M-15. Moreover, we found a loss-of-function mutation in OmpK35 caused by a frameshift mutation (E42fs), resulting in a premature stop. In OmpK36, we identified a duplication of a glycine and aspartate pair (G134D135) in loop 3, which has been shown to narrow the porin channel and thereby limit the entry of β-lactams (108,208,209). The premature stop in OmpK35 and the glycine-aspartate duplication in OmpK36 have previously been identified in collections of *K. pneumoniae* ST258 (108,142,208–210). The strain was susceptible to both CAZ-AVI (MIC 8 mg/L; with a fixed concentration of avibactam at 4 mg/L) and COL (MIC 0.5 mg/L). We also determined the susceptibility to CAZ-AVI in a 4:1 concentration ratio (denoted MIC_{ratio}), which is how it is administered to patients. However, there was no significant difference between MIC (8:4 mg/L) and MIC_{ratio} (16:4 mg/L) for this strain.

CAZ-AVI at 8:2 mg/L in combination with COL at 0.25 or 0.5 mg/L showed synergy in static time-kill experiments. We therefore decided to explore this combination further in 32-hour dynamic time-kill experiments. In the dynamic experiment, the pharmacokinetic profile of CAZ-AVI was designed to mimic the free concentration in plasma of a typical pneumonia patient receiving a standard dose (2/0.5g q8h as a 2-hour infusion, corresponding to C_{max} CAZ 70 mg/L, AVI 15 mg/L). COL was added to 1 mg/L corresponding to the free steady-state concentration in plasma. All three treatment regimens (CAZ-AVI, COL, and CAZ-AVI + COL) resulted in substantial bacterial killing following the first dose. While regrowth was observed with CAZ-AVI and COL alone, bacterial growth was undetectable from 10 h onwards during exposure to CAZ-AVI in combination with COL. The combination showed synergistic effects at all time points between 14 and 32 h. Population analysis profiling showed the emergence of resistance to COL during single-drug exposure already at 16 h. The resistant populations had significant (64-fold) MIC increases compared to the original strain. Despite the marked

regrowth during exposure to CAZ-AVI alone, no emergence of resistance was detected in the population analysis profiling.

In conclusion, this study demonstrated synergy with ceftazidime-avibactam and colistin at clinically relevant concentrations against a clinical KPC-2-producing *K. pneumoniae* with porin deficiencies. Our findings illustrate that combination therapy with ceftazidime-avibactam and colistin may be useful when MIC values for ceftazidime-avibactam are very close to the clinical breakpoint (R > 8 mg/L). More research is warranted to understand the mechanisms of synergistic interaction, genotypic-phenotypic associations, and the reasons for regrowth with ceftazidime-avibactam alone.

Paper IV

Impact of porin deficiency on the synergistic potential of colistin in combination with β-lactam/β-lactamase inhibitors against *Klebsiella pneumoniae*

Based on the mechanism of action with colistin, it is often hypothesized that the membrane-disrupting effect of colistin may facilitate the entry of other drugs through the outer membrane (65). In **paper III,** we showed synergistic and bactericidal effects in static and dynamic *in vitro* time-kill experiments with ceftazidime-avibactam (CAZ-AVI) in combination with colistin (COL) against a clinical KPC-2-producing *K. pneumoniae* strain with porin deficiencies. However, other *in vitro* studies show conflicting results regarding the synergistic potential and prevention of resistance in clinical strains with this combination (182–185). This may, in part, be due to differences in susceptibility and resistance mechanisms in the clinical strains studied. In this study, we explored if the loss of porins OmpK35 and OmpK36 influences the synergistic potential of COL in combination with CAZ-AVI or meropenem-avibactam (MEM-AVI) against β-lactamase-producing *K. pneumoniae*.

We constructed strains from *K. pneumoniae* strains (ATCC 35657) in which genes encoding CTX-M-15, KPC-2 or OXA-48 were introduced chromosomally into *galK*, and the porin-encoding genes (*ompK35*, *ompK36*) were either kept intact or knocked-out. Porin loss led to significant elevations in MICs (4 to 129-fold) for β-lactams and BLBLI combinations in the β-lactamase-producing strains compared to the corresponding β-lactamase-producing strains with intact porins. This observation highlights the importance of combined resistance mechanisms. Also, 4-16 times higher avibactam concentrations were required for strains with porin loss in order to inhibit the enzymatic activity enough to reach β-lactam susceptibility levels similar to the parental strain.

CTX-M-15 is recognized for its efficient hydrolysis of ceftazidime but has limited activity against meropenem (84). Conversely, KPC-2 and OXA-48 exhibit weaker hydrolysis of ceftazidime but typically greater efficiency in hydrolysing meropenem (96,211). Consequently, in order to evaluate the potential enhancement of bacterial killing when combined with colistin, avibactam was paired with ceftazidime for strains producing CTX-M-15 and with meropenem for strains producing KPC-2 and OXA-48. The *in vitro* activity of COL combined with CAZ-AVI or MEM-AVI was evaluated by 24-hour time-lapse microscopy screening complemented with cell viability testing (spot assay), and in static time-kill experiments.

In the screening experiments, we used a fixed avibactam concentration (0.125 or 0.5 mg/L) alongside a concentration range for β-lactams and colistin. We observed synergistic effects with the three-drug combination in at least

one case against all strains. Against strains with porin loss producing CTX-M-15 or KPC-2, there were more cases of synergy in total compared to the strains with intact porins. Synergistic effects were most frequently observed against the KPC-2-producing strains and occurred with COL concentrations below the MIC.

In time-kill experiments, combination effects were evaluated with COL concentrations at 0.5x, 1x or 2x MIC and with BLBLI at a concentration of 0.5x MIC_{ratio}. The MIC_{ratio} was determined using concentration ratios of BLBLI. Ceftazidime-avibactam is available for clinical use at a 4:1 ratio (2:0.5 g), which was therefore used in the MIC_{ratio} determination. A 2:1 ratio was selected for meropenem-avibactam based on standard dosing of meropenem (1 g) and avibactam (0.5 g) (57). The combination effects with the three-drug combination were less pronounced in time-kill experiments compared to screening, and synergy was rarely detected. We did not find any clear association between porin loss and synergy. The three-drug combinations were often bactericidal at the higher colistin concentrations (1x or 2x MIC) against both β-lactamase-producing strains with and without porin loss. Yet, the antibacterial effect was typically not superior to colistin alone or in combination with the β-lactam. Of note, there were large variations in the activity of colistin alone in both methods, which influenced the interpretation of the frequency of synergy.

In conclusion, our findings clearly illustrate the impact of porin loss on susceptibility towards avibactam in combination with ceftazidime or meropenem. Although some synergy with colistin was observed in combination with the BLBLIs, no apparent association was found between combination effects and porin loss. More research is needed to better understand the determinants of combination effects with colistin and BLBLI.

Concluding remarks

Antibiotic resistance is increasing among pathogenic bacteria worldwide, but few novel antibacterial agents are reaching the market. To address the shortage of new treatment options, antibiotic combination therapy is employed to enhance the effects of the existing drugs against multidrug-resistant bacteria. Yet, there is a lack of definitive data regarding which antibiotics to combine to achieve the best effect. Previous research has predominantly focused on clinical strains, which often exhibit significant genetic variability, which makes it challenging to pinpoint the determinants of combination effects. By examining the performance of combinations against both clinical and engineered isogenic laboratory strains, we can gain valuable insights into which resistance profiles can be potentially circumvented by specific antibiotic combinations. Such knowledge may facilitate the search for effective combination treatments tailored to the infecting bacteria and guide future clinical studies. In this thesis, we have explored drug combinations aiming to enhance the activity of existing β-lactam antibiotics. We have gained knowledge on resistance mechanisms for β-lactam/β-lactamase inhibitor combinations and genotype-phenotype associations of antibiotic combinations against multidrug-resistant *E. coli* and *K. pneumoniae*. These studies provide insight on the therapeutic potential and limitations of combinations including β-lactam antibiotics against strains with different setups of resistance genes. More research is required to understand how to best use the newly introduced β-lactam/β-lactamase inhibitor combinations to preserve their activity and enhance the value of the available antibiotics for future generations.

Populärvetenskaplig sammanfattning på svenska

Antibiotika används för att behandla pågående bakteriella infektioner men är också nödvändig för flera medicinska ingrepp där patienter löper ökad risk för bakteriella infektioner, såsom kirurgi, cancerbehandling och vård av för tidigt födda barn. Antibiotikaresistens hos sjukdomsframkallande bakterier är ett allvarligt och växande hot mot vår hälsa, då dessa läkemedel blir mindre effektiva. Det mest skrämmande är förekomsten av multiresistenta bakterier. Dessa bakterier är motståndskraftiga mot många av de antibiotika som vi har tillgängliga, vilket gör dem svåra att behandla. Samtidigt som antibiotikaresistens ökar världen över så är det få nya antibiotika som når marknaden. För att bemöta den ökande bristen på behandlingsalternativ vid svåra bakterieinfektioner kombinerar man två eller flera antibiotika med förhoppningen att förstärka behandlingseffekten. Det saknas dock kunskap om vilka antibiotika som bör kombineras för att uppnå bästa effekt. Vi behöver mer kunskap för att vägleda framtida kliniska studier och underlätta sökandet efter effektiva kombinationsbehandlingar skräddarsydda för de infekterande bakterierna. En viktig grupp av antibiotika som har fått ökad uppmärksamhet i detta sammanhang är beta-laktamantibiotika. Dessa antibiotika är effektiva mot en rad olika bakterier, men vissa bakterier har utvecklat förmågan att bryta ner antibiotikan och blir då resistenta. I denna avhandling har vi utforskat antibiotikakombinationer som syftar till att förbättra aktiviteten hos befintliga beta-laktamantibiotika. En strategi är att slå mot resistensmekanismerna som bakterierna har. Här har vi testat att öka genomsläppligheten av antibiotika in i bakteriecellen och att stoppa möjligheten för bakterierna att bryta ner antibiotika. Vi ser att effekterna är beroende av många olika faktorer, inklusive vilka specifika resistensmekanismer bakterierna bär på. Dessa studier ger ökad kunskap om potentialen och begränsningarna hos kombinationer som inkluderar beta-laktamantibiotika mot bakterier med olika resistensuppsättningar. Mer forskning krävs för att förstå hur de kombinationerna bäst kan användas för att bevara deras aktivitet och öka värdet av de tillgängliga antibiotikan för framtida generationer.

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