

# New Structural Templates for Clinically Validated and Novel Targets in Antimicrobial Drug Research and Development

Philipp Klahn and Mark Brönstrup

**Abstract** The development of bacterial resistance against current antibiotic drugs necessitates a continuous renewal of the arsenal of efficacious drugs. This imperative has not been met by the output of antibiotic research and development of the past decades for various reasons, including the declining efforts of large pharma companies in this area. Moreover, the majority of novel antibiotics are chemical derivatives of existing structures that represent mostly step innovations, implying that the available chemical space may be exhausted. This review negates this impression by showcasing recent achievements in lead finding and optimization of antibiotics that have novel or unexplored chemical structures. Not surprisingly, many of the novel structural templates like teixobactins, lysocin, griselimycin, or the albicidin/cystobactamid pair were discovered from natural sources. Additional compounds were obtained from the screening of synthetic libraries and chemical synthesis, including the gyrase-inhibiting NTBI's and spiropyrimidinetrione, the tarocin and targocil inhibitors of wall teichoic acid synthesis, or the boronates and diazabicyclo[3.2.1]octane as novel  $\beta$ -lactamase inhibitors. A motif that is common to most clinically validated antibiotics is that they address hotspots in complex biosynthetic machineries, whose functioning is essential for the bacterial cell. Therefore, an introduction to the biological targets—cell wall synthesis, topoisomerases, the DNA sliding clamp, and membrane-bound electron transport—is given for each of the leads presented here.

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P. Klahn (✉) · M. Brönstrup (✉)  
Department of Chemical Biology, Helmholtz Centre for Infection Research,  
Inhoffenstrasse 7, 38124 Braunschweig, Germany  
e-mail: philipp.klahn@helmholtz-hzi.de

M. Brönstrup  
e-mail: mark.broenstrup@helmholtz-hzi.de

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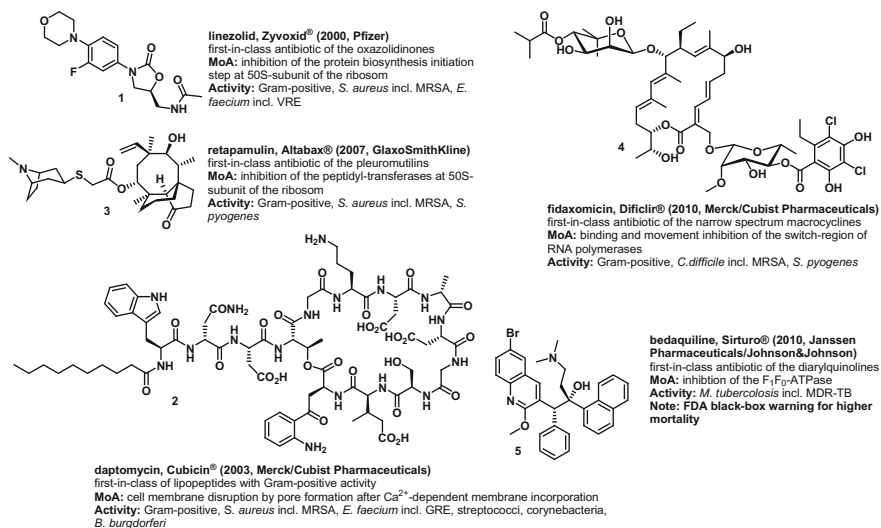
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## 1 Introduction

The “golden era of antibiotic drug discovery” (Davies 2006) commenced with the breakthrough discovery of the sulfonamides and  $\beta$ -lactams in the 1930s and lasted o the mid 1960s, as the majority of the antibiotics used today had been discovered in that period. Since then, the speed of novel antibiotic discovery has become slower. This is expressed by a declining absolute number of launched antibiotics, and by the fact that only few of those addressed a new mode of action (Policy 2010; Boucher et al. 2013; O’Connell et al. 2013; Butler et al. 2013b). Indeed, most antibiotics launched in the past 30 years are chemical analogs of well-established drug classes. It has to be clearly stated that most of such analogs (so-called ‘step-innovations’) confer a medical benefit (Wright et al. 2014), e.g., through enlarged safety windows, improved ADMET properties, circumvented bacterial efflux or degradation systems (Brötz-Oesterheld and Brunner 2008), and sometimes impressive shifts in antibacterial activity from a distinct activity against Gram-positive or Gram-negative bacteria to a broad-spectrum antibiotic activity, as demonstrated by the fourth and fifth generation of cephalosporins, advanced tetracycline, or fluoroquinolone analogs (Wright et al. 2014). However, the evolution of bacterial resistance against these analogs is often faster due to cross resistances (Coates et al. 2011).

Since 2000 five new classes of antibiotics have entered the market (Scheme 1): linezolid (2000), daptomycin (2003), retapamulin (2007), fidaxomicin (2010), and bedaquiline (2012) (Butler et al. 2013b).

These successes are a contribution to improve the innovation gap in antibiotic research and development, but they are clearly insufficient to cover the increasing medical need of a sustainable supply with effective antibiotics (O’Neill 2015). All of these five antibiotics only address infections with Gram-positive pathogens. Furthermore, retapamulin is confined to topical administrations, and fidaxomicin and bedaquiline are narrow spectrum agents that are only approved for treatments of *C. difficile* and MDR-TB, respectively. There remains a particularly strong, unmet need for novel antibiotics effective against Gram-negative pathogens.



**Scheme 1** Antibiotics for the treatment of Gram-positive infections launched since 2000 (*GRE* Glycopeptide-resistant Enterococci, *VRE* Vancomycin-resistant Enterococci, *MRSA* Methicillin-resistant *Staphylococcus aureus*)

Because the lack of innovation (Policy 2010; Boucher et al. 2013; O'Connell et al. 2013) in antibiotic drug research coincides with an increasing occurrence of infections with multi-drug-resistant pathogens (Boucher et al. 2009; Peterson 2009) associated with high mortality and morbidity (Pendleton et al. 2013), there are concerns that the increasing lack of effective therapeutics could develop into a serious threat for public health, or even into a fallback to a so-called pre-antibiotic era (Pidcock 2012; Ventola 2015; Wright 2015b).

The anthropological reasons for this situation are manifold; they include the inadequate clinical use of existing antibiotics (Gilbert 2015; Sanchez and Demain 2015; Shiva 2015), extended misuse of antibiotics in intensive animal husbandry for food production (Bengtsson and Greko 2014; Littmann et al. 2015), and the economically driven exodus of big pharma companies from the antibiotics research field that contributed to the innovation gap mentioned above (Lowther 1979; Powers 2003; Projan 2003; Spellberg et al. 2004; Taubes 2008; Torres 2010; O'Connell et al. 2013).

Beyond these anthropological acceleration forces (Breu et al. 2001; Gillings 2013), we have to accept that bacterial resistance to antibiotics is not a side effect of modern drug therapy, but an inherent part of bacterial evolution to fight for their evolutionary niche with other bacteria and further organisms (Wright 2012; Wright and Poinar 2012; Rodríguez-Rojas et al. 2013).

It has been estimated that bacteria-producing antibacterial metabolites originated at least hundreds of millions of years ago (Baltz 2008; Wright and Poinar 2012). During the evolution of antibacterial metabolites, these bacteria had to intrinsically

coevolve resistance mechanisms for self-preservation (Wright 2012; Wright and Poinar 2012; Perry et al. 2014), since in most cases they probably possess the same biological target for the drug. Therefore, it can be assumed that resistance mechanisms have existed for just as long as the corresponding antibacterial metabolites (O'Connell et al. 2013). This hypothesis is supported by a metagenomic analysis of DNA found in permafrost sediments, which was determined to be 30,000 years old and led to the discovery of genes encoding for resistance to  $\beta$ -lactams, tetracyclines, and glycopeptide antibacterials (D'Costa et al. 2011). This is most probably also true for purely synthetic antibiotics, as nature provides natural product antibiotics directed to nearly every known druggable target in bacteria (Lin et al. 1997; Keller et al. 2007; Johnston et al. 2016). Furthermore, we have to consider that every time an antibiotic is administered there is a significant influence on the resistome (Gillings 2013), which is defined as the collection of all genes in pathogenic and non-pathogenic bacteria that could contribute to a phenotype of antibiotic resistance (Frankel et al. 2006; Wright 2007, 2010; Forsberg et al. 2012; Gillings 2013; Nesme and Simonet 2015). This influence can lead to the evolution of resistance also in non-pathogenic bacteria in the patient or after clearance of the drug into the general environment, i.e., in organisms which have not been originally targeted by the treatment. The resistance genes can easily spread via horizontal gene transfer (HGT) (Syvanen 2012; Baltrus 2013; Polz et al. 2013) by mobile plasmids (Smillie et al. 2010; Harrison and Brockhurst 2012; Carattoli 2013), transposons (Casacuberta and Gonzalez 2013), or outer membrane vesicle (OMV) (Berleman and Auer 2013; Brown et al. 2015; Perez-Cruz et al. 2015; Schwechheimer and Kuehn 2015) transport through the whole pan-genome (Medini et al. 2005; Tettelin et al. 2008; Lapierre and Gogarten 2009) of the global microbiome (Whitman et al. 1998).

These facts imply that in pronounced contrast to other medical indications, the efficacy of antibacterial drugs deteriorates over time. Therefore, the identification of novel antimicrobials, especially with new modes of action (Fischbach and Walsh 2009; Wattal and Goel 2011), is a continuous, necessary task to keep a life-saving headway in the permanent race between bacterial evolution and the protection of human health (Rodríguez-Rojas et al. 2013). In addition, the way antibiotics are handled today should be seriously revised, since studies have shown that smart policies for the prudent use of antibiotics in the clinic and throughout agriculture can make a significant difference in the occurrence and the level of resistance (European Centre for Disease Prevention and Control: Annual Report of the European Antimicrobial Resistance Surveillance Network, EARS-Net 2012).

The threat of multi-drug-resistant pathogens has already been recognized and gained international political attention, leading to several national and international programs and initiatives for the research and development of novel antibiotics (Policy 2010; Boucher et al. 2013; Rex 2014; Bush 2015; Eichberg 2015). At this stage, most of the compounds that have recently been launched or that are currently undergoing phase II/III clinical trials are analogs of existing classes (Pucci and Bush 2013; Hestekamp 2016).

As the marketed classes of antibiotics have been extensively reviewed before (Butler et al. 2013b; Zetts 2014; Paris 2015), we would like to focus this review on the feasibility to discover novel structural templates with antibiotic activity, and to advance such compounds to late-stage pre-clinical as well as clinical development (Boucher et al. 2013; O'Connell et al. 2013; Butler et al. 2013b; Brown et al. 2014; Xu et al. 2014a; Bush 2015; Paris 2015). For this purpose, a brief overview of existing druggable targets and common features among them is given, followed by a review of promising novel scaffolds that address existing as well as novel targets. The presented scaffolds result from a personal and non-comprehensive selection of compounds addressing essential bacterial machineries, i.e., DNA replication, cell wall synthesis, and membrane components.

## 2 Lessons Learned from Druggable Targets in Bacteria

In the over one hundred years of antibiotic research, numerous different bacterial targets and their corresponding interaction with antibiotics have been investigated (Tommasi et al. 2015). As in other indications, the majority of drug-like, optimized compounds did not reach the clinics, as they addressed non-valid targets hampered by an insufficient conservation in the microbial spectrum, unexpected metabolic bypass mechanisms, non-essentiality under *in vivo* conditions or unexpectedly rapid resistance formations (Payne et al. 2007). A special characteristic of antibiotic R&D is the dominance of natural products as the source of approved drugs (Koehn and Carter 2005; Bérdy 2012; Newman and Cragg 2012; Kirst 2013; Bauer and Brönstrup 2014; Butler et al. 2014; Schaefer 2014; Harvey et al. 2015). In fact, the far majority of antibiotics on the market are derived from secondary metabolites of bacteria (Gerth et al. 2003; Clardy et al. 2006; Diez et al. 2012; Schäberle et al. 2014; Elshahawi et al. 2015), marine microorganisms (Blunt et al. 2011), fungi (Schueffler and Anke 2014; Stadler and Hoffmeister 2015), and (in rare cases) plants (Gibbons 2004; Savoia 2012; Upadhyay et al. 2014). This is no surprise, considering that nature provides a huge diversity of structures, which have been evolutionary optimized for binding their biological targets and used by their producers to fight for their ecological niche against competing or harmful microbes. Even though most of the antibacterial natural products need further semi-synthetic optimization to fulfill the ADME (Absorption, Distribution, Metabolism, and Excretion) properties required for a drug applied to humans, nature is still the most effective source for novel antibacterial lead structures (Cragg and Newman 2013; Brown et al. 2014). Natural products have also often disclosed novel, relevant biological targets, which were subsequently addressed by synthetic compounds.

In Table 1, the most important approved antibiotic classes are summarized according to the metabolic pathway they address, their molecular targets, and their distinct mechanism of action. The targets they address share important common

**Table 1** Bacterial targets and approved antimicrobial compounds addressing them (Brötz-Oesterheld and Brunner 2008; Kohanski et al. 2010; Lewis 2013; Butler et al. 2013b; Wright et al. 2014; Bush 2015)

Targeted biological pathway	Molecular target	Class of antibiotics (year of discovery)	Mode of action/mechanism of action	First-in-class (year of introduction)	Approved representatives of the class	Origin	Activity	
Peptidoglycan biosynthesis (PGS)	Penicillin binding proteins (PBPs), transpeptidases	Penicillins (Miller 2002; Zaffiri et al. 2012) (1928)	Inhibition of different transpeptidases	Penicillin (1938)	Ampicillin, oxacillin, methicillin, carbencillin,	Natural	Gram+/ <sup>a</sup>	
		Cephalosporins (Kallman and Barriere 1990; Zaffiri et al. 2012) (1955)		Cephalothin (1964)	Cefbiprole, cefepime, ceftazoxime, ceftazolin, ceftoxin,	Natural	Gram+/ <sup>a</sup>	
		Carbapenems (Singh 2004; Nicolau 2008; Papp-Wallace et al. 2011; Hawkey and Livermore 2012) (1976)		Imipenem (1985)	Doripenem, meropenem, erapenem	Natural	Gram+/ <sup>a</sup>	
		Monobactams (Singh 2004) (1978)		Aztreonam (1986)	Tabtoxin, nocardicin A, tigemonam	Natural	Gram+/ <sup>a</sup>	
DNA replication and transcription	MurA1 and MurA2 and D-Ala-D-Ala ligases.	Fosfomycins (Michalopoulos et al. 2011) (1969)	Blocking the formation of N-acetylmuramic acid	Fosfomycin (1971)	-	Natural	Gram+/ <sup>a</sup>	
		-		Competitive enzyme inhibition	D-cycloserine	-	Natural	<i>M. tuberculosis</i>
		Glycopeptides (Hammes and Neuhaus 1974; Watanakakorn 1984; Williams and Bardsley 1999; Kahne et al. 2005) (1953)		Inhibition of peptidoglycan cross linkage by binding to Acyl-D-Ala-D-Ala dipeptide terminus of Lipid II	Vancomycin (1955)	Teicoplanin, bleomycin, telavancin	Natural	Gram <sup>+</sup>
		Lanthibiotics Type A (*Hart et al. 2016) (1928)		Binding to Lipid II by recognition of the PP unit leading pore formation	Nisin* (2009)	-	Natural	Gram+
Folic acid metabolism	Dihydrofolate reductase (DHFR)	Fluoroquinolones and quinolones (Lipsky and Baker 1999; Olliphant and Green 2002; Aldred et al. 2014; Redgrave et al. 2014) (1961)	Formation of ternary complex with DNA and the Gyra subunit of topoisomerase II or IV	Nalidixic acid (1962)	Norfloxacin, moxifloxacin, gemifloxacin, ciprofloxacin	Synthetic	Gram+/ <sup>a</sup>	
		Aminocoumarines (Hooper et al. 1982; Héride 2014) (1955)		Binding to the ATP binding site (GyrB subunit)	Novobiocin (1965)	Chlorobiocin, coumermycin A	Natural	Gram+
		Benzyl diamino pyrimidines (Hawser et al. 2006; Sharma and Chauthan 2012) (1962)		Inhibition of DHFR (synergistic effects with sulfonamides)	Trimethoprim (1962)	-	Synthetic	Gram+/ <sup>a</sup>
	Dihydropteroate synthetase (DHPS)	Sulfonamides (Henry 1943; Maren 1976; Connor 1998) (1932)	Inhibition of DHPS (strong synergistic effects with benzyl/diamino pyrimidines)	Prontosil (1932) sulfanilamid (1936)	Sulfamethylthiazol, sulfadiazine, sulfamethoxazole, sulfasalazine	Synthetic	Gram+	

(continued)

**Table 1** (continued)

Targeted biological pathway	Molecular target	Class of antibiotics (year of discovery)	Mode of action/mechanism of action	First-in-class (year of introduction)	Approved representatives of the class	Origin	Activity
Protein biosynthesis (PBS)	30S ribosomal subunit	Aminoglycosides (Davis 1987; Kotra et al. 2000) (1943)	Binding to the 16S rRNA near A site of ribosomal 30S subunit, disturbance of proofreading	Streptomycin (1946)	Gentamicin, tobramycin, kanamycin, neomycin	Natural	Gram+/- <sup>a</sup>
		Tetracyclines (Chopra and Roberts 2001; Thaker et al. 2010) (1944)	Binding to 16S rRNA at A site of the ribosomal 30S subunit, blocking of attachment of charged aminoacyl-tRNA	Aureomycin (1948)	Doxycycline, minocycline, tigecycline, eavacycline	Natural	Gram+/- <sup>a</sup>
	50S ribosomal subunit	Lincosamides (Giguère 2013) (1963)	Binding to domain V of 23S rRNA in PTC of ribosomal 50S subunit at A and P site, peptidyl transferase reaction blocked	Clindamycin (1967)	Priflmycin, lincomycin	Natural	Gram+, anaerobic bacteria
	50S ribosomal subunit	Chloramphenicols (Yunis 1988) (1946)	Binding to A2451 and A2452 residues in the 23S rRNA of the 50S subunit	Chloramphenicol (1948)	Thiamphenicol	Natural	Gram+/- <sup>a</sup>
	Macrolides (Kamman and Mankin 2011) (1948)		Binding in the NPET close to PTC, blocking of elongation	Erythromycin (1951)	Clarithromycin, azithromycin, telithromycin, solithromycin	Natural	Gram+/- <sup>a</sup>
	Streptogramins (Giguère 2013; Mast and Wohleben 2014) group A (1963) Macrolactones		Binding to a tight pocket within the PTC (synergistic effects with group B)	Pristinamycin IIA (1998)	Dalfopristin	Natural	Gram+
	Streptogramins (Giguère 2013; Mast and Wohleben 2014) group B (1952) cyclic hexapeptides		Binding to the 23S rRNA within NPET (synergistic effects with group A)	Virginiamycin S1	Quinuapristin		
	Oxazolidinones (Bozhogian and Appelbaum 2004) (1955)		Binding to domain V of 23S rRNA in PTC of the ribosomal 50S subunit, initiation blocked	Linezolid (2000)	Radezolid, tedizolid	Synthetic	Gram+
	Pleuromutilins (Novak 2011; Giguère 2013) (1950)		Inhibition of peptidyl transferase at 50S ribosomal subunit	Repamulin (2007)	-	Natural	Gram+
	Fusidanes (Godfredsen et al. 1962; ZHao et al. 2013) (1962)		Binding to EF-G at the interface between domains G, II and IV, release of EF-G/GDP complex blocked	Fusidic acid (1968)	-	Natural	Gram+

(continued)

Table 1 (continued)

Targeted biological pathway	Molecular target	Class of antibiotics (year of discovery)	Mode of action/mechanism of action	First-in-class (year of introduction)	Approved representatives of the class	Origin	Activity
DNA transcription	RNA polymerase (RNAP)	Ansamycins (Floss and Yu 2005) (1957)	Binding to $\beta$ -subunit of RNAP and blocking the path of the growing RNA chain	Rifampicin (1971)	Rifampin, rifapentine	Natural	Gram+, <i>M. tuberculosis</i>
Cell membrane Biogenesis and functionality	Lipopolysaccharid (LPS) of outer membrane Cell membrane	Macrocyclines (Mullane and Gorbach 2011; Scott 2013; Butler et al. 2013b; Zhanel et al. 2015) (1948) Polypeptides (Yahav et al. 2011; Biswas et al. 2012) (1959)	Binding to the switch region of RNAP, movement inhibition Binding to LPS leads to electrostatic interaction and displacement of divalent cations	Fidaxomicin (2010)	–	Natural	Gram+
DNA integrity	Anaerobic electron transport proteins	Lipopeptides (Anderson et al. 2012; Butler et al. 2013b; Epaand et al. 2016) (1986) Nitroimidazoles (Edwards 1993; Mital 2009) (1955)	$\text{Ca}^{2+}$ -dependent incorporation to the lipid bilayer and oligomerization leads to pore formation Reactive nitroso radicals in vivo, (prodrugs)	Daptomycin (2003)	–	Natural	Gram+
Enzyme functionality	Cysteine-containing proteins	Arsenicals (Zalifri et al. 2012) (1905)	Oxidation to arsenic oxide in vivo, which crosslink sulphydryl groups of proteins	Azomycin (1955)	Metronidazol, tinidazol, nimorazol, dimetridazol, megazol, ornidazol	Natural/synthetic	<i>T. vaginalis</i> , Anaerobic Gram+/– <sup>a</sup>
Energy supply	F <sub>1</sub> F <sub>0</sub> -ATPase	Diarylquinolines (Cole and Riccardi 2011; Butler et al. 2013b) (1997)	Inhibition of ATP synthesis	Salvarsan (1911)	Neosalvarsan, solusalvarsan	Synthetic	Gram+/– <sup>a</sup>
				Bedaquiline (2012)	–	Synthetic	<i>M. tuberculosis</i> <sup>b</sup>

<sup>a</sup>Broad spectrum activity<sup>b</sup>Narrow spectrum activity<sup>c</sup>FDA approved as additive for food use

PTC Peptidyl transferase catalytic centre, NPET Nascent peptide exit tunnel, PP Pyrophosphate



features, i.e., essentiality for the survival of the pathogen, an evolutionary conservation in a variety of pathogens, and a high evolutionary distance to the mammalian counterparts (Brötz-Oesterhelt and Brunner 2008). Antibiotics mainly applied in monotherapies such as  $\beta$ -lactams, glycopeptides, or fluoroquinolones show additional common characteristics: The most successful antibiotics all interact with large biological structures consisting of multi-enzyme complexes like the protein or cell wall biosynthesis machinery, whose impairment has drastic consequences for the bacterial cell that are hard to repair or compensate (Table 1) (Silver 2007; Brötz-Oesterhelt and Brunner 2008). In contrast, the few antibiotics targeting single enzymes such as sulfonamides and benzyl diaminopyrimidines have to be applied in combination therapies to avoid rapid resistance.

Furthermore, it is noticeable that there are fewer antibiotics available for the treatment of infections caused by Gram-negative pathogens compared to Gram-positive ones. The main reason for that issue is the difficulty for antibiotics to cross the outer membrane of Gram-negative bacteria (Zgurskaya et al. 2015). The surface of the outer membrane of the Gram-negative bilayer is covered with lipopolysaccharides (LPS), lipidic structures embedded and anchored into the bilayer that consist of three, covalently connected structural elements: A proximal hydrophobic lipid A, a core oligosaccharide region, and a distal O-antigen polysaccharide (Cohen 2011). It has been shown that LPS molecules interact with each other on the cell surface through divalent cations, thereby forming a permeability barrier (Nikaido 2003; Zhang et al. 2013). Due to the embedded cations, hydrophobic molecules have been shown to partition poorly into LPS and to permeate across the outer membrane bilayer with extremely low rates. Therefore, the LPS-containing bilayer serves as an efficient barrier for diffusion-mediated uptake of many chemical scaffolds known to be active against Gram-positive bacteria (Zhang et al. 2013). The uptake of most antibiotics into Gram-negative bacteria is mediated by porins,  $\beta$ -barrel-shaped proteins that act as a pore through which small, polar molecules can diffuse. Because the physicochemical constraints for porin diffusion are hardly met by existing compound collections designed for high affinity binding of canonical eukaryotic targets and for assuring oral bioavailability, attempts to discover novel lead structures active against Gram-negative pathogens have been by-and-large unsuccessful (Payne et al. 2007; Tommasi et al. 2015). Thus, the searches for relevant chemical matter (e.g., natural products preoptimized by microorganisms) or for alternative strategies [e.g., active transport machineries of Gram-negative pathogens (Ji et al. 2012; Górska et al. 2014; Mislin and Schalk 2014; Saha et al. 2013; Wang et al. 2014; Johnstone and Nolan 2015; Page 2013; Górska et al. 2014; Mislin and Schalk 2014; Xu et al. 2014b; Zgurskaya et al. 2015)] need to be intensified significantly.

### 3 Lipid II and Lipid III—The Bottlenecks in Peptidoglycan and Wall Teichoic Acid Synthesis

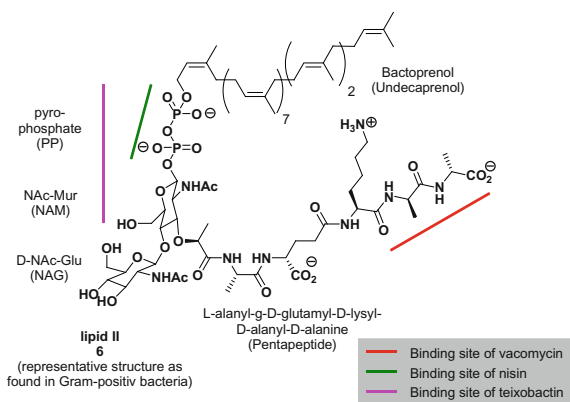
Known, clinically validated targets represent the most promising choice for the development of novel antibiotics, in particular when novel chemical scaffolds that address different binding sites compared to existing drugs break resistance.

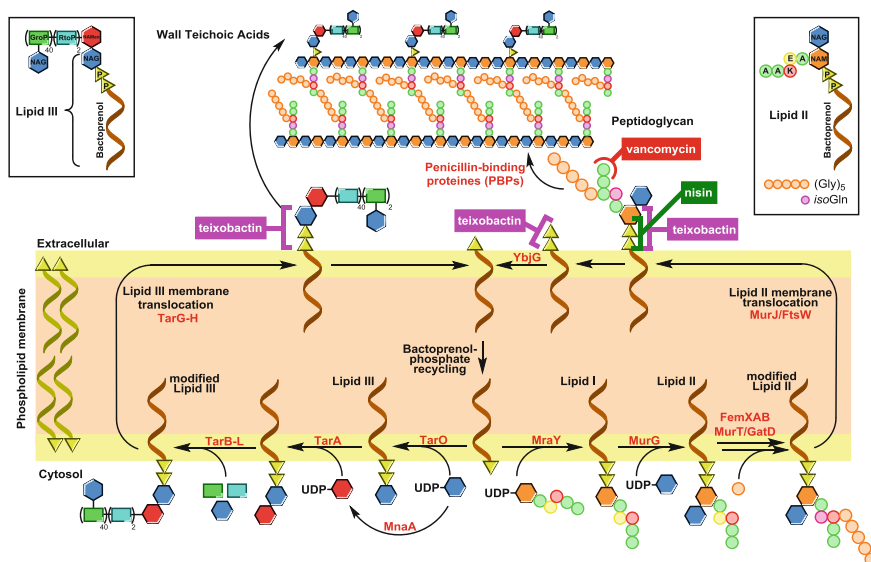
An excellent example for such a target is lipid II **6** (Scheme 2) (Breukink and de Kruijff 2006; de Kruijff et al. 2008), an amphiphilic, membrane-anchored peptidoglycan precursor molecule that is essential for cell membrane functionality.

The cell walls of all bacteria contain a layer of peptidoglycan, a biopolymer of the alternating amino sugars *N*-acetylglucosamine (D-NAc-Glu, NAG), and *N*-acetylmuramic acid (NAc-Mur, NAM), which is modified with pentapeptides of the sequence L-alanyl- $\gamma$ -D-glutamyl-L-lysyl-D-alanyl-D-alanine (in the case of *S. aureus*) that is attached to the 3-hydroxy group of the NAc-Mur sugar (Schwartz et al. 2001; Breukink and de Kruijff 2006; de Kruijff et al. 2008). The cross linkage of the peptides by penicillin-binding proteins (PBPs) to macromolecules confers a structural rigidity and mechanical strength that prevents the bacterial protoplast to burst under the osmotic internal pressure.

The synthesis of lipid II as the precursor monomer of the peptidoglycan layer begins on the cytoplasmic site of the plasma membrane (Scheme 3, right side). *N*-Acetyl-muramic-acid-pentapeptide uridiny pyrophosphate (UDP-NAc-Mur) is attached by the enzyme phosphor-MurNAc-pentapeptide translocase (MraY) (Chung et al. 2013) to bactoprenol phosphate, which is embedded in the phospholipid membrane. The resulting membrane-anchored lipid I is then further glycosylated by the peripherally membrane-associated enzyme MurG (Mohammadi et al. 2007) leading to the formation of lipid II. Next, the FemXAB transpeptidases catalyze the coupling of a pentaglycine-moiety to the D-lysine of the pentapeptide unit and furthermore, the amidation of the  $\gamma$ -D-glutamate of the pentapeptide unit by the MurT/GatD two-enzyme complex leading to the corresponding *D*-isoglutamin (Münch et al. 2012; Zapun et al. 2013). This modified lipid II now contains the

**Scheme 2** Structures of lipid II and binding sides of selected antibiotics





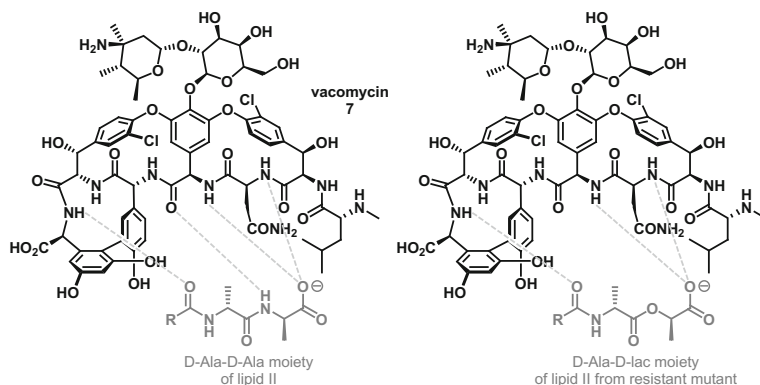
**Scheme 3** Peptidoglycan and wall teichoic acid biosynthesis in Gram-positive bacteria, interactions with selected antibiotics

complete peptidoglycan monomer linked via a pyrophosphate to the bactoprenol membrane anchor, which is translocated through the phospholipid membrane by the MurJ flippase and presented on the extracellular membrane surface (Mohammadi et al. 2011; Butler et al. 2013a; Sham et al. 2014; Meeske et al. 2015).

On the extracellular membrane surface the peptidoglycan precursor is released, incorporated into the growing peptidoglycan layer and crosslinked (Zapun et al. 2013) with each other catalyzed by the PBPs (Sauvage et al. 2008).

The peptidoglycan layer in Gram-positive bacteria is generally around 20–80 nm thick and contains up to 20 sublayers with vast amounts of peptidoglycan subunits, which all have to be translocated through the membrane in the form of the membrane-anchored lipid II after assembly on the cytosolic membrane surface. Since supply with bactoprenol phosphate in bacteria is restricted (Storm and Strominger 1974; Kramer et al. 2004), the amount of lipid II that can be generated is limited. Thus, the lipid II cycle is a highly dynamic process that represents the bottleneck in bacterial cell wall synthesis. Therefore, the lipid II cycle is an ideal target for antibiotics (Breukink and de Kruijff 2006).

The first approved antibiotic targeting lipid II was the glycopeptide vancomycin 7 (Scheme 4). Glycopeptide antibiotics are active against a broad variety of Gram-positive pathogens and act by binding to the D-Ala-D-Ala-dipeptide terminus in lipid II on the extracellular membrane surface via several hydrogen bonds and ionic interactions (Jia et al. 2013; Münch et al. 2015).



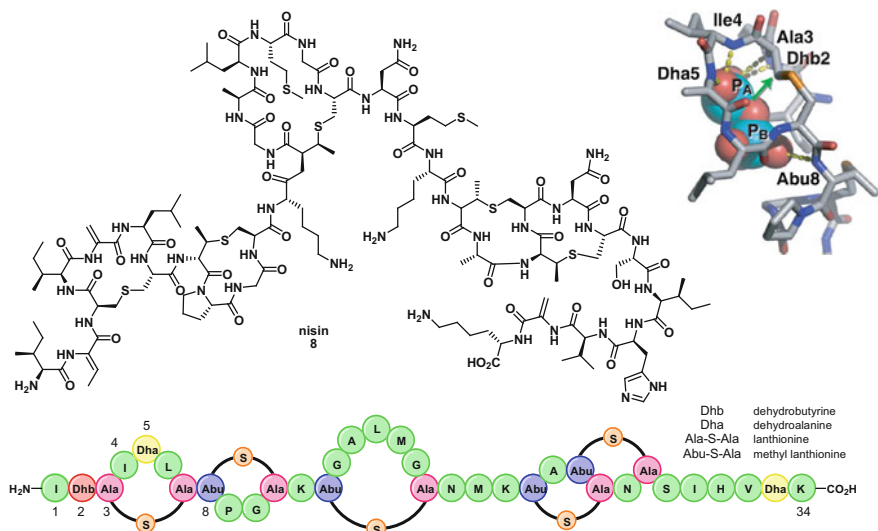
**Scheme 4** Vancomycin and its binding mode to the D-Ala-D-Ala moiety of lipid II

Vancomycin has been for a long time the antibiotic of last resort against Gram-positive pathogens (Williams and Bardsley 1999). Due to the difficulty for bacteria to circumvent binding to an essential metabolite (rather than a protein target), the first significant levels of resistance to vancomycin took almost three decades to occur (Boneca and Chiosis 2003). However, nowadays there is an increasing prevalence of resistance against vancomycin (Williams and Bardsley 1999), which is caused in most cases by the exchange of the terminal D-alanine of the lipid II structure by a D-lactate (Williams and Bardsley 1999; Kahne et al. 2005), which results in the loss of a single hydrogen bond to the D-Ala residue, and a 100-fold drop in binding affinity (Mccomas et al. 2003).

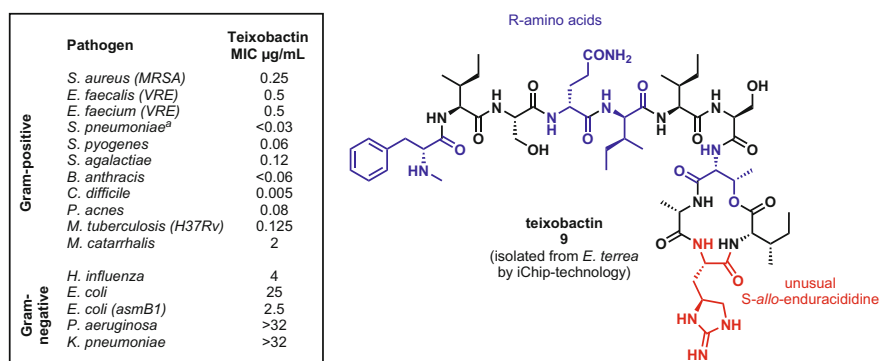
Another well-investigated antibiotic interacting with lipid II is the peptide I antibiotic nisin **8** (Scheme 5). Nisin, obtained by fermentation of *Lactobacillus lactis* on natural substrates such as milk or dextrose, is widely used as a safe food preservation additive (Cleveland et al. 2001), since orally administered nisin is completely degraded by digesting enzymes in the stomach.

Nisin binds to the pyrophosphate moiety of lipid II (Scheme 5) forming a phosphate cage that prevents the release of the peptidoglycan subunits and leads to the formation of a pore in the membrane via inter-membrane assembly of a nisin–lipid II 8:4 complex (Hsu et al. 2004; Bauer and Dicks 2005; Martin and Breukink 2007).

The first isoprene unit and the pyrophosphate in lipid II and the N-terminal part of nisin have been identified as determinants for the nisin–lipid II interaction (Chan et al. 1989; Kuipers et al. 1993; Brötz et al. 1998; Wiedemann et al. 2001). Additionally, several other lipid II interacting compounds are currently under preclinical and clinical investigation such as ramoplanin, mannopeptimycin, lyso-bactin (Lee et al. 2016b), and plusbacin A<sub>3</sub> (Breukink and de Kruijff 2006).



**Scheme 5** Structure and amino acid sequence of nisin and its binding mode to lipid II



**Scheme 6** Structure and antibacterial activity of teixobactin

Recently, a novel lipid II interacting cyclodepsipeptide has been isolated from the Gram-negative  $\beta$ -proteobacterium *Eleftheria terrae* (Ling et al. 2015). Teixobactin 9 (Scheme 6) is an undecapeptide bearing four R-amino acids and seven S-amino acids including the unusual S-allo-enduracididine. The N-terminal R-phenylalanine is mono methylated, and the C-terminus of the molecule is cyclized to the hydroxyl function of a R-threonine side chain in the sequence, forming a 13-membered depsipeptide ring.

Teixobactin was discovered within a screen of so far uncultured soil bacteria by in situ cultivation in diffusion chambers using the so-called iChip technology (Nichols et al. 2010; Piddock 2015). The compound showed highly potent antibacterial activity

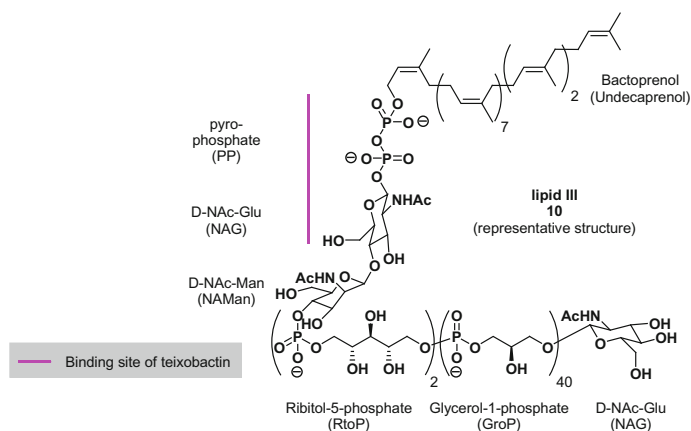
against a broad variety of Gram-positive pathogens including several multi-drug-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* (VRE). Teixobactin was also highly active against *C. difficile* (MIC = 0.005  $\mu\text{g}/\text{mL}$ ) and *M. tuberculosis* (MIC = 0.125  $\mu\text{g}/\text{mL}$ ). The compound was ineffective against Gram-negative bacteria with the exception of *E. coli* (asmB1), a mutant having a defective outer membrane barrier.

In vitro experiments have shown that teixobactin bound under formation of stable 2:1 drug:target complexes to lipid I and lipid II by interaction with the pyrophosphate sugar moiety (Schemes 2 and 3) (Ling et al. 2015). Furthermore, at higher concentrations teixobactin was able to completely inhibit the YbjG-catalyzed mono-dephosphorylation of bactoprenol pyrophosphate, which is an essential step of the bactoprenol phosphate recycling with in the lipid II cycle of peptidoglycan biosynthesis (Scheme 3).

In vivo the binding to lipid II is believed to be the primary effect of teixobactin, since lipid I is not exposed to the extracellular site. Nevertheless, the exact nature of the sugar in the binding motif seems to be less important, since it has been demonstrated that teixobactin efficiently bound lipid III **10** (Scheme 7), bearing a NAc-Glu instead of NAc-Mur attached to the pyrophosphate.

Lipid III is a precursor molecule in the synthesis of wall teichoic acids (WTA). Wall teichoic acids are glycol-phosphopolyol biopolymers that are attached to the NAc-Mur moieties on the surface of the peptidoglycan layer of Gram-positive bacteria (Pereira et al. 2008; Brown et al. 2013), rendering the bacterial surface strongly anionic (Scheme 3).

WTA biosynthesis inhibition is not per se lethal for Gram-positive bacteria. Early-stage WTA inhibition can lead to cells showing enhanced sensitivity to certain drugs (see next chapter) (Sewell and Brown 2014; Lee et al. 2016a). In contrast, late-stage inhibition of membrane-bound WTA precursors is lethal due to accumulation of toxic intermediates and the depletion of cellular pools of

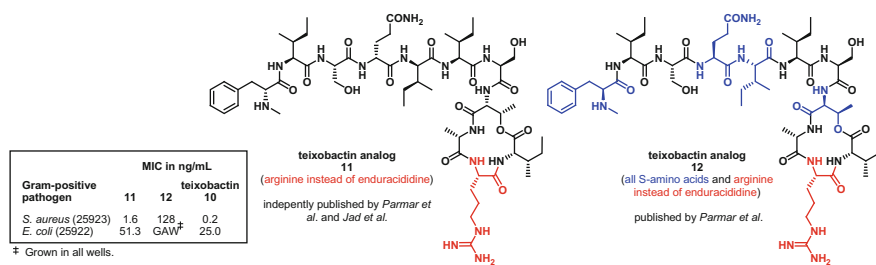


**Scheme 7** Structures of lipid II and binding side of teixobactin

bactoprenol phosphate (D'Elia et al. 2006b; Swoboda et al. 2011; Brown et al. 2013; Wang et al. 2013), which is essential for peptidoglycan biosynthesis as discussed above. Furthermore, it is known that teichoic acids anchor autolysins to prevent uncontrolled hydrolysis of peptidoglycan (Bierbaum and Sahl 1985; Dub  e et al. 2011). Thus, inhibition of WTA synthesis leads to enhanced concentrations of free autolysins, which significantly contributes to the outstanding antibacterial activity of teixobactin. With its binding to lipid II and lipid III, teixobactin is targeting two non-protein lipid structures that are bottlenecks of cell wall synthesis in Gram-positive bacteria (Wright 2015a). It is therefore no surprise that the escape strategies for the pathogens are limited. Whether a resistance mechanism already exists is not known, but so far it was not possible to obtain teixobactin-resistant mutants of *S. aureus* or *M. tuberculosis* at 4x MIC or in serial passaging experiments (Ling et al. 2015). As the teixobactin producer is a Gram-negative bacterium, it is intrinsically resistant against re-entry of the drug due to the impermeability of the outer membrane, and therefore does not encode a resistance mechanism that could be horizontally transferred to Gram-positive species (K  ahrstr  m 2015).

Preliminary pharmacokinetic and pharmacodynamics data from *in vivo* studies in mice were promising (100 % survival rate in a mouse efficacy study). The compound retained its potency in blood serum, showed overall good stability and low toxicity to mammalian cells. Therefore, teixobactin could become a valuable candidate to fight back the antibiotic crisis (Scheme 8).

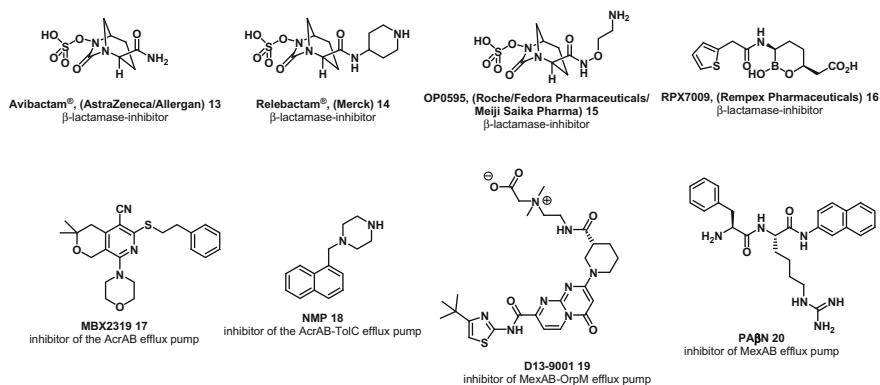
Not surprisingly, the compound has already motivated medicinal chemists to synthesize the first analogs (Jad et al. 2015; Parmar et al. 2016). Two research groups independently published the simple solid-supported synthesis of the teixobactin analog **11** (Scheme 7), where the unusual *S-allo*-enduracididine is substituted by the structurally related, simpler arginine. In a second analog (**12**), the four R-amino acids were additionally substituted by S-amino acids. Antibacterial tests demonstrated that substitution of *S-allo*-enduracididine leads to a 10-fold drop in activity for analog **11**. Furthermore, the R-configuration of several amino acids in the teixobactin sequence seems to be essential for the activity, since analog **12** leads to a 640-fold drop in activity compared to natural teixobactin. Nevertheless, more detailed structure–activity relationship (SAR) studies are needed to determine the exact binding interactions and to further optimize this emerging natural lead structure.



**Scheme 8** Recently published analogs of teixobactin and their antibacterial activity

## 4 Early and Late-Stage WTA Biosynthesis Inhibition— Restoring the Efficacy of $\beta$ -Lactams Against Gram-Positive Pathogens

The predominant resistance mechanisms toward  $\beta$ -lactams in Gram-negative and Gram-positive pathogens are different. Resistance in Gram-negative pathogens normally is achieved via two general mechanisms. One escape strategy is the production of degradation enzymes such as  $\beta$ -lactamases, rendering the antibiotic inactive due to hydrolysis of the  $\beta$ -lactam pharmacophore (Bush 2010; Rawat and Nair 2010). Alternatively, several Gram-negative pathogens overexpress membrane-embedded efflux pumps, which actively transport drugs out of the bacteria (Amaral et al. 2014; Blair et al. 2014). Therefore, considerable efforts have been made to restore the antibacterial activity of  $\beta$ -lactams against Gram-negative pathogens through inhibition of  $\beta$ -lactamases or of efflux pumps. While marketed  $\beta$ -lactamase inhibitors utilize a  $\beta$ -lactam motif themselves for suicide inhibition, chemists have recently identified and optimized novel structural templates: 1,6-diazabicyclo[3.2.1]octane-2-carboxamide-based, reversible  $\beta$ -lactamase inhibitors such as avibactam **13**, relebactam **14**, and OPO595 **15** (Scheme 9) have been advanced to clinical trials as additive in combination therapies with different  $\beta$ -lactam antibiotics (Hirsch et al. 2012; Livermore et al. 2013; Lapuebla et al. 2015a; Livermore et al. 2015; Paris 2015; Toussaint and Gallagher 2015). Avibactam has already been approved for combination therapy with ceftazidime and is in clinical trials for combination therapy with several other  $\beta$ -lactams (Zetts 2014; Paris 2015). Furthermore, RPX7009 **16**, a novel boronic acid-based  $\beta$ -lactamase inhibitor, is currently in phase III clinical trials in combination therapy with meropenem. Notably, some congeners of the boronic acid-based class of compounds also cover metallo- $\beta$ -lactamases like NDM-1 in addition to serine- $\beta$ -lactamases (Hecker et al. 2015; Paris 2015; Lapuebla et al. 2015b). The



**Scheme 9** Structures of advanced  $\beta$ -lactamase inhibitors and efflux pump inhibitors



significant progress achieved on this target class contributes to alleviate the upcoming antibiotic crisis in short to medium term.

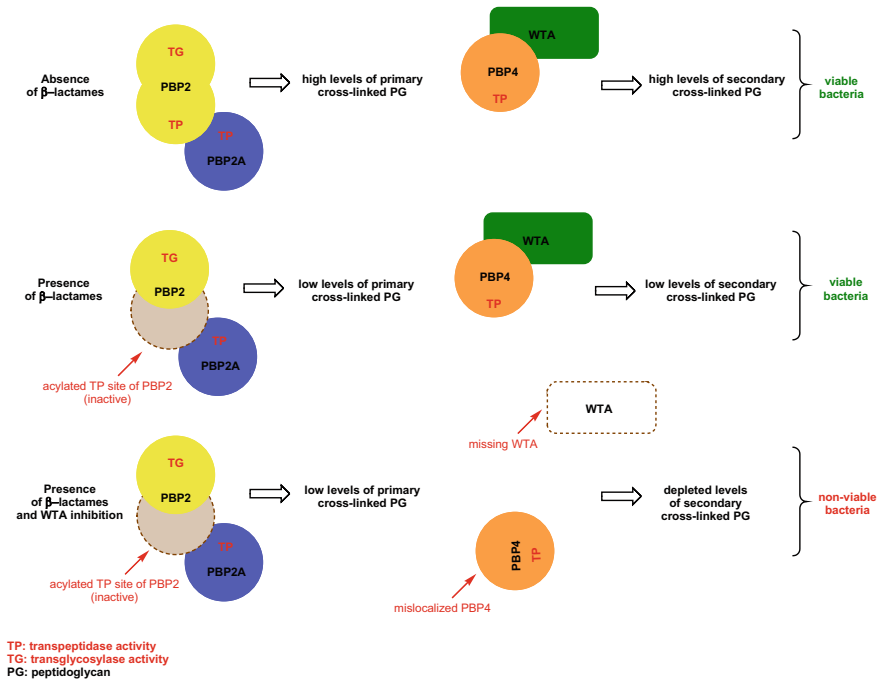
Also, several promising efflux pump inhibitors for the AcrAB-TolC and MexAB-OrpM efflux pumps have been discovered (Scheme 9) (Askoura et al. 2011; Tegos et al. 2011; Opperman and Nguyen 2015; Venter et al. 2015), but so far none of them has entered the clinic.

The predominant resistance mechanism against  $\beta$ -lactams in clinically important Gram-positive pathogens is different, thus requiring a different strategy: It has been shown that resistance in MRSA is achieved by the horizontally acquired penicillin binding protein PBP2A, which has a considerably lower affinity for  $\beta$ -lactam antibiotics compared to the original, bifunctional transglycosylase/transpeptidase PBP2 (Pinho and Errington 2005; Lim and Strynadka 2002).  $\beta$ -Lactams acylate the active site for transpeptidation on PBP2 and thereby inhibit the autonomous localization to the division septum. In MRSA, PBP2A can take over the transpeptidase activity and additionally, act as a structural scaffold to recruit acylated PBP2 to the division septum to just exert its essential transglycosylation function (Pinho et al. 2001; Pinho and Errington 2005). This PBP2/PBP2A cooperation leads to the formation of peptidoglycan strands with low level of crosslinking (Leski and Tomasz 2005; Atilano et al. 2010), which are substrates for PBP4, a secondary transpeptidase that catalyzes the formation of highly crosslinked peptidoglycan (Wyke et al. 1981; Atilano et al. 2010).

It has been demonstrated that the correct localization of PBP4 at the division septum, which is crucial for the resistance mechanism, depends on the presence of wall teichoic acid (WTA) polymers (Scheme 10) (Atilano et al. 2010; Brown et al. 2012). As WTAs are also responsible for the anchoring of autolysins of the peptidoglycan as discussed above, simultaneous inhibition of WTA during the presence of  $\beta$ -lactams leads to depleted levels of crosslinked peptidoglycan and to non-viable bacteria, thus circumventing the resistance mechanism toward  $\beta$ -lactams and restoring their antibacterial activity against Gram-positive bacteria (Maki et al. 1994; Swoboda et al. 2011; Farha et al. 2013; Wang et al. 2013; Sewell and Brown 2014; Lee et al. 2016a).

WTA biosynthesis, as summarized earlier in Scheme 3, is categorized into two distinct groups: The non-essential, WTA early-stage genes *tarO*, *tarA*, and *mnaA*, responsible for attachment of *N*-acetylglucosamine (NAG, D-NAc-Glu) and *N*-acetylmuramic acid (NAC-Mur, NAM) to membrane-anchored bactoprenol phosphate, and the conditionally essential WTA late-stage genes *tarB*, *tarD*, *tarF*, *tarI*, *tarJ*, *tarL*, *tarG*, and *tarH*, responsible for the further assembly of the WTA biopolymers and finally for translocation through the phospholipid membrane to the extracellular environment (Lazarevic and Karamata 1995; Meredith et al. 2008; Pereira et al. 2008; Brown et al. 2013).

As mentioned earlier, the inhibition of late-stage wall teichoic acid biosynthesis steps or deletions of the encoding genes leads to antibacterial effects due to accumulation of toxic intermediates and the depletion of cellular pools of bactoprenol phosphate (D'Elia et al. 2006b; Swoboda et al. 2011; Brown et al. 2013; Wang

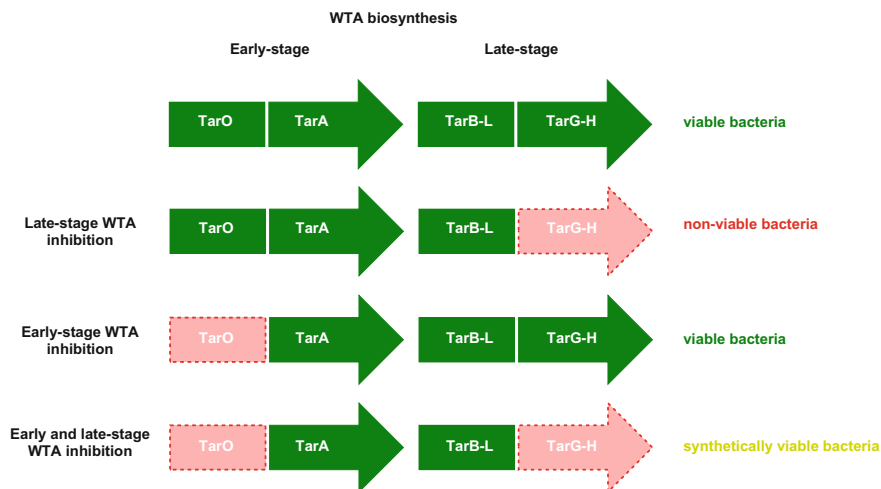


**Scheme 10** Model of  $\beta$ -lactam resistance in MRSA and role of WTA. Adapted from Sewell and Brown (2014)

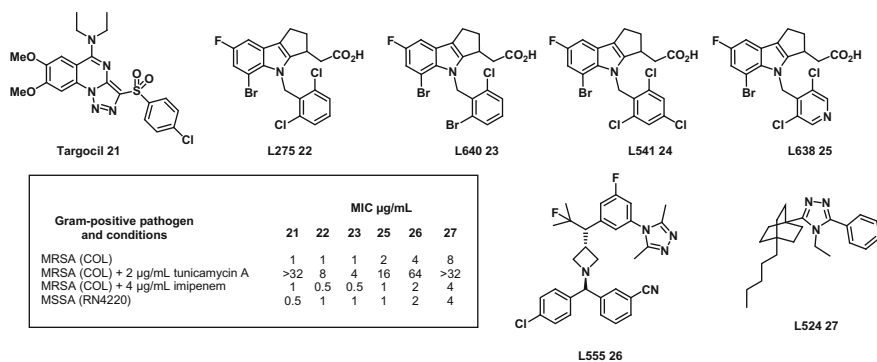
et al. 2013). In contrast, the inhibition of early-stage biosynthesis steps catalyzed by TarO and TarA or deletion of their corresponding genes leads to viable bacteria.

Paradoxically, it was demonstrated that the essentiality of late-stage WTA biosynthesis steps can be suppressed by the concomitant inactivation of early-stage WTA biosynthesis steps. This phenomenon has been referred to as the essential gene paradox (Scheme 11) (D'Elia et al. 2006a, b, 2009a, b; Farha et al. 2013; Sewell and Brown 2014; Lee et al. 2016a). Probably, concomitant inhibition of early-stage steps avoids depletion of the bactoprenol phosphate pool, which seems to be the lethal factor in late-stage WTA inhibition due to its significant effect on peptidoglycan biosynthesis. Bacteria under such early- and late-stage inhibitory pressure are synthetic-viable, but show significantly higher drug sensitivity. The concomitant early-stage inhibition of WTA synthesis, which represents also the major resistance mechanism toward late-stage WTA inhibitors, results in decreased fitness of the bacteria.

The first reported late-stage WTA biosynthesis inhibitor is targocil **21** (Scheme 12), which is a selective TarG inhibitor identified by the high throughput screening (HTS) of a library of 55000 small molecules against wild-type *S. aureus* and a tarO-deletion mutant (Lee et al. 2010; Swoboda et al. 2010). Targocil has been proven to induce significant cell wall stress (Campbell et al. 2012) and



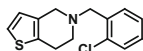
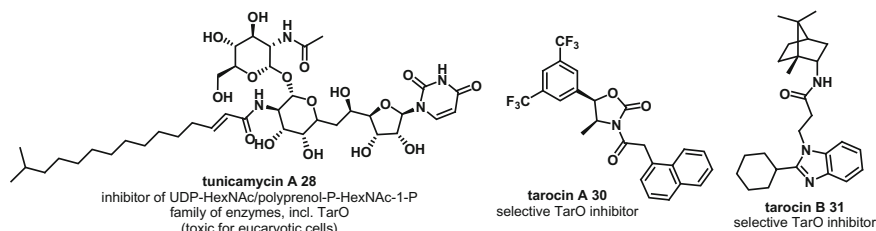
**Scheme 11** Schematic presentation of the essential gene paradox in WTA biosynthesis. Adapted from Sewell and Brown (2014)



**Scheme 12** Structures of reported TarG inhibitors and their antibacterial activities

displayed a submicromolar MIC against MRSA. Targocil treatment came with a high frequency of resistance selection (FOR  $7 \times 10^{-7}$  at  $8 \times \text{MIC}$  (Lee et al. 2010)), which was substantially reduced in co-application with the  $\beta$ -lactam oxacillin, suggesting that the mechanism of resistance is an early-stage WTA biosynthesis gene mutation.

In another screening of a focused library of 20000 small molecules bioactive against *S. aureus* performed by researchers from Merck, six additional TarG inhibitors were identified (Wang et al. 2013). Interestingly, none of these inhibitors was structurally related to targocil. Four of them, L275 22, L640 23, L541 24, and L638 25, contain a tricyclic 1,2,3,4-tetrahydrocyclopenta[b]indole core. Compound

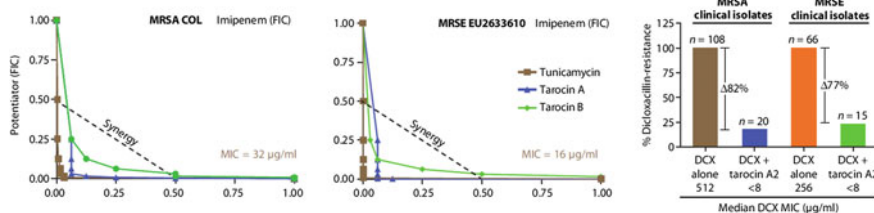


**ticlopidine, Ticlid® (Sanofi) 29**  
adenosin diphosphate receptor inhibitor  
(approved as antiplatelet drug)  
and TarO inhibitor

<i>in vitro</i> <i>S. aureus</i> strain <sup>a</sup>	μg/mL			
	cefuroxime MIC	cefuroxime FIC <sup>b</sup>	29 MIC	29 FIC <sup>b</sup>
MRSA (USA600)	>1024	0.008	>256	0.032
MRSA (USA100/800/NY)	1024	0.125	>256	0.125
MRSA (USA200/EMRSA16)	1024	0.032	>256	0.063
MRSA (EMRSA15)	512	0.063	>256	0.063

[a] hospital-associated isolated, [b] FIC: Fractional inhibitory concentration FIC=[X]/MICX, where [X] is the lowest inhibitory concentration of drug in the presence of a co-drug

Phenotypic assay [μM]	28	30	31
MRSA (COL)	32	>200	>200
TarG inhibitor suppression (EC <sub>50</sub> )	<0.1	26	6
Phage K resistance (EC <sub>50</sub> )	0.1	14	3
Imipenem potentiation (SIC)	<0.1	3	3
Intrinsic bioactivity [μM]			
MSSA (RNA4220)	32	>200	>200
MRSA (COL)	32	>200	>200
MRSE (CLB27217)	16	>200	>200
<i>C. albicans</i> (MY1055)	1.19	>200	>200
<i>H. influenzae</i> (ATCC 49247)	0.595	>200	>200
<i>S. pneumoniae</i> (M86357)	>2.38	>200	>200
<i>S. pyogenes</i> (CL10253)	1.19	>200	>200
<i>M. catarrhalis</i> (ATCC 25238)	>2.38	>200	>200
HeLa (IC <sub>50</sub> )	0.197	>100	>100



**Scheme 13** Structures of reported TarO inhibitors and their antibacterial activities. Adapted from Lee et al. (2016a) and Farha et al. (2013)

L555 **26** is an active member of a large group of screened 4-*N*-aryl-4H-1,2,4-triazoles all depicting two chiral centers. Compound L524 **27** is a 3C-aryl-4H-1,2,4-triazol bearing a bicycle[2.2.2]octane motif. All these hits had submicromolar antibacterial activity against MRSA. Furthermore, combination experiments with the β-lactam imipenem showed clear synergistic effects. In contrast, concomitant inhibition of the early-stage WTA biosynthesis enzyme TarO by addition of the specific inhibitor tunicamycin A **28** (see Scheme 13) led to suppression of TarG inhibition and increased MICs. Since the TarG inhibitory effect of L275, L638, L640, and L555 could not be fully suppressed, it is likely that these compounds display an off-target associated non-specific cellular toxicity or even address secondary targets (Wang et al. 2013). The frequencies of resistance development for compounds L275 **22** (FOR  $<3.6 \times 10^{-8}$  at  $8 \times$  MIC) and L638 **25** (FOR  $1.9 \times 10^{-10}$  at  $8 \times$  MIC) were significantly lower than for targocil.

Beyond tunicamycin A **28**, which displayed selective inhibitory activity for TarO in bacteria, but was cytotoxic for eukaryotic cells due to a promiscuous inhibition of UDP-HexNAc:polyprenol-P HexNAc-1-P family of enzymes including GlcNAc phosphotransferase (Swoboda et al. 2011), the first early-stage WTA biosynthesis inhibitor reported was ticlopidine **29**. Ticlopidine is an adenosine diphosphate receptor inhibitor approved as the antiplatelet drug Ticlid®, which has been identified as a TarO inhibitor in a screening of a library of 2080 previously approved drugs (Farha et al. 2013). Ticlopidine was demonstrated to have strong synergistic antibacterial activity against MRSA in combination with the  $\beta$ -lactam cefuroxime both in vitro and in vivo, while displaying no antibacterial activity when applied alone. In addition, ticlopidine as an approved drug has already optimized ADMET properties, a known side effect profile and established manufacturing processes. Nevertheless, it remains to be clarified whether the adenosine diphosphate receptor inhibition, the primary effect of ticlopidine, can be tolerated under the conditions of antibiotic therapy regimens. Recently, researchers at Merck reported a screen of 2.8 million synthetic small molecules for inhibitors of early-stage WTA biosynthesis in MRSA. Tarocin A **30** and tarocin B **31** (Scheme 13) were identified as compounds that could suppress TarG inhibition with  $IC_{50}$ 's of 26 and 6  $\mu$ M, respectively (Lee et al. 2016a). The compounds were subsequently proven to selectively inhibit TarO. Interestingly, none of them is structurally related to ticlopidine: While tarocin A has an oxazolidin-2-one core, tarocin B consists of a benzimidazol scaffold bearing a bicyclo[2.2.1]heptane side chain.

Both compounds lack any intrinsic growth inhibitory activity against MRSA, MRSE, MSSA, or several other pathogens. In contrast to tunicamycin A, tarocin A and B show no cytotoxic activity against HeLa cells.

Furthermore, it could be demonstrated that both compounds as well as derivatives with improved water solubility (tarocin A1, tarocinA2, structures not shown) were able to restore  $\beta$ -lactam efficacy against MRSA and MRSE, as strong synergistic effects with imipenem and dicloxacillin in vitro and in vivo were observed. For example, panels of 108 different dicloxacillin-resistant MRSA strains and 66 dicloxacillin-resistant MRSE strains had significantly higher susceptibilities against a combination of dicloxacillin and tarocin A2 versus dicloxacillin alone (Lee et al. 2016a). In summary, the inhibition of WTA synthesis represents a novel and innovative strategy to restore the efficacy of  $\beta$ -lactam antibiotics in Gram-positive pathogens; the clinical proof of this concept has yet to be established, though.

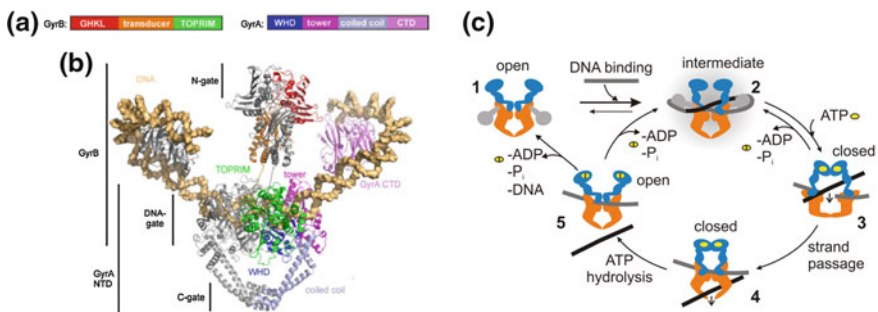
## 5 DNA Gyrase Inhibition—Blocking the Relaxation of Supercoiled Bacterial DNA

Bacterial topoisomerases constitute a target class that has been successfully addressed in antibiotic therapy. Topoisomerases are enzymes controlling the topological state of DNA within bacterial cells and are therefore an integral part of

essential processes such as DNA replication and transcription (Collin et al. 2011; Ehmann and Lahiri 2014). Topoisomerases in general are found in eukaryotic and prokaryotic cells and can be divided into two main types, type I and type II (Schoeffler and Berger 2008). Type I topoisomerases catalyze the relaxation of supercoiled DNA through the transient break of one DNA strand, while type II topoisomerases induce the relaxation through a transient double-strand breakage in an ATP hydrolysis-depending sequence (Liu et al. 1980). All topoisomerases are able to relax supercoiled DNA, but only the DNA gyrase (a type II topoisomerase in bacteria) can also introduce negative supercoils (Bates and Maxwell 2007; Nöllmann et al. 2007; Schoeffler and Berger 2008). There are significant differences between eukaryotic and prokaryotic type II topoisomerases. While eukaryotic type II topoisomerases are homodimers of large single-subunit enzymes, the prokaryotic type II topoisomerases, such as DNA gyrase (or topoisomerase II) and the structurally closely related topoisomerase IV, are  $A_2B_2$  complexes of the subunits GyrA and GyrB (Scheme 14) (Champoux 2001; Wang 2002).

The DNA gyrase has three interfaces that exist in a closed or open conformation: The N-terminal domain of GyrB (N-gate), the GyrA-GyrB-DNA interface presenting tyrosine residues in the active cleaving site (DNA gate) and the C-terminal area of coiled coils (C-gate). The details of the complex mechanism of supercoiling by DNA gyrase are still under investigation (Gubaev and Klostermeier 2014). Nevertheless, the so-called two-gate model (Roca and Wang 1992, 1994; Roca et al. 1996) (Scheme 14c) is strongly supported by biochemical and structural data.

The DNA binds with the G-segment (gate segment) to the gyrase at the interface of the N-terminus of the GyrA dimer and the TOPRIM domain of GyrB. Upon binding of two molecules adenosin triphosphate (ATP) the GyrB subunits dimerize, which closes the N-gate and traps the so-called T-segment (transport segment) of DNA. Then the DNA gyrase transiently cleaves the G-segment via a double-strand breakage of the DNA by the formation of covalent DNA-phosphotyrosyl bonds. The hydrolysis of one ATP molecule leads to the rotation of GyrB, opening of the DNA gate, and the T-segment is transported through the cleaved DNA site. The subsequent religation of the G-segment introduces two negative supercoils into the



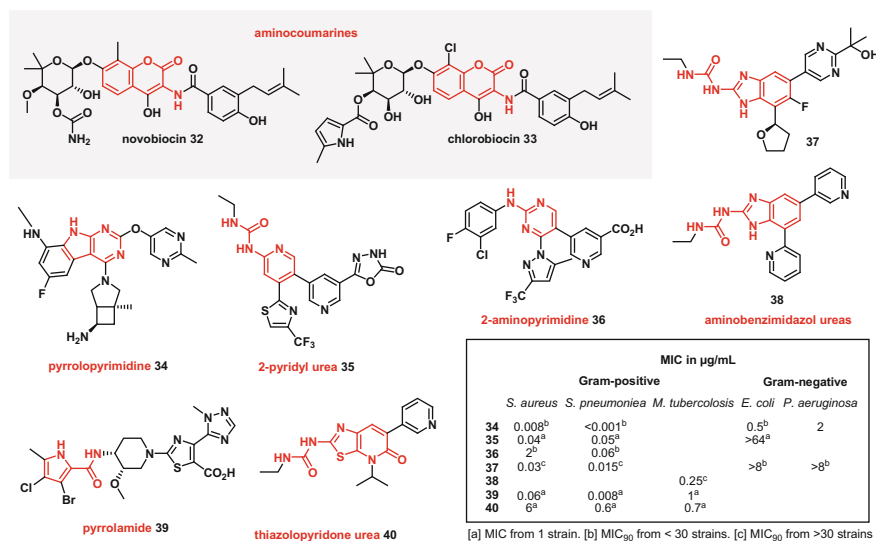
**Scheme 14** Structural architecture of the DNA gyrase and schematic mechanism. Adapted from Gubaev and Klostermeier (2014) and Costenaro et al. (2007)

DNA. Finally, the release of the T-segment and the hydrolysis of the second ATP molecule sets the gyrase into its starting position (Costenaro et al. 2007; Collin et al. 2011).

In general, inhibitors of the DNA gyrase can be divided into two groups: Those blocking the ATP binding site and those interfering with the DNA binding or the DNA strand passage within the gyrase. The latter are called catalytic site inhibitors or gyrase poisons. Due to the close structural similarity of DNA gyrase and topoisomerase IV, inhibitors possessing these mechanisms of action usually show activity against both targets. This dual-targeting phenomenon often results in low mutation frequencies for drug resistance and has probably significantly contributed to the success of antibacterial drugs targeting topoisomerases.

The first ATP-site inhibitor discovered was novobiocin **32** (Scheme 15), a bacterial metabolite produced by *Streptomyces niveus*, introducing the class of aminocoumarins as gyrase-inhibiting compounds.

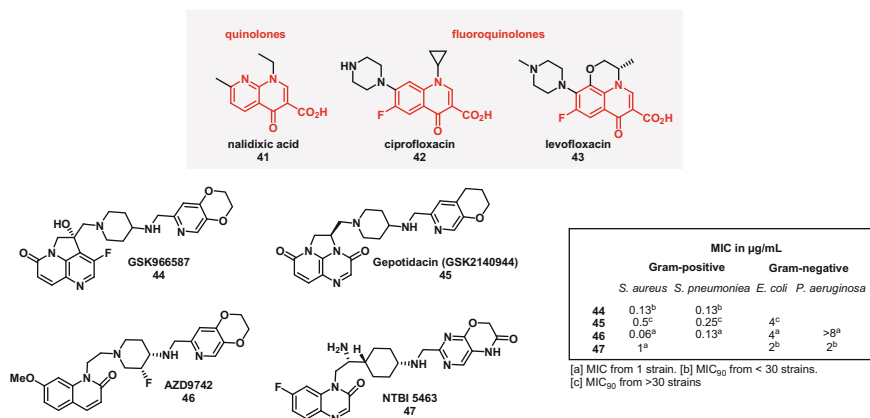
The compounds bind to the GyrB subunit near the ATP binding site with very high affinity and thereby block the functionality of the gyrase. Although the aminocoumarins show potent antibacterial activity against Gram-positive bacteria, their therapeutic application is limited due to poor activity against most Gram-negative pathogens and in particular due to poor water solubility and in vivo toxicities (Maxwell 1993; Maxwell and Lawson 2003). Therefore, novel ATP-site inhibitors possessing more drug-like scaffolds have been investigated in recent years, with some of them already demonstrating their efficacy in vivo (Scheme 15) (Ehmann and Lahiri 2014).



**Scheme 15** Structures of the aminocoumarins novobiocin and chlorobiocin as well as advanced ATP-site inhibitors with novel scaffolds (name-giving structural motifs are highlighted in red) and their antibacterial activities, adapted from Ehmann and Lahiri (2014)

Tari et al. presented the pyrrolopyrimidine **34** showing broad-spectrum antibacterial activity with impressive MIC<sub>90</sub> values of 0.008 µg/mL against *S. aureus* (Tari et al. 2013). Compound **34** displays also good activity against Gram-negative pathogens representing a major advancement for ATP-site inhibitors. Furthermore, the 2-pyridinyl urea **35** (Basarab et al. 2013), the 2-aminopyrimidine **36** (Uria-Nickelsen et al. 2013), aminobenzimidazols **37** (Finn 2013) and **38** (Chopra et al. 2012), the pyrrolamide **39** (Shahul et al. 2014) and the thiazolopyridone **40** (Kale et al. 2013, 2014) were reported as efficient ATP-site inhibitors against Gram-positive bacteria, and, for some of them (**38**, **39** and **40**) also moderate activities against *M. tuberculosis* were revealed.

The first catalytic site inhibitor of the DNA gyrase was nalidixic acid **41** (Scheme 16), introducing the antibacterial class of quinolones. The first generation of quinolones had relatively weak antimicrobial activity, but with the introduction of the fluoroquinolones such as ciprofloxacin **42** or levofloxacin **43** very potent antimicrobials were available, displaying broad-spectrum activity against Gram-positive and Gram-negative pathogens (Collin et al. 2011). Such catalytic site inhibitors stabilize the DNA cleavage complex of the gyrase or topoisomerase IV and thereby poison the enzyme. This has been shown to be a very effective mode of inhibition of type II topoisomerases, since very low concentration of the inhibiting compounds bound to their target can lead to sufficient protein-stabilized DNA breaks, initiating a cascade which cumulates in cell death (Anderson and Osheroff 2001; Kohanski et al. 2010). Nevertheless, significant occurrence of resistance toward fluoroquinolones has been reported (Jacoby 2005), mostly associated with mutations in the quinolone resistance-determining regions in GyrA and GyrB (or ParC and ParE, the equivalents in topoisomerase IV) (Yoshida et al. 1990, 1991; Jacoby 2005).



**Scheme 16** Structures of nalidixic acid and the fluoroquinolones ciprofloxacin and moxifloxacin as well as advanced catalytic site inhibitors with novel scaffolds and their antibacterial activities (adapted from Ehmman et al.) (Ehmman and Lahiri 2014)

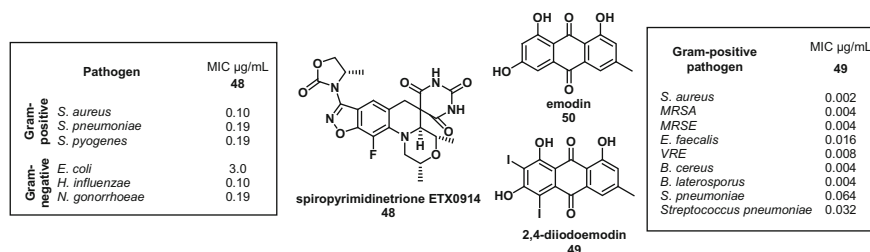


In the recent years, intensive research led to the discovery of a group of several non-fluoroquinolone inhibitors of the gyrase that bind near the catalytic site, but are mechanistically and microbiologically distinct from the fluoroquinolones (Ehmann and Lahiri 2014).

These compounds are simply called novel bacterial topoisomerase inhibitors (NBTIs). Most NBTIs possess a 4-aminopiperidine moiety bridging two aromatic motifs. Investigations on the mechanism of action have been shown that NBTIs also stabilize the DNA–protein complex, but in contrast to the fluoroquinolones they bind to the gyrase in the presence of intact, unbroken DNA strands (Bax et al. 2010).

The first frontrunner advanced to clinical trials was GSK966587 **44** developed by researchers at GlaxoSmithKline (Miles et al. 2013). The compound bears a tricyclic 3-fluoro-4,5-dihydro-7H-pyrrolo[3,2,1-de][1,5]naphthyridin-7-one core and showed potent activity against Gram-positive bacteria (Ehmann and Lahiri 2014). A follower of the same series, gepotidacin **45** (Mayer and Janin 2014), is in several clinical phase II trials investigating the treatment of respiratory tract infections caused by *S. pneumoniae*, acute bacterial skin and skin structure infections caused by *S. aureus* and uncomplicated urogenital gonorrhoea (Paris 2015). Additionally, researchers at AstraZeneca reported further NBTIs showing potent activity against Gram-positive bacteria such as AZD9742 **46** (Reck et al. 2012) and NTBI 5463 **47** (Dougherty et al. 2014). The latter presents a 4-aminocyclohexyl bridge between the two aromatic regions with promising activity against Gram-negative pathogens. Noteworthy, all of these compounds show activity against fluoroquinolone-resistant bacterial strains.

Recently, several catalytic site inhibitors of the DNA gyrase with novel unique scaffolds different from the early NBTIs (Scheme 16) have been discovered. Researchers at AstraZeneca reported compound ETX0914 **48** (Scheme 17) with an unusual spiropyrimidinetrione scaffold that showed potent broad-spectrum activity against Gram-positive as well as Gram-negative bacteria (Jacobsson et al. 2014; Alm et al. 2015; Kern et al. 2015; Su et al. 2016). The compound was active against fluoroquinolone-resistant strains, suggesting that the exact mode of inhibition is also different from the fluoroquinolones (Basarab et al. 2015). Genetic analysis of

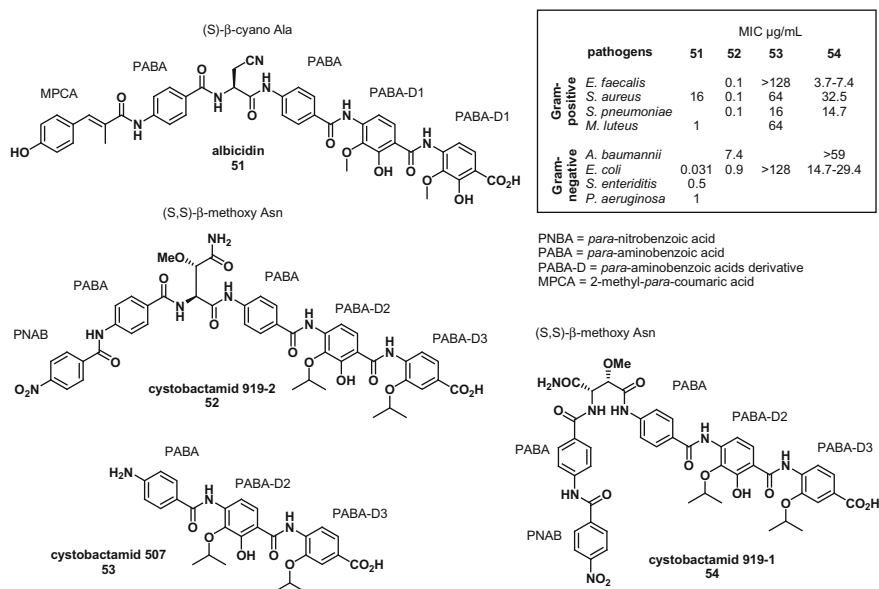


**Scheme 17** Structures and antibacterial activities of spiropyrimidinetrione ETX0914 and 2,4-diiodoemodin

resistant mutants showed that mutations in the conserved GyrB TOPRIM domain are correlated to decreased susceptibilities to ETX0914 (Alm et al. 2015). Currently, the compound is in clinical phase II trials for the treatment of uncomplicated gonorrhea (Paris 2015). Another compound, which has recently been shown to have extremely potent antibacterial activities (up to MIC = 0.002  $\mu\text{g}/\text{mL}$ ) against Gram-positive pathogens including several multi-drug-resistant and also fluoroquinolone-resistant strains, is 2,4-diiodoemodin **49** (Chen et al. 2014), a semi-synthetic derivative of the secondary metabolite emodin **50**, which naturally occurs in plants and fungi. Interestingly, **49** has been shown to be an inhibitor of DNA gyrase and bacterial topoisomerase I, which is a unique dual-targeting mode of this compound. Additionally, unlike emodin, which caused cytotoxic effects through strong inhibition of the human topoisomerase II $\alpha$ , 2,4-diiodoemodin showed only very little inhibitory effect against this enzyme. Nevertheless, a further understanding of this new class of antibacterial haloemodins, for example with respect to target promiscuity and the associated side effects, is needed to advance this structural template.

A novel structural class of gyrase inhibitors of proteobacterial origin, consisting of oligomeric pseudopeptides formed by chains of coupled *para*-aminobenzoic acids, has recently been discovered by two research groups from two different Gram-negative producer strains.

Albicidin **51** (Scheme 18) has been isolated from *Xanthomonas albilineans* (Cociancich et al. 2015), a xylem-invading plant pathogen that causes leaf scald



**Scheme 18** Structures and antibacterial activities of albicidin and the cystobactamids, novel unusual PABA-oligomeric pseudopeptides

disease in sugarcane (Royer et al. 2004). The compound is a phytotoxin that blocks the DNA gyrase in sugarcane chloroplasts (Birch and Patil 1987a, b). Additionally, albicidin has been demonstrated to be a nanomolar inhibitor of bacterial DNA gyrase with a broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens (Hashimi et al. 2007; Cociancich et al. 2015; Kretz et al. 2015). Albicidin stabilizes the DNA gyrase cleavage complex with relaxed or supercoiled DNA in the presence of ATP via binding to the GyrA subunit, leading to a similar DNA fragmentation pattern as ciprofloxacin. Nevertheless, only low levels of cross resistance to albicidin in some fluoroquinolone- and coumerymycin-resistant strains have been observed (Hashimi et al. 2007). The existence of a potent gyrase inhibitor produced by *X. albilineans* has been known for decades, but since the bacterium produces only very small amounts of the compound, the compound's structure could be elucidated only recently, when Süssmuth and coworkers expressed heterologously in *X. axonopodis* sp. *vesicatoria* and established an extended purification protocol (Cociancich et al. 2015).

The structure of albicidin consists of the non-proteinogenic  $\alpha$ -amino acid (*S*)- $\beta$ -cyanoalanine, the two aromatic  $\delta$ -amino acids *para*-aminobenzoic acid (PABA) and 2-hydroxy-3-methoxy-*para*-aminobenzoic acid (PABA-D1) as well as 2-methyl-*para*-coumaric acid (MPCA) that represents the N-terminus of this oligomeric pseudopeptide. Biosynthetically albicidin is a product of the polyketide synthase-nonribosomal peptide synthase (PKS-NRPS) machinery (Huang et al. 2001; Vivien et al. 2007; Piel 2010). Albicidin has been successfully targeted by a total synthesis by Süssmuth and coworkers, thus confirming the unusual structure (Kretz et al. 2015).

In parallel, the structure of the cystobactamid 919-2 **52** (Scheme 18) was published by Müller and coworkers (Baumann et al. 2014; Herrmann et al. 2016).

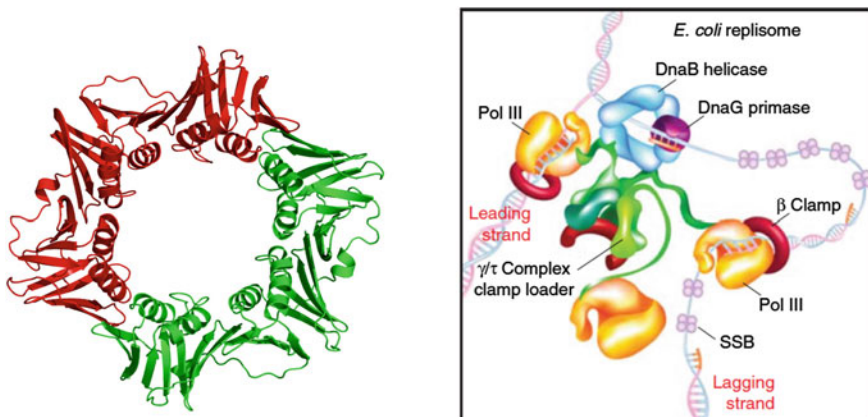
The compound was isolated together with the truncated homolog cystobactamid 507 **53** (Moreno et al. 2015) and the isomer cystobactamid 919-1 **54** from cultivations of myxobacterium *Cystobacter* sp. Cbv34. Cystobactamid 919-2 shares the general PABA-derived oligomeric, pseudopeptidic structure of albicidin, but the central amino acid is a non-proteinogenic (*S,S*)- $\beta$ -methoxy asparagine, and it possesses a different oxidation pattern on the right-hand site of the molecule. Additionally, there is a *para*-nitrobenzoic acid (PNBA) unit at the N-terminus. In accordance with the structural similarity of cystobactamids and albicidin, also cystobactamid 919-2 displayed very potent broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens due to inhibition of the DNA gyrase at the GyrA subunit with low levels of cross resistance to fluoroquinolone-resistant strains (Baumann et al. 2014). Both compounds were regarded as “natural quinolones” representing the first natural products inhibiting the GyrA subunit of DNA gyrase (Baumann et al. 2014; Johnston and Magarvey 2015). Currently, chemical optimization studies of albicidin and cystobactamid 919-2 to improve their properties as antibacterial drugs are ongoing (personal communication, M. Brönstrup).

## 6 Bacterial DnaN Polymerase Sliding Clamp—A Highly Conserved, Multi-enzyme Interacting Core Architecture of the Bacterial Replisome

As mentioned earlier, multi-enzyme complexes or structures synthesized by multi-enzymatic pathways are less prone to bacterial resistance development, since their malfunctioning is hard to bypass.

In this context the bacterial replisome machinery, which consists of at least twelve interacting enzymes that are highly conserved in bacteria, is a good structure to target (Robinson et al. 2012). Next to the DNA gyrase, which also belongs to the bacterial replisome and has already been discussed, the bacterial DnaN polymerase sliding clamp has recently attracted attention as an antibacterial target (Georgescu et al. 2008b; Kjelstrup et al. 2013; Wolff et al. 2014; Yin et al. 2014a, b; Holzgrabe 2015; Kling et al. 2015; Yin et al. 2015).

The bacterial DnaN or  $\beta$ -clamp is a ring-shaped homodimer, with each monomer composed of three globular domains, which functions as a crucial subunit of the DNA polymerase III holoenzyme (Scheme 19). The general protein–DNA interactions between polymerases and DNA templates are weak (Mizrahi et al. 1985). Therefore, the association of the polymerase to the DNA strand is one of the rate-limiting steps of the replication. The assembly of the two  $\beta$ -subunits of the bacterial  $\beta$ -clamp around the DNA, mediated by ATP hydrolysis, leads to strong and specific protein–protein interactions between the  $\beta$ -clamp and the polymerase III (Pol III), thereby preventing polymerase dissociation and dramatically increasing the polymerase activity (Stukenberg et al. 1991; Argiriadi et al. 2006; Georgescu et al. 2008a; Donnell et al. 2013; Cho et al. 2014). Therefore, the



**Scheme 19** Structure of the DnaN/ $\beta$ -clamp in *E. Coli* (left) and architecture of the bacterial replisome (right). Adapted from Oakley et al. (2003) and Donnell et al. (2013); SSB stands for single-strand binding protein

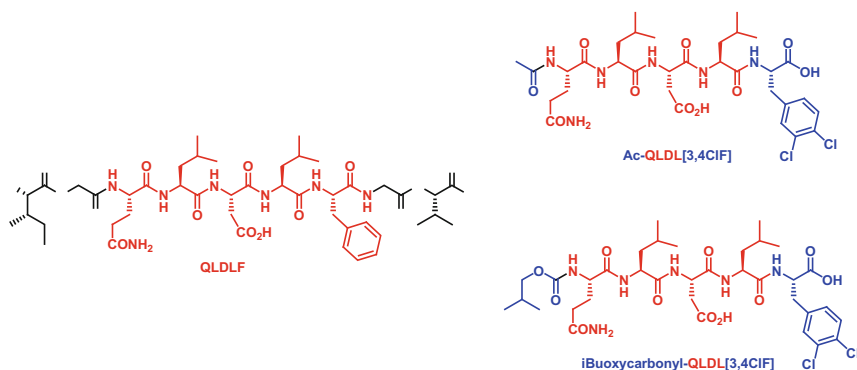
$\beta$ -clamp serves as a processivity-promoting factor in DNA replication (Stukenberg et al. 1991).

Furthermore, as the  $\beta$ -clamp shows direct protein–protein interactions with a multitude of other enzymes involved in the replication process such as the  $\alpha$ -polymerase, the  $\varepsilon$ -proofreading subunits (Ozawa et al. 2013), or the clamp loader  $\gamma/\tau$  complex (Stukenberg et al. 1991), it can be seen as the linchpin of the replisome (Robinson et al. 2012). The important functionality within the replication, its moderate copy number per cell (330–600 homodimers) (Leu et al. 2000), its highly conserved structure across the bacterial species (Burnouf et al. 2004; Argiriadi et al. 2006; Georgescu et al. 2008a; Gui et al. 2011; Wolff et al. 2014; Niiranen et al. 2015), and its significant structural divergence to the mammalian counterpart (proliferating cell nuclear antigen, PCNA) (Bloom 2009; Robinson et al. 2012; Donnell et al. 2013) make the  $\beta$ -clamp a particularly attractive antibacterial target.

In 2001, Jennings and coworkers discovered that the general protein–protein interaction between the  $\beta$ -clamp and several  $\beta$ -clamp-interacting proteins is based on a consensus sequence of amino acids in a linear pentapeptidic-binding motif, given as QL[S/D]LF and QLxLx[L/F][S/D] (Scheme 20) (Dalrymple et al. 2001).

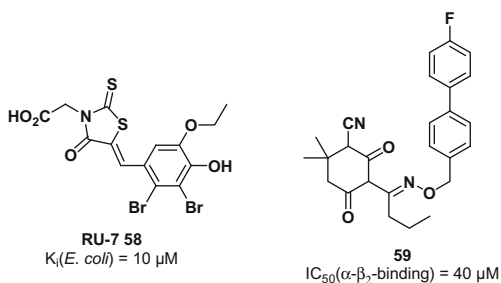
Jennings and coworkers were able to identify the first synthetic nonapeptides containing these linear binding motifs that inhibited either the binding of the  $\alpha$ -subunit or of the  $\delta$ -subunit of *E. coli* Pol III to the corresponding *E. coli*  $\beta$ -clamp, displaying IC<sub>50</sub> values of 1.33–14.6  $\mu$ M or 8.8–12.9  $\mu$ M, respectively.

It was demonstrated in further SAR studies in 2004 (Wijffels et al. 2004) and 2011 (Wijffels et al. 2011) that lipophilic N-terminal extensions of the pentapeptidic linear binding motif as well as substitutions with more lipophilic phenylalanine derivatives led to tremendous increases of the inhibitory effect of the binding of the  $\alpha$ -subunit of *E. coli* Pol III to the corresponding *E. coli*  $\beta$ -clamp (IC<sub>50</sub> up to 20 nM). These linear motifs (LM) bind to the LM-binding pocket, which is highly conserved across Gram-negative and Gram-positive bacteria (Argiriadi et al. 2006) and consists of two subsites (I and II) at the interface of the two domains on each



**Scheme 20** Early and advanced peptidic inhibitors of  $\alpha$ - $\beta_2$  binding

**Scheme 21** Small molecule inhibitors of the  $\beta$ -clamp



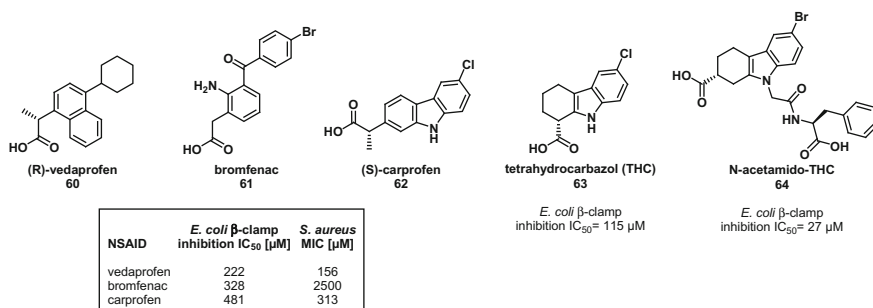
monomer of the  $\beta$ -clamp homodimer (Dalrymple et al. 2001; Bunting et al. 2003; Burnouf et al. 2004; Georgescu et al. 2008a, b).

In 2008, *O'Donnell* and coworkers published the first non-peptidic small molecule inhibitor of the  $\beta$ -clamp, RU-7 **58** (Scheme 21) (Georgescu et al. 2008b). The compound was identified by screening a library of 30000 polar small molecules for their inhibitory effect on  $\alpha$ - $\beta_2$ -binding by a fluorescence anisotropy titration assay. RU-7 inhibited the *E. coli* relication system with a  $K_i$  of 10  $\mu\text{M}$  by binding to the subunit I of the LM-binding pocket, but showed no inhibitory effect on the PCNA-dependent eukaryotic system in the yeast *S. cerevisiae*.

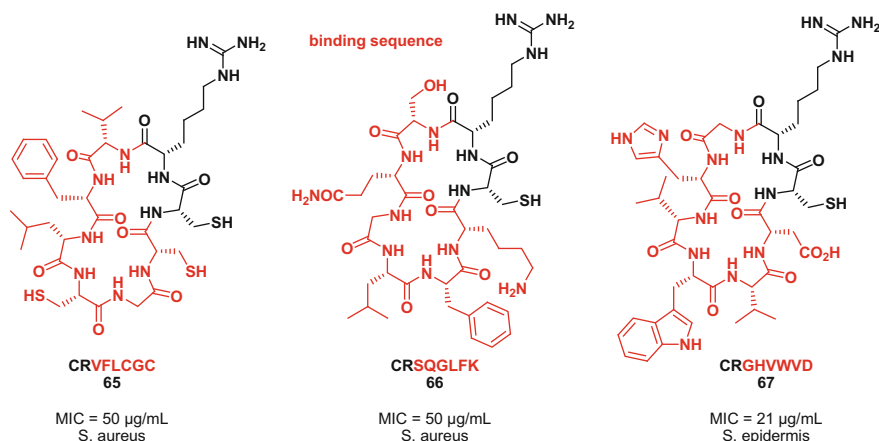
An other small molecule, the oxim ether **59**, was identified by an in silico screen of 32000 compounds followed by an  $\alpha$ - $\beta_2$ -binding assay (Wijffels et al. 2011). The compound, which also bound to the subunit I of the LM-binding pocket, displayed an  $IC_{50}$  value for inhibition of  $\alpha$ - $\beta_2$ -binding of 40  $\mu\text{M}$ .

In 2014, *Oakley* and coworkers reported that the weak antibacterial effects of non-steroidal anti-inflammatory drugs (NSAIDs) such as (*R*)-vedaprofen **60**, bromfenac **61**, or (*S*)-carprofen **62** (Scheme 22) are due to their binding to the subunit I of the LM-binding pocket of the  $\beta$ -clamp (Yin et al. 2014a).

Recently, a fragment-based screening of 352 compounds utilizing X-ray crystallography led to the discovery of tetrahydrocarbazol **63** (Scheme 22) as a  $\beta$ -clamp binding small molecule, which is structurally closely related to carprofen and was



**Scheme 22** Structures of NSAIDs, their antibacterial activities, and tetrahydrocarbazoles as  $\beta$ -clamp binding lead compounds



**Scheme 23** Cyclic octapeptides inhibiting the DnaN–DnaN protein interaction

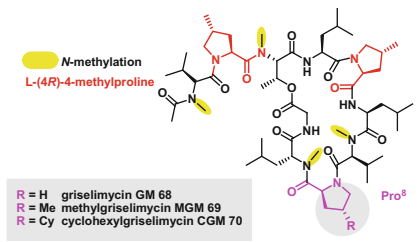
also shown to bind into the subunit I of the  $\beta$ -clamp (Yin et al. 2014b). Further, SAR studies led to the identification of N-acetamido-THC **64** with an 10-fold decreased  $\text{IC}_{50}$  for the inhibition of the  $\beta$ -clamp (Yin et al. 2015).

Whereas all compounds discussed so far were binding to the LM-binding pocket of the  $\beta$ -clamp, Lobner-Olesen and coworkers recently reported cyclic octapeptides (**65–67**, Scheme 23) inhibiting the DnaN–DnaN monomer interaction of the homodimer (Kjelstrup et al. 2013).

The compounds were identified using the SICLOPPS technology (Scott et al. 1999) for the intracellular in vivo production of cyclic 21-mer peptides. A six-amino-acid-long randomized sequence was used to build a library of over 900000 combinations, which were subsequently screened for their ability to inhibit DnaN–DnaN interactions in the *S. aureus* replisome. The initial six-amino-acid-long hit sequences were incorporated in an octapeptide and synthesized utilizing the Impact Twin System (New England Biolabs) and evaluated for their antibacterial activity. Interestingly, while cyclic octapeptides displayed MIC values of 50  $\mu\text{g}/\text{mL}$  and 21  $\mu\text{g}/\text{mL}$ , respectively, the corresponding linear peptides were totally inactive.

An issue with all of the synthetic DnaN binders described above is that their antibacterial potency is mostly weak. This again illustrates that the ability to penetrate into the bacterial cell is a crucial hurdle for new antibiotic templates.

This is fundamentally different for griselimycin (GM) **68** (Scheme 24), a long known natural product from bacterial ferments that attracted renewed interest as an anti-tuberculosis drug (Holzgrabe 2015; Kling et al. 2015). Griselimycin is a cyclic decadepsipeptide containing two unusual L-(4*R*)-4-methylproline and one unsubstituted L-proline in the linear sequence, which is cyclized with its C-terminal glycine onto the hydroxyl group of an internal threonine side chain. Additionally, the compound possesses several posttranslational N-methylations, and the



	GM	MGM	CGM
MIC ( $\mu\text{g/mL}$ ) for <i>M. tuberculosis</i> in liquid culture	1	0.6	0.06
MIC ( $\mu\text{g/mL}$ ) for <i>M. tuberculosis</i> in liquid culture containing 25% human serum	1.2	0.9	0.3
MIC ( $\mu\text{g/mL}$ ) for <i>M. tuberculosis</i> in macrophages	6.2	2.1	0.2
Unbound fraction (%) in human plasma	nd	7.9	0.3
Metabolic stability in human liver microsomes (% remaining compound after 20 min)	62	100	86
Metabolic degradation/clearance for human liver microsomes ( $\mu\text{L/min per mg}$ )	187.5	3	6.75
$C_{\text{max}}$ (ng/mL) in plasma	3900	2820	2620
$C_{\text{max}}$ (ng/mL) in lung tissue	2580	8550	8850
AUC (ng hour/mL) in plasma	5100	6500	23000
AUC (ng hour/mL) in lung tissue	5900	12000	70000
Clearance (liters/hour per kg)	2.4	2.1	1.1
$V_{\text{d}}$ (liters/kg)	1.2	1.6	5.5
$t_{1/2}$ (hours) in plasma	2.0	2.4	4.3
$t_{1/2}$ (hours) in lung tissue	3.9	4.3	4.3
Oral bioavailability (F)(%)	48	47	89

$C_{\text{max}}$ , maximum concentration; AUC, area under the concentration curve;  $V_{\text{d}}$ , volume of distribution;  $t_{1/2}$ , half-life; nd, not determined. Pharmacokinetic parameters ( $C_{\text{max}}$ , AUC, and  $t_{1/2}$ ) were determined after a single oral administration of 30mg/kg of the test compound in mice. For oral bioavailability, a single oral dose was compared to a single intravenous dose of 3 mg/kg.  $F = [\text{AUC}_{\text{oral}}]/[\text{AUC}_{\text{iv}}]$ , the ratio of exposure of an equivalent dose after nonintravenous (in this case, oral) and intravenous administration as a measure of bioavailability

**Scheme 24** Griselimycin, novel analogs, and their pharmacokinetic properties and anti-TB activities

N-terminus is acetylated. Griselimycin was isolated from two strains of *Streptomyces* in the 1960s (Terlain and Thomas 1969, 1971a, b), and was found to exhibit antibacterial activity specifically against *Corynebacteria* including *M. tuberculosis*, though the exact mode of action and target were unknown. The pharmaceutical company Rhône-Poulenc started with early investigations for the development of griselimycin as an anti-tuberculosis drug. The first studies in humans were promising, despite poor pharmacokinetics of the compound (Bénazet et al. 1966; Noufflard-Guy-Loé and Berteaux 1965). But first derivatization programs to find analogs with improved pharmacokinetics (Bouchaudon 1964; Jolles 1971) were soon terminated in the 1970s after rifampin became available for tuberculosis treatment. Since novel tuberculosis drugs are desperately needed and GM was reported to be highly active against multi-drug-resistant *M. tuberculosis*, researchers from Sanofi-Aventis, Müller, and coworkers re-investigated the use of griselimycin as anti-tuberculosis drug (Kling et al. 2015).

First, a metabolic stability profiling of natural, less-abundant analogs of GM led to the identification of Pro<sup>8</sup> as main site of metabolic degradation responsible for the poor pharmacokinetic parameters of griselimycin. This result was supported by the finding that the natural derivative methylgriselimycin (MGM) 69 (Scheme 24) showed a remarkably increased stability toward degradation compared to griselimycin. Since MGM is produced in only small amounts, a solid-supported total synthesis for griselimycins was established to provide access to several GM analogs bearing Pro<sup>8</sup> modifications, supposed to improve potency and metabolic stability of the lead structure. From biological evaluation of these analogs cyclohexylgriselimycin (CGM) 70 (Scheme 24) was identified, displaying MIC values of 0.06  $\mu\text{g/mL}$  and 0.2  $\mu\text{g/mL}$  in the drug susceptible *M. tuberculosis* strain H37Rv and within macrophage-like RAW264.7 cells, respectively. CGM revealed a



time-dependent *in vitro* activity, and although the unbound fraction in plasma was very low, the MIC dropped only fivefold in the presence of human plasma. CGM showed high-level activity against a panel of *M. tuberculosis* strains covering a broad geographical and evolutionary diversity, including strains mono-resistant to first- and second-line anti-tuberculosis drugs. The overall ADMET properties of CGM also improved; in particular CGM displayed a high-level oral bioavailability of 89 %, a moderate clearance (1.1 L/h/kg), and an expanded half-life and drug exposure, enabling a once daily administration.

*In vivo* activity studies in a mouse model of tuberculosis revealed a minimum-effective dose (MED) of 50 mg/kg, and mice treated with 600 mg/kg daily dose were proven culture-negative after 4 weeks of treatment. Giving the promising result of CGM administered in monotherapy, a combination therapy with first-line anti-tuberculosis drugs such as rifampin (RIF), pyrazinamide (PZA), and isoniazid (INH) was investigated. In an *in vivo* mouse model of TB, CGM was administered at MED of 100 mg/kg alone and in combination with other TB drugs in a model of chronic TB. CGM alone was demonstrated to be as active as INH, the most bactericidal first-line anti-tuberculosis drug, and showed significantly improved activity in combination with RIF compared to the standard combination therapy (INH/RIF/PZA).

The analysis of the genes of a GM- and MGM-producing *Streptomyces* strain, which is naturally resistant to GM, led to the identification of *griR*, a homolog of the *dnaN* gene (51 % of identity). Introduction of the *griR* gene to *Streptomyces coelicolor*, a strain susceptible to GM, allowed the strain to survive in the presence of GM, suggesting that overexpression of GriR mediates GM resistance and that the griselimycins interact with DnaN polymerase sliding clamp ( $\beta$ -clamp) of the replisome. Further, genomic-based investigations on the resistance mechanisms *in vitro* and *in vivo* in *M. smegmatis* and *M. tuberculosis* revealed that resistance in these species is achieved by overexpression of DnaN. However, this process occurred with very low rate and was accompanied by considerable loss of fitness, expressed in a negative correlation of growth to increasing level of resistance.

Finally, the interaction between GM, MGM, and CGM with DnaN from *M. smegmatis*, *M. tuberculosis*, and *E. coli* as well as human sliding clamp (PCNA) was analyzed and characterized by surface plasmon resonance (SPR) and by a crystal structure analysis. SPR analysis demonstrated a high affinity binding of GM, MGM, and CGM to mycobacterial sliding clamps in the picomolar range, whereas significantly lower binding to the sliding clamp of *E. coli* and no binding between PCNA and griselimycins was detected. The analysis of co-crystals of GM and CGM with DnaN from *M. smegmatis* and *M. tuberculosis* revealed that the griselimycins bind to the LM-binding pocket, filling both subsite I and II. The linear N-terminal segment and the adjacent half of the macrocycle of GM and CGM superimpose very well with the natural linear peptidic-binding motif of the LM-binding pocket.

Since there is no preexisting resistance of griselimycins due to the different modes of action compared to the approved anti-tuberculosis drugs, and since resistance occurs at an extremely low frequency associated with fitness loss of the

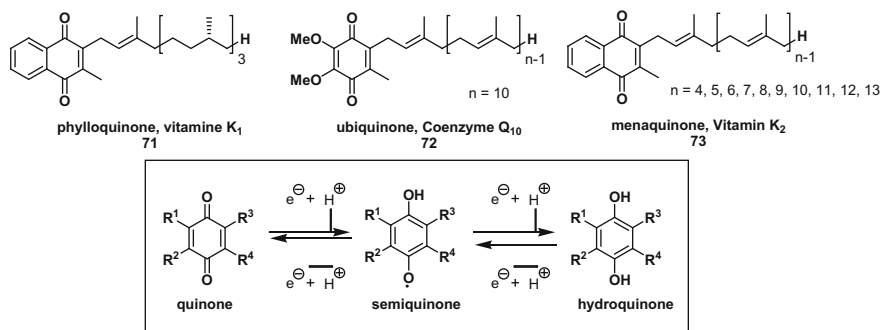
pathogens, the griselimycins are attractive candidates for the treatment of drug-sensitive and multi-drug-resistant tuberculosis. Clinical trials with CGM have not been reported yet, though.

## 7 Menaquinone as a Small Molecule Antibacterial Target

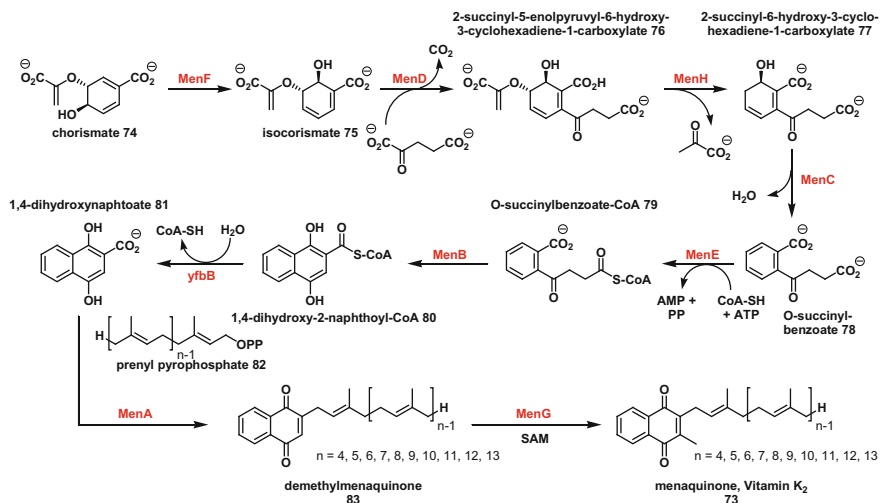
Isoprenoid quinones such as phyloquinone (vitamin K<sub>1</sub>) **71**, ubiquinone (coenzyme Q<sub>10</sub>) **72**, and menaquinone **73** (Scheme 25) are present in almost all living organisms as plasma membrane-anchored electron carriers or antioxidants (Kawamukai 2002; Aguilaniu et al. 2005; Kurosu and Begari 2010; Nowicka and Kruk 2010; Cervellati and Greco 2016). These compounds are involved in several essential, live-sustaining metabolic processes such as the generation of adenosine triphosphate (ATP) in the respiratory chain. The general structure of these compounds consists of a polar, quinone-containing head and an unpolar, hydrophobic side chain, which allows the molecules to anchor in the phospholipid bilayer of cell membranes.

While phyloquinone and menaquinone show a methylnaphtoquinone core, the polar head group of ubiquinone consists of a dimethoxy-benzoquinone. The length and saturation of the side chain can differ in the particular species. The quinone moiety in these compounds is crucial for their biological activity as electron carriers in cellular redox processes, as it is reduced by a two-step reversible reduction process via the intermediate semiquinone to the corresponding hydroquinone. While ubiquinone is utilized as coenzyme for the ATP generation in the mammalian respiratory chain, in most Gram-positive and anaerobic Gram-negative bacteria menaquinone is the major isoprenoid quinone, responsible for the electron transfer in the bacterial respiratory chain and a multitude of other metabolic redox processes (Kurosu and Begari 2010; Fujimoto et al. 2012).

The biosynthesis of menaquinone in *E. coli* is catalyzed by seven menaquinone-specific enzymes, MenA-G, which are encoded in two gene clusters (Young 1975;



**Scheme 25** Structures of menaquinone, phyloquinone, and ubiquinone

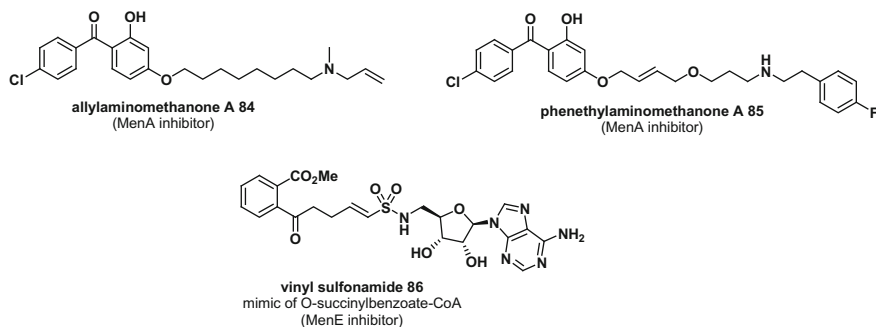


**Scheme 26** Biosynthesis of menaquinone

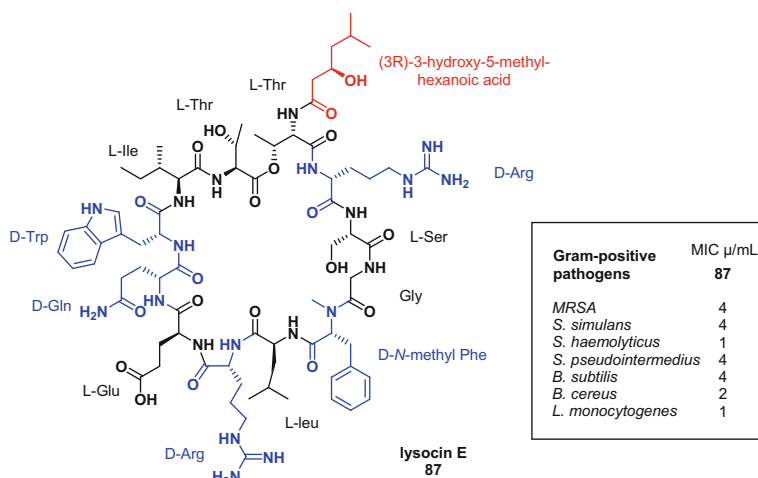
Schoepp-Cothenet et al. 2009). It starts from chorismate **74** (Scheme 26), an intermediate of the shikimate pathway. First, MenF isomerizes chorismate **74** to isochorismate **75**. MenD, a thiamin diphosphate-dependent enzyme, then mediates the *Stetter*-type 1,4-conjugate addition of  $\alpha$ -ketoglutarate to isochorismate, leading to the formation of **76**. The elimination of the pyruvate moiety is then catalyzed by MenH, yielding 2-succinyl-6-hydroxy-3-cyclohexadiene-1-carboxylate **77**, and subsequently MenC forces the elimination of water providing *O*-succinylbenzoate **78**. The *O*-succinyl-CoA ligase MenE attaches **78** to coenzyme A and a *Diekmann*-type cyclization catalyzed by MenB and subsequent thioester hydrolysis by yfbB leads to formation of 1,4-dihydroxy-2-naphthoate **81**. Finally, prenylation by MenA and methylation mediated by MenG gives menaquinone **73** (Kurosu and Begari 2010). The most common menaquinones contain 7, 8, or 9 isoprene units; however, menaquinones with 4, 5, 6, 10, 11, 12, or 13 isoprene units have been isolated from bacteria.

Since it has been demonstrated that menaquinone is crucial for bacterial growth (Dhiman et al. 2009; Fujimoto et al. 2012) and colony development (Pelchovich et al. 2013), it is not a surprise that menaquinone biosynthesis has already been investigated as a target for the development of novel antibiotics (Kurosu and Begari 2010).

In the last years, a few inhibitors of individual enzymes of menaquinone biosynthesis have been reported (Kurosu and Begari 2010). For example, allylaminomethanone A **84** and phenylethylaminomethanone A **85** (Scheme 27) are inhibitors of MenA, which mimic the structure of the product of MenA catalysis, demethylmenaquinone, and display MIC values of 1.5 and 12.5  $\mu\text{g/mL}$ , respectively, against *M. tuberculosis* (Kurosu et al. 2007; Debnath et al. 2012).



**Scheme 27** Structures of representative inhibitors of menaquinone biosynthesis



**Scheme 28** Lysocin E and its antibacterial activity

Furthermore, the vinylsulfonamide **86**, which was designed as mimic of O-succinylbenzoate-CoA ester, was reported to efficiently inhibit MenE at low nanomolar concentrations (Lu et al. 2012).

Recently, the novel natural cyclodepsipeptide lysocin E **87** (Scheme 28) was reported to target the redox metabolism in bacteria by directly binding to menaquinone (Hamamoto et al. 2014). Since the biosynthesis of menaquinone is a multi-enzymatic pathway as described above and the role of menaquinone is crucial for viability of bacteria, menaquinone seems to be a much smarter choice for an antibacterial target than the individual enzymes of the biosynthesis, even more when considering that an alternative biosynthetic pathway for menaquinones starting from futasoline exists in several Gram-positive bacteria (Hiratsuka et al. 2008).

The cyclic structure of **87** displays a head-to-tail lactonization of the hydroxyl function of the N-terminal threonine to the carboxylic acid of the C-terminal

threonine. Furthermore, the compound contains five D-amino acids, (3R)-3-hydroxy-5-methyl-hexanoic acid capping the N-terminal amino group and a N-methylation at the present phenylalanine.

Lysocin E was discovered by an extensive screening campaign in invertebrates, where culture supernatants of soil bacteria were tested in a silkworm infection model. The compound has shown good antibacterial activity against several Gram-positive pathogens including MRSA.

Lysocin E induced potassium leakage through the bacterial membrane, which led to a rapid loss of membrane potential and bacteriolysis. The hemolytic activity against mammalian cells was very low, and further investigations demonstrated that the MIC value of **87** against *S. aureus* increased in a dose-dependent manner upon addition of menaquinone. The direct binding of menaquinone in a 1:1 complex was further indicated by micro-calorimetric measurements, detecting an exothermic response upon addition of lysocin to menaquinone. Interestingly, no binding of the compound to the structurally similar ubiquinone was observed, suggesting that the naphthoquinone core in menaquinone is crucial for the interaction.

Furthermore, the first in vivo studies in *S. aureus* infected mice showed a promising ED<sub>50</sub> value of 0.5 mg/kg body weight, even smaller than that of vancomycin. This is remarkable in view of the moderate–good MIC value in vitro. Even a dose of 400 mg/kg body weight administered by intraperitoneal injection was tolerated, and no increase of biochemical markers for tissue damage of liver and kidneys was observed. Lysocin E has already been successfully addressed in a solid-supported total synthesis by *Inoue* and coworkers, and the first structure–activity relationship studies have revealed that the N-methylation at the phenylalanine in lysocin E is crucial for its activity (Murai et al. 2015).

In summary, lysocin E demonstrates that the depletion of essential small molecule mediators of energy metabolism is a viable, but yet underexplored antibiotics strategy. It also highlights that in vivo phenotypic assays, a highly successful discovery strategy of pharmaceutical research before the advent of in vitro assay systems, are still a powerful way to disclose urgently needed, novel antibiotic lead compounds.

## 8 Conclusions

Antibiotic lead finding suffers from insufficient volume and scale since the pharmaceutical industry has considerably reduced its efforts in this indication, inter alia due to an unsatisfying outcome of a target-centric, high throughput screening-based antibiotic research strategy (Payne et al. 2007; Tommasi et al. 2015), and due to uncertain economic revenues. But it does not suffer from a shortage of validated biological mechanisms, or from an exhaustion of chemical matter, as demonstrated by the showcases of this review. Microbial natural products continue to play a dominant role as sources of novel antibiotics, and their discovery is facilitated by multiple advances in natural product research (Harvey et al. 2015): Classical

strategies like the screening of extracts in relevant phenotypic assays (e.g., lysocin), or a re-investigation of neglected compounds of the past with modern chemical and genetic methods (as with griselimycin), are complemented by the cultivation of underexplored sources (e.g., teixobactin, cystobactamid), the heterologous expression of (almost) silent gene clusters (e.g., albicidin) or genome mining techniques (Doroghazi et al. 2014). Apart from being promising leads and drug candidates, natural products also teach important lessons and question existing ‘rules’ of drug discovery. Griselimycin, for example, demonstrates that peptides of elevated (>1000 Da) molecular weight can not only disrupt protein–protein interactions at picomolar concentrations, but at the same time exhibit high oral bioavailability, and high stability in plasma. Teixobactin, on the other hand, confirmed the rule that a dual molecular mode of action is advantageous with respect to suppression of resistance. It also underlines the common principle that natural antibiotics preferably target hotspots in complex biosynthetic machineries, as their malfunctioning is hard to circumvent by bacterial mutations.

New structural templates also arise from synthetic libraries that are screened against innovative targets, as exemplified by the inhibitors of WTA biosynthesis, the DNA sliding clamp, the DNA gyrase, or the  $\beta$ -lactamases. While many synthetic hits from the past suffered from insufficient translocation or permeation across bacterial membranes, the examples above demonstrate how an early incorporation of whole cell assays in the optimization cascade (or directly in the primary screening assay) assured the successful optimization of synthetic structures to antibiotics.

Our selection of case studies has been subjective and non-comprehensive, as it did not discuss the numerous additional innovative approaches to fight bacterial infections that include antibiotics (e.g., translation inhibitors, antimicrobial peptides, or actively transported conjugates), non-antibiotic pathogenicity-blocking small molecules [e.g., secretion system inhibitors, quorum sensing inhibitors, immune stimulants (Rasko and Sperandio 2010)], biomolecules [e.g., lysins, toxin binders (Morrison 2015)], or even larger entities [e.g., bacteriophages, probiotics] (Czaplewski et al. 2016). However, the few examples provide sufficient arguments that from a scientific point of view, the discovery and development of novel antibiotics is highly rewarding.

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