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Antibiotics with novel mode of action as new weapons to fight antimicrobial resistance

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Abstract: Antimicrobial resistance (AMR) is a major public health issue, causing 5 million deaths per year. Without any action plan, AMR will be in a near future the leading cause of death ahead of cancer. AMR comes from the ability of bacteria to rapidly develop and share resistance mechanisms towards current antibiotics, rendering them less effective. To circumvent this issue and avoid the phenomenon of cross-resistance, new antibiotics acting on novel targets or with new modes of action are required. Today, the pipeline of potential new treatments with these characteristics includes promising compounds such as gepotidacin, zoliflodacin, ibezapolstat, MGB-BP-3, CRS-3123, afabycin and TXA-709, which are currently in clinical trials, and lefamulin, which has been recently approved by FDA and EMA. In this review, we report the chemical synthesis, mode of action, structure-activity relationships, *in vitro* and *in vivo* activities as well as clinical data of these eight small molecules listed above.

Keywords: Antimicrobial resistance – New drugs – Novel mode of action – Novel target

Abbreviations: 3-MBA, 3-methoxybenzamide; ABSSSI, acute bacterial skin and skin structure infection; ACP, acyl carrier protein; ADME, absorption, distribution, metabolism, excretion; AE, adverse event; AMR, antimicrobial resistance; ATP, adenosine triphosphate; AUC, area under curve; BCS, biopharmaceutics classification system; CABP, community-acquired bacterial pneumonia; CDAD, *Clostridioides difficile*-associated diarrhea; CDI, 1,1'-carbonyldiimidazole / *C. difficile* infections; CFU, colony-forming unit; dGTP, deoxyguanosine triphosphate; DISARM, developing an innovative strategy for antimicrobial resistant microorganisms; DMF, dimethylformamide; DNSSA, daptomycin non-susceptible *S. aureus*; DPPA, diphenylphosphoryl azide; ED, effective dose; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELF, epithelial lining fluid; EMA, european medicines agency; EPI, efflux pump inhibitors; ESBL, extended spectrum β -lactamase; FDA, food and drug administration; HMDS, hexamethyldisilazane; HOBt, hydroxybenzotriazole; Ki, inhibitory constant; LC-MS, liquid chromatography-mass spectrometry; LDA, lithium diisopropylamide; LEAP, lefamulin evaluation against pneumonia; LNSSA, linezolid non-susceptible *S. aureus*; MDR, multidrug-resistant; MetRS1, type 1 methionyl-tRNA synthetase; MGBs, minor groove binders; MIC, minimum inhibitory concentration; MOA, mode of action; MPK, milligrams per kilograms; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; NADH, nicotinamide adenine dinucleotide hydrogen; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; NBTI, novel bacterial topoisomerase inhibitors; NCS, N-chlorosuccinimide; PA β N, phenylalanine-arginine- β -naphthylamide; PAE, post-antibiotic effect; PD, pharmacodynamic; pH, potential of hydrogen; PK, pharmacokinetic; PPB, plasma protein binding; PRSP, penicillin-resistant *S. pneumoniae*; PTC, peptidyl transferase center; PTSA, p-toluenesulfonic acid; QIDP, qualified infectious disease product; SAR, structure-activity relationship; S_N2, nucleophilic 2nd order substitution; S_NAr, nucleophilic aromatic substitution; STI, sexually transmitted infections; t_{1/2}, half-life; TEA, triethylamine; THF, tetrahydrofuran; UTI, urinary tract infection; VISA, vancomycin-intermediate *S. aureus*; VRE, vancomycin-resistant *E. faecium*; VRSA, vancomycin-resistant *S. aureus*.

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1. Introduction

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Antimicrobial resistance (AMR) is one of the major public health problems of this century, with an estimated 10 million people killed annually by 2050 [1]. Recently, predictive statistical models have estimated 5 million AMR-related deaths in the year 2019 [2]. This phenomenon is mainly due to the extensive misuse of antibiotics which leads to the selection of resistant bacteria [3,4]. The resulting infections become more difficult to treat, causing serious illness, with the risk of spreading the disease and facing death.

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Drug resistance in bacteria can be classified into four different mechanisms: (1) enzymatic inactivation of the drug, (2) export of the drug by efflux pumps, (3) limitation of drug uptake by changes in membrane permeability, and (4) modification of the drug target [4]. Due to the lack of lipopolysaccharide in the outer membrane, Gram-positive bacteria are more likely to enzymatically inactivate the drug or modify its target rather than limit the uptake or express efflux pumps. This is not the case for Gram-negative bacteria, which can acquire resistance through all the mechanisms described above [5].

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Considering the different types of mechanisms that confer drug resistance, the alteration of the drug target is a key feature. Therefore, efforts have been made to develop drugs acting on a novel target and/or with a new mode of action (MOA), especially in academia or in small biotech companies [6,7]. Interestingly, the identification of novel drug targets may come with the discovery and development of first-in-class drugs [8].

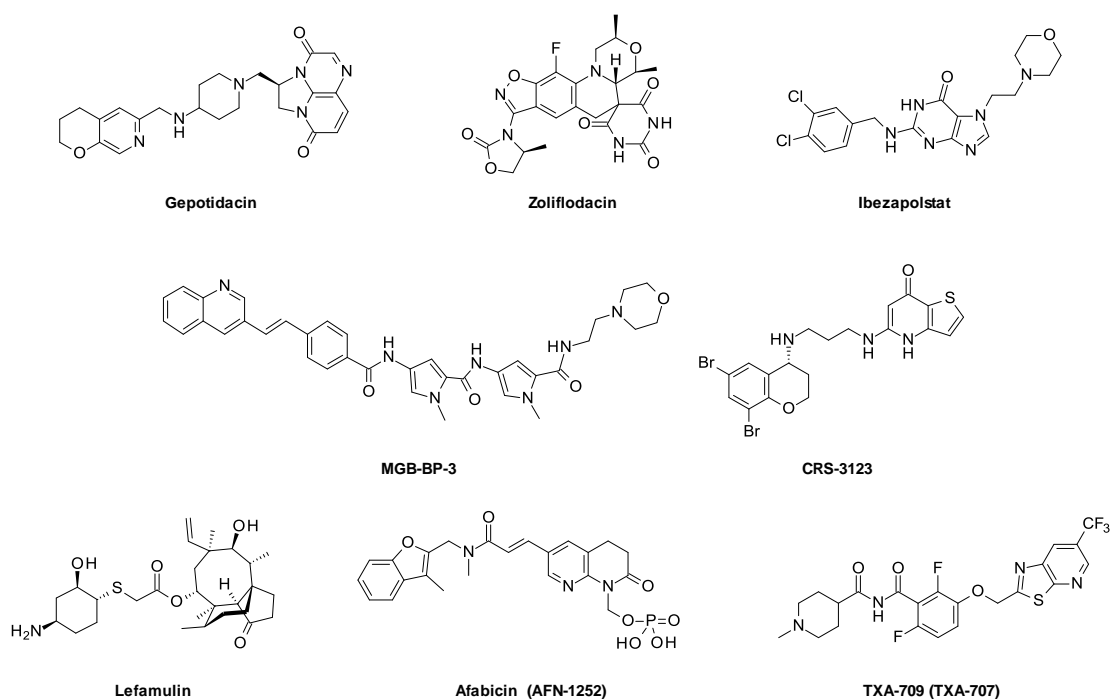
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In this review, we focus on new drugs in clinical development or recently approved, characterized by novel targets and/or MOA, that are of interest for the treatment of infections caused by multidrug-resistant bacteria (Figure 1 and Table 1). We have deliberately chosen not to discuss compounds at the pre-clinical stage.



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Figure 1. New drugs in the clinical pipeline or recently approved for the treatment of infections caused by resistant bacteria. For prodrugs, the name of the active pharmaceutical ingredient is written into brackets.

Table 1. Main information and calculated chemical properties of the eight drug candidates with their progression toward clinical development.

Name	Gepotidacin	Zoliflodacin	Ibezapolstat	MGB-BP-3
Family	Triazaacenaphthylene/NBTI	Spiropyrimidinetrione	DCBG ([dichlorobenzyl] guanine)	Distamycin
Mode of action	Inhibition of DNA gyrase and topoisomerase IV	Inhibition of topoisomerase II	Inhibition of DNA polymerase III C	Binding to the minor groove of DNA
Chemical features	MW: 448.22 cLogP: 0.12 H-bond acc.: 7 H-bond don.: 1 PSA: 90.37	MW: 487.44 cLogP: 1.79 H-bond acc.: 8 H-bond don.: 2 PSA: 143.31	MW: 423.30 cLogP: 2.49 H-bond acc.: 6 H-bond don.: 2 PSA: 83.78	MW: 631.72 cLogP: 3.79 H-bond acc.: 6 H-bond don.: 3 PSA: 122.52
Development phase	Phase III	Phase III	Phase II	Phase II
Relevant references	[9, 14, 17]	[44, 45, 48, 55, 56]	[68, 71, 72, 80, 81]	[83, 85, 86, 87, 89]
Name	CRS-3123	Lefamulin	Afabicin (prodrug of AFN-1252)	TXA-709 (prodrug of TXA-707)
Family	Diaryldiamine	Tricyclic diterpenoid pleuromutilin	Benzofuran naphthyridine	Benzamide
Mode of action	Inhibition of methionyl-tRNA synthetase (MetRS1)	Inhibition of 50S ribosomal subunit	Inhibition of FabI	Favoring uncontrolled FtsZ polymerization
Chemical features	MW: 513.25 cLogP: 4.02 H-bond acc.: 5 H-bond don.: 3 PSA: 90.63	MW: 507.73 cLogP: 2.77 H-bond acc.: 7 H-bond don.: 3 PSA: 135.15	MW: 485.43 cLogP: 2.57 H-bond acc.: 7 H-bond don.: 2 PSA: 143.22	MW: 514.47 cLogP: 3.69 H-bond acc.: 6 H-bond don.: 1 PSA: 112.66
Development phase	Phase II	Approved in 2019 (FDA) and 2020 (EMA)	Phase II	Phase I
Relevant references	[97, 98, 100, 104, 105]	[105, 108, 114, 119, 130]	[140, 141, 143, 145-147, 149]	[153, 156, 157, 161]

2. DNA topoisomerases as drug targets

2.1. Introduction

DNA topoisomerases are enzymes able to induce negative supercoil to compensate positive supercoil occurring during cellular events such as replication, transcription, recombination and DNA

89 repair processes. Concerning bacteria, there are four types of topoisomerases: DNA gyrases, topoi-
90 somerases I, topoisomerases IV and reverse gyrases.

91 Currently, DNA topoisomerases are targeted by fluoroquinolones, a well-known class of antibi-
92 otics. However, more and more fluoroquinolone-resistant strains are being reported. This critical
93 point urges the need to develop topoisomerase inhibitors with a distinct mode of action to avoid
94 cross-resistance. Two of these antibiotics have reached clinical trials: gepotidacin and zoliflodacin.

95 **2.2. Gepotidacin**

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98 Gepotidacin (GSK2140944) is a triazaacenaphtylene developed by GSK and belongs to the class
99 of Novel Bacterial Topoisomerase Inhibitors (NBTI). This new antibiotic is currently being investigated
100 in three phase 3 clinical trials.

101 Gepotidacin is derived from the analogue GSK299423 described by Bax *et al.* [9], which results
102 from a medicinal chemistry program initiated after an unbiased antibacterial screening [10].

103 **2.2.1. Chemical synthesis**

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106 The synthesis of gepotidacin has been described in two patents in 2008 and 2016 and comprises
107 11 steps (Figure 2) [11,12]. First, 2-chloro-6-methoxy-3-nitro-pyridine reacts with
108 2-amino-propane-1,3-diol through nucleophilic aromatic substitution (S_NAr). The resulting diol is then
109 protected with 2,2-dimethoxypropane in presence of p-toluenesulfonic acid (PTSA) followed by the
110 reduction of the nitro group with hydrogen and 10% Pd/C. The aniline thus formed is then alkylated
111 with ethyl bromoacetate. Cyclization is performed in basic conditions using sodium hydride, followed
112 by oxidation using manganese dioxide. The acetal is then cleaved and the released diol reacts with
113 methanesulfonic anhydride to form the third cycle of the triazaacenaphtylene core. Substitution with
114 Boc-amino-piperidine, followed by deprotection and subsequent purification by chiral chromatogra-
115 phy affords the primary amine derivative, which can be condensed by reductive amination with the
116 corresponding aldehyde to give the free base of gepotidacin. The mono-hydrochloride salt is ob-
117 tained by reaction with one equivalent of HCl 1M in diethylether [13].

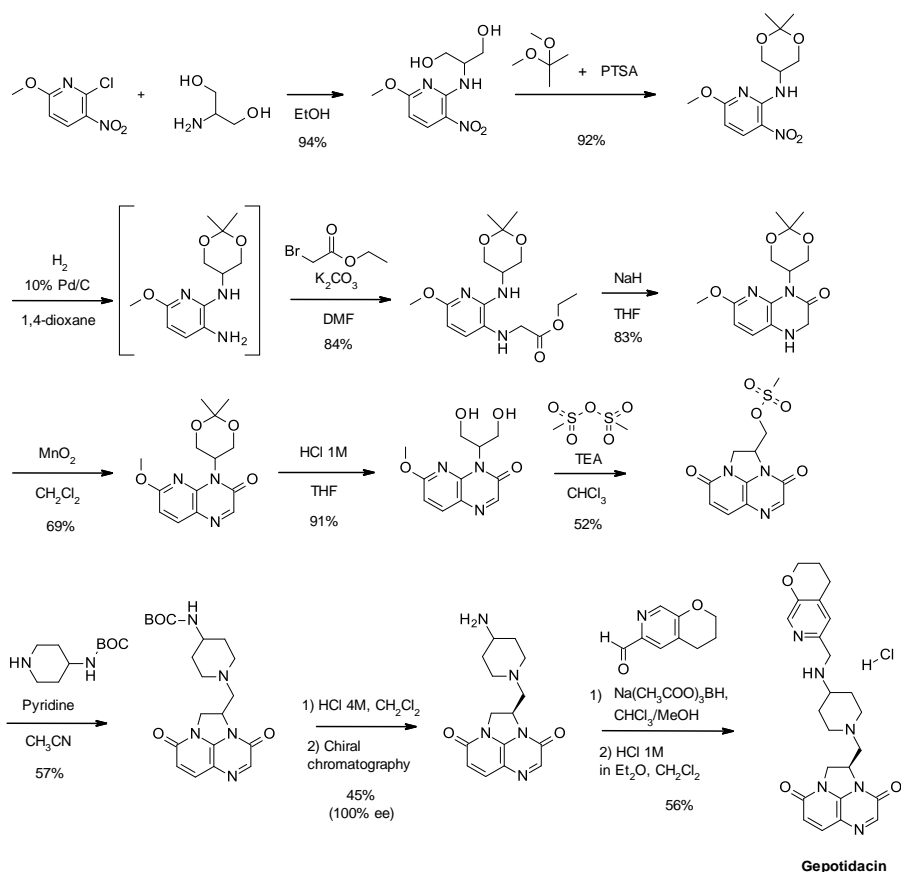


Figure 2. Description of the synthetic pathway leading to gepotidacin [11,12].

2.2.2. Target and mechanism of action

Gepotidacin inhibits both DNA gyrase and topoisomerase IV. More specifically, gepotidacin binds to the GyrA subunit of DNA gyrase and to the ParC subunit of topoisomerase IV. With its novel chemical scaffold, gepotidacin can form a ternary complex with DNA and two GyrA or ParC subunits (Figure 3) [9,14]. The structure of gepotidacin can be divided in three parts: the left-hand side (LHS), the linker and the right-hand side (RHS) (Figure 4). The polar tricyclic core of the LHS intercalates between DNA bases. Its position and structure can affect the spatial orientation of the linker and of the RHS. The linker possesses a basic amine able to interact with both GyrA/ParC subunits of DNA gyrase/topoisomerase IV. Lastly, the RHS binds to a hydrophobic pocket of the bacterial gyrase/topoisomerase IV [15].

With this new binding mode, gepotidacin shows potent antibacterial activity against a broad spectrum of Gram-positive and Gram-negative pathogenic bacteria, including clinical isolates with fluoroquinolone resistance mediated by DNA gyrase and topoisomerase IV mutations.

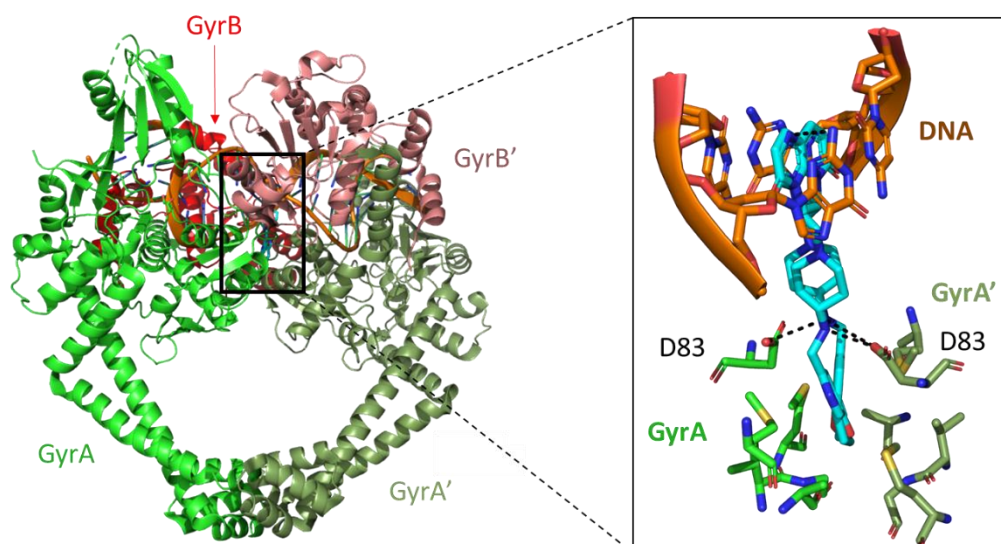


Figure 3. Views of the gepotidacin complex formed with *S. aureus* DNA gyrase and uncleaved DNA (PDB code: 6QTP). Due to a relative movement of the two GyrA subunits, the two existing positions of gepotidacin are represented.

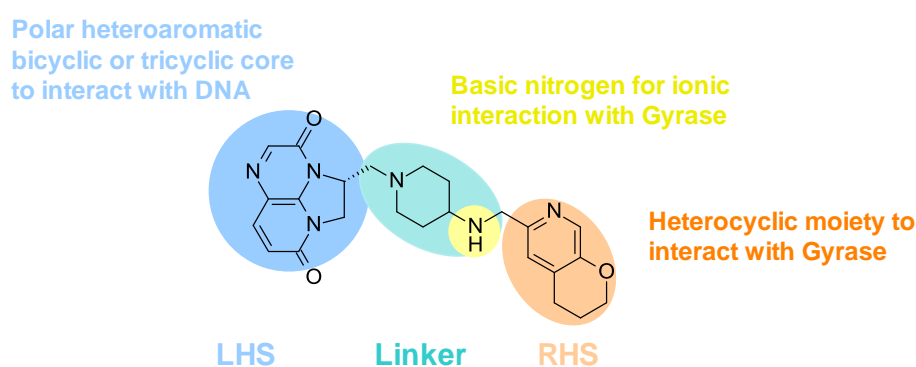


Figure 4. Summary of the key pharmacophore elements of gepotidacin.

2.2.3. *In vitro* activity

Gepotidacin displayed a bactericidal activity against a wide variety of pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant isolates of *Streptococcus pneumoniae* and *Escherichia coli* bacteria expressing extended spectrum β -lactamase (ESBL). Gepotidacin was also found active against different levofloxacin-resistant strains of *S. aureus* and *S. pneumoniae*, with MIC₉₀ of 0.5 μ g/mL. This compound was also potent against levofloxacin-resistant Gram-negative bacteria such as *E. coli* with MIC₉₀ of 4 μ g/mL [16,17].

2.2.4. *In vivo* experiments

The potency of gepotidacin against MRSA was demonstrated in a neutropenic murine lung infection model. Mice received different doses of gepotidacin, ranging from 1.56 to 400 mg/kg, administered every 6 h and starting 3 h post inoculation with six different clinical isolates of MRSA. The maximum decreases in CFU observed after 24 h were 1.4 to 3.1 log₁₀ CFU compared to the initial lung bacterial load in the neutropenic mice (between 5.6 and 6.3 log₁₀ CFU) [18].

159 Using neutropenic mouse models of thigh and lung infection, the pharmacoki-
160 netic-pharmacodynamic (PK-PD) parameters of gepotidacin against *S. aureus* and *S. pneumoniae*
161 were characterized. The free plasma AUC/MIC ratio was identified as the PK-PD index most closely
162 associated with efficacy, implying that gepotidacin activity was concentration dependent [19].

163 In a recent study, the predicted human exposure was used to assess the efficacy in a rat pyelo-
164 nephritis model with four multidrug-resistant (MDR) *E. coli* isolates. Overall, treatment with
165 gepotidacin resulted in a significant reduction in CFU in the kidney and bladder [20].

166 Regarding toxicity, gepotidacin did not demonstrate arthropathy in a juvenile rat model, contrary
167 to fluoroquinolones [21].

168 2.2.5. Phase 1

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171 Since 2012, gepotidacin has been evaluated in thirteen phase 1 clinical trials [22–34], assessing
172 the safety and tolerability of single and repeated oral or IV doses (100 mg to 6 g per day) in adults
173 and adolescents. In these studies, the authors also evaluated the PK parameters in plasma and lungs,
174 cardiac conduction, food effect, drug-drug interactions and penetration into prostate and tonsil tis-
175 sue. Finally, the PK was established in patients with various renal and hepatic insufficiencies. Re-
176 cently, another phase 1 study started to compare the PK properties of the powder for oral suspen-
177 sion to the existing gepotidacin tablets [35].

178 2.2.6. Phase 2

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181 Gepotidacin was tested against three types of bacterial infections in phase 2 clinical trials. It was
182 studied as a treatment for Gram-positive Acute Bacterial Skin and Skin Structure Infections (ABSSSI),
183 for uncomplicated urogenital gonorrhoea caused by *Neisseria gonorrhoeae*, as well as for uncompl-
184 icated Urinary Tract Infections (UTI).

185 In the ABSSSI phase 2 trial, 122 patients with wound infection, major cutaneous abscess or cellu-
186 litis were enrolled. Three dose regimens were evaluated: 750 mg every 12 h (q12h), 1000 mg every
187 12 h and 1000 mg every 8 h (q8h). Success was observed for the 750 mg q12h and the 1000 mg q8h
188 regimens. The majority of adverse events (AE) declared were gastrointestinal events, principally
189 nausea and diarrhoea [36].

190 In a second phase 2 trial, 69 participants with urogenital gonorrhoea received a single oral dose
191 of 1500 or 3000 mg. 96% of patients achieved microbiological cure (bacterial eradication of *N. gon-*
192 *orrhoeae*). Of note, 2 out of 3 failures were due to a high-level resistance. AEs were mostly gastroin-
193 testinal events (diarrhoea, flatulence, abdominal pain, nausea). Two patients described severe gas-
194 trointestinal AE (flatulence or dizziness) but did not discontinue the study. One treatment-related
195 cardiovascular AE (mild tachycardia) was reported by a patient with no underlying cardiovascular
196 history [37].

197 The goal of the third phase 2 trial was to explore the PK properties in UTI patients. 22 women
198 with uncomplicated UTI received 1500 mg gepotidacin orally twice daily for 5 days. The test of cure
199 was performed from days 10 to 13 and the follow-up visits were planned at day 28 +/- 3. With a dose
200 of 1500 mg twice daily, the maximum plasma concentration was reached 1 h to 1.5 h after dosing.
201 Steady state was reached on day 3. Clinical success was validated for 18 patients (82%) at follow-up
202 visits. 21 patients (95%) experienced mild to moderate AEs, primarily gastrointestinal events. Two
203 patients (9%) reported vulvovaginal mycotic infection after treatment, but no AE prevented treat-
204 ment [38].

205 2.2.7. Current advances

208 Gepotidacin is currently being evaluated in three phase 3 clinical trials. Two studies aim to com-
209 pare gepotidacin (1500 mg orally twice a day for 5 days) and nitrofurantoin (100 mg orally twice a
210 day for 5 days) for the treatment of uncomplicated UTI in approximately 2500 patients each [39,40].
211 The third trial will assess the efficacy of gepotidacin (3000 mg orally twice) compared with the com-
212 bination ceftriaxone/azithromycin (single doses of 500 mg intramuscular and 1000 mg orally, respec-
213 tively) for the treatment of uncomplicated urogenital gonorrhoea on an estimated 600 patients [41].

214 In a recent study, gepotidacin was also found efficacious in an inhalational model of pneumonic
215 plague in the African green monkey, a non-human primate, at exposures of the drug equivalent to
216 those achieved in humans. In this experiment, gepotidacin provided a 75 to 100% survival benefit
217 with doses ranging from 28 to 48 mg/kg. These data open up the possibility of also using gepotidacin
218 as a treatment for pneumonic plague caused by *Yersinia pestis* [42].

220 **2.3. Zoliflodacin**

221
222 Zoliflodacin (previously known as ETX0914 or AZD0914) is a novel spiropyrimidinetrione antibi-
223 otic being developed as single oral dose for the treatment of uncomplicated gonorrhoea [43–46].

225 **2.3.1. Spectrum of activity**

226
227 Zoliflodacin displays activity against Gram-positive bacteria including *S. pneumoniae*, *Strepto-*
228 *coccus pyogenes*, and methicillin-resistant fluoroquinolone-resistant *S. aureus*, as well as against fas-
229 tidious Gram-negative bacteria including *Haemophilus influenzae* and *N. gonorrhoea* [47].

231 **2.3.2. Chemical synthesis**

232
233 The synthesis of zoliflodacin described below was reported in 2015 [47]. The first step, starting
234 from 2,3,4-trifluorobenzaldehyde, consists of the protection of the aldehyde function to an acetal
235 group. After deprotonation using *n*-BuLi, formylation is performed with DMF to introduce an alde-
236 hyde group, which is then converted to oxime using hydroxylamine. Chlorination with
237 *N*-chlorosuccinimide (NCS), followed by reaction with *L*-alaninol and intramolecular S_NAr allows the
238 formation of the benzisoxazole ring. The oxazolidinone moiety is obtained using
239 1,1'-carbonyldiimidazole (CDI). The deprotection of the aldehyde is then performed in acidic condi-
240 tions followed by another S_NAr at the *ortho* position of the aldehyde using
241 (2*R*,6*S*)-2,6-dimethylmorpholine. Finally, a Knoevenagel condensation between the aldehyde and
242 hexahydropyrimidine-2,4,6-trione is performed, followed by an intramolecular rearrangement con-
243 sisting in an [1-5] hydride shift and then intramolecular cyclization leading to zoliflodacin (Figure 5).

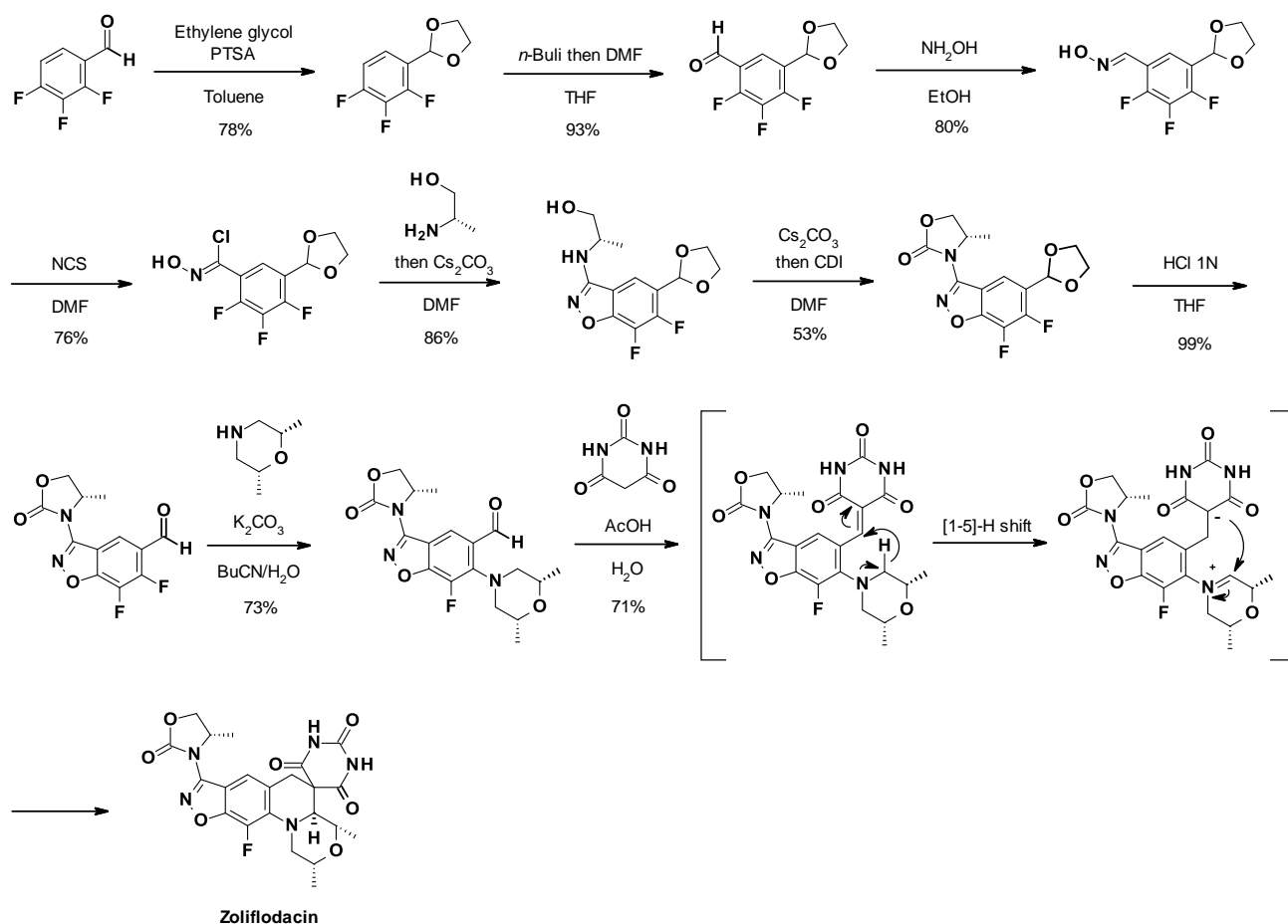


Figure 5. Description of the synthetic pathway leading to zoliflodacin [47].

2.3.3. Target and mechanism of action

Zoliflodacin is a topoisomerase II inhibitor that presents a new mode of action compared with previous inhibitors, such as fluoroquinolones [48]. Zoliflodacin binds to the GyrB subunit of the bacterial DNA gyrase whereas fluoroquinolones target the GyrA subunit [49]. Briefly, the binding enables to stop the cleaved covalent complex of gyrase which thus blocks the religation of the double-strand cleaved DNA to form fused circular DNA. This process leads to the inhibition of DNA biosynthesis and the accumulation of double-strand cleavages in bacteria [50]. Interestingly, there is no cross-resistance with the existing fluoroquinolones [51].

2.3.4. Structure-activity relationship (SAR) studies

After a high-throughput screening at Pharmacia & Upjohn, QPT-1 was found as the first spiroimidinetrone inhibiting bacterial DNA gyrase and topoisomerase IV with no cross-resistance with fluoroquinolones (Figure 6). A benzisoxazole scaffold was then introduced by scientists at AstraZeneca [52]. However, due to toxicity and genotoxicity other variations were studied:

- **Spirocyclic pyrimidinetrone:** the pyrimidinetrone was kept constant in all the synthesized analogues.
- **Oxazolidinone introduction:** the oxazolidinone ring leading to zoliflodacin was introduced replacing the methyl substituent of the previous lead to mitigate mammalian toxicity and to obtain compounds characterized by a greater safety [44]. Highest potency was found for derivatives

with substituents at both the 4- and 5- positions orientated below the plane of the oxazolidinone ring [53].

- **Benzisoxazole substitution:** the replacement of the oxazolidinone ring with an ethyloxadiazole and triazole rings led to analogues with higher activity than zoliflodacin. Moreover, the introduction of heterocycles linked to the benzisoxazole through an ether oxygen in a two-atom bridge and the replacement of the fluorine atom with a chlorine resulted in compounds with a similar activity to zoliflodacin [44,54]. In contrast, pyrrolidine benzisoxazole substituents led to derivatives 200-fold less active against *S. aureus*.
- **Morpholine modifications:** overall, the morpholine ring showed little scope for variations and the modifications resulted in less active compounds. The tested analogues were synthesized replacing the morpholine with substituted piperidines and piperazines. However, the introduction of a *N*-cyano piperazine resulted in a *S. aureus* active compound [54]. Furthermore, the biological evaluation of the corresponding enantiomers and diastereoisomers showed that the specific stereochemistry and the two methyl groups around the morpholine are crucial for the antibacterial activity [44,53].

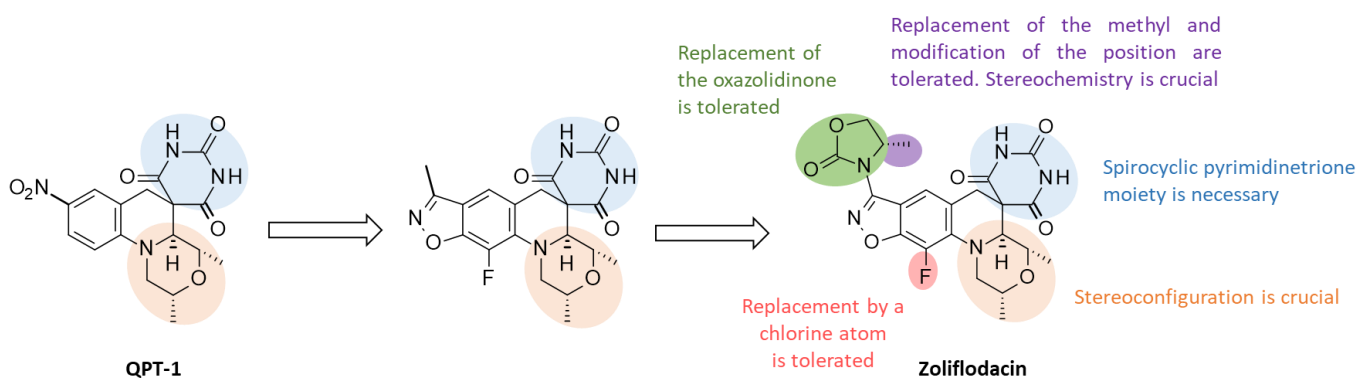


Figure 6. Summary of the SARs of zoliflodacin.

2.3.5. *In vitro* activity

Zoliflodacin is a potent and very selective inhibitor of bacterial topoisomerases. Indeed, *in vitro* assays measuring the growth inhibition of myeloid and erythroid cell lines in mammals showed no genotoxicity or bone marrow toxicity for zoliflodacin at the highest concentrations tested. These data indicate that zoliflodacin presents reduced risks of hematologic toxicity compared with some other fluoroquinolones (such as gemifloxacin, for instance), which have been associated with anemia, leukopenia and bone marrow suppression [48].

Zoliflodacin is bactericidal, with a low frequency of resistance and a potent *in vitro* activity against *N. gonorrhoeae* (MIC₉₀ of 0.125 µg/mL) [45,48]. To date, no clinical zoliflodacin-resistant *N. gonorrhoeae* isolates have been found [48]. However, zoliflodacin resistant mutants have been selected *in vitro*, and all of them contained nonsynonymous mutations in amino acid codons D429 or K450 of GyrB, which leads to increased MICs of zoliflodacin in the mutants [49]. Zoliflodacin also presented potent *in vitro* activity against *Chlamydia pneumoniae*, certain Gram-positive organisms (*S. aureus* and *S. pneumoniae*; including fluoroquinolone- and vancomycin-resistant staphylococci), fastidious Gram-negative organisms (*Moraxella catarrhalis* and *Haemophilus* spp., in particular *H. influenzae*), and atypical anaerobic organisms [44,51].

Zoliflodacin also displayed potent *in vitro* activity against *Mycoplasma genitalium* and 11 azithromycin- and fluoroquinolone-susceptible isolates with MICs < 1 mg/L [55]. Furthermore, zoliflodacin showed activity against *Chlamydia trachomatis* with the highest zoliflodacin MIC of 0.5 mg/L among 10 strains.

Zoliflodacin was also fully active against laboratory strains of *S. aureus* and *S. pneumoniae* resistant to novobiocin. It was also more active against *S. aureus* (including MRSA) than linezolid, which is the current standard of care for skin infections caused by this pathogen [47].

More recently, zoliflodacin demonstrated potent *in vitro* antibacterial activity against a collection of clinical gonococcal isolates from China, including isolates with a high-level of resistance to ciprofloxacin, azithromycin, and extended-spectrum cephalosporins [56]. Due to a distinct mechanism of action, cross-resistance between zoliflodacin and fluoroquinolones has not been observed [57].

2.3.6. *In vivo* experiments

In vitro and *in vivo* studies across species showed that zoliflodacin was extensively metabolized in humans via the cytochrome P450 and non-P450-mediated pathways [58]. The assessment of the *in vivo* efficacy of zoliflodacin was defined using a murine thigh infection model in immunocompetent and neutropenic mice using a methicillin-susceptible strain of *S. aureus* [59]. This study showed that the compound was efficient in a dose-dependent manner in both mouse models and a 3-log reduction in CFU was observed when the exposure (AUC) achieved 70 µg.h/mL in immunocompetent mice and 120 µg.h/mL in immunosuppressed mice.

2.3.7. Phase 1

Two phase 1 studies have been conducted with single oral doses in healthy volunteers [58]. The first study determined the safety, tolerability and PK parameters of zoliflodacin in both fed and fasted states [60], whereas the second study assessed the PK and ADME properties using [¹⁴C] zoliflodacin [61].

In the first study, zoliflodacin showed a rapid absorption, with a T_{max} observed between 1.5 and 2.3 h, and a T_{1/2} range of 5.3 to 6.3 h. Interestingly, the absorption of the drug was delayed (T_{max} at 4 h) in

336 subjects who were dosed following ingestion of a high-fat meal. Finally, no significant AEs were ob-
337 served in this study.

338 In the second study, the ADME properties were defined after a single oral dose of 3000 mg of [¹⁴C]
339 zoliflodacin. The data demonstrated that the PK profile of zoliflodacin (AUC and C_{max}) was similar to
340 the one obtained in the first phase 1 study.

341 Overall, both phase 1 studies showed linear PK data, good oral bioavailability and no significant
342 safety findings.

344 2.3.8. Phase 2

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346 The safety and efficacy of zoliflodacin was then assessed in a randomized phase 2 study on 179
347 participants with uncomplicated urogenital gonorrhoea [62]. The subjects were randomized to receive
348 a single oral dose of zoliflodacin (either 2 or 3 g) or a single 500 mg intramuscular dose of ceftriax-
349 one. In summary, oral zoliflodacin has successfully treated most uncomplicated urogenital and rectal
350 gonococcal infections, but has shown little efficacy in treating pharyngeal infections.

352 2.3.9. Phase 3

353
354 Following promising results from one of the phase 2 clinical trials, a phase 3 for the treatment of
355 uncomplicated urogenital gonorrhoea is currently underway [63]. The study is expected to include ap-
356 proximately 1000 adults with urogenital gonorrhoea from different sites (USA, The Netherlands, Thai-
357 land, and South Africa) and results are now expected in 2023 (current status). This open-label trial
358 compares a single 3 g oral dose of zoliflodacin to a combination of ceftriaxone and azithromycin (2:1
359 randomization). Recently, a parallel study [64] demonstrated that a single oral dose of zoliflodacin
360 was positively evaluated for cardiac safety, supporting further clinical experiments with single 3 g
361 doses in patient with gonorrhoea [46].

363 3. DNA polymerase as a drug target

365 3.1. Introduction

366
367 DNA replication is carried out by the DNA polymerase, a massive enzymatic complex highly orga-
368 nized and therefore susceptible to disruption. Inhibitors of DNA polymerase are widely used to treat
369 viral infections, such as aciclovir for the treatment of herpes virus. However, there is currently no an-
370 tibiotic in clinic which targets the bacterial DNA polymerase, whereas human and bacterial DNA
371 polymerases have many differences [65]. To date, ibezapolstat is the first bacterial specific DNA
372 polymerase inhibitor in clinical development.

374 3.2. Ibezapolstat

375
376 Ibezapolstat (formerly ACX-362E) is a member of the 6-anilinouracil family of dGTP analogues
377 [66]. Developed by Acurx Pharmaceuticals, it selectively inhibits DNA polymerase III α of several
378 Gram-positive bacteria, through competition with dGTP [67]. In 2012, Dvoskin *et al.* demonstrated
379 that ibezapolstat was highly effective against *Clostridioides difficile* on *in vitro* and *in vivo* models
380 [68]. The compound is currently being evaluated in a phase 2 clinical study [69].

382 3.2.1. Chemical synthesis

Ibezapolstat was first synthesized in 2010 in a 3-step procedure, following a protocol described by Wright *et al.* (Figure 7) [70]. Firstly, 2-chloro-6-methoxypurine is alkylated with 1,2-dibromoethane to afford a mixture of 7- and 9- isomers. After isolation of the 7-isomer, a S_N2 reaction with morpholine leads to the corresponding intermediate, which is then subjected to reaction with 3,4-dichlorobenzylamine to give ibezapolstat after the concomitant demethylation due to HCl release.

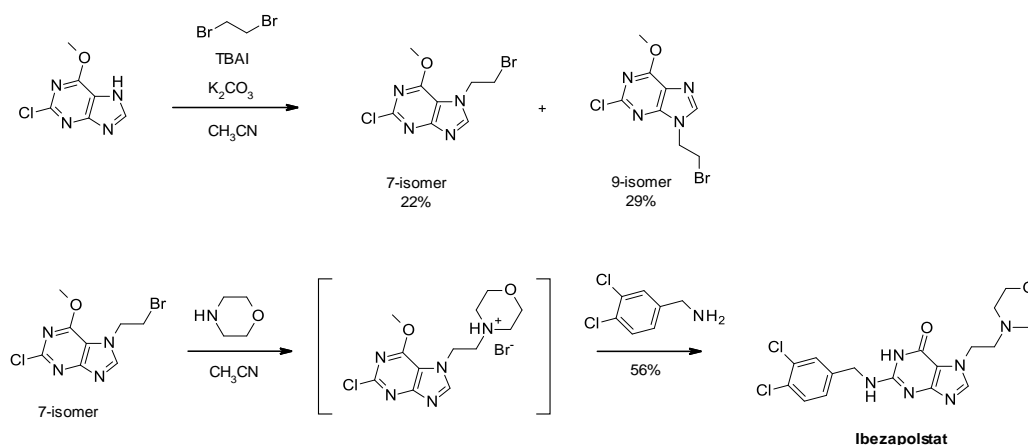


Figure 7. Description of the synthetic pathway leading to ibezapolstat [70].

3.2.2. Target and mechanism of action

Ibezapolstat inhibits the DNA polymerase III_C (DNA pol III_C), an enzyme essential for bacterial DNA replication [71], from low G + C Gram-positive germs (DNA containing less than 50 % of cytosine and guanine). Ibezapolstat is also an analogue of dGTP, allowing the compound to pair with dCTG of DNA. In addition, the aryl part of the molecule can bind to DNA pol III_C. The combination of these two pro-perties leads to the formation of an inactive ternary complex between DNA, ibezapolstat and DNA pol III_C (Figure 8). To note, the compound inhibits the DNA pol III_C of *C. difficile* with a K_i of 0.325 μM [72]. Moreover, there is only little homology between DNA pol III_C and mammalian polymerase [73].

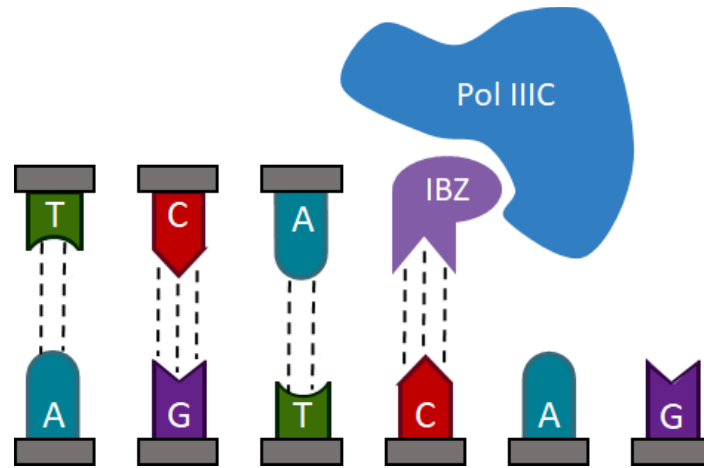


Figure 8. Binding mode of ibezapolstat (IBZ) to dCTP and DNA pol III C. Adapted from Acurx Pharmaceuticals [74].

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406

407

3.2.3. Structure-activity relationship studies

Ibezapolstat is a 6-anilinouracil derived from 6-(p-hydroxyphenylhydrazino)-uracil (H₂-HPUra), itself identified in the late 1970's as an inhibitor of DNA pol IIIc. In order to improve potency, structure-activity relationship studies were performed [75,76] (Figure 9):

- **Replacement of the uracile core by a guanine core:** H₂-HPUra binds to cytosine via its uracil core. The latter was replaced by the natural DNA base guanine. A loss of activity was observed, except when the 3,4-dichlorobenzyl moiety was introduced.

- **Modification of the 3,4-dichlorobenzyl moiety:** replacement by other groups and/or modification of the position of the two chlorine atoms led to less potent compounds.

- **Alkylation of the guanine:** the alkylation of the nitrogen at position 7 resulted in more potent derivatives. On the contrary, the alkylation of the nitrogen at position 9 led to a total loss of potency.

- **Modification of the linker:** even if compounds with a longer linker are active, the ethylene linker results in the most potent compound.

- **Modification of the morpholino part:** replacement of the morpholino part by esters or alcohols is tolerated. However, the presence of a protonated nitrogen at physiological pH probably explains why the morpholine ring was kept.

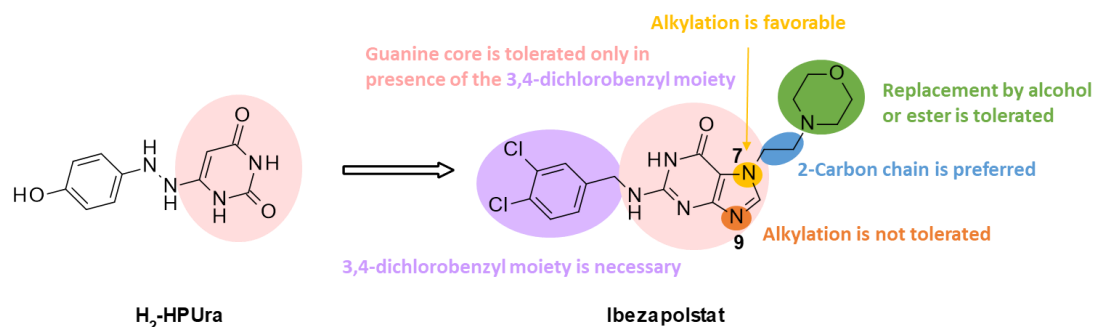


Figure 9. Summary of the SARs of ibezapolstat from H₂-HPUra.

3.2.4. *In vitro* activity

Against 23 isolates of *C. difficile*, ibezapolstat (MIC₅₀ of 2 µg/mL) was shown as potent as metronidazole and vancomycin (MIC₅₀ of 1 µg/mL for both compounds), which are the treatment of choice for *C. difficile* infections (CDI) [77]. More recently, ibezapolstat was evaluated against 104 isolates of *C. difficile* [71]. MIC range of the molecule (1-8 µg/mL) was higher than metronidazole (0.25-16 µg/mL), vancomycin (0.5-4 µg/mL) and fidaxomicin (0.015-1 µg/mL). However, due to the mechanism of action of ibezapolstat targeting DNA pol IIIc, which is not expressed by Gram-negative bacteria, the compound has a narrow spectrum compared to metronidazole and vancomycin. Indeed, ibezapolstat has low activity against representatives of the normal colonic flora, such as *Bacteroides fragilis*, while metronidazole is active against some of them [72]. Overall, ibezapolstat exhibits an interesting spectrum profile, as the compound is unlikely to disrupt the endogenous bacterial flora, a property that is preferable for avoiding recurrence of CDI [78,79].

3.2.5. *In vivo* experiments

An efficacy study was performed in a golden Syrian hamster model by calculating the percentage of survivors after 5 days. Animals were completely protected at a dose of 12.5 mg/kg administered twice daily for 3 days. However, 67 % of the animals died after 15 days, due to recurrence of the infection. The recurrence was less pronounced with animals treated for more than 3 days. Indeed, only

447 40 % of the animals died after 7 days of treatment. Administration of ibezapolstat during 14 days did
448 not result in recurrence in any of the animals infected during the 34 days of the experiment.

449 In the hamster model of *Clostridioides difficile*-associated diarrhea (CDAD), ibezapolstat showed
450 no signs of toxicity at an oral dose of 1 g/kg [68]. Moreover, at the oral dose of 75 mg/kg, only 5 % is
451 absorbed. Because the site of CDI is exclusively located in the colon, a lower absorption results in
452 higher concentrations in the intestine.

453 454 3.2.6. Phase 1

455
456 Ibezapolstat has completed a phase 1 study in the United States [80]. The randomized, dou-
457 ble-blind, placebo-controlled clinical trial involved 62 healthy adults and was divided into three parts.

458 The first consisted of oral administration of a single ascending dose of ibezapolstat at doses of
459 150, 300, 600 and 900 mg. In the second part, a single dose of 300 mg was given with a meal. Finally,
460 the third part was a multi-day ascending dose study of ibezapolstat 300 mg and 450 mg given twice
461 daily or vancomycin 125 mg given four times daily.

462 The drug candidate was found to be safe and well-tolerated. Consistent with the hamster phar-
463 macokinetics, ibezapolstat was poorly absorbed, resulting in no systemic accumulation. Of note, fae-
464 cal concentrations of ibezapolstat after 4 days of multiple ascending doses were higher than those of
465 vancomycin. Vancomycin also decreased the diversity of the microbiome while ibezapolstat targeted
466 only low G+C organisms. Moreover, vancomycin increased the proportion of proteobacteria, which is
467 associated with susceptibility to CDI [81].

468 469 3.2.7. Phase 2

470
471 A phase 2a study was conducted in 10 adults with CDI, as a single-arm, open-label multicenter
472 clinical trial [82]. Ibezapolstat was orally given 450 mg twice daily, during 10 days. Safety and phar-
473 macokinetic profiles were consistent with the data from the phase 1 study. *C. difficile* was eradicated
474 after 3 days, resulting in 100 % cure of all 10 patients. In addition, none of the patients experienced a
475 recurrence during the 28-day study.

476 Regarding the microbiome, an increased proportion of healthy microbiota was observed, includ-
477 ing bacteria capable of metabolizing primary bile acids into secondary bile acids [83]. This change in
478 the microbiome resulted in an increased proportion of secondary bile acids, which is associated with
479 protection against CDI [84]. Ibezapolstat is currently being evaluated in phase 2b study to compare
480 efficacy of the drug candidate against vancomycin [69].

481 482 4. DNA minor groove as a drug target

483 484 4.1. Introduction

485
486 Small molecules can manifest their effect on DNA through various binding mechanisms such as
487 intercalation, alkylation as well as major and minor groove binding [85]. These interactions can cause
488 a variety of important biological responses. Minor groove binders (MGBs) are well-known for their
489 applications as anticancer drugs, although their importance as anti-infective agents is growing [86].
490 The majority of synthetic MGBs are linked to the two natural occurring antibiotics, netropsin and
491 distamycin A, and are generally characterized by the presence of an alkene, a non-polar N-terminal
492 amide isostere, and N-alkyl chains on the pyrrole rings [87].

493 494 4.2. MGB-BP-3

MGB-BP-3 is a synthetic polyamide belonging to the large family of minor groove binders, small molecules capable of binding to the minor groove of DNA. Hundreds of MGBs were synthesized and tested for various biological activities by Khalaf *et al.* at the University of Strathclyde. In the end, MGB-BP-3 was found to be the most active against Gram-positive bacteria. Collaboration with MGB Biopharma enabled the preclinical and clinical development of MGB-BP-3 for the treatment of gastrointestinal infections caused by *C. difficile* [87–89].

4.2.1. Chemical Synthesis

MGB-BP-3 was synthesized by Anthony *et al.* by combining two moieties (synthon A and synthon B) [90]. To obtain the synthon A, 1-methyl-4-nitro-pyrrole-2-carboxylic acid is first converted into an acyl chloride which reacts with aminoethyl morpholine to give the corresponding amide. After reduction of the nitro group the intermediate is reacted with the previous acyl chloride to deliver the synthon A. The synthon B is synthesized in three steps starting from methyl 4-(bromomethyl)benzoate. At first, an Arbuzov reaction using triethylphosphite gives the corresponding phosphonate which reacts with quinoline-3-aldehyde *via* a Horner Wadsworth Emmons reaction. Finally, the saponification of the methyl ester delivers the synthon B. Eventually, the reduction of the nitro group of the synthon A and the subsequent coupling with the synthon B lead to MGB-BP-3 (Figure 10).

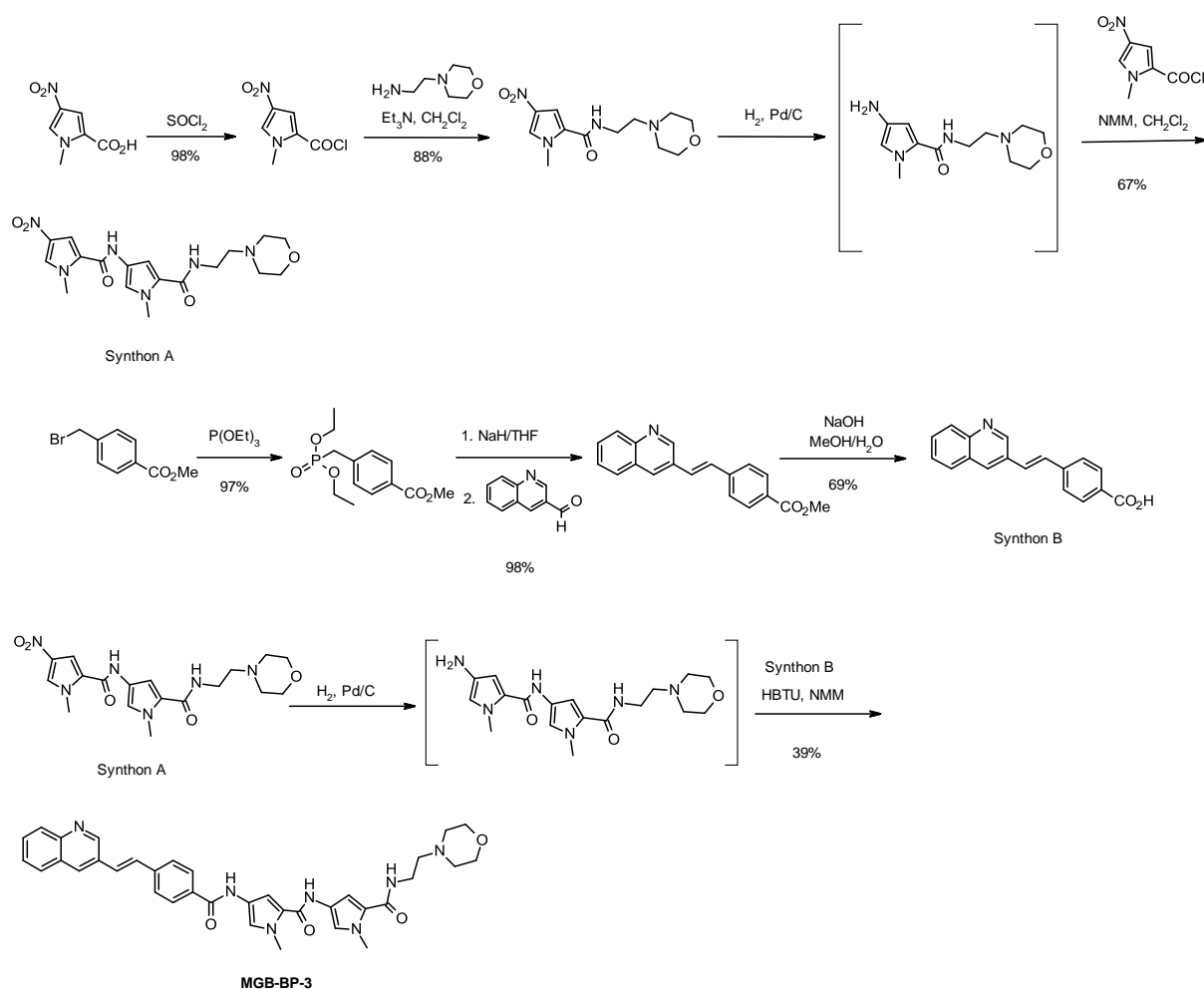


Figure 10. Description of the synthetic pathway leading to MGB-BP-3 [90].

4.2.2. Target and mechanism of action

MGB-BP-3 binds non-covalently to the minor groove of the DNA, most readily to AT-rich regions [89]. Probably, MGB-BP-3 binds as a dimer, with two molecules of ligand in the minor groove with an antiparallel configuration. Thermal melt analysis, capillary electrophoresis, fluorescence intercalator displacement assay, mass spectrometry and NMR techniques have been exploited to investigate and confirm the binding of MGB-BP-3 to DNA [91,92]. MGB-BP-3 binding to DNA interferes with gene expression at the transcription level. Recently, Hind *et al.* found out that MGB-BP-3 interferes with other DNA-centric processes, such as the action of topoisomerase and gyrase, with a different mechanism of action than fluoroquinolones [92].

4.2.3. Structure-activity relationship studies

Suckling *et al.* synthesized several MGB-BP-3-like compounds and reported the following structure-activity relationships against Gram-positive bacteria (Figure 11) [88]:

- **Amino tail group:** substituents such as piperazines, piperidines, pyrrolidines were introduced and led to significantly active but more toxic compounds.
- **Aromatic head group:** aromatic head groups such as meta-methoxybenzene and para-methoxybenzene led to active compounds. However, the introduction of a naphthalene and isoquinoline head groups resulted in less active compounds.
- **Linker:** to increase further the non-polar interactions an alkene between the benzene ring and the quinoline was successfully introduced and opened the way for the discovery of the lead compound MGB-BP-3. In contrast, when an alkene was introduced between the two pyrroles the activity was totally lost.
- **N-substitution:** the introduction of isopropyl or bulkier groups on the nitrogen of pyrrole rings led to active compounds.

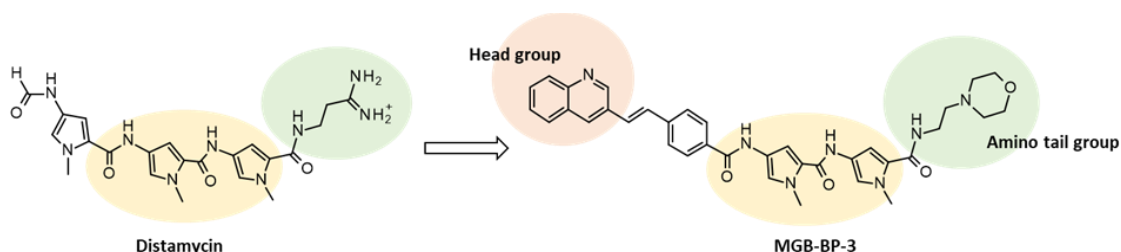


Figure 11. Summary of the SARs of MGB-BP-3 from distamycin.

4.2.4. *In vitro* activity

MGB-BP-3 showed substantial antibacterial activity against many Gram-positive bacteria such as *S. aureus* ($MIC_{80} < 0.2 \mu M$) [89]). No evident toxicity was found on a mammalian cell line (HS27 murine fibroblast). Thanks to fluorescence microscopy, Suckling *et al.* showed that there was no penetration of MGB-BP-3 into several mammalian cell lines and concluded that the minor groove binder targeted selectively the bacterial cell [88].

MGB-BP-3 is highly active against *C. difficile*, killing the vegetative bacterium cell before sporulation. In particular, MGB-BP-3 exhibits strong bactericidal activity against BI/NAP1/027, the most virulent strain that is highly resistant to current therapies [93].

555 In addition, *S. aureus* was grown at sub-MIC₈₀ concentrations of MGB-BP-3 or rifampicin (used as
556 a control) for 80 generations. No resistant strain towards MGB-BP-3 was observed, contrary to ri-
557 fampicin [89].

558 MGB-BP-3 did not show significant activity against Gram-negative bacteria. Interestingly, signifi-
559 cant activity was found when *Pseudomonas aeruginosa* bacteria were treated with MGB-BP-3 in as-
560 sociation with an efflux pump inhibitor (PAβN, Phenylalanine-Arginine-β-naphthylamide) or a mem-
561 brane permeabilizer (polymyxin B nonapeptide). These results suggest that the major obstacle to the
562 antibacterial activity of MGB-BP-3 is the expression of efflux pumps [89].

563 4.2.5. *In vivo* experiments

564
565
566 MGB-BP-3 was able to protect against death and prolonged post-treatment survival in a hamster
567 model of CDI [93]. In the study, the efficacy of MGB-BP-3 (free base suspension and colonic delivery
568 microparticles) was compared to that of vancomycin in the treatment of clindamycin-induced *C.*
569 *difficile* infection in a hamster CDAD model [83].

570 The animals received 30 mg/kg oral clindamycin, followed 24 hours later by *C. difficile* (strain BI1)
571 by gavage. The treatment started 20 hours post *C. difficile* infection and was administered orally for 5
572 days. The hamsters received MGB-BP-3 as a free base suspension or as colonic delivery
573 microparticles (10 mg bid), vancomycin (25 mg/kg bid) or a vehicle. The animals were observed for
574 up to 13 days post *C. difficile* administration and sacrificed when severe infection developed.

575 All untreated and vehicle treated hamster died within 36 hours post infection. On the contrary,
576 MGB-BP-3 free base suspension, MGB-BP-3 microparticle formulation and vancomycin treated ham-
577 sters survived throughout the treatment period. 3 days after the end of the treatment, hamsters that
578 received MGB-BP-3 free base deteriorated clinically (wet tail and hypothermia). Conversely,
579 MGB-BP-3 microparticle formulation and vancomycin treated hamsters remained healthy and sur-
580 vived to the end of the study.

581 Moreover, the rates of regrowth of *C. difficile* isolates in hamster following the end of
582 vancomycin and MGB-BP-3 treatment were compared. *C. difficile* burden in the colon and caecum
583 was much higher in animals treated with vancomycin compared to MGB-BP-3 microparticles. Finally,
584 MIC values of MGB-BP-3 against *C. difficile* before and after infection were measured and no change
585 was observed in their values, indicating that resistance did not develop during therapy.

586 In another study, the same hamster model of severe CDAD was used to assess the disposition of
587 two MGB-BP-3 formulations (free base suspension and microparticles) in plasma and intestines after
588 oral administration [84]. The results indicated that MGB-BP-3 has a favorable PK profile for the
589 treatment of *C. difficile* infections.

590 4.2.6. Phase 1

591
592
593 A group of 40 healthy volunteers took part in a phase 1 study in which MGB-BP-3 exhibited an
594 excellent safety and tolerability profile with no serious AEs. Only one subject in the 125 mg dose co-
595 hort experienced transient dizziness [94].

596 4.2.7. Phase 2

597
598
599 A phase 2 study was conducted to assess the safety, tolerability, and efficacy of incremental
600 doses of MGB-BP-3 in patients with CDAD. Three sequential ascending doses of 125 mg, 250 mg, and
601 500 mg given twice daily were investigated. The study showed that a 10 day-administration of 250
602 mg of MGB-BP-3 given twice daily resulted in an initial cure and 100% durable cure, with no disease
603 recurrence measured four weeks after treatment. This regimen has been selected for further clinical
604 trials [95].

4.2.8. Current Advances

MGB-BP-3 has been granted “qualified infectious disease product” status (QIDP) by the FDA, allowing it to receive fast track designation and priority review. The molecule will also be eligible to participate in the US program DISARM (Developing an Innovative Strategy for Antimicrobial Resistant Microorganisms), which addresses the lack of financial incentives for pharmaceutical companies to develop new antibiotics [93]. In addition, a phase 3 head-to-head trial has been agreed with the FDA, comparing MGB-BP-3 to vancomycin [89].

5. tRNAs and ribosomes as drug targets

5.1. Introduction

tRNAs are small non-coding RNAs that play a key role in the translation process by delivering the amino acids to the ribosome for incorporation into a growing polypeptide chain during the protein synthesis [96]. Any component of this essential process is thus an interesting target for the development of new antibiotics. This will be highlighted with the development of CRS-3123 and lefamulin.

5.2. CRS-3123

CRS-3123 (formerly REP-3123) targets the methionyl-tRNA synthetase of Gram-positive bacteria, in particular *C. difficile* [97]. The drug candidate is being developed by Creston Inc. and has completed two phase 1 studies in 2022 [98,99].

5.2.1. Chemical synthesis

CRS-3123 was initially synthesized by Replidyne Inc, [100] following a convergent strategy described in Figure 12 [101]. Synthon A is prepared in 6 steps, by first condensing 3-thienyl isocyanate and Meldrum acid through a Knoevenagel-type reaction. The thienolate intermediate is then alkylated with iodomethane. Substitution with 3,3-diethoxypropylamine allows the introduction of the alkyl chain. The obtained intermediate is then heated in presence of HMDS (hexamethyldisilazane) to perform an intramolecular cyclization and obtain thieno[3,2-b]pyridine bicycle, bearing a carboxylic acid. Decarboxylation is achieved with sodium methoxide at reflux to give the pyridinolate intermediate, which is next acylated with benzoic anhydride. Lastly, the acetal group is cleaved in acidic conditions to give the aldehyde A.

In parallel, synthon B is obtained in 3 steps. 6,8-dibromochroman-4-one is stereoselectively reduced using (1S,2S)-(+)-*N*-*p*-tosyl-1,2-diphenylethylene diamine and dichloro(*p*-cymene)-ruthenium (II) dimer to give the *S*-alcohol intermediate. S_N2 with DPPA (diphenylphosphoryl azide) allows the replacement of the hydroxy group with an azido group, while achieving inversion of configuration of the chiral carbon. The *R*-azido derivative is then reduced *via* a Staudinger reaction to give the synthon B.

Reductive amination between synthons A and B with sodium triacetoxyborohydride gives the corresponding intermediate, which is then deprotected using ammonia in methanol to cleave the benzoyl group. Finally, salification with HCl in ethanol affords CRS-3123 as a dihydrochloride salt.

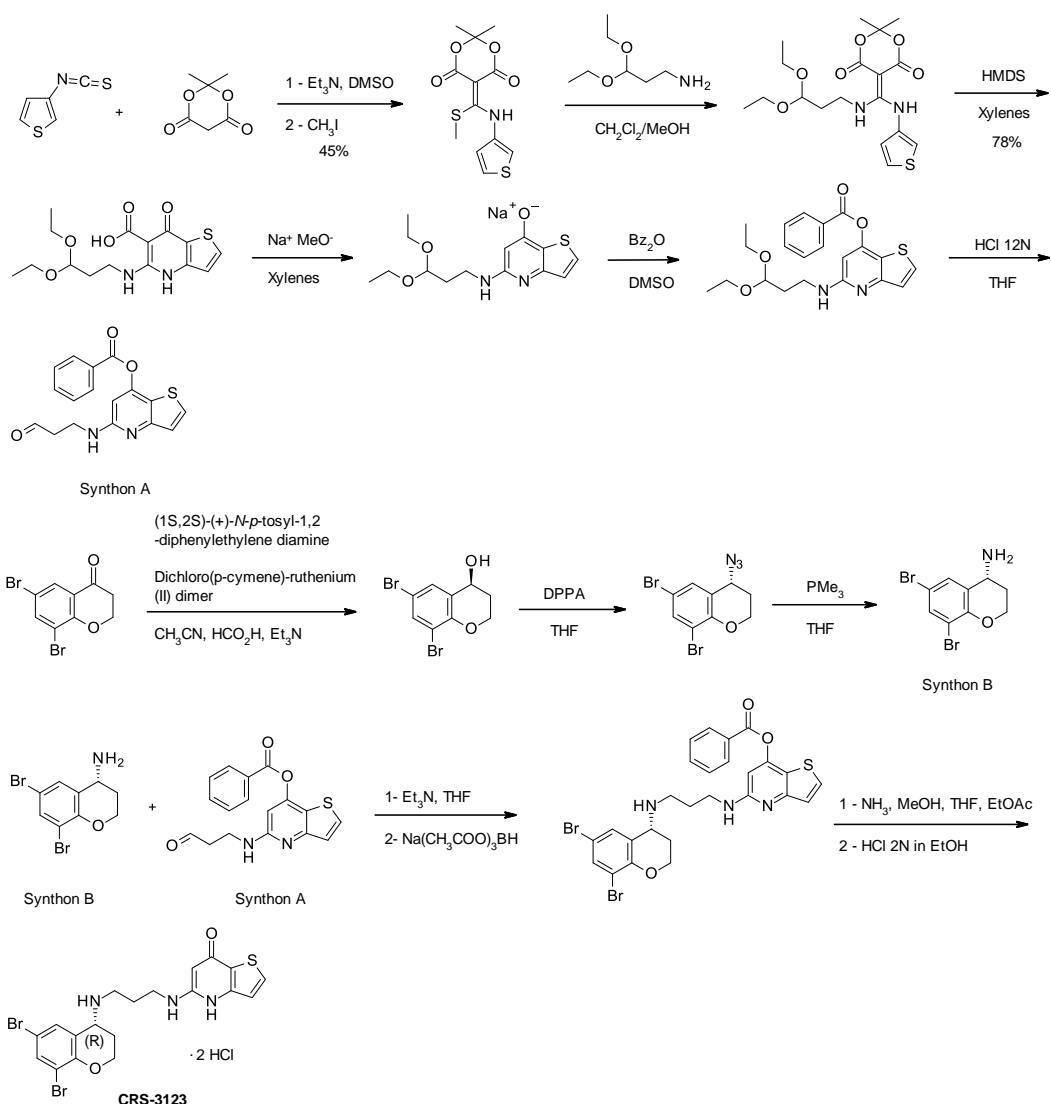


Figure 12. Description of the synthetic pathway leading to CRS-3123 [101]. Where known, yields are indicated.

5.2.2. Target and mechanism of action

CRS-3123 inhibits bacterial protein synthesis by targeting type 1 methionyl-tRNA synthetase (MetRS1) of Gram-positive bacteria [100]. This enzyme catalyzes two consecutive reactions [102]: the formation of the reactive methionyl adenylate species, resulting from the condensation of methionine and ATP, and the transfer of the amino acid on tRNA *via* the activated intermediate. The charged tRNA is then delivered to the ribosome to initiate translation.

Interestingly, the R-enantiomer of CRS-3123 is the most potent stereoisomer against the *C. difficile* MetRS1, with a K_i of 0.02 nM. CRS-3123 inhibits also MetRS1 of other Gram-positive bacteria, such as *S. aureus* ($K_i = 0.017$ nM) and *S. pneumoniae* ($K_i = 0.08$ nM). Notably, the compound is 1000 times less potent on the human mitochondrial MetRS1 ($K_i = 28$ nM). CRS-3123 is less potent against MetRS2, which is present among Gram-negative bacteria, such as *H. influenzae* ($K_i = 178$ nM) and *E. coli* ($K_i = 1900$ nM). To note, the human cytoplasmic type 2 enzyme is not inhibited ($K_i > 20000$ nM).

5.2.3. *In vitro* activity

669 In agreement with the inhibition data of MetRS1 described above, CRS-3123 is potent against
670 more than 100 isolates of *C. difficile* with MIC ranging from 0.5 to 1 µg/mL [97,100]. Moreover, the
671 compound is as potent as vancomycin (MIC = 0.25-4 µg/mL). CRS-3123 is also active against other
672 Gram-positive bacteria, such as MRSA (MIC = 0.015-0.5 µg/mL) or vancomycin-resistant *Enterococcus*
673 *faecium* (MIC < 0.004 µg/mL). However, the drug is not potent against Gram-negative bacteria, with
674 MICs > 32 µg/mL for *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa* or *Acinetobacter baumannii*.
675 Moreover, CRS-3123 is not active against various *Lactobacillus* spp., *Actinomyces* spp. and
676 *Bifidobacterium* spp. (MIC > 32 mg/mL for each) which are Gram-positive anaerobic bacteria of gut
677 microbiota. Even if no pre-existing resistance was observed for *C. difficile*, mutants with decreased
678 susceptibility towards CRS-3123 were selected, with spontaneous resistance frequencies of 10⁻⁹-10⁻⁸
679 at 16x MIC and 4x MIC respectively [103].

680 Contrary to vancomycin and metronidazole, CRS-3123 is able to decrease production of toxins A
681 and B from *C. difficile* [104], which are at the origin of the pathogenicity of the bacterium [105].

682 Finally, CRS-3123 shows reduction of spore production after 4 days, even at 0.25x MIC [104]. In
683 contrast, metronidazole and vancomycin are not able to decrease the formation of spores, which is
684 even promoted for some strains. The inhibition of spore production observed for CRS-3123 is an in-
685 teresting property, as spores are very difficult to eliminate and are the main source of new infections
686 and recurrences [105].

687 5.2.4. *In vivo* experiments

689 CRS-3123 was shown more effective than vancomycin *in vivo* [104]. In a hamster model of CDI,
690 CRS-3123 at daily doses of 0.5 mg/kg and 5 mg/kg greatly reduced the mortality of the animal, with
691 62 % and 75 % survival after 33 days, respectively. In this experiment, CRS-3123 was also superior to
692 vancomycin, where no animal survived after 25 days. The increased survival of CRS-3123 is likely due
693 to the inhibition of spore formation, preventing re-infection of animals during the study.

694 5.2.5. Toxicity and pharmacokinetics

695 In healthy uninfected hamsters, CRS-3123 displayed a bioavailability of 0.6 %, leading to low
696 systemic exposure and maximal concentrations in the gut [104]. The bioavailability is also low in
697 Sprague-Dawley rats (1 to 10.5 %) and in beagle dogs (< 1 to 7.3 %). CRS-3123 safety was sufficient
698 for progression of the drug into clinical studies [98].

699 5.2.6. Phase 1

700 CRS-3123 has completed two phase 1 clinical studies. A double-blind, single-center, placebo-
701 controlled single-ascending dose study was conducted in 40 healthy adults, with a dose of 100,
702 200, 400, 800 or 1200 mg [98]. The drug candidate was found safe and well tolerated, with the same
703 frequency and severity of AEs between the drug group and the placebo group. Although bioavailabil-
704 ity has not been precisely determined, it was found that plasma concentration at higher dose of
705 CRS-3123 were above MICs of Gram-positive pathogenic bacteria. By LC-MS analysis, the
706 *N*-glucuronide metabolite of CRS-3123 was observed in plasma and urine, indicating that the metab-
707 olite is excreted in urine. This is also the case for the non-metabolized CRS-3123.

708 The second phase 1 trial was a double-blind, single-center, placebo-controlled multiple-ascending
709 dose study, conducted in 30 healthy adults with doses of 200, 400 and 600 mg administered twice
710 daily during 10 days [99]. As for the first phase 1 study, CRS-3123 was safe and well tolerated. In par-
711 ticular, no prolongation of the QT interval was noticed. After administration, a large proportion of
712 the drug was retained in the gut and excreted in feces. Indeed, the percentage of free drug and
713 glucuronide metabolite in urine was inferior to 2 %. The low absorption of CRS-3123 leads to no sys-
714 temic exposure.

719 temic accumulation. Moreover, high concentrations of the drug are recovered in feces at any doses
720 (2115 – 8280 $\mu\text{g/g}$), which are superior to the MIC_{90} value against *C. difficile* (1 $\mu\text{g/mL}$). However,
721 even with high concentrations, CRS-3123 has a minimal effect on the normal microbiota and does
722 not increase the proportion of Proteobacteria. With this narrow spectrum of activity, CRS-3123 is
723 likely to reduce recurrences after treatment of CDI, as it was demonstrated for fidaxomicin [106].

724 725 5.2.7. Current advances

726
727 With a promising pharmacokinetic and bacterial selectivity profile for therapeutic use, CRS-3123
728 is currently evaluated in a phase 2 study, to compare efficacy of the drug against vancomycin in
729 adults with CDI [107].

730 731 **5.3. Lefamulin**

732
733 Lefamulin is a semisynthetic derivative of the naturally occurring tricyclic diterpenoid
734 pleuromutilin class of antibiotics [108,109]. The compound (formerly known as BC-3781) was devel-
735 oped by Nabriva Therapeutics and is currently marketed under the brand name Xenleta. The drug
736 was approved by the FDA and the EMA in 2019 and 2020, respectively, as a short-course
737 monotherapy for the treatment of community-acquired bacterial pneumonia (CABP) in adults
738 [110–112]. This is the first agent within the Pleuromutilin derivative class to be developed for sys-
739 temic use in humans by oral and intravenous administration [108,113].

740 741 5.3.1. Chemical synthesis

742
743 The synthetic route was patented in 2008 by Mang *et al.* [114]. Lefamulin is synthesized in two
744 step from pleuromutilin thiol [115] and syn-3,4-epoxycyclohexyl-carbamic acid tert-butyl ester [116].
745 After a $\text{S}_{\text{N}}2$ reaction leading to a mixture of diastereomers (1R, 2R, 4R) and (1S, 2S, 4S), the Boc pro-
746 tecting group is cleaved in acidic conditions. The mixture is then separated by chiral column chroma-
747 tography to afford lefamulin (Figure 13).

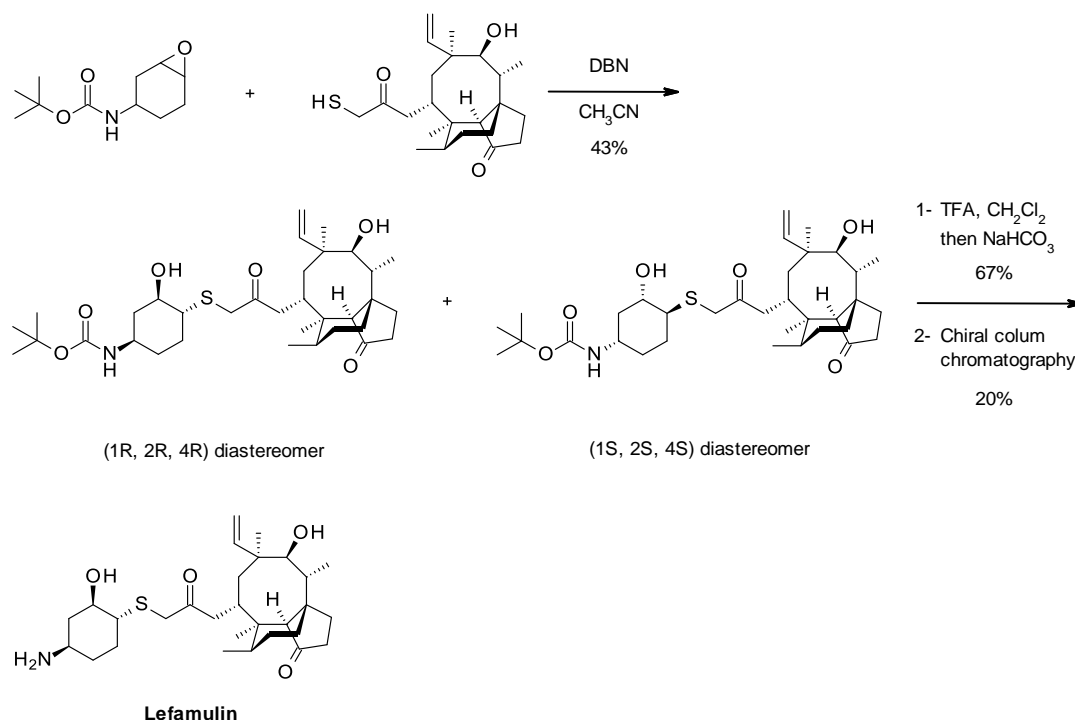


Figure 13. Description of the synthetic pathway leading to Lefamulin [114].

5.3.2. Target and mechanism of action

Lefamulin selectively inhibits bacterial ribosomal translation without affecting the eukaryotic process through a unique mechanism of tight-fit binding to the peptidyl transferase center (PTC) on the 50S ribosomal subunit [108,117]. More precisely, the tricyclic core and the side chain of lefamulin interact respectively with the A (aminoacyl) and P (peptidyl) sites of the subunit, through hydrophobic and hydrogen bond interactions (Figure 14) [108,117,118]. A binding pocket is then formed and the bacterial ribosome closes around the tricyclic core, preventing the correct positioning and binding of tRNA at the A and P sites, which leads to protein synthesis inhibition. However, once elongation has started, lefamulin is ineffective [109,113].

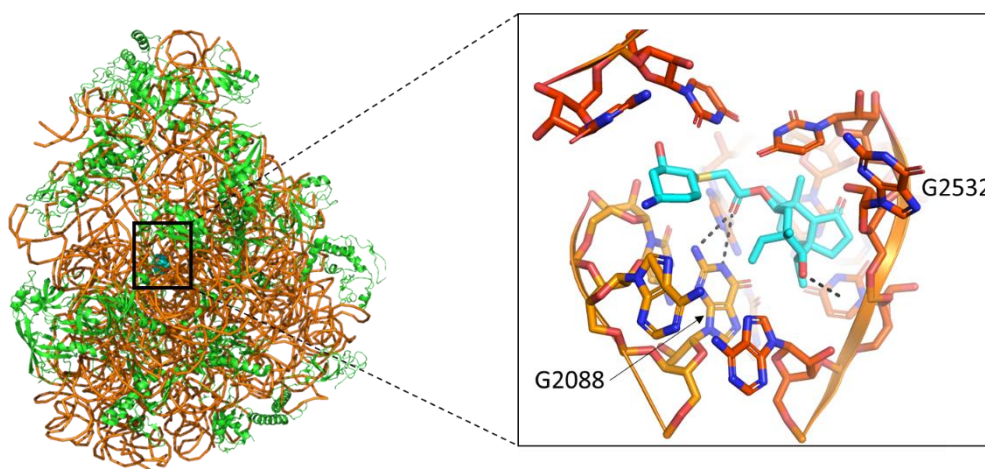


Figure 14. Views of lefamulin complex formed with *S. aureus* DNA gyrase and uncleaved DNA (PDB code: 5HL7).

5.3.3. Structure-activity relationship studies

Lefamulin was designed after a comprehensive study of the possible modifications at the C14-side chain of pleuromutilins [119] (Figure 15).

- **R side chain:** Replacement of the cyclohexyl moiety with an aromatic or non-aromatic heterocycle was tolerated. The thioether function resulted in a gain in potency and water solubility [120].
- **Linker:** the ester linker was favorable for the antibacterial activity. Indeed, the replacement with an amide or a methoxy linker resulted in less potent compounds.
- **Tricyclic core:** the tricyclic moiety together with its hydroxyl and vinyl groups are essential for the antimicrobial activity.

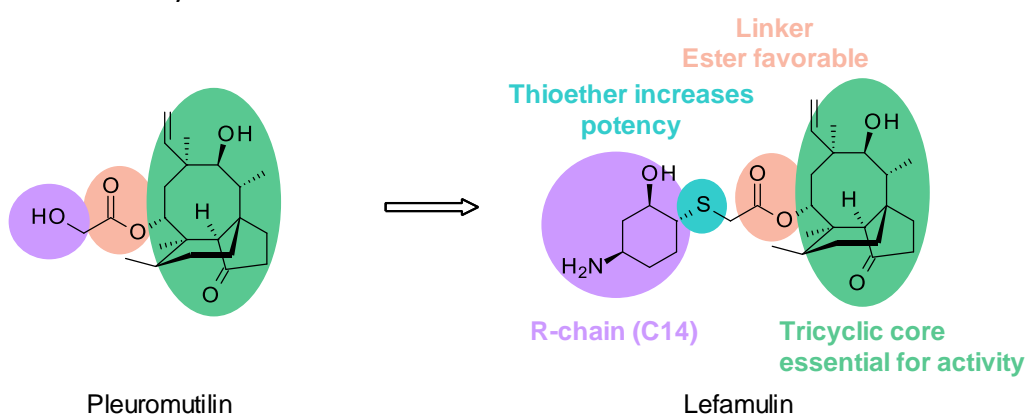


Figure 15. Summary of the SARs of Lefamulin starting from pleuromutilin.

5.3.4. *In vitro* activity

Lefamulin selectively inhibits prokaryotic ribosomal protein synthesis [117]. This selectivity is due to the double layer nucleotides around the pleuromutilin tricyclic core, which creates a structurally and chemically different binding pocket between the prokaryotes and the eukaryotes/archaea. [121] Lefamulin has a potent activity against Gram-positive (*S. pneumoniae*) and resistant Gram-positive (vancomycin-intermediate *S. aureus* (VISA), heterogeneous VISA (hVISA), vancomycin-resistant *S. aureus* (VRSA), penicillin-resistant *S. pneumoniae* (PRSP), MDR *S. pneumoniae*, and vancomycin-resistant *E. faecium* (VRE)) microorganisms. Moreover, the compound shows a potent activity against some Gram-negative pathogens such as *H. influenzae*, *Moraxella catharralis* and *Neisseria* spp.; highly effective against *N. gonorrhoeae* as well as against mycoplasmas (*Mycoplasma pneumoniae* and *Mycoplasma genitalium*) and intracellular organisms (*Legionella pneumophila*, *Chlamydia trachomatis*, *C. pneumoniae* and spp.) [108,111,117,122,123]. Lefamulin is also fully active against MRSA, β -hemolytic streptococci (group A: *Streptococcus pyogenes* and group B: *Streptococcus agalactiae*) and other drug-resistant organisms such as macrolide-resistant *M. pneumoniae*, for instance [111,117]. Other potent activities against coagulase-negative staphylococci and viridans group streptococci have also been demonstrated [117,122]. Pleuromutilins suppress bacterial protein translation with excellent specificity, but has no effect on eukaryotic protein synthesis, as shown by *in vitro* transcription/translation assays using bacterial ribosomes

In vitro studies revealed that lefamulin is both a substrate and an inhibitor of the CYP3A isoenzyme [124]. Lefamulin has shown *in vitro* activity against aerobic Gram-positive bacteria, a limited number of fastidious Gram-negative bacteria, and atypical bacteria [122,125]. Interestingly, the compound is bactericidal against *S. pneumoniae*, *H. influenzae* and *M. pneumoniae* at concentrations of 0.06, 0.5 and 0.008 $\mu\text{g/ml}$, respectively, and bacteriostatic against *S. aureus* and *S. pyogenes* [118].

In addition, lefamulin exhibits excellent activity against drug-resistant strains of *S. pneumoniae*, the most prevalent bacterial cause of CABP. The drug-resistant strains include isolates resistant to penicillins, cephalosporins, clarithromycin, doxycycline, and trimethoprim–sulfamethoxazole, as well as MDR isolates [126]. Lefamulin also displayed potent *in vitro* activity against Gram-positive organisms commonly associated with ABSSIs, which include *S. pyogenes*, *S. aureus* and MRSA strains [127–129]. Although not indicated for this use, lefamulin has *in vitro* activity against *C. trachomatis*, susceptible and MDR *M. genitalium* and susceptible and resistant *N. gonorrhoeae*, which are the most relevant bacterial pathogens that cause sexually transmitted infections (STI) [130].

5.3.5. *In vivo* experiments

Lefamulin has shown a potent *in vivo* efficacy in pulmonary infection mouse model [131]. The study showed that the compound is not affected by pulmonary surfactant and is able to quickly penetrate and accumulate in J774 mouse macrophages, with a plateau reached in 3 h. The PK parameters were assessed in mice (35 to 70 mg/kg), and demonstrated a fast distribution of lefamulin from the plasma to the target tissue (the epithelial lining fluid (ELF) in lungs), with a 2-fold increase in the drug exposure in the ELF during the 5.5 h period. Moreover, the study displayed desirable PD data in the neutropenic lung infections experiments, where mice received cyclophosphamide before being infected with single *S. pneumoniae* or *S. aureus* strains, and subcutaneous lefamulin (1.25–160 mg/kg) given twice daily post-infection.

The same authors reported that lefamulin exhibits time- and concentration-dependent killing in a neutropenic murine thigh infection model [132]. In this study, free drug values (~20% free fraction) were also established, ranging from 1.98–6.42 and 8.04–16.5 for the *S. pneumoniae* or *S. aureus* strains, respectively.

5.3.6. Phase 1

A four-period crossover study was performed to assess the bioavailability of lefamulin [133]. Moreover, a single-dose study was performed to evaluate the penetration of lefamulin into the ELF. The latter was performed on healthy human volunteers and demonstrated the good penetration into the ELF (fAUC_{0–24} of 8978 ng·h/mL and C_{max} 706 ng/mL) but also in other tissues such as the skeletal muscle and adipose tissue [134]. Last but not least, the PK parameters of IV lefamulin and its major metabolite BC-8041 were assessed in subjects with different degrees of hepatic impairment as well as healthy individuals. Overall, lefamulin was well tolerated, and no significant pharmacokinetic difference was observed between the compound and its metabolite [112].

5.3.7. Phase 2

A double-blind, parallel group, multicenter phase 2 study was performed to compare the efficacy of IV lefamulin to IV vancomycin in 210 adults with ABSSIs. Similar clinical success rates across pathogens were observed between the two groups, including methicillin-susceptible *S. aureus* (MSSA) and MRSA [135]. To date, no subsequent phase 3 trials have been pursued for the treatment of ABSSIs.

5.3.8. Phase 3

Although no phase 2 study for the indication of CABP was performed, two multicentre, randomized, double-blind, double-dummy phase 3 trials were conducted for this infectious disease: the Lefamulin Evaluation Against Pneumonia 1 and 2 (LEAP 1 and LEAP 2). The LEAP 1 trial assessed the

850 efficacy and safety of IV lefamulin compared to IV moxifloxacin +/- linezolid in 551 adults patients
851 with moderate to severe CABP [136,137]. The LEAP 2 study compared the safety and efficacy of
852 600mg oral lefamulin twice daily for 5 days versus 400 mg of oral moxifloxacin once daily for 7 days
853 in 738 adults patients with moderate CABP [138,139]. Data from both trials have shown noninferior
854 efficacy with short course therapy compared with moxifloxacin in the treatment of CABP [113].

855 5.3.9. Safety and tolerability

856
857
858 Intravenous and oral lefamulin was generally well tolerated in phase 1 and phase 2 clinical stud-
859 ies [124,134]. From the phase 2 trial, the most frequently reported AEs were headache, nausea, and
860 diarrhea [135]. In both LEAP 1 and LEAP 2 trials, most AEs were mild or moderate in severity for both
861 arm groups [137,140]. Lefamulin remains contraindicated in patients with known hypersensitivity to
862 pleuromutilins [141]. Interestingly, the concomitant IV or oral administration of lefamulin with strong
863 and/or moderate CYP3A or P-glycoprotein inducers can reduce the efficacy of the drug and should be
864 then avoided. On the other hand, IV or oral coadministration of lefamulin with drugs that strongly or
865 moderately inhibit CYP3A or P-glycoprotein inhibitors might increase the risk of AEs.

866 5.3.10. Current advances

867
868
869 Lefamulin is currently marketed as treatment for CABP caused by susceptible microorganisms.
870 Moreover, lefamulin could be an option for the treatment of other serious bacterial infections
871 caused by MRSA and vancomycin-resistant *E. faecium*, but also by MDR organisms associated with
872 STI (*N. gonorrhoeae* and *M. genitalium*). The use of lefamulin could be also considered for patients
873 with high-risk/recurrent CDI [109].

874 6. FabI enzyme as a drug target

875 6.1. Introduction

876
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878
879 FabI is an essential NADH-dependent enzyme which catalyzes the reduction of enoyl-acyl carrier
880 protein (enoyl-ACP) in the last step of the bacterial fatty acid biosynthesis pathway (FAS-II), involved
881 in the synthesis of phospholipids. Contrary to the multifunctional mammalian type I fatty acid syn-
882 thase (FAS-I), each reaction of FAS-II is catalyzed by a different enzyme [142]. This structural differ-
883 ence between the two pathways is the reason why inhibitors of the FAS-II pathway are selective and
884 safe for use in humans. On the other hand, the complexity in the bacterial fatty acid metabolism can
885 narrow the spectrum of activity of FabI inhibitors [142]. However, their development has continued
886 since FabI represents an essential and validated target in multidrug-resistant *S. aureus*.

887 FabI inhibitors can be divided in two groups, depending on their ability to form a covalent or
888 non-covalent adduct with the NAD⁺ cofactor. The covalent inhibitors include the diazaborines and
889 the first-line tuberculosis drug isoniazid whereas inhibitors that do not form a covalent adduct in-
890 clude for instance AFN-1252, the preclinical drug candidate fabimycine [143], and the preservation
891 agent triclosan, forming a much broader and heterogeneous group [142].

892 6.2. AFN-1252

893
894
895 AFN-1252 is a selective inhibitor of *S. aureus* FabI. This molecule belongs to the benzofuran
896 naphthyridines, a novel class of antibiotics. The effort to identify potent and selective FabI inhibitors
897 began with the screening of the GSK proprietary compound collection. From a first high-throughput
898 screening on the *S. aureus* FabI enzyme, Miller *et al.* identified a 1,4-benzodiazepine derivative as a

low micromolar inhibitor. A structure-guided optimization process led to aminopyridine and oxotetrahydronaphthyridine derivatives and to the selection of AFN-1252 as a lead development candidate [144,145].

6.2.1. Chemical Synthesis

The synthesis of AFN-1252 was patented in 2008 by Affinium Pharmaceuticals [145]. The strategy consists of combining two different moieties. The first 3-methylbenzofuran moiety (synthon A) is synthesized starting from 3-methylbenzofuran-2-carboxylic acid. The addition of aqueous methylamine to the acyl chloride intermediate leads to the formation of the corresponding amide, which is then reduced using LiAlH_4 . The second oxotetrahydronaphthyridine moiety (synthon B) is synthesized starting from 6-bromo-3,4-dihydro-1,8-naphthyridin-2(1H)-one bromide. A Heck reaction is performed with *tert*-butylacrylate. The desired carboxylic acid is then obtained as a HBr salt by treating the previously obtained ester with HBr in glacial acetic acid. The coupling between the two moieties by using HOBt and EDCI leads to the formation of AFN-1252 (Figure 16). Afabycin, the corresponding prodrug of AFN-1252, is obtained in three more steps. Firstly, AFN-1252 is reacted with chloromethyl bis(2-trimethylsilylethyl)phosphate, then the two protecting groups are removed in acidic conditions. Finally, the addition of the desired base (ethanolamine, NH_4OH , NaOH) leads to the corresponding salt of afabycin [146].

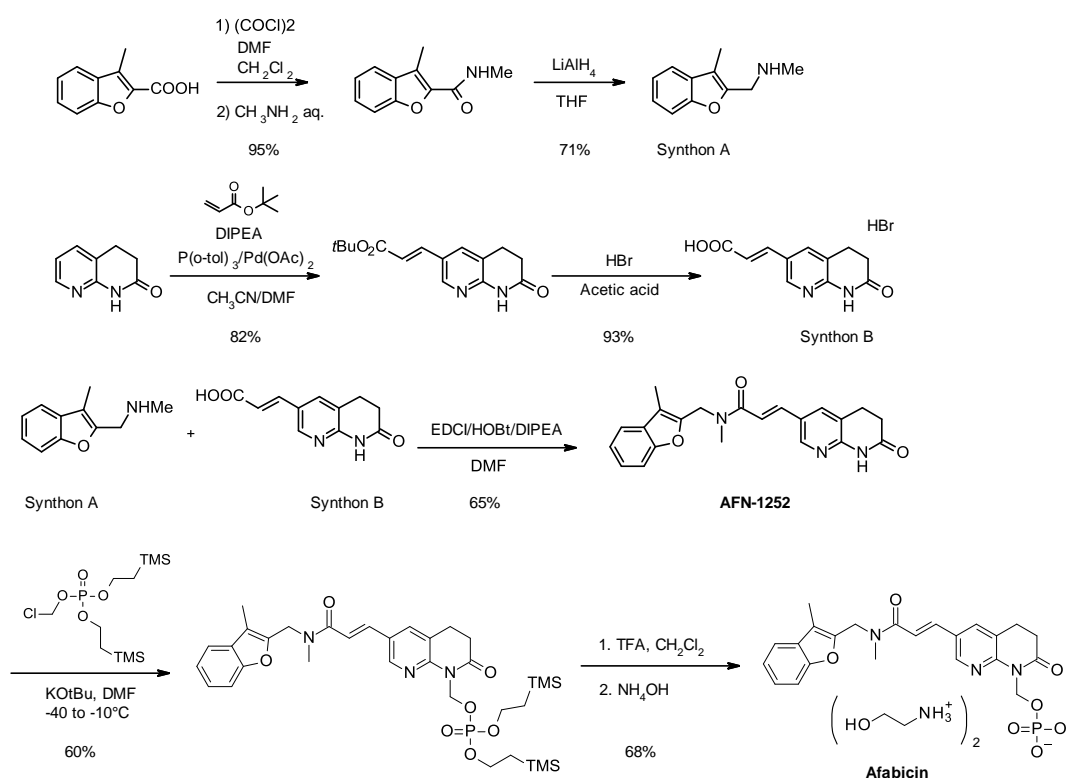


Figure 16. Description of the synthetic pathway leading to AFN-1252 and the diethanol ammonium salt of afabycin [145,146].

6.2.2 Target and mechanism of action

AFN-1252 interrupts fatty acid biosynthesis at the enoyl-acyl carrier protein reductase (FabI) step. FabI was confirmed to be the target of AFN-1252 by using biochemistry, macromolecular synthesis, genetics and co-crystallization of AFN-1252-FabI complex (Figure 17). The 3-methylbenzofuran moiety of AFN-1252 occupies a hydrophobic pocket of the enzyme created by residues F204, Y157 and

Y147. The oxotetrahydronaphthyridine moiety of the molecule occupies a more solvent exposed region and is involved in two hydrogen bond interactions with A97. The carbonyl of the linking cis-amide form two hydrogen bond interactions with the 2'-hydroxyl of the cofactor and the hydroxyl group of Y157 [147].

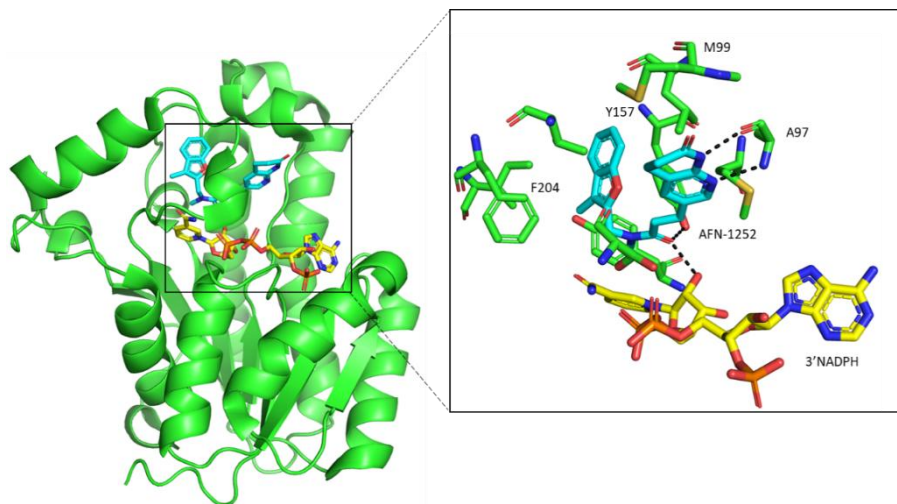


Figure 17. Views of AFN-1252 complex formed with *S. aureus* FabI and the NADPH cofactor (PDB code: 4FS3) .

6.2.3 Structure-activity relationship studies

High-throughput screening of 305,189 compounds from the GSK compound collection identified the benzodiazepine derivative as a low micromolar inhibitor of *S. aureus* FabI [144,148]. In order to improve potency, SAR studies were performed (Figure 18):

- **Removal of the ester chain:** the synthesis of compounds without the ester chain showed that the chain is not important for the inhibition of FabI.
- **Ring-opened variants:** ring-opened variants of the benzodiazepine derivative maintained the activity against *S. aureus*, indicating that the seven membered ring is not necessary for the antibacterial activity.
- **Importance of the *para* amino group:** removal of the *para* amino group caused a substantial decrease in activity.
- **Replacement of the aniline moiety with aminopyridine:** aminopyridine is an essential moiety engaged in two hydrogen-bonding interactions with Ala 97 from the cofactor.
- **Introduction of the unsaturated two-carbon linker:** acrylamide moiety was introduced to maximize the overlapping with previously synthesized promising compounds. The synthesis of the corresponding saturated derivatives led to inactive compounds.

In 2020, Parker *et al.* were able to re-design AFN-1252 into a derivative, AFN-1252-NH₃, that accumulates in *Escherichia coli* and possesses an antibacterial activity against Gram-negative bacteria [143].

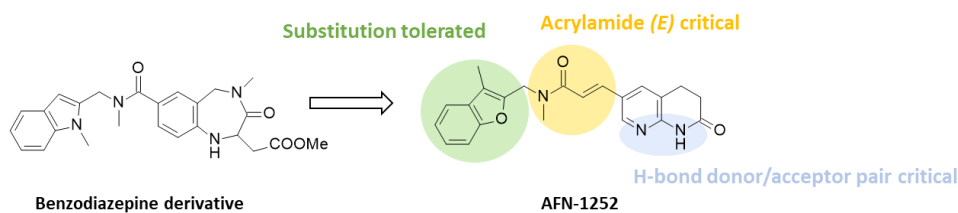


Figure 18. Summary of the SARs of AFN-1252 from the benzodiazepine series.

6.2.4 *In vitro* activity

Kaplan *et al.* showed that AFN-1252 exhibits MIC₉₀ values < 0.015 µg/ml against diverse clinical isolates of *S. aureus*. The research group also reported a high binding (> 95% from equilibrium dialysis) to serum proteins of mouse, rat, dog and humans. This parameter is in line with the eight-fold increase in AFN-1252 MICs observed in the presence of 50% of serum against MSSA, as well as with the long elimination half-life observed in pharmacokinetic studies (mice and early phase 1 studies). Similar MICs were also observed in the presence and absence of lung surfactant. Moreover, AFN-1252 exhibited a short dose-dependent post-antibiotic effect (PAE) of 1.1 hours against both MSSA and MRSA at 128 x MIC, and of 0.1 hour at 32 x MIC, following a 4-hour exposure. AFN-1252 did not show interaction with major antibiotic classes, with the exception of gentamicin, with which it demonstrated synergy [147,149].

6.2.5 *In vivo* experiments

AFN-1252 was orally available in mouse pharmacokinetic studies, where a linear relationship was observed between the administered dose (0.3, 0.6, and 1.0 mg/kg), the observed C_{max} (154, 196, and 304 ng/ml) and AUC_{0-t} (367, 806, and 1.030 ng-h/ml). The long elimination half-life measured (5–7 h) indicated the potential for once- or twice-daily administration in human. Kaplan *et al.* also showed that a single oral dose of 1 mg/kg was efficacious in a mouse model of septicemia, with 100% survival from an otherwise lethal peritoneal infection of *S. aureus*. The group also compared the *in vivo* efficacy of a single oral administration of AFN-1252 to linezolid, and an ED₅₀ of 0.15 mg/kg, suggesting that AFN-1252 was 12 to 24 times more potent than linezolid in this model [147].

6.2.6 Phase 1

A double-blind, randomized, placebo-controlled, two-part study was initiated to assess the safety, tolerability and pharmacokinetic properties of an immediate release tablet formulation of AFN-1252 in healthy subjects, following oral administration in an ascending dose trial [150]. In the first part, single doses of 100, 200, 300, or 400 mg of AFN-1252 were administered. In the second part, subjects received 100, 200, 300 and 400 mg twice a day. This study showed oral doses of AFN-1252 to be safe and well tolerated and highlighted the potential of the drug for once or twice-a-day dosing to treat staphylococcal infections. AFN-1252 was well-absorbed with C_{max} at 3–4 h when given once per day and 2.5–9 h when administered twice daily.

6.2.7 Phase 2

A phase 2 trial was designed to evaluate the efficacy and safety of 200 mg of AFN-1252, given orally twice-a-day in the treatment of ABSSSI. This study showed that AFN-1252 is well tolerated and highly effective in the treatment of ABSSSI caused by *S. aureus*, including MRSA. The overall early re-

995 response rate at day 3 was 97.3%, where 82.9% of patients had a >20% decrease in the area of ery-
 996 thema, and 77.9% of patients had a >20% decrease in the area of induration. Microbiologic eradica-
 997 tion rates for MRSA and MSSA were around 90% at short- and long-term follow-ups. The main
 998 drug-related AEs reported, which were mostly mild or moderate, were headache (26.2%) and nausea
 999 (21.4%) [151,152].

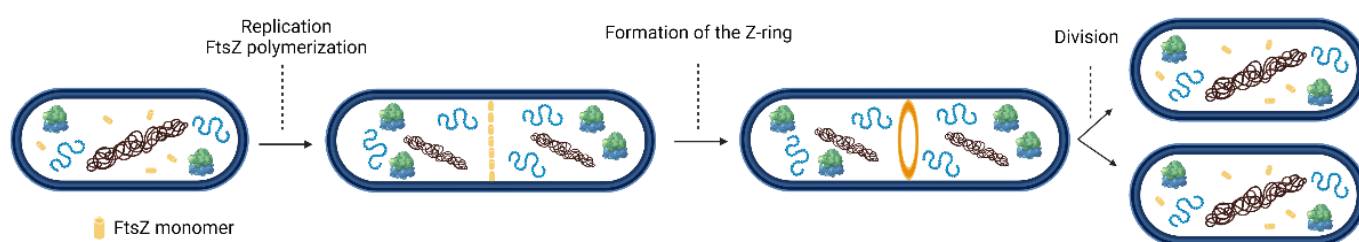
1000 6.2.8 Prodrug

1003 AFN-1252 belongs to class II biopharmaceutics classification system (BCS), presenting a limited
 1004 aqueous solubility and oral bioavailability. Therefore, Debiopharm invested in the development of its
 1005 prodrug Afabacin desphosphono (formerly Debio1450, AFN-1720). As expected, the prodrug showed
 1006 little to no activity against *S. aureus*, *E. coli* and other species. Afabacin was rapidly converted *in vivo*
 1007 to the active metabolite AFN-1252 and its antibacterial activity was better than AFN-1252 adminis-
 1008 tered by itself orally [153]. The prodrug was studied in a phase 2 clinical trial to compare its efficacy,
 1009 safety, and tolerability with those of vancomycin/linezolid in the treatment of ABSSSIs [154]. In a
 1010 phase 1 clinical trial, the penetration of afabacin into bones in patients undergoing elective hip re-
 1011 placement surgery was evaluated [155]. Moreover, a phase 2 clinical trial is underway to study the
 1012 safety and efficacy of afabacin in human subjects with bone and joint infections caused *S. aureus*.
 1013 [156].

1014 7. FtsZ protein as a drug target

1015 7.1. Introduction

1018 FtsZ is a bacterial protein involved in cell division forming single-stranded filaments by guanosine
 1019 5'-triphosphate (GTP) dependent polymerization. These FtsZ filaments form the cytokinetic ring
 1020 (called Z-ring) at midcell, able to recruit other critical elements for peptidoglycan synthesis and sep-
 1021 tum formation, which leads to the induction of cell division. Because FtsZ is essential for the germ
 1022 and is widely conserved throughout bacteria, it displays all the attractive features of a new target for
 1023 antibiotic compounds (Figure 19) [157].



1024 **Figure 19.** Involvement of the FtsZ protein towards the formation of the Z-ring.

1025 7.2. TXA707/TXA709

1026 TXA707 is the first antibacterial compound targeting FtsZ to reach clinical development. It is be-
 1027 ing developed as an oral prodrug (TXA709) by Taxis Pharmaceuticals to treat MRSA infections.

1028 TXA707 is derived from PC190723, which is coming from a fragment-based medicinal chemistry
 1029 program starting from 3-methoxybenzamide (3-MBA), a known FtsZ inhibitor in *Bacillus subtilis* [158].
 1030 To improve the pharmacokinetic properties of PC190723, the chlorine atom on the pyridyl ring was
 1031 replaced with a trifluoromethyl group, resistant to metabolism by cytochrome P450 enzymes.
 1032
 1033
 1034

7.2.1. Chemical synthesis

The commercial synthetic route for TXA709 is a convergent strategy described in a 2018 patent by Taxis Pharmaceuticals [159]. Synthon A is prepared starting from 2-chloro-3-nitro-5-(trifluoromethyl)pyridine. After the substitution of the chlorine atom with a sulfur atom brought by thiourea, the nitro group is reduced to form the aniline. The intermediate finally reacts with 2-chloroacetyl chloride to give the corresponding thiazolo[4,5-*b*]pyridine synthon A. The synthesis of synthon B begins with the nucleophilic substitution between 2,4-difluorophenol and benzyl bromide. After deprotonation with LDA, the carbanion reacts with CO₂ to give the corresponding carboxylic acid, further transformed into primary amide using thionyl chloride and ammonia. The benzyl group is then removed by hydrogenolysis to give synthon B. The phenol ring from synthon B can then react with synthon A to form the active compound TXA707. A coupling reaction is finally performed between the latter and 1-methylpiperidine-4-carboxylic acid using EDCI to obtain the imide prodrug TXA709 (Figure 20).

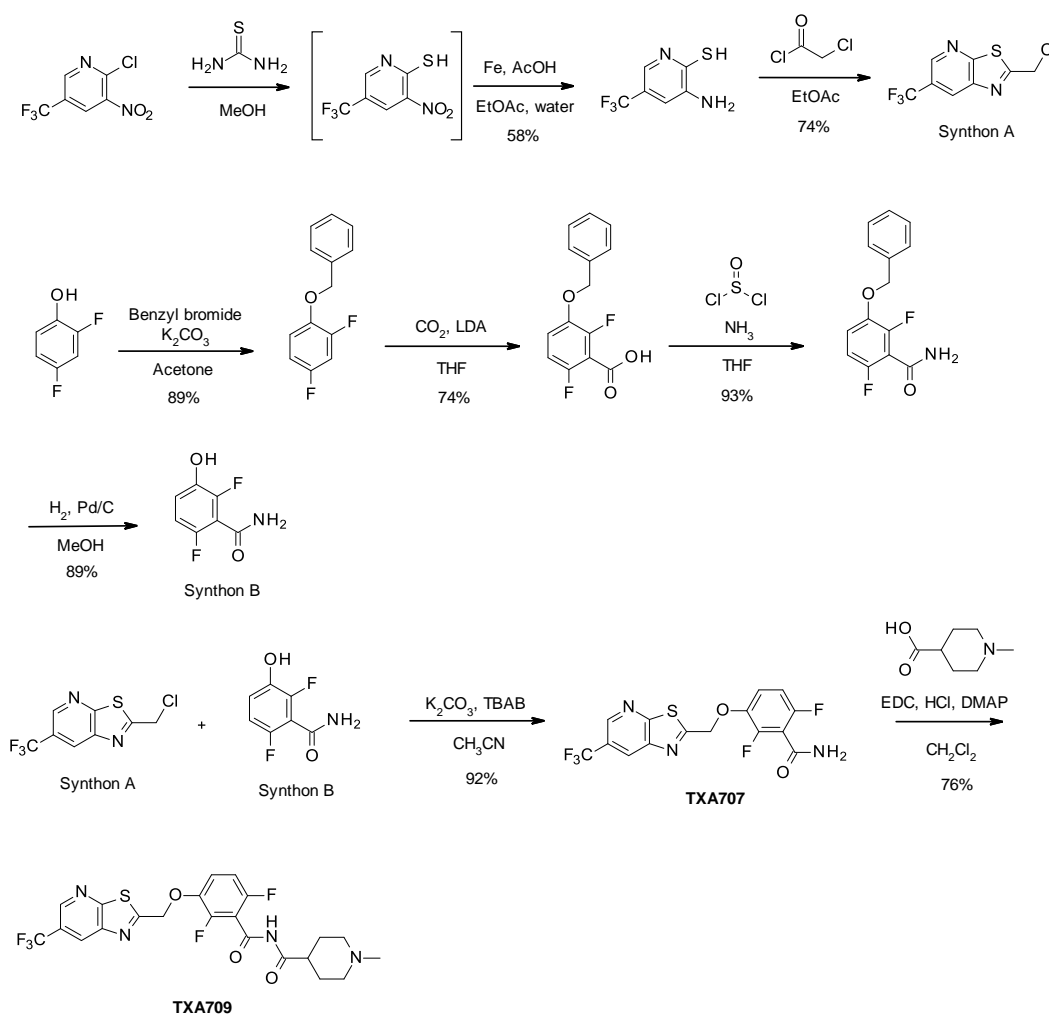


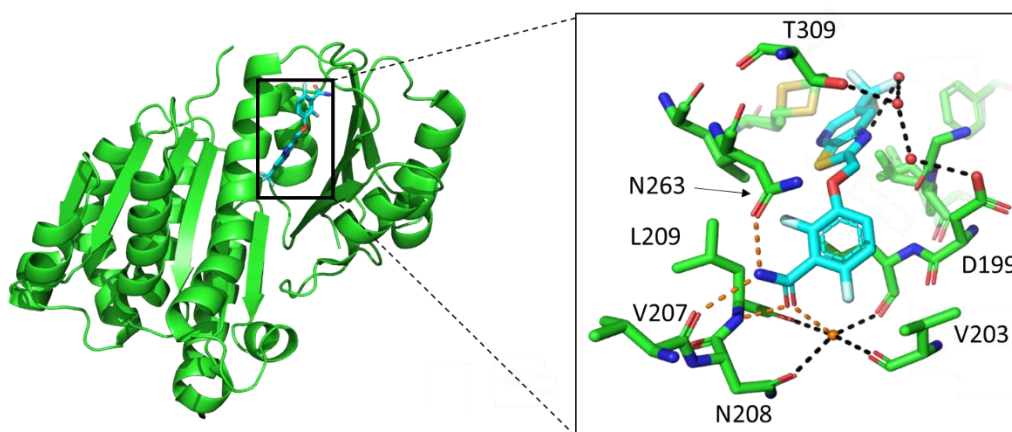
Figure 20. Description of the synthetic pathway leading to TXA709 [159].

7.2.2. Target and mechanism of action

Several biological experiments were performed to validate FtsZ as the target of TXA707. The drug stimulates FtsZ polymerization in a concentration-dependent manner and induces a mislocalization of FtsZ to multiple sites throughout the cell, preventing the formation of the Z-ring.

1057 Transmission electron microscopy studies showed that within 1 hour of treatment with TXA707, *S.*
 1058 *aureus* cells became enlarged enough, resulting to cell lysis. Furthermore, TXA707 was co-crystallized
 1059 in complex with FtsZ (Figure 21) [160]. Eventually, the target was validated with the selection of
 1060 TXA707-resistant MRSA mutants (frequencies of resistance: $3 \cdot 10^8$ in MRSA ATCC 43300; $1 \cdot 10^8$ in an
 1061 MRSA clinical isolate). Twenty resistant clones were isolated and the *ftsZ* gene was sequenced. Data
 1062 showed that all TXA707-resistant strains carried a mutation within this gene [161].

1063 It is worthwhile noting that the mechanism of action of TXA707 on FtsZ echoes that of paclitaxel
 1064 (tradename TAXOL) on tubulin, the eukaryotic structural homologue of FtsZ. Indeed, both com-
 1065 pounds prevent polymers disassembly, eventually leading to cell division halt.



1066
 1067
 1068 **Figure 21.** Views of TXA707 complex formed with *S. aureus* FtsZ 12-316 (PDB code: 5XDT).

1069 7.2.3. Structure-activity relationship studies

1070 The starting point for the development of TXA707 was 3-methoxybenzamide, proven to be an FtsZ
 1071 inhibitor in *B. subtilis* [158]. The methoxy and amide moieties in their respective positions were shown
 1072 to be essential for the activity on FtsZ (Figure 22). Authors then investigated modifications around the
 1073 3-methoxybenzamide core:

- 1074 • **Substitution of the phenyl ring:** substitution was detrimental, except for the addition of halogen
 1075 atoms in *ortho* position to the amide.
- 1076 • **Substitution of the methoxy moiety:** substitution with other alkoxy chains was possible. It was
 1077 shown that increasing the length of the alkyl chain up to nine carbon atoms allowed an 8000-fold
 1078 improvement in potency on *B. subtilis* compared to the methoxy moiety [162]. However, this also
 1079 led to an increase in PPB. Subsequent replacement of the alkyl chain with various heterocycles was
 1080 investigated, where the benzothiazole gave interesting results.
- 1081 • **Substitution of the benzothiazole ring:** substitution in position 4 was detrimental. In position 6,
 1082 substitution was tolerated with small moieties (methyl, chlorine) but larger groups led to inactive
 1083 compounds (phenyl, methoxy). Lastly, substitution in position 5 was favorable, particularly with a
 1084 chlorine or bromine atom, phenyl ring, propyl or ethoxy chain.
- 1085 • **Modification of the benzothiazole ring:** to further decrease the plasma protein binding,
 1086 thiazolopyridine analogues were synthesized. This allowed the identification of PC190723, which
 1087 displayed a good potency on *S. aureus* (MIC = 1 $\mu\text{g}/\text{mL}$) and a relatively low PPB (85.4%
 1088 bound)[163].

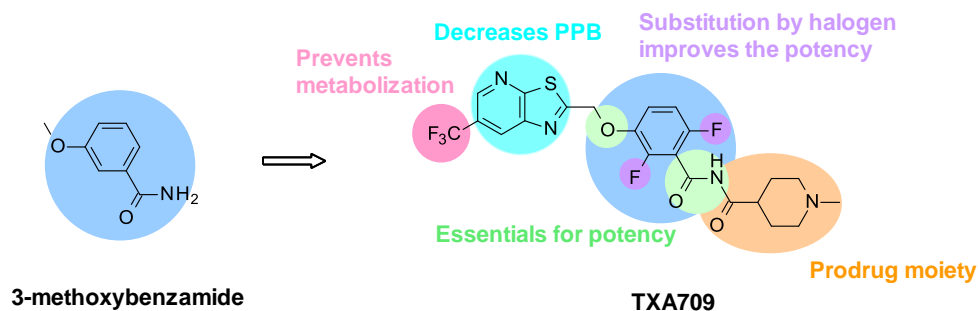


Figure 22. Summary of the SARs of TXA709.

Because PC190723 displayed a low solubility, the maximum dose that could be administered orally was not sufficient to display any efficacy. To circumvent this issue, an imide prodrug with a basic piperidine, TXY541, was synthesized, allowing a great improvement in solubility [164]. Administration of TXY541 proved to be efficacious *in vivo* but required high doses (192 mpk against a MRSA strain). To reduce administered doses, metabolization was decreased by replacement of the chlorine atom on the pyridyl ring with a trifluoromethyl group, yielding TXA707 and the corresponding imide prodrug TXA709 [161].

7.2.4. *In vitro* activity

TXA707 exhibits potent bactericidal activity against a panel of 84 *S. aureus* clinical strains with various levels of resistance against current treatment options. The most prevalent MIC for TXA707 was 1 $\mu\text{g}/\text{mL}$ against the five types of clinical isolates tested: MRSA, VISA, VRSA, DNSSA (Daptomycin Non-Susceptible *S. aureus*), and LNSSA (Linezolid Non-Susceptible *S. aureus*). The prodrug TXA709 also showed antibacterial activity against all *S. aureus* strains tested so far, however its modal MICs were 2- to 4-fold higher than those of TXA707. This is explained by a slower conversion from TXA709 to TXA707 in the culture medium [161]. Overall, these results make TXA707 and its prodrug TXA709 a promising alternative for the treatment of multidrug resistant *S. aureus*.

Regarding selectivity, it was shown *in vitro* that PC190723, the parent analogue of TXA707, did not impact positively or negatively the polymerization of tubulin, the eukaryote counterpart of FtsZ [157]. Moreover, TXA709 demonstrated minimal toxicity on mammalian cells (HeLa and MDCK, $\text{IC}_{50} > 120 \mu\text{g}/\text{mL}$) [161].

7.2.5. *In vivo* experiments

Firstly, the pharmacokinetic properties of TXA709 were measured in male BALB/c mice following oral (32 mpk) or IV (24 mpk) administration. TXA709 was not detectable in plasma, indicating a rapid conversion into the active compound TXA707. Maximum plasmatic concentrations were attained after 1h following oral administration, and the bioavailability was calculated to be 95% [161].

The efficacy of TXA709 was demonstrated in a mouse peritonitis model, both with MSSA and MRSA strains. It was demonstrated that a dose of 36 mpk by intravenous administration and a dose of 48 mpk by oral administration were sufficient for the survival of all mice infected by the MRSA strain. TXA709 and TXA707 were further evaluated in a mouse thigh model of MRSA infection. After intravenous administration of TXA709, volume of distribution at steady state for TXA707 was measured at 2.02 L/kg. This value is three times higher than the total water volume of the mouse (0.7 L/kg), indicating that TXA707 is well distributed in the tissues. Moreover, an oral dose of 120 mg/kg of TXA709 in the same mouse thigh infection model resulted in a 2-log reduction in bacterial CFU [161].

1129 Lastly, PK/PD parameters were evaluated in a murine neutropenic thigh infection model and the
1130 ratio AUC/MIC was deemed to be the PK/PD index most closely linked to efficacy [165].
1131

1132 7.2.6. Current advances 1133

1134 TXA709 has been evaluated in a non-registered phase 1 clinical trial and no serious AE was re-
1135 ported [166].
1136

1137 8. Conclusion and perspectives 1138

1139 For the first time in decades, pharmaceutical companies are refuelling the pipeline of antibiotics.
1140 To date, at least eight compounds with new targets and/or MOA are in clinical trials, including two in
1141 phase 3, or have been recently approved. They represent a new hope to fight the bad bugs that are
1142 becoming more and more resistant to current antibiotics.

1143 Beside these clinical candidates, other promising antibiotics and/or new targets have been re-
1144 ported in the literature. This is the case for a series of novel tetrazole ligands targeting LpxA, the first
1145 enzyme in the lipid A pathway [167]. Moreover, over twenty potential candidates targeting the bac-
1146 terial RNA polymerase enzyme have been designed [168,169].

1147 In addition, other strategies are being explored such as pathoblockers to disarm bacteria or ad-
1148 juvants like efflux pump inhibitors (EPI) to potentialize current antibiotics.

1149 Unlike antibiotics, which kill bacteria or inhibit growth and so exert a selection pressure,
1150 pathoblockers inhibit virulence factors. Virulence is the ability of bacteria to cause disease and is me-
1151 diated by factors such as toxins, proteases or cytolysins [170]. Therefore, antivirulence drugs disrupt
1152 the relation between the host and the pathogen, causing the disarmament of bacteria which can
1153 then be eliminated by the immune system. Because the bacterium viability is not impacted,
1154 pathoblockers are believed to reduce resistance development. To date, only one small molecule act-
1155 ing as a pathoblocker has been evaluated in clinical trials: fluorothiazinon (formerly CL-55), devel-
1156 oped by the Gamaleya Research Institute of Epidemiology and Microbiology in Russia. Fluorothiazinon
1157 inhibits the type III secretion system, a well conserved system among Gram-negative
1158 bacteria allowing the secretion of several virulence factors [171–174]. After completing a phase 1
1159 study [175], fluorothiazinon is currently evaluated in a phase 2 clinical study for the treatment of
1160 complicated urinary tract infections caused by *P. aeruginosa* [176]. Moreover, recent studies report-
1161 ed quorum sensing inhibitors to reduce virulence in *A. baumannii* [177,178].

1162 EPIs are compounds able to block bacterial multidrug efflux transporters. These pumps are able
1163 to extrude several classes of antibiotics from the bacteria such as tetracyclines, macrolides,
1164 β -lactams, fluoroquinolones, therefore decreasing their intra-bacterial concentration and eventually
1165 rendering them ineffective [179]. (Over)expression of multidrug efflux transporters, notably from the
1166 resistance-nodulation-cell division family, is one of the most worrying resistance mechanisms re-
1167 garding multidrug antibacterial resistance. One strategy to override this defense mechanism is the
1168 conception of EPIs [180]. Administrating an EPI in combination with an antibiotic effluxed by the tar-
1169 geted bacterial transporters could allow to restore the antibiotic activity and decrease the necessary
1170 antibiotic dose, thus reducing the selection pressure [181].
1171

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