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- 1 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 9 year. Without any action plan, AMR will be in a near future the leading cause of death ahead of can-10 cer. AMR comes from the ability of bacteria to rapidly develop and share resistance mechanisms to-11 wards current antibiotics, rendering them less effective. To circumvent this issue and avoid the phe-12 nomenon of cross-resistance, new antibiotics acting on novel targets or with new modes of action 13 are required. Today, the pipeline of potential new treatments with these characteristics includes 14promising compounds such as gepotidacin, zoliflodacin, ibezapolstat, MGB-BP-3, CRS-3123, afabicin 15 and TXA-709, which are currently in clinical trials, and lefamulin, which has been recently approved 16 by FDA and EMA. In this review, we report the chemical synthesis, mode of action, structure-activity 17 relationships, in vitro and in vivo activities as well as clinical data of these eight small molecules listed 18 above. 19

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

Abbreviations: 3-MBA, 3-methoxybenzamide; ABSSSI, acute bacterial skin and skin structure infec-23 tion; ACP, acyl carrier protein; ADME, absorption, distribution, metabolism, excretion; AE, adverse 24 event; AMR, antimicrobial resistance; ATP, adenosine triphosphate; AUC, area under curve; BCS, 25 biopharmaceutics classification system; CABP, community-acquired bacterial pneumonia; CDAD, 26 Clostridioides difficile-associated diarrhea; CDI, 1,1'-carbonyldiimidazole / C. difficile infections; CFU, 27 colony-forming unit; dGTP, deoxyguanosine triphosphate; DISARM, developing an innovative strate-28 gy for antimicrobial resistant microorganisms; DMF, dimethylformamide; DNSSA, daptomycin 29 non-susceptible S. aureus; DPPA, diphenylphosphoryl azide; ED, effective dose; EDCI, 30 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELF, epithelial lining fluid; EMA, european medi-31 cines agency; EPI, efflux pump inhibitors; ESBL, extended spectrum β-lactamase; FDA, food and drug 32 administration; HMDS, hexamethyldisilazane; HOBt, hydroxybenzotriazole; Ki, inhibitory constant; 33 LC-MS, liquid chromatography-mass spectrometry; LDA, lithium diisopropylamide; LEAP, lefamulin 34 evaluation against pneumonia; LNSSA, linezolid non-susceptible S. aureus; MDR, multidrug-resistant; 35 MetRS1, type 1 methionyl-tRNA synthetase; MGBs, minor groove binders; MIC, minimum inhibitory 36 concentration; MOA, mode of action; MPK, milligrams per kilograms; MRSA, methicillin-resistant S. 37 aureus; MSSA, methicillin-susceptible S. aureus; NADH, nicotinamide adenine dinucleotide hydrogen; 38 NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; NBTI, novel bacterial topoisomer-39 ase inhibitors; NCS, N-chlorosuccinimide; PAβN, phenylalanine-arginine-β-naphthylamide; PAE, 40 post-antibiotic effect; PD, pharmacodynamic; pH, potential of hydrogen; PK, pharmacokinetic; PPB, 41 plasma protein binding; PRSP, penicillin-resistant S. pneumoniae; PTC, peptidyl transferase center; 42 PTSA, p-toluenesulfonic acid; QIDP, qualified infectious disease product; SAR, structure-activity rela-43 tionship; S_N2, nucleophilic 2nd order substitution; S_NAr, nucleophilic aromatic substitution; STI, sex-44 ually transmitted infections; t_{1/2}, half-life; TEA, triethylamine; THF, tetrahydrofuran; UTI, urinary tract 45 infection; VISA, vancomycin-intermediate S. aureus; VRE, vancomycin-resistant E. faecium; VRSA, 46 vancomycin-resistant S. aureus. 47

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50 **1. Introduction**

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.

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].

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].

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.



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	Triazaacenaphtylene/NBTI	Spiropyrimidinetrione	DCBG ([dichlorobenzyl] guanine)	Distamycin
Mode of action	Inhibition of DNA gyrase and topoisomerase IV	Inhibition of topoisomerase II	Inhibition of DNA polymerase IIIC	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)
Name 	CRS-3123 Diaryldiamine	Lefamulin Tricyclic diterpenoid pleuromutilin	Afabicin (prodrug of AFN-1252) Benzofuran naphthyridine	TXA-709 (prodrug of TXA-707) Benzamide
Name Family Mode of action	CRS-3123 Diaryldiamine Inhibition of methio- nyl-tRNA synthetase (MetRS1)	Lefamulin Tricyclic diterpenoid pleuromutilin Inhibition of 50S ribosomal subunit	Afabicin (prodrug of AFN-1252) Benzofuran naphthyridine Inhibition of Fabl	TXA-709 (prodrug of TXA-707) Benzamide Favoring uncontrolled FtsZ polymerization
Name Family Mode of action Chemical features	CRS-3123 Diaryldiamine Inhibition of methio- nyl-tRNA synthetase (MetRS1) MW: 513.25 cLogP: 4.02 H-bond acc.: 5 H-bond don.: 3 PSA: 90.63	Lefamulin Tricyclic diterpenoid pleuromutilin Inhibition of 50S ribosomal subunit MW: 507.73 cLogP: 2.77 H-bond acc.: 7 H-bond don.: 3 PSA: 135.15	Afabicin (prodrug of AFN-1252) Benzofuran naphthyridine Inhibition of Fabl MW: 485.43 cLogP: 2.57 H-bond acc.: 7 H-bond don.: 2 PSA: 143.22	TXA-709 (prodrug of TXA-707) Benzamide Favoring uncontrolled FtsZ polymerization MW: 514.47 cLogP: 3.69 H-bond acc.: 6 H-bond don.: 1 PSA: 112.66
Name Family Mode of action Chemical features Development phase	CRS-3123 Diaryldiamine Inhibition of methio- nyl-tRNA synthetase (MetRS1) MW: 513.25 cLogP: 4.02 H-bond acc.: 5 H-bond don.: 3 PSA: 90.63 Phase II	Lefamulin Tricyclic diterpenoid pleuromutilin Inhibition of 50S ribosomal subunit MW: 507.73 cLogP: 2.77 H-bond acc.: 7 H-bond don.: 3 PSA: 135.15 Approved in 2019 (FDA) and 2020 (EMA)	Afabicin (prodrug of AFN-1252) Benzofuran naphthyridine Inhibition of Fabl MW: 485.43 cLogP: 2.57 H-bond acc.: 7 H-bond don.: 2 PSA: 143.22 Phase II	TXA-709 (prodrug of TXA-707) Benzamide Favoring uncontrolled FtsZ polymerization MW: 514.47 cLogP: 3.69 H-bond acc.: 6 H-bond don.: 1 PSA: 112.66 Phase I

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2. DNA topoisomerases as drug targets

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2.1. Introduction

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DNA topoisomerases are enzymes able to induce negative supercoil to compensate positive supercoil occurring during cellular events such as replication, transcription, recombination and DNA repair processes. Concerning bacteria, there are four types of topoisomerases: DNA gyrases, topoi somerases I, topoisomerases IV and reverse gyrases.

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

96 **2.2. Gepotidacin**

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Gepotidacin (GSK2140944) is a triazaacenaphtylene developed by GSK and belongs to the class of Novel Bacterial Topoisomerase Inhibitors (NBTI). This new antibiotic is currently being investigated 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].

104 **2.2.1.** Chemical synthesis

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



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

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121 2.2.2. Target and mechanism of action

Gepotidacin inhibits both DNA gyrase and topoisomerase IV. More specifically, gepotidacin 123 binds to the GyrA subunit of DNA gyrase and to the ParC subunit of topoisomerase IV. With its novel 124 chemical scaffold, gepotidacin can form a ternary complex with DNA and two GyrA or ParC subunits 125 (Figure 3) [9,14]. The structure of gepotidacin can be divided in three parts: the left-hand side (LHS), 126 the linker and the right-hand side (RHS) (Figure 4). The polar tricyclic core of the LHS intercalates 127 between DNA bases. Its position and structure can affect the spatial orientation of the linker and of 128 the RHS. The linker possesses a basic amine able to interact with both GyrA/ParC subunits of DNA 129 gyrase/topoisomerase IV. Lastly, the RHS binds to a hydrophobic pocket of the bacterial 130 gyrase/topoisomerase IV [15]. 131

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.

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

- 143 **2.2.3**. *In vitro* activity
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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 µg/mL. This compound was also potent against levofloxacin-resistant
 Gram-negative bacteria such as *E. coli* with MIC₉₀ of 4 µg/mL [16,17].

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- 152 **2.2.4.** *In vivo* experiments
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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]. Using neutropenic mouse models of thigh and lung infection, the pharmacokinetic-pharmacodynamic (PK-PD) parameters of gepotidacin against *S. aureus* and *S. pneumoniae* were characterized. The free plasma AUC/MIC ratio was identified as the PK-PD index most closely associated with efficacy, implying that gepotidacin activity was concentration dependent [19].

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

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

169 **2.2.5.** Phase 1

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

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 uncompli-184 cated Urinary Tract Infections (UTI).

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

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

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

206 **2.2.7. Current advances**

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

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

2.3. Zoliflodacin

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

225 **2.3.1. Spectrum of activity**

Zoliflodacin displays activity against Gram-positive bacteria including *S. pneumoniae, Strepto-coccus pyogenes*, and methicillin-resistant fluoroquinolone-resistant *S. aureus*, as well as against fastidious Gram-negative bacteria including *Haemophilus influenzae* and *N. gonorrhea* [47].

231 **2.3.2.** Chemical synthesis

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





247 2.3.3. Target and mechanism of action

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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].

257 2.3.4. Structure-activity relationship (SAR) studies

After a high-throughput screening at Pharmacia & Upjohn, QPT-1 was found as the first spiropyrimidinetrione 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 pyrimidinetrione:** the pyrimidinetrione 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].



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Figure 6. Summary of the SARs of zoliflodacin.

288 2.3.5. In vitro activity

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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 296 against N. gonorrhoeae (MIC₉₀ of 0.125 µg/mL) [45,48]. To date, no clinical zoliflodacin-resistant N. 297 gonorrhoeae isolates have been found [48]. However, zoliflodacin resistant mutants have been se-298 lected in vitro, and all of them contained nonsynonymous mutations in amino acid codons D429 or 299 K450 of GyrB, which leads to increased MICs of zoliflodacin in the mutants [49]. Zoliflodacin also 300 presented potent in vitro activity against Chlamydia pneumoniae, certain Gram-positive organisms (S. 301 aureus and S. pneumoniae; including fluoroquinolone- and vancomycin-resistant staphylococci), fas-302 tidious Gram-negative organisms (Moraxella catarrhalis and Haemophilus spp., in particular H. 303 *influenzae*), and atypical anaerobic organisms [44,51]. 304

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].

- 318 **2.3.6.** *In vivo* experiments
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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.

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- 328 **2.3.7. Phase 1**

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

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

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

344 **2.3.8.** Phase 2

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

2.3.9. Phase 3

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

363 **3. DNA polymerase as a drug target**

3.1. Introduction

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.

3.2. Ibezapolstat

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

382 3.2.1. Chemical synthesis

³⁸³ Ibezapolstat was first synthesized in 2010 in a 3-step procedure, following a protocol described by ³⁸⁴ Wright *et al.* (Figure 7) [70]. Firstly, 2-chloro-6-methoxypurine is alkylated with 1,2-dibromoethane to ³⁸⁵ afford a mixture of 7- and 9- isomers. After isolation of the 7-isomer, a S_N2 reaction with morpholine ³⁸⁶ leads to the corresponding intermediate, which is then subjected to reaction with ³⁸⁷ 3,4-dichlorobenzylamine to give ibezapolstat after the concomitant demethylation due to HCl re-³⁸⁸ lease.

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

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394 **3.2.2.** Target and mechanism of action

Ibezapolstat inhibits the DNA polymerase IIIC (DNA pol IIIC), an enzyme essential for bacterial DNA 395 replication [71], from low G + C Gram-positive germs (DNA containing less than 50 % of cytosine and 396 guanine). Ibezapolstat is also an analogue of dGTP, allowing the compound to pair with dCTG of DNA. 397 In addition, the aryl part of the molecule can bind to DNA pol IIIC. The combination of these two 398 pro-perties leads to the formation of an inactive ternary complex between DNA, ibezapolstat and 399 DNA pol IIIC (Figure 8). To note, the compound inhibits the DNA pol IIIC of C. difficile with a K_i of 400 0.325 µM [72]. Moreover, there is only little homology between DNA pol IIIC and mammalian poly-401 merase [73]. 402



Figure 8. Binding mode of ibezapolstat (IBZ) to dCTP and DNA pol IIIC. Adapted from Acurx Pharma ceuticals [74].

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408 **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, struc ture-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.

427 **3.2.4**. *In vitro* activity

Against 23 isolates of *C. difficile*, ibezapolstat (MIC₅₀ of 2 µg/mL) was shown as potent as metro-429 nidazole and vancomycin (MIC₅₀ of 1 μ g/mL for both compounds), which are the treatment of choice 430 for C. difficile infections (CDI) [77]. More recently, ibezapolstat was evaluated against 104 isolates of 431 C. difficile [71]. MIC range of the molecule (1-8 µg/mL) was higher than metronidazole (0.25-16 432 μ g/mL), vancomycin (0.5-4 μ g/mL) and fidaxomicin (0.015-1 μ g/mL). However, due to the mechanism 433 of action of ibezapolstat targeting DNA pol IIIC, which is not expressed by Gram-negative bacteria, 434 the compound has a narrow spectrum compared to metronidazole and vancomycin. Indeed, 435 ibezapolstat has low activity against representatives of the normal colonic flora, such as Bacteroides 436 fragilis, while metronidazole is active against some of them [72]. Overall, ibezapolstat exhibits an in-437 teresting spectrum profile, as the compound is unlikely to disrupt the endogenous bacterial flora, a 438 property that is preferable for avoiding recurrence of CDI [78,79]. 439

- 441 3.2.5. *In vivo* experiments
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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.

In the hamster model of *Clostridioides difficile*-associated diarrhea (CDAD), ibezapolstat showed 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 absorbed. Because the site of CDI is exclusively located in the colon, a lower absorption results in higher concentrations in the intestine.

- 454 **3.2.6.** Phase 1
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Ibezapolstat has completed a phase 1 study in the United States [80]. The randomized, double-bind, placebo-controlled clinical trial involved 62 healthy adults and was divided into three parts.

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

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

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

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

482 **4. DNA minor groove as a drug target**

484 **4.1.** *Introduction*

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

- 493494**4.2. MGB-BP-3**
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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].

503 4.2.1. Chemical Synthesis

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MGB-BP-3 was synthesized by Anthony et al. by combining two moieties (synthon A and synthon 504 B) [90]. To obtain the synthon A, 1-methyl-4-nitro-pyrrole-2-carboxylic acid is first converted into an 505 acyl chloride which reacts with aminoethyl morpholine to give the corresponding amide. After reduc-506 tion of the nitro group the intermediate is reacted with the previous acyl chloride to deliver the 507 The synthon B is synthesized in three steps starting from synthon Α. methyl 508 4-(bromomethyl)benzoate. At first, an Arbuzov reaction using triethylphosphite gives the corre-509 sponding phosphonate which reacts with quinoline-3-aldehyde via a Horner Wadsworth Emmons 510 reaction. Finally, the saponification of the methyl ester delivers the synthon B. Eventually, the reduc-511 tion of the nitro group of the synthon A and the subsequent coupling with the synthon B lead to 512 MGB-BP-3 (Figure 10). 513



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

516 4.2.2. Target and mechanism of action

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MGB-BP-3 binds non-covalently to the minor groove of the DNA, most readily to AT-rich regions [89]. 518 Probably, MGB-BP-3 binds as a dimer, with two molecules of ligand in the minor groove with an 519 antiparallel configuration. Thermal melt analysis, capillary electrophoresis, fluorescence intercalator 520 displacement assay, mass spectrometry and NMR techniques have been exploited to investigate and 521 confirm the binding of MGB-BP-3 to DNA [91,92]. MGB-BP-3 binding to DNA interferes with gene 522 expression at the transcription level. Recently, Hind et al. found out that MGB-BP-3 interferes with 523 other DNA-centric processes, such as the action of topoisomerase and gyrase, with a different 524 mechanism of action than fluoroquinolones [92]. 525

527 4.2.3. Structure-activity relationship studies

528 Suckling *et al.* synthesized several MGB-BP-3-like compounds and reported the following struc-529 ture-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.

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545 **4.2.4**. *In vitro* activity

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⁵⁴⁷ MGB-BP-3 showed substantial antibacterial activity against many Gram-positive bacteria such as *S.* ⁵⁴⁸ *aureus* (MIC₈₀ < 0.2 μ M) [89]). No evident toxicity was found on a mammalian cell line (HS27 murine ⁵⁴⁹ fibroblast). Thanks to fluorescence microscopy, Suckling *et al.* showed that there was no penetration of ⁵⁵⁰ MGB-BP-3 into several mammalian cell lines and concluded that the minor groove binder targeted ⁵⁵¹ selectively the bacterial cell [88].

552 MGB-BP-3 is highly active against *C. difficile*, killing the vegetative bacterium cell before sporula-553 tion. In particular, MGB-BP-3 exhibits strong bactericidal activity against BI/NAP1/027, the most viru-554 lent strain that is highly resistant to current therapies [93]. In addition, *S. aureus* was grown at sub-MIC₈₀ concentrations of MGB-BP-3 or rifampicin (used as a control) for 80 generations. No resistant strain towards MGB-BP-3 was observed, contrary to rifampicin [89].

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

4.2.5. In vivo experiments

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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].

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

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

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

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

4.2.6. Phase 1

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

4.2.7. Phase 2

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

606 4.2.8. Current Advances

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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

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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

626 CRS-3123 (formerly REP-3123) targets the methionyl-tRNA synthetase of Gram-positive bacteria, 627 in particular *C. difficile* [97]. The drug candidate is being developed by Creston Inc. and has complet-628 ed two phase 1 studies in 2022 [98,99].

5.2.1. Chemical synthesis

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CRS-3123 was initially synthesized by Replidyne Inc, [100] following a convergent strategy de-632 scribed in Figure 12 [101]. Synthon A is prepared in 6 steps, by first condensing 3-thienyl isocyanate 633 and Meldrum acid through a Knoevenagel-type reaction. The thienolate intermediate is then alkyl-634 ated with iodomethane. Substitution with 3,3-diethoxypropylamine allows the introduction of the 635 alkyl chain. The obtained intermediate is then heated in presence of HMDS (hexamethyldisilazane) to 636 perform an intramolecular cyclization and obtain thieno[3,2-b]pyridine bicycle, bearing a carboxylic 637 acid. Decarboxylation is achieved with sodium methoxide at reflux to give the pyridinolate interme-638 diate, which is next acylated with benzoic anhydride. Lastly, the acetal group is cleaved in acidic con-639 ditions to give the aldehyde A. 640

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).

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

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

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

5.2.4. In vivo experiments

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690 CRS-3123 was shown more effective than vancomycin *in vivo* [104]. In a hamster model of CDI, 691 CRS-3123 at daily doses of 0.5 mg/kg and 5 mg/kg greatly reduced the mortality of the animal, with 692 62 % and 75 % survival after 33 days, respectively. In this experiment, CRS-3123 was also superior to 693 vancomycin, where no animal survived after 25 days. The increased survival of CRS-3123 is likely due 694 to the inhibition of spore formation, preventing re-infection of animals during the study.

5.2.5. Toxicity and pharmacokinetics

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

5.2.6. Phase 1

CRS-3123 has completed two phase 1 clinical studies. A double-bind, single-center, place-705 bo-controlled single-ascending dose study was conducted in 40 healthy adults, with a dose of 100, 706 200, 400, 800 or 1200 mg [98]. The drug candidate was found safe and well tolerated, with the same 707 frequency and severity of AEs between the drug group and the placebo group. Although bioavailabil-708 ity has not been precisely determined, it was found that plasma concentration at higher dose of 709 CRS-3123 were above MICs of Gram-positive pathogenic bacteria. By LC-MS analysis, the 710 N-glucuronide metabolite of CRS-3123 was observed in plasma and urine, indicating that the metab-711 olite is excreted in urine. This is also the case for the non-metabolized CRS-3123. 712

The second phase 1 trial was a double-bind, single-center, placebo-controlled multiple-ascending dose study, conducted in 30 healthy adults with doses of 200, 400 and 600 mg administered twice daily during 10 days [99]. As for the first phase 1 study, CRS-3123 was safe and well tolerated. In particular, no prolongation of the QT interval was noticed. After administration, a large proportion of the drug was retained in the gut and excreted in feces. Indeed, the percentage of free drug and glucuronide metabolite in urine was inferior to 2 %. The low absorption of CRS-3123 leads to no systemic accumulation. Moreover, high concentrations of the drug are recovered in feces at any doses (2115 – 8280 μ g/g), which are superior to the MIC₉₀ value against *C. difficile* (1 μ g/mL). However, even with high concentrations, CRS-3123 has a minimal effect on the normal microbiota and does not increase the proportion of Proteobacteria. With this narrow spectrum of activity, CRS-3123 is likely to reduce recurrences after treatment of CDI, as it was demonstrated for fidaxomicin [106].

725 5.2.7. Current advances

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

5.3. Lefamulin

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

- 5.3.1. Chemical synthesis
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The synthetic route was patented in 2008 by Mang *et al.* [114]. Lefamulin is synthesized in two step from pleuromutilin thiol [115] and syn-3,4-epoxycyclohexyl-carbamic acid tert-butyl ester [116]. After a $S_N 2$ reaction leading to a mixture of diastereomers (1R, 2R, 4R) and (1S, 2S, 4S), the Boc protecting group is cleaved in acidic conditions. The mixture is then separated by chiral column chromatography to afford lefamulin (Figure 13).



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

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751 5.3.2. Target and mechanism of action

Lefamulin selectively inhibits bacterial ribosomal translation without affecting the eukaryotic pro-752 cess through a unique mechanism of tight-fit binding to the peptidyl transferase center (PTC) on the 753 50S ribosomal subunit [108,117]. More precisely, the tricyclic core and the side chain of lefamulin in-754 teract respectively with the A (aminoacyl) and P (peptidyl) sites of the subunit, through hydrophobic 755 and hydrogen bond interactions (Figure 14) [108,117,118]. A binding pocket is then formed and the 756 bacterial ribosome closes around the tricyclic core, preventing the correct positioning and binding of 757 tRNA at the A and P sites, which leads to protein synthesis inhibition. However, once elongation has 758 started, lefamulin is ineffective [109,113]. 759



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

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- 5.3.3. Structure-activity relationship studies 764

Lefamulin was designed after a comprehensive study of the possible modifications at the C14-side chain 765 of pleuromutilins [119] (Figure 15). 766

- R side chain: Replacement of the cyclohexyl moiety with an aromatic or non-aromatic 767 heterocycle was tolerated. The thioether function resulted in a gain in potency and water solu-768 bility [120]. 769
- Linker: the ester linker was favorable for the antibacterial activity. Indeed, the replacement with ٠ 770 an amide or a methoxy linker resulted in less potent compounds. 771
- **Tricyclic core:** the tricyclic moiety together with its hydroxyl and vinyl groups are essential for the 772 antimicrobial activity. 773



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

5.3.4. In vitro activity 776

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Lefamulin selectively inhibits prokaryotic ribosomal protein synthesis [117]. This selectivity is due 778 to the double layer nucleotides around the pleuromutilin tricyclic core, which creates a structurally 779 and chemically different binding pocket between the prokaryotes and the eukaryotes/archaea. [121] 780 Lefamulin has a potent activity against Gram-positive (S. pneumoniae) and resistant Gram-positive 781 (vancomycin-intermediate S. aureus (VISA), heterogeneous VISA (hVISA), vancomycin-resistant S. 782 (VRSA), penicillin-resistant S. pneumoniae (PRSP), MDR S. pneumoniae, aureus and 783 vancomycin-resistant E. faecium (VRE)) microorganisms. Moreover, the compound shows a potent 784 activity against some Gram-negative pathogens such as H. influenzae, Moraxella catharralis and 785 Neisseria spp.; highly effective against N. gonorrhoeae) as well as against mycoplasmas (Mycoplasma 786 pneumoniae and Mycoplasma genitalium) and intracellular organisms (Legionella pneumophila, 787 Chlamydia trachomatis, C. pneumoniae and spp.) [108,111,117,122,123]. Lefamulin is also fully active 788 against MRSA, β-hemolytic streptococci (group A: Streptococcus pyogenes and group B: Streptococ-789 cus agalactiae) and other drug-resistant organisms such as macrolide-resistant M. pneumoniae, for 790 instance [111,117]. Other potent activities against coagulase-negative staphylococci and viridans 791 group streptococci have also been demonstrated [117,122]. Pleuromutilins suppress bacterial pro-792 tein translation with excellent specificity, but has no effect on eukaryotic protein synthesis, as shown 793 by in vitro transcription/translation assays using bacterial ribosomes 794

In vitro studies revealed that lefamulin is both a substrate and an inhibitor of the CYP3A 796 isoenzyme [124]. Lefamulin has shown in vitro activity against aerobic Gram-positive bacteria, a lim-797 ited number of fastidious Gram-negative bacteria, and atypical bacteria [122,125]. Interestingly, the 798 compound is bactericidal against S. pneumoniae, H. influenzae and M. pneumoniae at concentrations 799 of 0.06, 0.5 and 0.008 µg/ml, respectively, and bacteriostatic against S. aureus and S. pyogenes [118]. 800

In addition, lefamulin exhibits excellent activity against drug-resistant strains of S. pneumoniae, the 801 most prevalent bacterial cause of CABP. The drug-resistant strains include isolates resistant to peni-802 cillins, cephalosporins, clarithromycin, doxycycline, and trimethoprim-sulfamethoxazole, as well as 803 MDR isolates [126]. Lefamulin also displayed potent in vitro activity against Gram-positive organisms 804 commonly associated with ABSSSIs, which include S. pyogenes, S. aureus and MRSA strains 805 [127–129]. Although not indicated for this use, lefamulin has in vitro activity against C. trachomatis, 806 susceptible and MDR M. genitalium and susceptible and resistant N. gonorrhoeae, which are the 807 most relevant bacterial pathogens that cause sexually transmitted infections (STI) [130]. 808

810 5.3.5. *In vivo* experiments

Lefamulin has shown a potent in vivo efficacy in pulmonary infection mouse model [131]. The 812 study showed that the compound is not affected by pulmonary surfactant and is able to quickly pen-813 etrate and accumulate in J774 mouse macrophages, with a plateau reached in 3 h. The PK parame-814 ters were assessed in mice (35 to 70 mg/kg), and demonstrated a fast distribution of lefamulin from 815 the plasma to the target tissue (the epithelial lining fluid (ELF) in lungs), with a 2-fold increase in the 816 drug exposure in the ELF during the 5.5 h period. Moreover, the study displayed desirable PD data in 817 the neutropenic lung infections experiments, where mice received cyclophosphamide before being 818 infected with single S. pneumoniae or S. aureus strains, and subcutaneous lefamulin (1.25-160 819 mg/kg) given twice daily post-infection. 820

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

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A four-period crossover study was performed to assess the bioavailability of lefamulin [133]. 828 Moreover, a single-dose study was performed to evaluate the penetration of lefamulin into the ELF. 829 The latter was performed on healthy human volunteers and demonstrated the good penetration into 830 the ELF (fAUC₀₋₂₄ of 8978 ng·h/mL and C_{max} 706 ng/mL) but also in other tissues such as the skeletal 831 muscle and adipose tissue [134]. Last but not least, the PK parameters of IV lefamulin and its major 832 metabolite BC-8041 were assessed in subjects with different degrees of hepatic impairment as well 833 as healthy individuals. Overall, lefamulin was well tolerated, and no significant pharmacokinetic dif-834 ference was observed between the compound and its metabolite [112]. 835

5.3.7. Phase 2

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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.

845 **5.3.8.** Phase 3

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

5.3.9. Safety and tolerability

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

5.3.10. Current advances

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

6. Fabl enzyme as a drug target

6.1. Introduction

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

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

6.2. AFN-1252

AFN-1252 is a selective inhibitor of *S. aureus* Fabl. This molecule belongs to the benzofuran naphthyridines, a novel class of antibiotics. The effort to identify potent and selective Fabl inhibitors began with the screening of the GSK proprietary compound collection. From a first high-throughput screening on the *S. aureus* Fabl 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].

903 6.2.1. Chemical Synthesis

The synthesis of AFN-1252 was patented in 2008 by Affinium Pharmaceuticals [145]. The strategy 904 consists of combining two different moieties. The first 3-methylbenzofuran moiety (synthon A) is 905 synthesized starting from 3-methylbenzofuran-2-carboxylic acid. The addition of aqueous methyla-906 mine to the acyl chloride intermediate leads to the formation of the corresponding amide, which is 907 then reduced using LiAlH₄. The second oxotetrahydronaphthyridine moiety (synthon B) is synthesized 908 starting from 6-bromo-3,4-dihydro-1,8-naphthyridin-2(1H)-one bromide. A Heck reaction is per-909 formed with tert-butylacrylate. The desired carboxylic acid is then obtained as a HBr salt by treating 910 the previously obtained ester with HBr in glacial acetic acid. The coupling between the two moieties 911 by using HOBt and EDCI leads to the formation of AFN-1252 (Figure 16). Afabicin, the corresponding 912 prodrug of AFN-1252, is obtained in three more steps. Firstly, AFN-1252 is reacted with chloromethyl 913 bis(2-trimethylsilylethyl)phosphate, then the two protecting groups are removed in acidic conditions. 914 Finally, the addition of the desired base (ethanolamine, NH₄OH, NaOH) leads to the corresponding 915 salt of afabicin [146]. 916



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

921 6.2.2 Target and mechanism of action

AFN-1252 interrupts fatty acid biosynthesis at the enoyl-acyl carrier protein reductase (Fabl) step. Fabl was confirmed to be the target of AFN-1252 by using biochemistry, macromolecular synthesis, genetics and co-crystallization of AFN-1252-Fabl 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 oxotetrahydronaphtyridine moiety of the molecule occupies a more solvent exposed re gion 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].

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

- 934 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* Fabl [144,148]. In order to im prove 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 Fabl.
- **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].





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

955 **6.2.4** *In vitro* activity

Kaplan et al. showed that AFN-1252 exhibits MIC_{90} values < 0.015 µg/ml against diverse clinical 957 isolates of S. aureus. The research group also reported a high binding (> 95% from equilibrium dialy-958 sis) to serum proteins of mouse, rat, dog and humans. This parameter is in line with the eight-fold in-959 crease in AFN-1252 MICs observed in the presence of 50% of serum against MSSA, as well as with the 960 long elimination half-life observed in pharmacokinetic studies (mice and early phase 1 studies). Simi-961 lar MICs were also observed in the presence and absence of lung surfactant. Moreover, AFN-1252 962 exhibited a short dose-dependent post-antibiotic effect (PAE) of 1.1 hours against both MSSA and 963 MRSA at 128 x MIC, and of 0.1 hour at 32 x MIC, following a 4-hour exposure. AFN-1252 did not show 964 interaction with major antibiotic classes, with the exception of gentamicin, with which it demon-965 strated synergy [147,149]. 966

6.2.5 In vivo experiments

AFN-1252 was orally available in mouse pharmacokinetic studies, where a linear relationship was 970 observed between the administered dose (0.3, 0.6, and 1.0 mg/kg), the observed C_{max} (154, 196, and 971 304 ng/ml) and AUC_{0-t} (367, 806, and 1.030 ng-h/ml). The long elimination half-life measured (5–7 h) 972 indicated the potential for once- or twice-daily administration in human. Kaplan et al. also showed 973 that a single oral dose of 1 mg/kg was efficacious in a mouse model of septicemia, with 100% survival 974 from an otherwise lethal peritoneal infection of S. aureus. The group also compared the in vivo effi-975 cacy of a single oral administration of AFN-1252 to linezolid, and an ED₅₀ of 0.15 mg/kg, suggesting 976 that AFN-1252 was 12 to 24 times more potent than linezolid in this model [147]. 977

6.2.6 Phase 1

A double-blind, randomized, placebo-controlled, two-part study was initiated to assess the safe-981 ty, tolerability and pharmacokinetic properties of an immediate release tablet formulation of 982 AFN-1252 in healthy subjects, following oral administration in an ascending dose trial [150]. In the 983 first part, single doses of 100, 200, 300, or 400 mg of AFN-1252 were administered. In the second 984 part, subjects received 100, 200, 300 and 400 mg twice a day. This study showed oral doses of 985 AFN-1252 to be safe and well tolerated and highlighted the potential of the drug for once or 986 twice-a-day dosing to treat staphylococcal infections. AFN-1252 was well-absorbed with Cmax at 3-4 h 987 988 when given once per day and 2.5–9 h when administered twice daily.

990 6.2.7 Phase 2

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

- 1001 6.2.8 Prodrug
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AFN-1252 belongs to class II biopharmaceutics classification system (BCS), presenting a limited 1003 aqueous solubility and oral bioavailability. Therefore, Debiopharm invested in the development of its 1004 prodrug Afabicin desphosphono (formerly Debio1450, AFN-1720). As expected, the prodrug showed 1005 little to no activity against S. aureus, E. coli and other species. Afabicin was rapidly converted in vivo 1006 to the active metabolite AFN-1252 and its antibacterial activity was better than AFN-1252 adminis-1007 tered by itself orally [153]. The prodrug was studied in a phase 2 clinical trial to compare its efficacy, 1008 safety, and tolerability with those of vancomycin/linezolid in the treatment of ABSSSIs [154]. In a 1009 phase 1 clinical trial, the penetration of afabicin into bones in patients undergoing elective hip re-1010 placement surgery was evaluated [155]. Moreover, a phase 2 clinical trial is underway to study the 1011 safety and efficacy of afabicin in human subjects with bone and joint infections caused S. aureus. 1012 [156]. 1013

- 1015 **7. FtsZ protein as a drug target**
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1017 **7.1. Introduction**

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



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

1026 **7.2. TXA707/TXA709**

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

1030TXA707 is derived from PC190723, which is coming from a fragment-based medicinal chemistry1031program starting from 3-methoxybenzamide (3-MBA), a known FtsZ inhibitor in *Bacillus subtilis* [158].1032To improve the pharmacokinetic properties of PC190723, the chlorine atom on the pyridyl ring was1033replaced with a trifluoromethyl group, resistant to metabolization by cytochrome P450 enzymes.

1035 **7.2.1. Chemical synthesis**

The commercial synthetic route for TXA709 is a convergent strategy described in a 2018 patent by 1036 Taxis Pharmaceuticals [159]. Synthon А is prepared starting from 1037 2-chloro-3-nitro-5-(trifluoromethyl)pyridine. After the substitution of the chlorine atom with a sulfur 1038 atom brought by thiourea, the nitro group is reduced to form the aniline. The intermediate finally 1039 reacts with 2-chloroacetyl chloride to give the corresponding thiazolo[4,5-b]pyridine synthon A. The 1040 synthesis of synthon B begins with the nucleophilic substitution between 2,4-difluorophenol and 1041 benzyl bromide. After deprotonation with LDA, the carbanion reacts with CO₂ to give the corre-1042 sponding carboxylic acid, further transformed into primary amide using thionyl chloride and ammo-1043 nia. The benzyl group is then removed by hydrogenolysis to give synthon B. The phenol ring from 1044 synthon B can then react with synthon A to form the active compound TXA707. A coupling reaction is 1045 finally performed between the latter and 1-methylpiperidine-4-carboxylic acid using EDCI to obtain 1046 the imide prodrug TXA709 (Figure 20). 1047



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

1052 **7.2.2.** Target and mechanism of action

1054 Several biological experiments were performed to validate FtsZ as the target of TXA707. The 1055 drug stimulates FtsZ polymerization in a concentration-dependent manner and induces a 1056 mislocalization of FtsZ to multiple sites throughout the cell, preventing the formation of the Z-ring. 1057 Transmission electron micrography 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: 3.10⁸ in MRSA ATCC 43300; 1.10⁸ 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.



- 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

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

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



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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].

1099 **7.2.4**. *In vitro* activity

TXA707 exhibits potent bactericidal activity against a panel of 84 S. aureus clinical strains with 1101 various levels of resistance against current treatment options. The most prevalent MIC for TXA707 1102 was 1 µg/mL against the five types of clinical isolates tested: MRSA, VISA, VRSA, DNSSA (Daptomycin 1103 Non-Susceptible S. aureus), and LNSSA (Linezolid Non-Susceptible S. aureus). The prodrug TXA709 1104 also showed antibacterial activity against all S. aureus strains tested so far, however its modal MICs 1105 were 2- to 4-fold higher than those of TXA707. This is explained by a slower conversion from TXA709 1106 to TXA707 in the culture medium [161]. Overall, these results make TXA707 and its prodrug TXA709 a 1107 promising alternative for the treatment of multidrug resistant S. aureus. 1108

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, IC₅₀ > 110 μ g/mL) [161].

1114 **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].

1120 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 1121 of 48 mpk by oral administration were sufficient for the survival of all mice infected by the MRSA 1122 strain. TXA709 and TXA707 were further evaluated in a mouse thigh model of MRSA infection. After 1123 intravenous administration of TXA709, volume of distribution at steady state for TXA707 was meas-1124 ured at 2.02 L/kg. This value is three times higher than the total water volume of the mouse (0.7 1125 L/kg), indicating that TXA707 is well distributed in the tissues. Moreover, an oral dose of 120 mg/kg 1126 of TXA709 in the same mouse thigh infection model resulted in a 2-log reduction in bacterial CFU 1127 [161]. 1128

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

1132 **7.2.6. Current advances**

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

1137 **8. Conclusion and perspectives**

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

Beside these clinical candidates, other promising antibiotics and/or new targets have been reported in the literature. This is the case for a series of novel tetrazole ligands targeting LpxA, the first enzyme in the lipid A pathway [167]. Moreover, over twenty potential candidates targeting the bacterial 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.

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

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

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