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Review Article

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Rational Drug Design Targeting Multidrug-Resistant Microorganisms: A Narrative Review

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Antimicrobial resistance remains a major global health challenge and continues to erode the clinical utility of many existing antibiotics. This review outlines recent advances in rational antibacterial discovery, with emphasis on fully synthetic, structure-guided scaffolds designed to target essential bacterial processes such as cell division, lipid A biosynthesis, DNA replication, and protein synthesis. While many candidate molecules show strong enzymatic inhibition and promising antibacterial activity in vitro, their progress is often limited by poor Gram-negative permeability, active efflux, a narrow spectrum, and off-target toxicity. Recent efforts are therefore focusing on scaffold optimization, prodrug and conjugation strategies, dual-target agents, and combination regimens to improve delivery, broaden activity, and reduce resistance emergence. In parallel, early evaluation of whole-cell potency, efflux liability, pharmacokinetics, and safety appears increasingly important for identifying viable leads. Overall, the evidence suggests that future success in antibacterial development will likely depend on integrating target potency with physicochemical and pharmacological properties from the earliest stages of design.

Keywords: Antimicrobial resistance, Drug design, Multidrug-resistant, Novel chemical entities, Bacterial membrane permeability

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1. Introduction

Antimicrobial resistance (AMR) has emerged as one of the foremost threats to global public health, driven by the rapid evolution and horizontal dissemination of resistance determinants among pathogenic bacteria [1,2]. The proliferation of multidrug-resistant (MDR) and extensively drug-resistant (XDR) phenotypes has rendered many empirical therapies obsolete, a crisis exacerbated by the inherent limitations of semisynthetic modification in generating novel scaffolds [1]. Epidemiological projections corroborate this exigency; the World Health Organization estimates that antimicrobial resistance, which was associated with approximately 4.95 million deaths in 2019, will cause 10 million deaths annually by 2050 [3].

The clinical threat is disproportionately attributed to the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species). This group necessitates the development of agents capable of circumventing established resistance mechanisms [4]. The ubiquity of resistance in Methicillin-resistant *S. aureus* (MRSA) isolates has precipitated a clinical environment in which standard-of-care antibiotics, including vancomycin and linezolid, increasingly fail, necessitating agents that act via novel mechanisms to prevent established resistance pathways [5]. The persistence of *S. aureus* in cystic fibrosis patients, facilitated by biofilm formation and adaptive virulence factors, underscores the limitations of current therapeutic regimens and highlights the urgent need for novel chemical entities that can disrupt bacterial cell-division machinery [6].

The contemporary stagnation in antimicrobial innovation is attributed not to a paucity of effort, but to the structural obsolescence of a discovery paradigm historically tethered to the finite chemical diversity of natural products [1]. Although natural product isolation defined the mid-20th-century discovery era, the subsequent reliance on semisynthetic modification has proven recalcitrant to the rapid evolution of resistance mechanisms [1]. Specifically, semisynthetic derivatization of the lincosamide class has failed to circumvent the methylation of ribosomal RNA conferred by *erm* and *cfr* enzymes, thereby necessitating fully synthetic strategies to access novel

chemical space [1]. Concurrently, the financial retrenchment of the pharmaceutical sector has created a critical gap in the development of agents with novel mechanisms of action [7]. Consequently, the ubiquity of derivative compounds in clinical pipelines, inherently susceptible to cross-resistance, has necessitated a paradigm shift toward fully synthetic, rationally designed scaffolds devoid of structural homology to extant pharmacophores [4].

The integration of whole-genome sequencing with high-throughput screening has illuminated a plethora of potential antibacterial targets; however, translating these genomic coordinates into viable clinical candidates remains hindered by high attrition rates in late-stage development [7]. While systems biology has successfully elucidated essential metabolic targets in *Mycobacterium tuberculosis*, specifically DfrA, FolB, and Tmk, the physicochemical recalcitrance of the bacterial envelope frequently renders these enzymatic inhibitors impotent *in vivo*, necessitating rigorous *in silico* validation to mitigate downstream failure [7]. The prohibitively high attrition rate in lead identification is frequently attributed to the physicochemical inability of candidate molecules to permeate the Gram-negative outer membrane or evade active efflux mechanisms, which constitute formidable barriers to intracellular accumulation [8]. To mitigate these physicochemical liabilities before synthesis, structure-based drug design protocols have been deployed to interrogate virulence factors, recently yielding novel coumarin-based antagonists targeting the *Salmonella typhi* effector protein SipA [9].

The permeation of the Gram-negative cell envelope constitutes the rate-limiting step in contemporary antibiotic development, necessitated by the dual-membrane architecture wherein the outer membrane functions as a stringent permeability checkpoint. Unlike Gram-positive counterparts, these organisms possess a lipopolysaccharide-rich outer leaflet and size-selective porin channels that severely restrict the passage of small molecules, necessitating the optimization of physicochemical properties to achieve adequate intracellular accumulation. Molecules that successfully traverse the outer membrane are frequently effluxed by efflux pumps, which prevent accumulation at the target site. To overcome this exclusion, rational design strategies have evolved to exploit active transport mechanisms via molecular mimicry. For instance, the conjugation of DNA gyrase inhibitors to siderophore mimics facilitates the hijacking of bacterial iron-uptake machinery, thereby causing active transport into the periplasm of *E. coli*. This "Trojan Horse" strategy exemplifies the paradigm shift from serendipitous discovery to molecular engineering, wherein permeation is structurally conferred rather than incidental [8].

This review delineates the trajectory of rational antimicrobial design, prioritizing novel chemical entities that exploit phylogenetically conserved yet underutilized targets to bypass established resistance mechanisms [10,11].

2. Search Strategy

A literature search was conducted in PubMed/MEDLINE, Scopus, Web of Science, and Google Scholar to identify studies on the rational design and discovery of novel antimicrobial agents against MDR bacterial pathogens. Publications from 2012 to 2026 were searched, with selected seminal earlier studies included where relevant.

Search terms covered three domains: (i) MDR pathogens and antimicrobial resistance (e.g., MRSA, VRE, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*); (ii) design approaches (e.g., rational drug design, structure-based drug design, virtual screening, machine learning, and antimicrobial peptide design); and (iii) molecular targets and antimicrobial classes (e.g., FtsZ, LpxC, DNA gyrase, GyrB, dihydrofolate reductase, ATP synthase, antimicrobial peptides, polymyxins, vancomycin, and lincosamides).

Articles were selected based on relevance, scientific significance, recency, and representation of major targets and design strategies, with emphasis on studies reporting activity against clinically relevant MDR pathogens. Reference lists of key articles were also screened to identify additional relevant studies. All the references were checked for validity [12].

3. Rational Design of Small Molecules

3.1 Cell Division Inhibitors (FtsZ)

Filamenting temperature-sensitive mutant Z (FtsZ), the prokaryotic homologue of eukaryotic tubulin, functions as the central orchestrator of bacterial cytokinesis and represents a validated target for antimicrobial intervention [5]. Driven by Guanosine triphosphatase (GTP) hydrolysis, FtsZ polymerizes at the cellular midline to assemble the Z-ring, a dynamic scaffold that recruits divisome components to facilitate septal constriction [6]. Despite preserving the structural fold characteristic of eukaryotic tubulin, FtsZ shares negligible sequence homology (<20%) with its mammalian counterpart, a divergence that minimizes the risk of antimitotic-related neurotoxicity and provides a high margin of selectivity for host safety [6]. This evolutionary distance is particularly significant from a safety-engineering perspective: it implies that potent FtsZ binders need not engage tubulin at therapeutically relevant concentrations, thereby circumventing the peripheral neuropathy and myelosuppression that have hampered repositioning of classical antimitotic agents as antibacterials. As of early 2025, the Protein Data Bank contains 97 FtsZ structures from 12 bacterial species, furnishing an increasingly detailed structural atlas that is driving structure-based inhibitor design across multiple pharmacophore classes [13]. Pharmacological interrogation of FtsZ typically exploits two distinct modalities: orthosteric blockade of the GTP-binding pocket to arrest polymerization, or allosteric modulation of the interdomain cleft (PC pocket), which often stabilizes aberrant polymeric filaments, precipitating cytokinetic failure [14, 15].

The benzamide scaffold remains the most clinically advanced pharmacophore targeting the interdomain cleft, recently optimized to prevent the metabolic instability and solubility limitations inherent to earlier generations. The structure-activity relationship (SAR) of TXH9179, a thiazolopyridine-benzamide derivative incorporating a 6-acetylene functionality, was elucidated via crystallographic analysis (PDB: 8HTB), revealing that the acetylene moiety enables the molecule to adopt a "straight" binding conformation within the *S. aureus* FtsZ cleft. This conformation maximizes hydrophobic contacts without necessitating the steric rearrangement of residues Met226, Thr309, and Ile311 required by the bent conformation of the trifluoromethyl analogue, TXA707. This structural optimization translated into superior potency, with TXH9179 exhibiting a minimum inhibitory concentration (MIC) of 0.25 μg/mL against a panel of MRSA, vancomycin-intermediate *S. aureus*, and vancomycin-resistant *S. aureus* isolates, representing a 4-fold improvement over the comparator, TXA707. To address formulation challenges, the carboxamide prodrug TXH1033 was synthesized for enzymatic hydrolysis by serum acetylcholinesterases. Oral administration of this agent (72 mg/kg) in a murine peritonitis model resulted in 100% survival, significantly outperforming the previous lead prodrug TXA709 [5].

Extending this strategy to Gram-negative pathogens, the second-generation FtsZ inhibitor/acinetobactin conjugate RUP7 linked an oxazole-benzamide pharmacophore to acinetobactin, the native siderophore of *A. baumannii* [16]. Under iron-limiting conditions, RUP7 exhibited enhanced uptake, likely via the BauABCDEF acinetobactin transport system, and showed potent bactericidal synergy with PBP3-targeting β-lactams such as aztreonam and ceftazidime [16]. This "Trojan horse" strategy exploits bacterial iron-acquisition pathways to overcome the outer membrane permeability

barrier that has historically limited benzamide activity against Gram-negative organisms.

Efforts to expand the benzamide chemotype also identified four benzodioxane-benzamide derivatives (FZ95, FZ100, FZ116, and FZ118) as the first members of this class with bactericidal activity against Streptococcus pneumoniae [17]. With MICs of 25-80 µg/mL, these compounds disrupted both cell division and elongation, consistent with inhibition of FtsZ polymerization at the interdomain cleft [17]. Together with earlier activity against efflux-deficient E. coli, these findings highlight the benzodioxane-benzamide scaffold as a versatile platform for broader-spectrum FtsZ inhibitor development. Parallel efforts to rigidify the benzamide scaffold involved the introduction of non-planar heterocyclic linkers, specifically through a series of 4,5-dihydroisoxazole-containing benzamides. This campaign identified racemate A16 as a potent polymer stabilizer, and subsequent chiral resolution demonstrated that the S-enantiomer (A16-S) is the eutomer, with an MIC of ≤ 0.125 µg/mL against MRSA ATCC 43300. These findings confirm that stereochemical geometric constraints are critical for maintaining an optimal fit within the hydrophobic cleft [18]. This principle appears broadly applicable: the equivalent chiral selectivity observation has been made independently across the dihydroisoxazole, thiazolopyridine, and benzodioxane series, implying that the PC pocket imposes a stringent chiral filter on binding partners regardless of the linker chemotype employed.

Natural products provide a complementary source of FtsZ-targeting scaffolds, although their clinical development has often been limited by mammalian cytotoxicity arising from off-target interactions with eukaryotic tubulin [6]. Sanguinarine and berberine exemplify this challenge: both isoquinoline alkaloids possess antibacterial activity but also exhibit significant toxicity due to their ability to inhibit tubulin polymerization [6]. Recent studies have shown that sanguinarine acts through a distinctive mechanism, inducing unfolding of *S. aureus* FtsZ and reducing the conformational flexibility required for polymer assembly, rather than simply inhibiting GTPase activity. This mechanism translated into MIC values of 8-16 µg/mL against multidrug-resistant MRSA isolates [19]. Such findings suggest that compounds capable of destabilizing FtsZ structure may represent an underexplored allosteric strategy distinct from the polymer-stabilizing activity of benzamide derivatives.

Another promising natural product is magnolol, a hydroxylated biphenyl neolignan derived from *Magnolia officinalis*. Identified through cell-based screening, magnolol exhibited activity against Gram-positive pathogens, including MRSA and vancomycin-resistant *Enterococcus* (MIC 8-16 µg/mL), with biochemical studies confirming inhibition of both FtsZ GTPase activity and polymerization. Its antibacterial efficacy was further supported in a *Galleria mellonella* infection model [20]. Although natural products generally remain less potent than optimized synthetic benzamides, they serve as valuable starting points for medicinal chemistry. For example, alkoxy benzene-berberine derivatives achieved MIC values of 2-16 µg/mL against MRSA and vancomycin-resistant *Enterococcus* while partially reducing the cytotoxicity associated with the parent alkaloid [20]. Collectively, these studies highlight natural products as a rich source of structurally distinct FtsZ inhibitors and attractive templates for semi-synthetic optimization.

While benzamide inhibitors traditionally target the interdomain cleft, recent pharmacophore hybridization strategies have yielded cationic indolyl-acrylamides designed to target the GTP-binding site of FtsZ and reduce the outer membrane impermeability of Gram-negative pathogens. Among these novel scaffolds, the indolyl-acrylamide derivative 12e was synthesized to optimize hydrophobic and electrostatic interactions within the nucleotide-binding pocket. The incorporation of an ethylenediamine tail conferred superior potency; 12e achieved an MIC of 1.2 µg/mL against *A. baumannii* BAA-747, and 4.3 µg/mL against the XDR clinical isolate A-564, representing a 13-fold improvement over cefepime. Mechanistic characterization attributed this efficacy to a dual mode of action: the compound inhibited FtsZ GTPase activity by 51% at 32 µg/mL and simultaneously caused membrane permeabilization. This dual mode of action, inhibiting FtsZ GTPase activity by 51% at 32 µg/mL while simultaneously permeabilizing the bacterial membrane, is clinically significant because it reduces the probability that single-step mutations in ftsZ will generate full resistance. Subsequent homologation to the butyl-derivative 12j enhanced potency against XDR *A. baumannii* (MIC 1.2 µg/mL) despite attenuated GTPase inhibition (30.5%), implying that increased lipophilicity shifts the primary mode of action toward membrane disruption [3]. This mechanistic fluidity complicates resistance prediction but may be advantageous in the context of extensively drug-resistant pathogens, where any additional membrane stress is bactericidal.

The exploration of fused heterocyclic systems has yielded additional high-potency inhibitors targeting the "Phosphatidylcholine hydrophobic pocket". Compound C9, a 1-methylquinazoline derivative decorated with a 4-fluorostyryl side chain that interacts with the T7-loop, exhibited potent bactericidal activity against clinical isolates of *S. aureus* (MIC 0.125 µg/mL) and *Bacillus subtilis* (MIC 0.0625 µg/mL), with time-kill kinetics confirming rapid bacterial eradication [14]. However, C9 was identified as a substrate of the *acrB* efflux pump in *E. coli*, evidenced by a dramatic MIC shift from 256 µg/mL in wild-type strains to 16 µg/mL in ΔAcrB mutants [14]. Expanding on the quinolinium scaffold, Compound A3, a 4-methylpiperidine derivative that functions as a polymer stabilizer, promoted lethal filamentation with an MIC of 1.0 µg/mL against MRSA and 4.0 µg/mL against Vancomycin-Resistant *E. faecium*, while demonstrating strong synergy with methicillin (FICI = 0.31) [21]. The mechanistic basis of this synergy is interpretable: FtsZ-mediated filamentation sensitizes the bacteria by extending and distorting the division septum, thereby increasing access of the cell wall-active agent. Moving to tricyclic architectures, the benzofuroquinolinium derivative Compound 5 was reported to target the GTP-binding pocket rather than the interdomain cleft, a binding-site alteration that conferred broad-spectrum activity, inhibiting NDM-1-producing *E. coli* (MIC = 1.0 µg/mL) with no observed resistance after 25 passages [15]. Similarly, Compound 16e, a hybrid of 1-methyl-2-phenylpyridin-1-ium and indole, was introduced as an agent operating via a dual mechanism, allosteric stabilization of the FtsZ T-state and dissipation of the proton motive force, resulting in MIC values of 0.0625 µg/mL for *S. aureus* and 1.0 µg/mL against *A. baumannii* [22].

Concurrently, structure-based virtual screening campaigns targeting the allosteric interdomain cleft of FtsZ have precipitated the identification of non-canonical scaffolds. C11, a benzothiophene-oxazole derivative that binds to the allosteric interdomain cleft, exhibited a potent MIC of 2 µg/mL against *S. aureus* and demonstrated remarkable synergy with carbapenems to restore susceptibility in MRSA strains. This compound inhibited FtsZ polymerization with an IC50 of 47.97 µM and efficiently eradicated mature biofilms at 4 µg/mL [6]. The therapeutic viability of these next-generation inhibitors is further corroborated by their favorable selectivity profiles; C11 maintained >90% viability in A549 epithelial cells at 25 µM, while the quinolinium derivative A3 exhibited negligible hemolytic toxicity (IC5 = 64 µg/mL), thereby circumventing the eukaryotic cytotoxicity often associated with tubulin-homologous targets [6,21]. Critically, the low risk of on-target host toxicity resulting from the limited (<20%) structural homology between FtsZ and tubulin contrasts sharply with the mammalian toxicity observed for natural product inhibitors such as sanguinarine and berberine, highlighting the advantages of structure-guided synthetic drug discovery over direct natural product

development [13,23]. Current evidence suggests that the most promising route toward clinically viable FtsZ inhibitors integrates high-resolution structural characterization of inhibitor-FtsZ complexes for rational potency optimization, prodrug or conjugation approaches to overcome pharmacokinetic and permeability barriers, and mechanism-based combination therapies, particularly with cell wall-active antibiotics, to limit resistance development and expand activity against challenging Gram-negative pathogens.

3.2 Lipid A Biosynthesis (LpxC)

The zinc-dependent metalloenzyme LpxC (UDP-3-O-acyl-N-acetylglucosamine deacetylase) constitutes a critical therapeutic target, as it catalyzes the first committed step of the Raetz pathway, an obligate sequence for Lipid A biogenesis and the structural integrity of the Gram-negative outer membrane. Distinguished by its structural conservation across diverse pathogenic species and the absence of mammalian homologues, LpxC presents a theoretical framework for high-selectivity inhibition [24]. However, the medicinal chemistry of LpxC inhibition has historically been plagued by a reliance on hydroxamate-based zinc-binding groups, which often exhibit poor selectivity against host metalloproteins, leading to severe cardiovascular toxicity [25]. The persistence of this toxicity liability across structurally diverse hydroxamate series has led to a broad consensus in the field that the warhead itself, rather than the hydrophobic tail, is the primary driver of off-target metalloprotein engagement, necessitating either radical scaffold departures or precisely engineered steric constraints around the chelating motif [26]. To overcome these off-target effects, contemporary discovery efforts have bifurcated: one trajectory pursues the structural rigidification of the hydroxamate scaffold to enhance selectivity [27]. While the other necessitates the complete dereplication of the hydroxamate warhead in favor of alternative chelating architectures [25].

The therapeutic potential of the hydroxamate pharmacophore was first evaluated clinically via ACHN-975, a novel inhibitor of UDP-3-O-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) that progressed to Phase 1 trials. *In vitro* characterization revealed potent enzymatic inhibition (IC50: 0.68 nM) concomitant with broad-spectrum activity against 250 clinical *P. aeruginosa* isolates (MIC90: 0.25 μg/mL). Despite achieving rapid bactericidal kinetics, defined by a 3-log10 reduction in colony-forming units within 4 hours, the clinical progression of this agent was terminated due to dose-limiting hypotension driven by peak plasma concentrations (Cmax) rather than total exposure. Optimization campaigns subsequently produced LPXC-516 (IC50: 0.71 nM), a lead candidate exhibiting superior efficacy in neutropenic murine lung infection models; however, development was precluded by the reemergence of cardiovascular toxicity, suggesting this liability is intrinsic to the hydroxamate zinc-binding motif utilized in this scaffold series [24]. The mechanistic interpretation is that hydroxamate geometry permits bidentate chelation of zinc in a manner geometrically compatible with both LpxC and human matrix metalloproteinases, and that no amount of peripheral lipophilic tail engineering can fully overcome this promiscuity when the warhead retains full conformational flexibility [26].

To circumvent the promiscuity inherent to flexible hydroxamate linkers, structure-based design was employed to engineer Compound 1(−), a derivative anchored by a chiral L-threonine scaffold. Optimization of the hydrophobic tail with a rigid propargyl ether ensured tunnel occupancy, while a beta-gem-dimethyl group stabilized the zinc-binding group. The resulting compound demonstrated exceptional affinity (IC50 = 0.001 μM) and efficacy against *P. aeruginosa* (MIC90 = 2 μg/mL), with excellent selectivity against human matrix metalloproteinases (MMPs) and histone deacetylases (IC50 > 30 μM). Yet, a critical limitation persisted. Despite dissociating LpxC inhibition from mammalian cytotoxicity (CC50 > 295 μM in HepG2 cells), the compound's spectrum remained recalcitrant to expansion, exhibiting negligible activity against broader *Enterobacteriaceae* [27]. This spectrum gap is particularly consequential given the 2024 WHO priority pathogen list update, which added multiple MDR Gram-negative Enterobacteriaceae as critical priority organisms, underscoring the need for LpxC inhibitors with pan-Gram-negative coverage rather than species-selective activity [26].

The cardiovascular toxicity intrinsic to the hydroxamate pharmacophore necessitated structural divergence, culminating in the engineering of TP0586532, an inhibitor that utilizes an *N*-hydroxyformamide zinc-binding group anchored to a 2-hydroxymethyl imidazole scaffold. This compound exhibited an IC50 of 0.101 μM against *Escherichia coli* LpxC and demonstrated broad-spectrum potency against carbapenem-resistant *K. pneumoniae* (MIC90 = 4 μg/mL) and *mcr-1* positive colistin-resistant *E. coli* (MIC = 2 μg/mL). Notably, the replacement of the hydroxamate warhead resulted in a selectivity index exceeding 700-fold against human MMPs, and *in vivo* safety assays corroborated the absence of hypotension or QT prolongation in guinea pig models [25]. The crystallographic structure of TP0586532 bound to P. aeruginosa LpxC (PDB: 7DEM) provided the foundation for a second generation of 2-(1-S-hydroxyethyl)imidazole inhibitors, now the most actively developed non-hydroxamate LpxC chemotype [26]. Building on this scaffold, Forge Therapeutics disclosed compounds 46 and 47 in 2022, both exhibiting improved antibacterial activity over TP0586532 in preclinical pneumonia models, while Blacksmith Pharmaceuticals reported compound 48 in 2024, optimized specifically for P. aeruginosa lung infections [26]. In parallel, incorporation of an aromatic acetylene substituent into the P4 region generated compound 49, which displayed enhanced oral bioavailability against Gram-negative pathogens [26]. Together, these advances reflect growing industrial commitment to non-hydroxamate LpxC inhibitors, motivated by the favorable safety and efficacy profile first demonstrated by TP0586532.

Academic efforts have simultaneously pursued scaffold-hopping strategies to identify alternative zinc-binding group architectures. Chen and colleagues recently reported hydrazone-containing derivatives of the clinical reference inhibitor CHIR-090 that retained antibacterial activity against E. coli while exhibiting low HepG2 cytotoxicity and favorable plasma protein binding [28]. The hydrazone moiety was designed to engage the hydrophobic tunnel of LpxC and modulate physicochemical properties, with docking studies indicating key interactions with Trp191 and Cys63 [28]. Notably, these same residues are targeted by the oxazolidinone-based inhibitor 23j, previously identified by Kurasaki and colleagues through a scaffold-hopping campaign that yielded nanomolar LpxC inhibition and potent activity against both *E. coli* and *K. pneumoniae* with low susceptibility to efflux [29]. The recurrent engagement of Trp191 and Cys63 across structurally distinct inhibitor classes suggests that these residues constitute a pharmacophoric hotspot that may serve as a valuable focus for future LpxC inhibitor design.

While the non-hydroxamate strategy appears to have resolved the toxicity bottleneck, the genetic plasticity of the target persists as a challenge. Resistance frequencies as high as 9.3×10^{-7} were observed in *K. pneumoniae*, attributed to mutations in the *fabZ* gene, which regulates the supply of fatty acid substrates to the LpxC pathway [25]. The mechanistic basis of FabZ-mediated resistance is now well established. Increased FabZ activity redirects acyl-acyl carrier protein (ACP) flux toward phospholipid biosynthesis, thereby reducing competition for LpxC substrates and restoring the lipid A/phospholipid balance disrupted by LpxC inhibition. As a result, bacterial cells can compensate for the enzymatic blockade without directly modifying the drug target itself [26]. Notably, this resistance mechanism appears to be chemotype-independent, having been reported across hydroxamate, N-hydroxyformamide, and

sulfonamide-hydroxamic acid LpxC inhibitor classes. These findings suggest that FabZ upregulation represents a broadly conserved adaptive response to LpxC inhibition and highlight the potential need for combination therapies or FabZ co-targeting strategies to overcome resistance [26].

3.3 DNA Replication (Gyrase B & ParE Inhibitors)

The maintenance of genomic topology, a prerequisite for replication, transcription, and decatenation, is governed by the bacterial type IIA topoisomerases, DNA gyrase and topoisomerase IV [8]. While the clinical utility of fluoroquinolones relies on the stabilization of the DNA-enzyme cleavage complex, the emergence of resistance has necessitated the exploration of inhibitors targeting the N-terminal ATPase domains of GyrB and ParE [4,8]. These subunits are integral to the transduction of free energy derived from ATP hydrolysis, a process that drives the introduction of negative supercoils into the DNA lattice [30]. Consequently, competitive inhibition at the ATPase active site, a locus distinct from the fluoroquinolone binding pocket, circumvents the resistance mechanisms conferred by mutations in *gyrA* and *parC*, thereby restoring potency against recalcitrant MDR isolates [31]. Despite the strong mechanistic rationale for targeting GyrB and ParE, clinical translation has proven challenging. Numerous ATPase inhibitor series have advanced into preclinical or clinical development, yet most have ultimately been limited by toxicity concerns, unfavorable pharmacokinetic properties, insufficient antibacterial spectrum, or the rapid emergence of resistance. Consequently, no GyrB/ParE ATPase inhibitor has achieved regulatory approval to date, highlighting the difficulty of converting potent target-based inhibition into safe, durable, and broadly effective antibacterial therapy [32].

Recent structural interrogations of the benzothiazole scaffold have necessitated a granular mapping of the *E. coli* GyrB ATP-binding pocket to identify exploitable subsites for enhanced ligand affinity. Specifically, the topology of the "hydrophobic floor," a lipophilic region defined by residues Ile78, Pro79, Ile94, and Ala98, was delineated as offering a distinct trajectory for inhibitor extension. Through the synthesis of benzothiazole-pyrrole-2-carboxamide derivatives, it was elucidated that *N*-benzylation of the carboxamide moiety (Compound 3) enables the inhibitor to accommodate the conformational plasticity of this sub-site, a behavior corroborated by potential energy surface calculations. Compound 3 manifested superior potency against *E. coli* DNA gyrase (IC50 = 19 ± 3 nM); this efficacy is attributed to the maintenance of the canonical Asp73 hydrogen bond network concomitant with the establishment of stabilizing interactions within the hydrophobic floor, specifically involving Lys103 [30]. A complementary quantum theory of atoms in molecules analysis by Zidar and colleagues further refined the understanding of ligand–target interactions within this scaffold. Studying a series of N-(benzothiazol-2-yl)pyrrolamide derivatives, the authors quantified the electron-density contributions of individual binding contacts and demonstrated that substituents oriented toward the hydrophobic floor contribute disproportionately to overall binding affinity relative to their size. These findings provide a high-resolution mechanistic framework for structure-based optimization and suggest that strategic elaboration of floor-facing substituents may represent a particularly productive direction for future fragment-growing campaigns within this chemical series [33].

While benzothiazole scaffolds exhibit exceptional enzymatic affinity, their translation into whole-cell activity against Gram-negative pathogens is frequently abrogated by the synergistic exclusion of the outer membrane barrier and active efflux mechanisms. To overcome these permeation deficits, siderophore-mediated active transport was exploited, conjugating the benzothiazole core to a hydroxypyridinone moiety designed to mimic ferric-siderophore complexes and facilitate uptake via bacterial iron-transport machinery. The resultant conjugate, 18b, retained low-nanomolar inhibitory potency against *E. coli* GyrB (IC50: 22 ± 6 nM) and demonstrated confirmed iron(III) chelation capacity through nuclear magnetic resonance spectroscopic titration. Microbiological profiling revealed that while 18b remained recalcitrant against wild-type *E. coli* (MIC >50 μM), significant potency was restored against the efflux-deficient ΔtolC strain JW5503 (MIC 12.5 μM) under iron-depleted conditions. These data elucidate that although the siderophore moiety facilitates periplasmic access, the elevated topological polar surface area (149.4 Å²) conferred by the conjugate renders the molecule a substrate for rapid efflux, necessitating further physicochemical optimization to ensure intracellular accumulation [8]. This result mirrors an identical bottleneck encountered in benzamide FtsZ inhibitor conjugates and underscores a field-wide challenge: siderophore conjugation reliably improves outer membrane penetration but cannot compensate for poor efflux pump evasion when the conjugated payload is itself an efflux substrate. Future design iterations for both target classes will likely require explicit efflux liability profiling as a primary filter rather than a late-stage assay [8,16].

Ligand-based virtual screening has led to the discovery of diverse chemotypes targeting the ATPase domain, identifying the 2,3-diaminoquinoxaline scaffold as a potent inhibitor class. The lead compound, 6c, interacts electrostatically with Arg84 and forms hydrogen bonds with Lys118 and Glu58 within the *S. aureus* GyrB active site. This molecule demonstrated robust antibacterial activity against MRSA clinical isolates (MIC of 0.5 μg/mL), equivalent to that of vancomycin. Conversely, the arylaminotriazine hit AG-205/33156020 exhibited superior enzymatic inhibition (IC50: 1.1 μM) but weaker whole-cell activity (MIC: 4–8 μg/mL), confirming the challenge of membrane permeation in this target class [4]. The disconnect between enzymatic potency and whole-cell MIC observed across these series is not surprising given the absence of a defined permeability pharmacophore in most ATPase inhibitor design campaigns; the field increasingly recognizes that lipophilicity and molecular weight must be treated as first-class design parameters, not afterthoughts [32].

To evade the metabolic instability and suboptimal pharmacokinetics of the clinical candidate zoliflodacin (ETX0914), a systematic derivatization of the benzisoxazole scaffold was undertaken, specifically targeting the oxazolidinone ring, which is prone to oxidative degradation. The introduction of a spirocyclopropane moiety at the C5 position yielded compound 33e, which demonstrated potent activity against MRSA (MIC <0.03–0.06 μg/mL) and *Neisseria gonorrhoeae* (MIC90: 0.125 μg/mL). Molecular docking elucidated that 33e retains the pharmacophoric hydrogen bond with Asp437 necessary to stabilize the DNA-cleavage complex, while the spirocyclopropane motif establishes additional hydrophobic contacts with Asn476. Subsequent bioisosteric modulation, replacing the oxazolidinone with a thiazolidinone core, culminated in compound 39e; this analogue exhibited superior metabolic stability and achieved a four-fold improvement in *in vivo* efficacy (ED50: 2.54 mg/kg) compared to the parent zoliflodacin in a murine model of systemic MRSA infection [31].

The clinical relevance of the zoliflodacin scaffold has since been directly validated: a global Phase III non-inferiority trial demonstrated that a single 3 g oral dose of zoliflodacin achieved a microbiological cure rate of 90.9% in urogenital gonorrhea, non-inferior to the ceftriaxone/azithromycin dual standard-of-care regimen, and the FDA accepted a New Drug Application for the agent in 2025 [34]. Crucially, zoliflodacin activity was unaffected by mutations in *gyrA*, *gyrB*, *parC*, or *parE* that elevate fluoroquinolone MICs, providing the first clinical-scale confirmation that the spiropyrimidinetrione binding mode on GyrB is genuinely orthogonal to fluoroquinolone resistance mechanisms [35]. This clinical milestone validates GyrB cleavage-complex stabilization as a viable antibacterial strategy and is likely to reinvigorate efforts to develop

next-generation spiropyrimidinetriones with improved Gram-negative activity.

A major challenge in GyrB/ParE inhibitor development has been mitigating mitochondrial toxicity without compromising antibacterial activity. Addressing this issue, Zhao and colleagues developed a hydroxyisopropyl pyridazine-substituted pyrrolamide (compound 28) that retained potent GyrB inhibition while markedly reducing mitochondrial toxicity, likely through improved selectivity over the human mitochondrial topoisomerase. This strategy provides a promising framework for future optimization of GyrB-targeting scaffolds [36].

Recent strategies have also explored dual-targeting agents to mitigate resistance development, leading to the synthesis of coumarin-1,3,4-thiadiazole hybrids designed to inhibit both GyrB and the enoyl-ACP reductase (FabI). Compound 7i, featuring a *meta*-nitro substitution, demonstrated significant activity against *A. baumannii* (MIC: 16 µg/mL). It has been suggested that this molecule occupies the ATPase domain of GyrB via π-anion interactions with Asp163, while simultaneously blocking the NADH-binding cleft of FabI. This dual-mechanism approach offers a viable strategy for overcoming the limitations of single-target ATPase inhibitors [37]

3.4 Ribosome Inhibitors

The bacterial 70S ribosome Peptidyl Transferase Center remains a validated target, yet the utility of traditional lincosamides is compromised by *erm*-encoded rRNA methyltransferases. These enzymes dimethylate nucleotide A2058, creating a steric clash that confers cross-resistance to MLS_B antibiotics. To overcome the limitations of semi-synthetic modifications, component-based total synthesis was employed to access novel chemical space that could bypass these barriers [1]. The absence of any new lincosamide entering clinical trials since 1970 highlights the formidable challenge posed by MLS_B resistance and supports the shift toward fully synthetic scaffolds capable of accessing chemical space beyond the reach of traditional semi-synthetic approaches [38].

This approach necessitated replacing the clindamycin pyrrolidine core with a rigidified oxepanoproline scaffold, designed to pre-organize the antibiotic for ribosomal binding. The initial rigidified prototype, OPP-1, failed to inhibit constitutively methylated strains, necessitating the addition of a hydrophobic moiety. This optimization yielded Iboxamycin (IBX), featuring a C7'-α-isobutyl extension. Crystallographic analysis revealed that IBX induces a conformational shift, displacing the methylated A2058 nucleotide by approximately 2 Å while anchoring into a hydrophobic cleft defined by residues A2451 and C2452. These compensatory hydrophobic interactions provide sufficient binding affinity to override the steric hindrance conferred by methylation [1]. Although iboxamycin substantially overcomes Erm- and Cfr-mediated resistance, its activity can still be partially compromised by ABCF ribosomal protection proteins, which displace the antibiotic through ribosome remodeling rather than target modification. Nevertheless, studies suggest that the high ribosomal affinity of iboxamycin limits the effectiveness of this resistance mechanism under clinically relevant conditions [39,40]. Additionally, Cfr-mediated C8-methylation of A2503 confers only moderate resistance to IBX (MIC of 2–8 µg/mL compared to 0.06 µg/mL for *cfr*-negative strains), a partial resistance profile that the Myers group explicitly used to guide the next cycle of optimization [40].

This active site remodeling conferred exceptional potency against MDR pathogens, including *c-ermA-positive S. aureus (MIC 1 µg/mL) and lsaA-expressing Enterococcus faecalis* (MIC 0.06 µg/mL). Departing from the spectrum of traditional lincosamides, IBX demonstrated expanded activity against Gram-negative pathogens, including carbapenem-resistant *A. baumannii* and *E. coli*. SAR analysis confirmed the critical role of the C7'-α stereochemistry; the β-epimer exhibited reduced potency against Gram-negative isolates, underscoring the need for precise geometric alignment to enhance target engagement [1].

The safety profile of IBX is attributed to the ubiquity of a G2058 residue in human mitochondrial rRNA, which precludes antibiotic binding. Consequently, the compound exhibited no cytotoxicity against HepG2 cells and did not inhibit mitochondrial protein synthesis. In a murine *model of Streptococcus pyogenes infection, IBX conferred 100% survival at 3 mg/kg, with 24% oral bioavailability, thereby* validating the displacement mechanism as a viable strategy for overcoming methylation-based resistance [1].

Building on the success of iboxamycin, subsequent generations have leveraged conformational pre-organization to further enhance potency, spectrum, and resistance evasion. Cresomycin introduced a bridged macrobicyclic scaffold that locks the molecule into its bioactive conformation, improving activity against multidrug-resistant Gram-positive and Gram-negative pathogens, including *Pseudomonas aeruginosa*, while retaining efficacy against multiple resistance mechanisms [41]. The latest analogue, BT-33, further optimized this strategy through structural modifications that strengthen ribosomal interactions and increase conformational rigidity, resulting in enhanced potency, improved pharmacokinetics, and favorable preclinical efficacy against multidrug-resistant pathogens [38]. Collectively, the progression from iboxamycin to cresomycin and BT-33 exemplifies a rational structure-guided medicinal chemistry program that has transformed a resistance-limited antibiotic class into a promising broad-spectrum antibacterial platform.

3.5 Cell Wall Biosynthesis & Metabolic Pathways

The escalating morbidity attributed to the "ESKAPE" pathogens, particularly MDR *S. aureus* and *A. baumannii*, has precipitated a paradigm shift away from conventional antibiotics toward novel targets that lack mammalian homologues. Concomitantly, rational drug design has necessitated the deployment of molecular hybridization, a strategy generating dual-mechanism scaffolds engineered to subvert established resistance pathways by simultaneously interrogating distinct pharmacological targets [37]. This strategic shift is further supported by the 2024 WHO Bacterial Priority Pathogen List, which continues to identify several ESKAPE pathogens, including carbapenem-resistant *Acinetobacter baumannii* and vancomycin-resistant *Enterococcus faecium*, as high-priority threats with limited treatment options, underscoring the urgent need for antibiotics with novel mechanisms of action [42].

To identify novel therapeutic candidates within the *S. aureus* core genome, researchers employed hierarchical subtractive genomics to filter for proteins that are essential to the pathogen yet non-homologous to the human host [43]. Through hierarchical subtractive genomics, two critical, non-host homologous targets were elucidated in MDR *S. aureus*: Aspartate-semialdehyde dehydrogenase (Asd) and Anthranilate synthase component II (TrpG). Asd is pivotal for the aspartate biosynthesis pathway, including the diaminopimelate and lysine biosynthetic processes, which are crucial for microorganisms' essential amino acids and cell biosynthesis. *In silico* screening of the Traditional Chinese Medicine library identified ZINC85492658 as the lead candidate, exhibiting a superior binding affinity (ΔGbind = −57.72 kcal/mol) for the Asd active site; notably, thermodynamic decomposition revealed this interaction is driven primarily by Van der Waals forces (−61.84 kcal/mol) rather than Coulombic attraction, which presented an unfavorable energy penalty [43]. Similarly, for the metabolic target TrpG, the Traditional Chinese Medicine library yielded ZINC70455378 (ΔGbind = -35.33 kcal/mol), suggesting that

targeting the biosynthesis of aromatic amino acids can effectively starve the bacterium of essential metabolic building blocks.

These findings corroborate the potential of small-molecule inhibitors to target essential metabolic pathways, thereby compromising bacterial growth and survival by disrupting the synthesis of critical amino acids and metabolites [43]. A key limitation of this approach is that computational predictions do not necessarily translate into whole-cell antibacterial activity. As neither lead compound has been validated by enzymatic inhibition or MIC testing, the subtractive genomics pipeline should be viewed as a target prioritization and hit-identification strategy rather than a complete drug discovery workflow. Nonetheless, it remains a valuable tool for efficiently narrowing the pool of potential drug targets in multidrug-resistant pathogens and guiding subsequent experimental validation [44].

A significant challenge in targeting the later stages of cell wall synthesis is the VanA-type resistance mechanism in *Enterococci*, where the D-Ala-D-Ala terminus is reprogrammed to D-Ala-D-Lac, reducing vancomycin affinity by 1000-fold. Researchers circumvented this resistance by engineering lipophilic vancomycin analogues that incorporate a quaternary ammonium cation. This modification confers a dual mechanism of action: the glycopeptide backbone retains Lipid II binding, while the lipophilic tail precipitates rapid membrane depolarization and permeabilization. The tetradecyl-derivative (Compound 6) demonstrated remarkable potency against vancomycin-resistant *E. faecium*, achieving an MIC of 0.7 µM, a >1000-fold improvement over native vancomycin. Furthermore, the octyl-derivative (Compound 4) exhibited superior safety profiles (non-hemolytic up to 1000 µM) and efficacy in a neutropenic mouse thigh infection model against MRSA, demonstrating a 3-log kill potency compared to the ED1-log kill potency of vancomycin. Crucially, this membrane-disrupting modality prevented the evolution of resistance in MRSA even after 52 serial passages, attributed to the difficulty of bacteria to remodel their entire membrane architecture [45].

This concept was further supported by the aryl sulfonium-modified vancomycin derivatives reported by Xie and colleagues, which displayed markedly enhanced activity against both MRSA and VRE through a dual mechanism involving membrane disruption and inhibition of cell wall biosynthesis. The lead compound demonstrated potent in vivo efficacy and favorable pharmacokinetic properties, highlighting the therapeutic potential of combining target engagement with membrane-directed activity [46]. Together with quaternary ammonium lipidated analogues, these findings suggest that introducing positively charged, membrane-interacting functionalities may represent a broadly applicable strategy for overcoming glycopeptide resistance and expanding the design space of next-generation vancomycin derivatives.

Beyond the peptidoglycan layer, the integrity of the bacterial membrane relies on the Type II Fatty Acid Synthesis pathway, specifically the enoyl-acyl carrier protein reductase (FabI). Researchers rationally designed novel coumarin-1,3,4-thiadiazole Schiff base hybrids to target FabI, exploiting the enzyme's hydrophobic active site. SAR analysis revealed that the electronic character of the phenyl ring substituents dictated strain selectivity. Compound 7i, featuring a meta-nitro substitution, demonstrated superior potency against carbapenemase-producing *A.baumannii* (MIC 16 µg/mL), effectively circumventing the resistance mechanisms that rendered the strain recalcitrant to ciprofloxacin (MIC >2 µg/mL) [37]. Molecular docking corroborated this activity, revealing that 7i occupies the NADH-binding cleft of the *A. baumannii* FabI reductase with a binding energy of -9.7 kcal/mol, stabilized by specific hydrogen bonding with Ile22 and π-cation interactions with Lys166. Conversely, the introduction of a thiophene bioisostere (Compound 7m) shifted the spectrum toward Gram-positive pathogens, achieving an MIC of 32 µg/mL against *S. aureus* via enhanced π-π stacking within the hydrophobic pocket. These data indicate that hybridization of pharmacophores can yield "broad-spectrum" scaffolds that are tunable to specific MDR pathogens through precise substituent modification [37]. The clinical advancement of afabicin has validated FabI and the FASII pathway as tractable antibacterial targets; however, its lack of Gram-negative activity highlights the persistent challenge of outer membrane permeability. This limitation was addressed through permeation-guided optimization, culminating in fabimycin, which demonstrated potent activity against multidrug-resistant Gram-negative pathogens and efficacy in multiple animal infection models [47]. These findings illustrate how rational permeability engineering can extend the utility of validated targets to Gram-negative bacteria and provide an important framework for future FabI inhibitor development.

4. Peptide Engineering & Peptidomimetics

4.1 De Novo Amphipathic Peptides

The renaissance of antimicrobial peptide (AMP) therapeutics is predicated on the transition from the isolation of natural products to the rational, *de novo* design of synthetic sequences [48, 49]. Unlike traditional antibiotics that target specific enzymatic pockets, *de novo* amphipathic peptides are engineered to exploit the fundamental electrostatic and hydrophobic disparities between prokaryotic and eukaryotic membranes [50]. The central design philosophy governs the segregation of cationic residues (typically Arginine or Lysine) to drive initial electrostatic attraction to anionic bacterial lipids (Lipid A, phosphatidylglycerol), and hydrophobic residues (Tryptophan, Leucine, Isoleucine) to facilitate insertion into the non-polar acyl core of the bilayer [11,51,52]. A key advantage of membrane-targeting agents is their high barrier to resistance. Because bacterial membrane composition is governed by multiple essential biosynthetic pathways, extensive remodeling is often biologically costly, making resistance less readily acquired than for antibiotics directed against single enzymatic targets. This property is increasingly recognized as an important contributor to the long-term therapeutic potential of antimicrobial peptides and other membrane-active agents [53].

The optimization of the "idealized amphipathic helix" requires a precise calibration of sequence length and amino acid composition to maximize the Minimum Optimal Length (MOL) parameter [51]. Excessive length is unnecessary for potency; by engineering the 12-residue peptide WR12 (composed exclusively of Arginine and Tryptophan), researchers achieved potent bactericidal activity (MIC: 4 µg/mL against MDR *P. aeruginosa*) comparable to the 24-residue parent peptide WLBU2 [48]. The positioning of Tryptophan residues at the hydrophilic-hydrophobic interface is critical, as the indole ring anchors the peptide to the membrane surface, facilitating the insertion of the helical core [51].

A critical divergence in SAR involves the selection of cationic residues. While Arginine provides robust membrane binding via bidentate hydrogen bonding with phosphate headgroups, this strong interaction often extends to zwitterionic mammalian membranes, resulting in toxicity [49]. In a systematic comparison of 12-mer helical peptides, the Arg-rich analogue (Peptide 8a) exhibited high hemolytic activity (HC50 = 45 µg/mL), whereas the Lysine-substituted analogue (Peptide 8b) retained broad-spectrum potency (MIC 3.1–6.2 µg/mL against MRSA and *A. baumannii*) but exhibited a significantly improved safety profile (HC50= 280 µg/mL) [49]. Building on the importance of amphipathic organization, Zhang and colleagues redesigned the classical (XXYY)n α-helical template to better align with the natural 3.6-residue-per-turn geometry of α-helices. The resulting template enhanced the segregation of cationic and hydrophobic faces, producing peptides with improved antimicrobial selectivity. The lead peptide, H-R, demonstrated potent

activity against multidrug-resistant bacteria and biofilms, low propensity for resistance development, and favorable in vivo efficacy and safety, highlighting a broadly applicable framework for antimicrobial peptide design [54]. Furthermore, to address proteolytic instability, a "leucine-centric" design was applied to the peptide P-α-02-B. By substituting specific Leucine residues with D-Isoleucine, the derivative H-10 was developed, which displayed exceptional stability (t1/2 > 6h against chymotrypsin) without compromising antimicrobial potency (MIC: 1.56–3.13 μM against MDR isolates) [11].

An important consideration in D-amino acid engineering is that complete enantiomerization does not necessarily improve therapeutic performance. Although full D-substitution can enhance proteolytic stability while preserving in vitro antimicrobial activity, it may also disrupt secondary structure formation in membrane-like environments and reduce in vivo efficacy. Consequently, selective D-substitution at protease-sensitive sites is often a more effective strategy than global inversion of peptide chirality [55].

The bactericidal efficacy of these *de novo* peptides is driven by rapid membrane permeabilization, often occurring within minutes of exposure. Confocal microscopy and scanning electron microscopy analyses of the Long Short-Term Memory-designed peptide NN2_0050 revealed massive cytoplasmic leakage and a flaccid, ridged morphology in *E. coli*, confirming a lytic mechanism of action [49]. Similarly, the engineered peptide PAX E35 induced irreversible membrane permeabilization in colistin-resistant *P. aeruginosa* (PA239) in under 10 minutes, as evidenced by propidium iodide uptake [51].

Molecular dynamics simulations provide an atomistic resolution of this interaction. It was observed that the lead peptide 8b undergoes a structural transition from a random coil in aqueous solution to a stable amphipathic α-helix upon contact with the lipid bilayer. The simulations indicated that hydrophobic side chains (Leu/Ile) drive deep insertion into the anionic bacterial membrane, whereas interactions with zwitterionic mammalian lipids remain superficial, explaining the selectivity [50]. These findings are consistent with recent machine learning-assisted AMP discovery studies, which have identified charge and hydrophobicity as the principal determinants of antimicrobial activity. By combining XGBoost-based prediction with physicochemical feature analysis, Bhangu and colleagues achieved high classification accuracy and successfully identified experimentally active novel peptides, demonstrating the utility of ML for accelerating AMP hit discovery while emphasizing the continued necessity of experimental validation to confirm biological activity [56]. Conversely, the peptide PEP-36, designed via a recurrent neural network, showed no *in vitro* activity due to helical unraveling at the membrane surface, yet achieved the highest survival rate (66.7%) in a murine sepsis model, suggesting an alternative *in vivo* mechanism potentially linked to immunomodulation [2].

This observation highlights a broader limitation of AMP discovery pipelines: conventional in vitro screening may overlook peptides whose primary activity depends on immunomodulatory or intracellular mechanisms rather than direct membrane disruption. Accordingly, future machine learning frameworks should incorporate predictions of host-directed and immunomodulatory activity alongside antimicrobial potency to better capture clinically relevant AMP candidates [53].

A major recent advance in the field has been the application of large-scale metagenomics and machine learning to mine the global microbiome for novel AMP sequences. Using this approach, Santos-Júnior and colleagues developed the AMPSphere resource, identifying more than 860,000 nonredundant candidate AMPs from environmental and host-associated microbiomes, most of which have no counterpart in existing databases [57]. Experimental validation confirmed antimicrobial activity for the majority of tested candidates, many of which were active against drug-resistant pathogens through membrane-disruptive mechanisms while sparing commensal bacteria. By dramatically expanding the pool of discoverable AMP sequences, AMPSphere represents a significant shift in the scale and efficiency of AMP hit identification and provides a valuable foundation for future antimicrobial development [57]. The clinical viability of *de novo* peptides is defined by the Therapeutic Index (TI), which is calculated as the ratio of the hemolytic concentration (HC50) to the antimicrobial potency (MIC). It was demonstrated that while maximizing cationicity increases potency, it often compromises safety; the Lysine-clustered analogue 6K-1426 exhibited drastic mammalian cytotoxicity (IC50 = 1.178 μM in HaCaT cells). In contrast, the rationally designed QUB-1426 achieved a perfect amphipathic balance, yielding a TI of 30.76 and 100% survival in an *in vivo Galleria mellonella* infection model [52]. Similarly, the D-amino acid substituted peptide H-10 demonstrated a TI of 30.33 and a favorable pharmacokinetic profile, reducing bacterial load by >3 log units in murine organs following intravenous administration [11]. Importantly, therapeutic indices exceeding 10 are generally regarded as a key benchmark for systemic development. The emergence of multiple independent AMP discovery strategies, including rational design, machine learning-guided optimization, D-amino acid engineering, and metagenomic mining, that consistently achieve therapeutic indices in the 10–30 range, suggests that the field is nearing the requirements for clinical translation. At present, the principal barriers are less related to antimicrobial potency or selectivity and more to pharmacokinetics, manufacturing scalability, and regulatory development pathways, emphasizing the need to complement sequence optimization with advances in formulation and translational development [53,56].

4.2 Peptidomimetics & Backbone Modifications

The clinical translation of natural AMPs is severely hampered by their rapid degradation by both bacterial proteases and host serum enzymes, resulting in poor pharmacokinetic profiles that preclude systemic administration. Furthermore, MDR pathogens frequently evolve surface modifications, such as the aminoarabinose (L-Ara4N) modification of Lipid A in *P. aeruginosa*, to electrostatically repel cationic peptides [58]. To overcome these barriers, peptidomimetics employ non-natural backbones that evade enzymatic recognition while retaining the specific amphipathic topology required for membrane disruption. By shifting the side chain from the α-carbon to the amide nitrogen, N-substituted glycines (peptoids) render the oligomer backbone invisible to proteases while maintaining the spatial arrangement of pharmacophores [59]. Peptoids achieve intrinsic, chemotype-independent protease resistance through their N-substituted glycine backbone, which is not recognized by endogenous proteases, unlike residue-specific D-amino acid substitution strategies [60].

Hybridization of these protease-resistant scaffolds with natural amino acids optimizes selectivity; notably, the alternating α-amino acid/α-peptoid hybrid 4 exhibited potent activity against NDM-1-producing *E. coli* (MIC = 2 μM) while maintaining a favorable therapeutic index (TI > 21) against HeLa cells, a profile superior to the significant cytotoxicity observed in homologous all-β3-amino acid oligomers (IC50 ≈ 31 μM) [59]. Extending peptoid applications to previously neglected pathogens, a 2024 study demonstrated potent activity against MDR ocular pathogens, including XDR *P. aeruginosa* resistant to all clinically available antibiotics, including polymyxins. Selected peptoids retained acceptable mammalian cell selectivity, highlighting their potential for topical ophthalmic therapy, where proteolytic stability and reduced concerns regarding systemic toxicity are particularly advantageous [61].

To circumvent the limitations of purely electrostatic targeting, a phosphate-recognition strategy was implemented by conjugating

antimicrobial peptoids with zinc-dipicolylamine (ZnDPA) and bivalent Zn2BPMP motifs. This design facilitates specific coordination with anionic bacterial surface components, such as lipopolysaccharides and lipoteichoic acids, thereby enhancing membrane selectivity independent of cationic charge density. The resulting conjugate, 8_Zn4, exhibited potent activity against Colistin-resistant *E. coli* (MIC = 6.3 μM) and Carbapenem-resistant *A. baumannii* (MIC = 1.6 μM). The Zn-mediated recognition mechanism conferred a superior safety profile, effectively abolishing hemolytic activity (0% at 100 μM) and yielding a selectivity index exceeding 32. In a murine sepsis model, the mono-BPMP analog 5_Zn2 (0.5 mg/kg) not only achieved 50% survival at 48 hours but also suppressed the cytokine storm (TNF-α/IL-6) associated with Gram-negative sepsis, validating the therapeutic potential of metal-coordinated peptidomimetics [62]. The dual antimicrobial and anti-inflammatory activity of 5_Zn2 is clinically significant because the morbidity and mortality of Gram-negative sepsis are driven largely by dysregulated host inflammation rather than bacterial burden alone. Consequently, an agent that simultaneously reduces bacterial load and mitigates immunopathology may offer advantages over conventional combinations of separate antimicrobial and anti-inflammatory therapies [62].

Stereochemical inversion and backbone modification constitute pivotal strategies for fortifying peptide stability while preserving the membrane-disruptive pharmacophore. A leucine-centric design was utilized to engineer H-10, a derivative containing a site-specific D-Ile substitution. This stereochemical modification conferred exceptional proteolytic stability, with a half-life (t1/2) exceeding 6 hours against chymotrypsin, compared to the rapid degradation of the L-isomer parent. H-10 displayed rapid bactericidal action against MDR *S. aureus* (MIC = 1.56 μM) and demonstrated a low propensity for resistance development; MIC values remained unchanged after 15 passages, whereas resistance to polymyxin B and rifampicin emerged rapidly under identical conditions [11]. Similarly, a "bond switch" strategy was introduced, replacing a standard amide bond with an isopeptide linkage at Lysine-9. This modification disrupted the α-helical structure locally, preventing the deep membrane insertion into zwitterionic mammalian bilayers that drives toxicity. The resulting analog, Amp1EP9, retained potent activity against *P. aeruginosa* (MIC = 3.12 μM) but exhibited 0% hemolysis at 80 μM and remained stable in human plasma for over 2 hours [63]. Complementing site-specific modifications, a 2024 study applied all-hydrocarbon stapling to the HIV-derived Tat peptide to generate membrane-active stapled antimicrobial peptides with broad-spectrum antibacterial activity. Stapling stabilized the helical conformation, enhanced membrane insertion, and markedly suppressed resistance development, with no resistance detected after 30 serial passages in *E. coli*, *K. pneumoniae*, or *A. baumannii*, while preserving low hemolytic activity [64]. Similar findings across multiple stapled peptide series suggest that helically pre-organized scaffolds disrupt bacterial membranes more uniformly and irreversibly than flexible linear analogues, thereby reducing the likelihood that adaptive mutations can restore membrane integrity and confer resistance [65].

The translational chasm between *in vitro* potency and *in vivo* efficacy remains a formidable barrier in peptidomimetic development, often precipitated by discordant serum stability and tissue kinetics. Compound 5x was developed as a polymyxin analogue, in which the diaminobutyric acid (Dab-3) residue was replaced with diaminopropionic acid (Dap-3) and a polar N-phenyl pyridone side chain. While this modification successfully eliminated *in vitro* renal cytotoxicity (hRPTEC TC50 > 100 μM compared to 22 μM for Polymyxin B), the compound failed to demonstrate superior efficacy in a murine neutropenic thigh model, highlighting that reductions in nephrotoxicity must be balanced against tissue accumulation and bactericidal kinetics [66]. Conversely, the polymyxin scaffold was successfully optimized by incorporating a hydrophobic octylglycine motif at position R7 (FADDI-002). This lipophilic modification circumvented the electrostatic repulsion conferred by lipid A L-Ara4N modifications, retaining potency against colistin-resistant *P. aeruginosa* (MIC 2–8 mg/L) and significantly attenuating bacterial burden in a lung infection model [58]. The most clinically advanced next-generation polymyxin analogue is SPR206 (EVER206), which was designed to retain antibacterial activity while reducing the nephrotoxicity associated with the fatty acyl tail of native polymyxins. In 2025, Outeda-García and colleagues reported potent activity against both colistin-susceptible and colistin-resistant *A. baumannii* clinical isolates, with MIC_{50}/MIC_{90} values of 0.12/0.25 mg/L, and observed resistance in only 1 of 118 isolates [67]. Notably, *pmrCAB* mutations that mediate colistin resistance did not affect SPR206 susceptibility, indicating evasion of a major resistance mechanism and underscoring its potential against emerging colistin-resistant strains [67]. Currently in Phase 2 clinical development for serious Gram-negative infections, SPR206 represents the most advanced example of rational optimization of the polymyxin scaffold.

Beyond peptide backbone modification, translating amphiphilic topologies into non-peptidic small-molecule scaffolds and high-molecular-weight polymers offers a strategy to circumvent the scalability and stability limitations inherent to traditional AMPs. Compound 27, an amphiphilic derivative of the cystic fibrosis drug ivacaftor, was synthesized to mimic the cationic/hydrophobic balance of AMPs. This compound demonstrated broad-spectrum activity (MIC = 0.195–3.125 μg/mL) and inhibited *S. aureus* biofilm formation (99.6% inhibition) [68]. The development of Compound 27 illustrates an attractive scaffold-repurposing strategy, as its parent molecule, ivacaftor, is already clinically approved and supported by extensive human safety data. Consequently, these amphiphilic derivatives may benefit from a more streamlined translational and regulatory pathway than entirely novel antimicrobial scaffolds [68]. More broadly, scaffold repurposing represents a complementary strategy to siderophore conjugation and AMP-mimetic polymers for overcoming the manufacturing costs and regulatory barriers that have historically limited AMP development [68].

In the polymeric domain, the guanidinium-functionalized acrylamide copolymer g-D50 demonstrated that environmental context dictates efficacy; contrary to the activity loss often observed with canonical peptides in complex media, g-D50 displayed a 4-fold increase in potency against *S. aureus* (MIC: 4.3 μM) in synthetic wound fluid relative to standard broth [69]. This counterintuitive enhancement of activity in wound fluid, likely driven by conformational changes induced by the ionic and protein-rich environment, highlights a key advantage of AMP-mimetic polymers over linear peptides. Unlike conventional AMPs, whose activity is often diminished under physiological conditions, polymeric architectures can be engineered to exploit these environments and maintain or even enhance membrane-disruptive activity, effectively transforming a major pharmacological constraint into a design opportunity [69].

5. Metabolic and Respiratory Inhibitors

The inhibition of oxidative phosphorylation and essential folate metabolism represents a strategic pivot from traditional replication-dependent targets, offering a mechanism to eradicate both metabolically active and non-replicating persister cells responsible for chronic infection relapse [10]. While the folate biosynthetic pathway remains a cornerstone of bacteriostatic therapy, the respiratory chain, specifically the F-ATP synthase complex, provides a bactericidal avenue by collapsing the proton motive force and depleting the intracellular ATP pool required for bacterial viability [10]. Clinical validation of bioenergetic targeting was established by bedaquiline (BDQ), the first-in-class diarylquinoline approved for

MDR tuberculosis. BDQ inhibits mycobacterial F_1F_o-ATP synthase through interactions with the c-subunit rotor ring and ε-subunit, blocking ATP production irrespective of bacterial metabolic state. This mechanism accounts for its activity against both actively replicating bacilli and dormant persisters, populations that are largely refractory to conventional antibiotics [70]. The rational design of novel chemical entities within this sphere focuses on overcoming two primary barriers: the structural evolution of plasmid-encoded resistance enzymes and the intrinsic impermeability of the bacterial cell envelope.

Targeting the mycobacterial F-ATP synthase (F0F1-ATPase) yielded the N-acyl phenothiazine derivative PT6, a hybrid scaffold integrating a benzimidazolone core to optimize interfacial binding between the enzyme's A and B chains [10]. It has been revealed that PT6 engages the target via critical π-π stacking interactions with the Phe69 residue of chain B and hydrogen bonding to Glu65, a binding mode that yields a potent IC50 of 0.788 μM against the isolated enzyme. Unlike traditional antibiotics that fail against dormant populations, this direct interference with bioenergetics results in rapid intracellular ATP depletion and bactericidal activity against MDR and rifampicin-resistant *M. tuberculosis* strains, with MIC values of 17.62 μM and 8.76 μM, respectively. The requirement for this pharmacophore hybridization is evidenced by the control compound PT1; lacking the fluoro-benzyl tail, PT1 exhibited an 8-fold potency reduction, corroborating the necessity of lipophilic optimization to access the membrane-embedded rotor complex [10]. An important advance in understanding ATP synthase selectivity came from a 2024 cryo-EM study of *M. tuberculosis* and human ATP synthase complexes with BDQ and its clinical analogue TBAJ-587. The structures showed that both compounds bind multiple sites at the bacterial a-subunit/c-ring interface, where their quinolinyl and dimethylamino moieties form extensive protein contacts. Comparison with the human enzyme revealed a structurally analogous binding pocket, providing a molecular explanation for BDQ-associated mitochondrial toxicity and a high-resolution framework for designing analogues that exploit species-specific residues to enhance bacterial selectivity [71]. A recent addition to the F-ATP synthase inhibitor pipeline is PRP020, a squaramide derivative reported in 2025 that targets mycobacterial ATP synthase at a site distinct from the BDQ-binding pocket, as demonstrated by ATP-driven inverted membrane vesicle acidification assays [72]. Structural studies showed that the aniline moiety engages Phe58 of the c-subunit through π–π interactions, while the methylenedioxy group and thiazol-5-ylmethanamine moiety establish additional contacts with Asn174, Phe69, and Tyr68. Unlike earlier squaramide analogues, PRP020 exhibited improved metabolic stability and low mammalian cytotoxicity, while retaining potent activity against diverse mycobacteria, including *M. avium*, an intrinsically drug-resistant pathogen of particular concern in cystic fibrosis patients [72]. Importantly, its non-overlapping binding site raises the possibility of combination therapy with BDQ, potentially enhancing target inhibition while reducing the impact of BDQ-resistance mutations [72].

In the metabolic domain, the rapid evolution of plasmid-encoded dihydrofolate reductase variants, such as DfrA1 and DfrA5, has necessitated the development of propargyl-linked antifolates (PLAs) that can accommodate active-site mutations such as D27E and L28Q [73]. Structural analysis of the PLA UCP1228 confirms that its flexible propargyl linker accommodates steric constraints to stabilize the ternary complex, maintaining nanomolar affinity (Ki = 20.08 nM) against the resistant DfrA5 isoform, where trimethoprim affinity is abrogated [73]. However, the utility of these lipophilic scaffolds is heavily modulated by efflux pump activity. In Gram-positive *S. aureus*, replacing the hydrophobic biphenyl moiety of early PLAs with a pyridyl ring (Compound 1) successfully evaded efflux, maintaining a low Mutant Prevention Concentration of 1.25 μg/mL [74]. Conversely, in Gram-negative *E. coli*, the biphenyl-containing PLAs (UCP1223 and UCP1228) remained susceptible to the AcrAB-TolC system, evidenced by a dramatic 16-fold improvement in MIC potency (from 10 μg/mL to 0.625 μg/mL) upon deletion of the *acrB* gene [73]. To overcome efflux-mediated limitations, Anderson, Wright, and colleagues developed zwitterionic PLA analogues inspired by the favorable Gram-negative penetration of zwitterionic antibiotics such as fluoroquinolones and tetracyclines. Incorporation of partial zwitterionic character improved mycobacterial permeability and yielded compounds with MIC values ≤1 μg/mL against MDR and XDR *M. tuberculosis*, including the most potent PLA derivatives reported to date against clinical MDR-TB isolates [75]. Building on these advances, a 2025 pharmacokinetic study systematically characterized the in vivo exposure, half-life, and tissue distribution of seven PLA antibiotics in mice, providing a quantitative framework for dose optimization and further scaffold refinement toward systemic therapeutic applications [76].

The clinical viability of these metabolic and respiratory inhibitors ultimately rests on their ability to distinguish between prokaryotic targets and their mammalian homologs. The respiratory inhibitor PT6 demonstrated a superior safety profile, achieving a selectivity index of >253 for mycobacterial ATP synthase over the mammalian mitochondrial homolog, and showed no cytotoxicity against Vero cell lines at concentrations up to 200 μg/mL [10]. The structural basis of this selectivity has been elucidated by the 2024 cryo-EM studies of Guo and colleagues, which revealed that residues flanking the BDQ-binding cavity differ between bacterial and human ATP synthases, creating exploitable determinants of species selectivity. These insights provide a high-resolution framework for the rational design of next-generation F-ATP synthase inhibitors beyond BDQ and its close analogues [71]. This high selectivity underscores the potential of targeting the distinct interfacial residues of bacterial F-ATP synthase without compromising mammalian cellular respiration. Collectively, these propargyl-linked antifolates demonstrate that while high-affinity binding to mutant enzymes is achievable, future optimization must prioritize physicochemical properties that reduce substrate recognition by MDR efflux transporters to ensure intracellular accumulation [73,74]. The convergence of cryo-EM structural data, zwitterionic permeability design rules, and comparative pharmacokinetic profiling represents a shift in the metabolic inhibitor field from largely empirical SAR optimization to genuinely mechanism-informed, structure-guided drug design, mirroring the paradigm transformation that crystallographic ribosome and topoisomerase structures brought to their respective fields in earlier decades.

6. Membrane-Active Small Molecules (Non-Peptides)

The clinical translation of host defense peptides has long been stalled by high production costs and susceptibility to proteolytic degradation. To bypass these hurdles, medicinal chemistry has shifted its focus to 'peptidomimetics.' The primary objective is to generate low-molecular-weight architectures with cationic domains for electrostatic attraction to anionic bacterial surfaces, coupled with hydrophobic moieties that facilitate lipid bilayer insertion and subsequent membrane permeabilization [77].

In a seminal investigation of this strategy, the design and synthesis of a novel class of bis-cyclic imidazolidine-4-one derivatives was elucidated. Synthesized through the dimerization of γ-substituted-N-acylated-N-aminoethyl peptides, Compound 3 relies on a rigid bis-imidazolidine core, cationic primary amines, and hydrophobic decyl (C10) chains. Critical SAR analysis revealed that the length of the lipophilic tail dictated the spectrum of activity. Unlike C12 analogs, which were excluded by the Gram-negative outer membrane, the optimized C10 chains enabled Compound 3 to retain broad-spectrum potency, with MICs of 2 μg/mL against MRSA

and 5 μg/mL against *E. coli* and *K. pneumoniae*. This finding reinforces a broader principle in the field: excessive hydrophobicity not only promotes hemolysis but can paradoxically narrow the antibacterial spectrum by triggering self-aggregation or by generating molecules too bulky to traverse the Gram-negative outer membrane barrier [77]. Membrane-targeting antimicrobial agents are probably less likely to promote bacterial resistance than conventional antibiotics, which makes fine-tuning of these physicochemical parameters especially strategically important.

To confirm membrane disruption as the primary mechanism, Wang and colleagues employed complementary fluorescence and imaging assays. The membrane potential-sensitive dye DiSC3(5) revealed rapid, concentration-dependent depolarization of the E. coli cytoplasmic membrane, while Propidium Iodide uptake confirmed inner membrane permeabilization and loss of membrane integrity [77].

Transmission electron microscopy further demonstrated extensive membrane disintegration in MRSA, providing direct morphological evidence of membrane lysis. Together, these findings established membrane disruption as the principal antibacterial mechanism of Compound 3. Mechanistic insights from related membrane-active compounds have further expanded understanding of this therapeutic strategy. In 2023, Melcrová and colleagues showed that the cationic tripeptide AMC-109 acts through a two-step process involving preferential association of self-assembled cationic aggregates with anionic bacterial membranes, followed by disruption of membrane nanodomains that govern protein organization and cell wall synthesis. Unlike classical pore-forming agents, AMC-109 exerts its effects through nanodomain dissolution, illustrating the mechanistic diversity available within membrane-targeting peptidomimetic scaffolds. A review by Ganesan and colleagues similarly highlighted membrane-targeting small molecules as a promising but underexploited strategy for combating drug-resistant S. aureus [78]. A notable feature of Compound 3 was its high selectivity for bacterial over mammalian membranes. The compound exhibited negligible hemolytic activity, with a hemolysis threshold exceeding 250 μg/mL and a selectivity index greater than 125 relative to its antibacterial MIC [77]. This selectivity reflects fundamental differences in membrane composition. Bacterial membranes are enriched in negatively charged phospholipids that promote electrostatic attraction of cationic compounds, whereas mammalian erythrocyte membranes are predominantly zwitterionic and contain substantial cholesterol, which enhances membrane rigidity and limits amphipathic insertion [78]. The reported selectivity index substantially exceeds the values generally considered necessary for therapeutic development.

The resistance profile of Compound 3 further distinguishes it from conventional antibiotics. No increase in MIC was observed against MRSA after 13 serial passages, whereas norfloxacin rapidly selected for resistance [77]. Similar findings were reported by Hamad and colleagues in 2024, where the membrane-disrupting compound SIMR 2404 failed to generate resistance after 24 serial passages, while ciprofloxacin resistance emerged readily under identical conditions [79]. These observations support the view that membrane-active agents impose a substantially higher evolutionary barrier than target-specific antibiotics because resistance would require extensive remodeling of membrane composition rather than mutation of a single cellular target. Nevertheless, resistance is not impossible. Alterations in membrane lipid composition, increased carotenoid production, and enhanced lysyl-phosphatidylglycerol biosynthesis have all been associated with reduced susceptibility in experimental systems, underscoring the importance of long-term serial passage studies during preclinical development. The comprehensive summary of the novel lead compounds and their targets is provided in Supplementary Table S1.

7. Future Perspectives

Future antibacterial discovery will likely need to move beyond target identification and focus on closing three recurring gaps: permeability, efflux, and toxicity. These barriers can be addressed more concretely by integrating physicochemical filtering, early whole-cell testing, and resistance profiling into the discovery pipeline at the start, rather than after target potency is established. First, compounds should be optimized for bacterial entry and intracellular retention by deliberately tuning molecular size, polarity, ionization, and scaffold rigidity. In parallel, transporter-hijacking strategies such as siderophore conjugation or prodrug design may help improve uptake into Gram-negative bacteria, although these approaches will probably require careful balancing against efflux liability.

Second, resistance risk can be reduced by favoring mechanisms that are less vulnerable to single-step escape and by using combination therapy where appropriate. Dual-target agents, target–membrane hybrid compounds, and pairing of new inhibitors with cell wall-active antibiotics may provide more durable activity than single-mechanism compounds alone.

Third, safety concerns should be addressed earlier through selectivity profiling against human homologues and off-target enzymes, especially for chemotypes with known toxicity liabilities. For example, non-hydroxamate replacements, stereochemical refinement, and prodrug strategies may help preserve antibacterial potency while improving tolerability.

Overall, the field will probably progress most efficiently if future candidates are evaluated as complete therapeutic systems rather than as isolated enzyme inhibitors. That shift would make it more likely that promising scaffolds advance from biochemical activity to clinically useful antibacterial agents.

8. Conclusion

The reviewed strategies suggest that future antibacterial development may be most successful when target potency is paired with improved permeability, reduced efflux susceptibility, and lower toxicity. A more integrated design approach, supported by combination therapy and early resistance assessment, will likely be important for translating promising scaffolds into clinically relevant candidates.

Declarations

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