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Research paper

Design, synthesis, and integrated biological and molecular modeling studies of ciprofloxacin–amino acid derivatives as multi-target antimicrobials

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ABSTRACT

Fluoroquinolones are broad-spectrum antibacterials that exert their effects by inhibiting DNA gyrase and topoisomerase IV, enzymes crucial for bacterial DNA replication. Here, we report the synthesis of 60 novel ciprofloxacin–amino acid derivatives, designed to inhibit multiple bacterial targets by the so-called poly-pharmacology approach. Some of the synthesized compounds retained or improved the antibacterial potency against Gram-positive, Gram-negative, and mycobacterial species. Notably, some derivatives demonstrated promising activity (MIC <10 μM) against multidrug-resistant strains. The most active derivatives were selected for *in silico*, *in vitro*, and *in vivo* mechanistic studies. Molecular modeling, enzyme assay, and phenotypic analysis supported the polypharmacological mode of action. These findings indicate inhibition of DNA gyrase, topoisomerase IV and reduced off-target potential on human topoisomerase II, along with engagement of LpxC and urease as supportive targets. Interference with bacterial cell division and transient membrane disruption in Gram-positive bacteria and aberrant penicillin binding protein activity and possible effects on the outer membrane in Gram-negative bacteria may additionally contribute to the observed antibacterial activity. Compounds **10b** and **12b** showed the most potent antibacterial activity, with MIC values ranging from 0.2 to 5.47 μM against the evaluated strains. Cytotoxicity studies further revealed favorable selectivity, with SI values of 10–25. Overall, these findings underscore new ciprofloxacin derivatives as promising multi-target antimicrobial agents with potential to combat multidrug-resistant pathogens.

1. Introduction

For the past 60 years, antibiotics have been exploited to combat infectious diseases caused by bacteria. Prevalence of antibiotic resistance is escalating faster than it can be controlled, especially in bacteria that cause community-acquired infections, specifically in staphylococci, pneumococci, *Escherichia coli*, and *Klebsiella pneumoniae* [1,2]. Reports of the World Health Organization (WHO) have shown high rates of resistance in *Mycobacterium tuberculosis* against rifampicin, isoniazid, and fluoroquinolones [3]. Generally, there are four main mechanisms of bacterial antibiotic resistance: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; and (4) active drug efflux [4,

5]. Increased antimicrobial resistance highlights the need to develop novel antimicrobial agents acting on unexploited biological targets [6].

One promising approach involves interfering with bacterial cell division and cell wall biosynthesis. FtsZ, the bacterial homolog of eukaryotic tubulin, is a conserved cytoskeletal protein important for bacterial cytokinesis. It possesses the same structural and GTP-dependent polymerization traits as eukaryotic tubulin. During cytokinesis, FtsZ filaments assemble into the Z-ring at the future division site. The formed Z-ring marks the septum location, where it recruits *divisome* proteins and coordinates septal cell wall synthesis [7,8]. FtsZ filaments treadmill, polymerizing at one end and depolymerizing at the other, to drive the movement of late division proteins involved in cell wall

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synthesis such as FtsW and penicillin-binding proteins (PBPs) [9]. Due to its central role in cytokinesis, FtsZ serves as a marker for cellular stress affecting DNA replication, division, and membrane integrity.

In addition to FtsZ, the unique outer membrane of Gram-negative bacteria, decorated with lipopolysaccharides (LPS), constitutes another attractive therapeutic target. Unlike Gram-positive bacteria, Gram-negative species are characterized by the presence of LPS [10]. LPS can be divided into three major components: lipid A, core oligosaccharide, and O-antigen polysaccharide [11]. The metalloamidase UDP-3-O-[(R)-3-hydroxymyristoyl]-N-acetylglucosamine deacetylase (LpxC) catalyzes the second irreversible step of lipid A biosynthesis where a free amino group is produced by cleaving the acetyl moiety from the lipid A precursor molecule. LpxC does not exhibit sequence homology to any mammalian protein and is highly conserved across all Gram-negative bacteria. These factors, along with its crucial function in lipid A production, make LpxC a promising target for the development of antibiotics that specifically target Gram-negative bacteria [12,13]. Most LpxC inhibitors share common pharmacophoric features (Fig. 1): a hydroxamate head group, a lipophilic tail, and a central linker. Based on structural data, LpxC consists of a hydrophobic tunnel that accommodates a side chain of myristate fatty acid and a catalytic metal ion. In order to occupy the hydrophobic tunnel, the majority of LpxC inhibitors have a lipophilic tail and a metal-binding group [14].

In addition to cell division and lipid A biosynthesis, bacterial survival mechanisms can be attractive targets. To survive acidic environments, bacteria hydrolyze urea via the nickel-dependent enzyme urease, producing ammonia and carbon dioxide, whereby ammonia neutralizes the surrounding acidic environment. Urease is found in various bacteria, including *Helicobacter pylori* and *Proteus mirabilis*. Urease acts as a virulence factor, contributing to infection stones, pyelonephritis, and peptic ulcers [15,16]. Many urease inhibitors have been developed, such as hydroxamic acid derivatives [17], phosphorodiamidates [18], imidazoles, and oxadiazoles [19]. However, clinical approval has so far been impeded due to toxicity, instability, or severe side effects. Therefore, research into novel inhibitors with high efficacy and low toxicity is needed [18].

While exploring new bacterial targets is critical, modifying existing scaffolds remains a valuable approach. Fluoroquinolones (FQs) represents an excellent starting point for developing novel antibacterials. They are broad-spectrum agents active against both Gram-positive and Gram-negative bacteria. They display good oral bioavailability, low toxicity, and favorable pharmacokinetics [20]. FQs primarily target DNA gyrase in Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*, and topoisomerase IV in Gram-positive bacteria like *Staphylococcus aureus*. FQs block DNA replication by forming a non-functional ternary complex with these enzymes and DNA, ultimately leading to cell death [21]. Moreover, some fluoroquinolones, including ciprofloxacin, have been reported to exert moderate activity against *H. pylori* and also have been observed to exert urease inhibition [6].

1.1. Design rationale

Collectively, these previous findings provide a strong rationale for

the development of multifunctional antibacterial agents based on FQ scaffolds. Inspired by our previous work and molecular modeling studies [22,23], as well as reported pharmacophore features of LpxC and urease inhibitors, we designed and synthesized new ciprofloxacin amino acid derivatives. The new derivatives incorporate hydrazide, hydroxamic, thiosemicarbazide, oxadiazole, and pyrrole functionalities, as well as various *N*-piperazinyl moieties (Fig. 2). The structural modifications represent non-classical bioisosteric replacements of the carboxyl group, enabling alternative binding modes and enhanced metal coordination. Additionally, integration of amino acid linkers was intended to mimic LpxC inhibitor geometry, improving binding interactions and enzymatic affinity. As a result, our compounds were designed as multi-target antibacterials. They could simultaneously inhibit DNA gyrase and topoisomerase IV as primary targets, while also targeting LpxC, and urease enzymes (polypharmacology strategy). This broad-spectrum activity is aimed at combating multidrug-resistant bacteria, as well as *M. tuberculosis*.

2. Results and discussion

2.1. Chemistry

The synthetic methods employed for preparation of the target derivatives are outlined in Schemes 1 and 2. The structures of all derivatives were confirmed by spectroscopic methods (^1H NMR, ^{13}C NMR, MS, IR for representative compounds), and their purity (>95%) was established by elemental analysis (within $\pm 0.3\%$ of theoretical values) and analytical HPLC (Figs. S1–140, Section S4.1). A series of *N*-acyl/sulphonyl ciprofloxacin derivatives **3a–f** were synthesized in 91–99% yields, by reacting ciprofloxacin **1** with various acyl and sulphonyl halides in presence of triethylamine in tetrahydrofuran [24,25]. The identity of obtained compounds was confirmed by their reported melting points (**3a** [26], **3b** [27], **3c** [28], **3d**, **3e** [29]). Further confirmation was achieved by ^1H NMR analysis.

The synthesis of amino acid ester derivatives **6–7a–f** was accomplished in yields ranging from 64% to 85% as outlined in Scheme 1. The reaction involved the activation of *N*-substituted piperazinyl ciprofloxacin derivatives **3a–f** with ethyl chloroformate in presence of triethylamine in dichloromethane to give the mixed anhydride intermediates, which subsequently reacted with amino acid ester hydrochlorides to yield the desired compounds. The obtained ester derivatives were purified through recrystallization from ethanol. Structural assignments were confirmed by IR, ^1H NMR, and ^{13}C NMR spectroscopy (see Supporting Information).

Hydrazinolysis of amino acid ester derivatives **6–7a–f** using hydrazine hydrate in methanol gave hydrazide derivatives **8–9a–f** in 77–96% yields (Scheme 1). The final products were purified by recrystallization from acetonitrile, followed by trituration with diisopropyl ether. Spectroscopic data were consistent with the proposed structures. To prepare hydroxamic acid derivatives **10a–f**, *N*-acyl/sulphonyl ciprofloxacin amino acid ester derivatives were treated with freshly prepared hydroxylamine in methanol at room temperature, affording hydroxamic acids as potassium salts. Neutralization with acetic acid, followed by

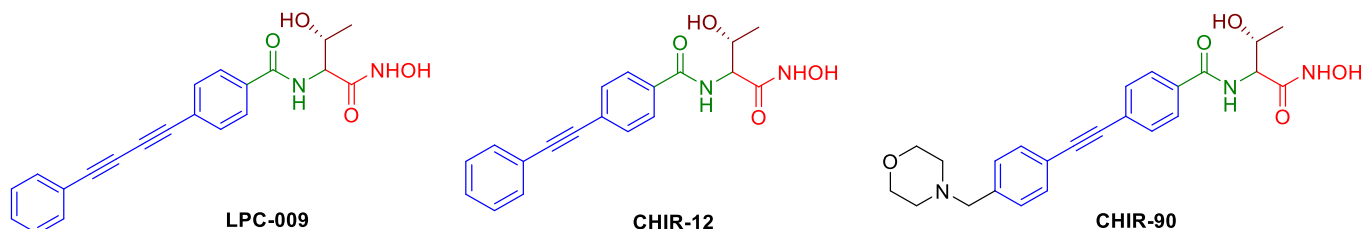


Fig. 1. Some reported LpxC enzyme inhibitors and common pharmacophoric features including a hydroxamate head group (red), a lipophilic tail (blue), and central linker (green).

Design rationale for multi-target inhibitors

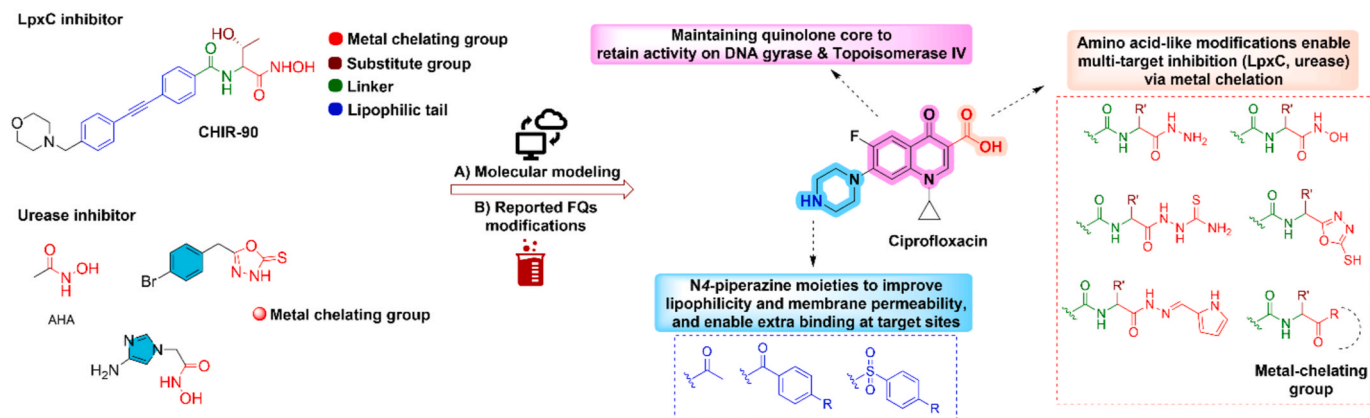
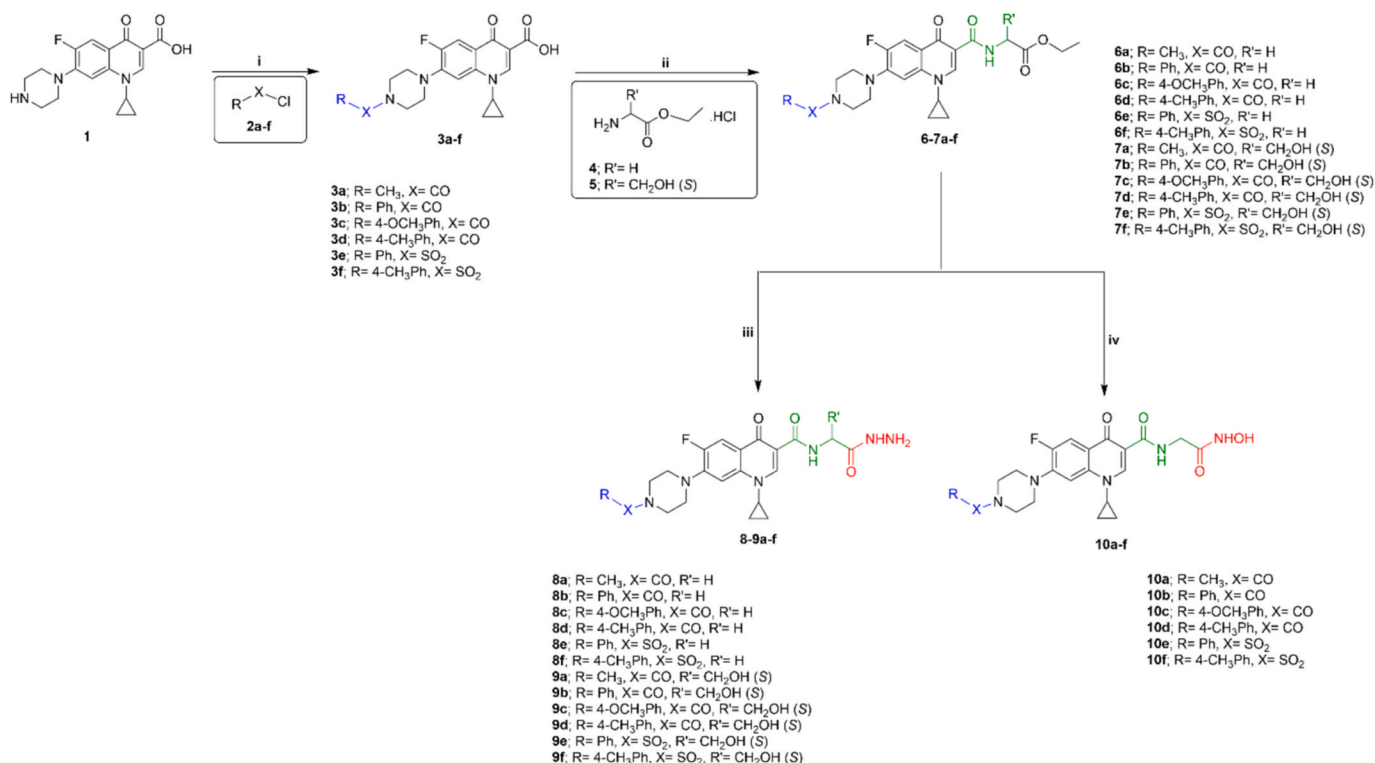


Fig. 2. Design rationale for the synthesis of multi-target ciprofloxacin derivatives.

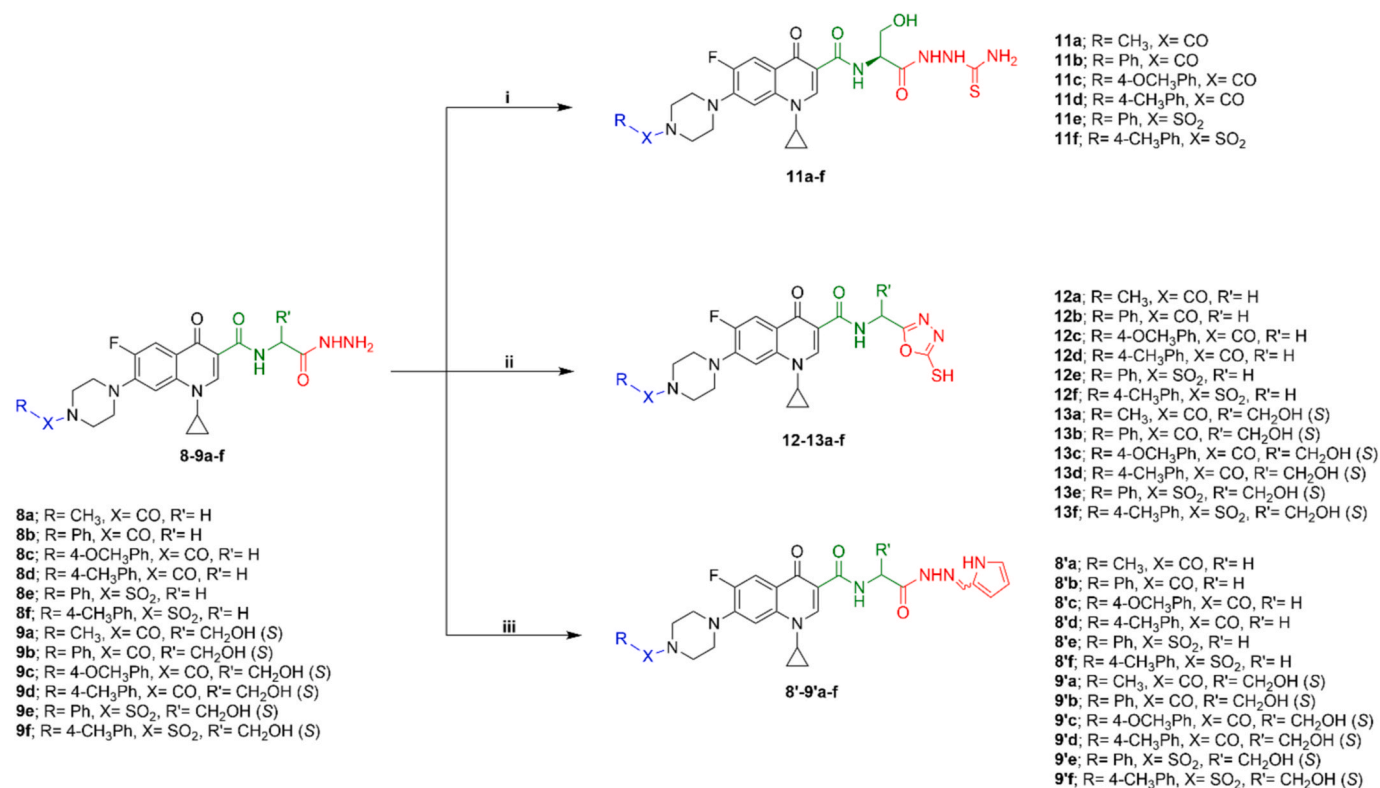
Scheme 1. Synthesis of the target derivatives 3–10a-f. Reagents and conditions: (i) Et₃N, THF, reflux, 6-8 h; (ii) Et₃N, ClCOOEt, DCM, rt, 24 h; (iii) H₂NNH₂·H₂O, MeOH, reflux, 8-12 h; (iv) 1. H₂NOH·HCl, KOH, MeOH, rt, 12-16 h, 2. CH₃COOH.

recrystallization from methanol/ethyl acetate and trituration with diethyl ether, gave the final compounds in 37–60% yields (Scheme 1). Structural characterization was established by IR, ¹H NMR, and ¹³C NMR analyses (Supporting Information).

Thiosemicarbazide derivatives (11a–f) were synthesized in 67–76% yield by treating hydrazides (9a–f) with ammonium thiocyanate in 2 M hydrochloric acid under reflux (Scheme 2). The obtained products were characterized by spectroscopic methods, which confirmed the incorporation of the thiosemicarbazide functionality. To further expand structural diversity, we next converted hydrazides 8-9a-f into oxadiazole derivatives (12–13a-f) by reaction with carbon disulfide under alkaline catalysis in methanol, followed by acidification with 10% hydrochloric acid as shown in Scheme 2. The resulting compounds were purified by recrystallization from ethanol to afford the desired oxadiazoles in yields ranging from 52% to 60%. The oxadiazoles were fully characterized by

IR, ¹H NMR, and ¹³C NMR spectroscopy (Supporting Information).

Finally, hydrazones 8'-9'a-f were synthesized by condensation of hydrazides 8-9a-f with pyrrole-2-carbaldehyde in methanol using glacial acetic acid as a catalyst (Scheme 2). The produced compounds were purified by recrystallization from ethanol, giving the desired hydrazones in 65–82% yield. NMR analysis revealed that isolated hydrazones exist as a mixture of *E/Z* geometrical isomers (ca. 3:1 in the case of glycine, ca. 1:1 in the case of serine) around the double bond (–N=CH–), as evidenced by the presence of duplicate sets of peaks for certain signals such as azomethine (N=CH), hydrazide (HNN=C), and pyrrole NH protons. The ratio of *E/Z* isomers, with the *E*-isomer being the predominant form, was estimated from integration of the azomethine (–N=CH–) and pyrrole NH signals, guided by predicted chemical shifts from density functional theory (DFT) calculations (Table S1). DFT results indicate that the *E*-isomer has lower total energy, making it more



Scheme 2. Synthesis of the target derivatives **11–13a-f** and **8'-9'a-f**. Reagents and conditions: (i) NH₄SCN, 2 M HCl in H₂O, reflux, 3 h; (ii) 1. KOH, CS₂, MeOH, reflux, 12–18 h, 2. 10% HCl; (iii) pyrrole-2-carbaldehyde, CH₃COOH, MeOH, reflux, 4–6 h.

stable and thermodynamically favored over the *Z*-isomer, consistent with previously reported data in the literature [30,31].

The representative lead compounds **8a**, **10b**, **11c**, and **12b** exhibited high chemical stability under physiological conditions (pH 7.4, 37 °C) for up to 14 days, retaining ≥97% of the parent compound as shown by HPLC (Table S2). No degradation products were detected, confirming the compounds' stability under conditions mimicking *in vivo* environments and supporting the reliability of the observed biological activities.

2.2. Biological investigation

2.2.1. In vitro antimicrobial activity

The minimal inhibitory concentrations (MICs) of the compounds were determined against *E. coli* ATCC 8739, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 10031 as Gram-negative wild type strains; *S. aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 19433 as Gram-positive wild type strains, and *H. pylori* ATCC 700392 as anaerobic strain. In addition, multidrug-resistant strains were tested, including methicillin-resistant *S. aureus* ATCC 43300 (MRSA; resistant to β-lactam antibiotics and fluoroquinolones) and *E. coli* NCTC 13476 (clinical isolate; resistant to third-generation cephalosporins and fluoroquinolones). Antimycobacterial activity was determined against *M. tuberculosis* MC26020 (attenuated strain) [32]. All assays were performed in triplicate using the broth microdilution method adapted from guidelines issued by the Clinical and Laboratory Standards Institute (CLSI) [33,34]. Ciprofloxacin (CIP), isoniazid (INH), and nitazoxanide (NTZ) were used as reference drugs (Table S3). For newly synthesized fluoroquinolone derivatives, a compound was considered a 'hit' if it exhibits an MIC ≤1–2 μM against wild-type strains and ≤5–10 μM against resistant strains, reflecting meaningful antibacterial activity suitable as starting point for further optimization.

Applying these criteria to the present study, compounds **8a**, **10b**, **10d**, **12b**, **12f**, and **13e** displayed potent antibacterial activity against *E. coli* ATCC 8739, being an average two-fold more active than

ciprofloxacin (MIC = 0.45 μM). The most promising compounds were **10b** (0.20 μM) and **12b** (0.27 μM), both below the clinical breakpoint for ciprofloxacin (0.25 mg/L, EUCAST, https://www.eucast.org/clinical_breakpoints). For *P. aeruginosa* ATCC 27853, compounds **10a**, **10d**, **12f**, and **13c** (MIC 1.12–1.67 μM) showed an average 1.5-fold lower MIC than ciprofloxacin (MIC = 2.26 μM). For *K. pneumoniae* ATCC 10031, compounds **7a**, **8d**, **9b**, **10f**, **12a**, and **13e** displayed an average 1.7-fold improvement compared to ciprofloxacin (MIC = 3.02 μM). Against *S. aureus* ATCC 6538, compounds **9e**, **11a**, **12b**, **12d** and **8'b** exhibited an average 2.4-fold lower MIC than ciprofloxacin (MIC = 2.26 μM). The most potent derivatives displayed MIC values ranging from 0.55 to 1.39 μM. Regarding *E. faecalis* ATCC 19433, compounds **7a**, **8f**, **11b**, **13a** and **13d** demonstrated approximately 1.8-fold greater potency on average than ciprofloxacin (MIC = 3.77 μM).

Compounds **9'f**, **10b**, **10d**, and **13e** retained reasonable activity against multidrug-resistant *E. coli* (MIC 3.45–6.02 μM), representing a substantial improvement over ciprofloxacin (MIC = 96.59 μM). Some derivatives, including **10c**, **11a**, and **12b**, displayed reasonable activity against MRSA, with MIC values ≤ 10 μM (ciprofloxacin, MIC = 145 μM). Notably, ciprofloxacin MIC values for the resistant strains were far above EUCAST resistance breakpoints, whereas some of the synthesized compounds showed activity in the low micromolar range. Against *H. pylori*, compounds **8d**, **10b**, **10f**, **11a**, and **13c** showed MIC values of 0.98–1.87 μM. These correspond to an average 2.5-fold improvement over ciprofloxacin (MIC = 3.02 μM) and 10.7-fold over nitazoxanide (MIC = 13.01 μM). Finally, selected compounds (**12a**, **12d**, and **13f**) showed antimycobacterial activity comparable to ciprofloxacin (MIC = 2.26 μM) and isoniazid (MIC = 1.82 μM), with an average 1.3-fold improvement. Overall, these results demonstrate that rational modifications of the ciprofloxacin scaffold can yield derivatives with enhanced potency across wild-type and resistant strains. This improvement supports their value for further optimization as broad-spectrum antimicrobial agents to overcome resistance mechanisms.

2.2.1.1. Structure-activity relationship (SAR) analysis. Systematic modification of ciprofloxacin successfully yielded new derivatives with enhanced antibacterial profiles. MIC values guided the following SAR analysis, which highlights how distinct functional groups influence activity against a panel of diverse bacterial strains (Table S3).

Compounds **3a-f** exhibited moderate activity, with **3b** and **3e** showing reasonable potency attributed to enhanced lipophilicity from benzoyl and phenylsulfonyl moieties, yet all showed low activity against multidrug-resistant strains. In contrast, compounds **3d** and **3f** exhibited lower activity despite the presence of a lipophilic methyl substituent. This observation indicated that lipophilicity alone does not govern antibacterial potency. The reduced activity of these derivatives may be due to the limited steric and electronic effects of the methyl group. Conversion of carboxylic acids to esters **6-7a-f** improved antibacterial activity compared to the parent acids, likely due to increased lipophilicity, enhancing cell membrane permeability. Potent activity for esters was observed against *E. coli*, *P. aeruginosa*, and *K. pneumoniae* (**6c**, **6e**, **7a**, **7b**). Compounds **6e** and **7b** also displayed moderate activity against resistant *E. coli*. Compound **7a** exhibited moderate activity against resistant MRSA, and **6e** was potent against *M. tuberculosis*. Serine esters **7a-f** generally showed greater potency than glycine esters **6a-f**. However, this trend was not observed consistently across all tested microorganisms, and some glycine derivatives exhibited comparable or superior activity against certain strains. This favorable activity of selected serine derivatives may be attributed to their balanced lipophilic-hydrophilic properties and the additional hydroxyl group acting as a hydrogen-bond donor/acceptor, facilitating interactions with DNA gyrase and topoisomerase IV enzymes. Molecular docking studies revealed that the hydroxyl group of the serine moiety engages in hydrogen-bonding interactions with Ser B84 in DNA gyrase and Ser B1084 and Arg A1123 in topoisomerase IV.

Hydrazide derivatives **8-9a-f** markedly enhanced antibacterial activity. The most potent compounds contained acetyl, benzoyl and phenylsulfonyl moieties, such as **8a**, **8b**, and **9e**, which were active against different strains. Serine hydrazides **9a**, **9b**, and **9e** demonstrated promising activity against multidrug-resistant strains and *M. tuberculosis*, whereas glycine derivatives were more effective against *P. aeruginosa*, *K. pneumoniae*, and *H. pylori*. The improved activity is attributed to the combination of a hydrophilic hydrazide moiety with lipophilic *N*⁴-piperazinyl substitutions, which enhance enzyme binding, and cellular uptake. Hydroxamic acid derivatives **10a-f** exhibited the most promising antibacterial activity due to the hydroxamic group's hydrogen-bonding and metal-chelating capacity, which allows multi-target inhibition. Compounds **10a**, **10b**, **10d**, and **10e** were most active against Gram-negative strains and *M. tuberculosis*. Compounds **10c** and **10f** were most effective against Gram-positive strains. Finally, compounds **10b** and **10f** showed the strongest activity against *H. pylori*, likely via urease inhibition.

Modification of serine hydrazides into thiosemicarbazides **11a-f** enhanced antibacterial activity, particularly against Gram-positive strains. Compounds **11a** and **11d** were potent against MRSA, while **11c** showed remarkable activity against Gram-negative bacteria. The thioamide moiety likely increases lipophilicity and enables stronger hydrogen-bonding and metal-chelating interactions with bacterial enzymes. Cyclization of hydrazides into oxadiazole thiols **12-13a-f** improved activity across tested strains, comparable to hydroxamic derivatives. Oxadiazoles, bioisosteres of the hydroxamic group, enhance potency through their structural versatility, and favorable pharmacokinetics. Aromatic *N*⁴-piperazinyl substituents (**12b**, **12d**, **13c**, **13e**) displayed promising activity against Gram-positive and Gram-negative bacteria. Compounds **12a** and **13f** showed the highest activity against *M. tuberculosis*, indicating that *N*⁴-acetyl and sulfonyl groups may enhance cell envelope penetration and target binding affinity. Against *H. pylori*, oxadiazoles showed enhanced activity, but slightly lower than hydroxamic derivatives.

Molecular hybridization of hydrazides with pyrrole-2-carbaldehyde

yielded hydrazones **8'-9'a-f**, which exhibited enhanced antibacterial activity. Sulfonyl-substituted derivatives **8'e** and **9'f** were potent against multidrug-resistant *E. coli*, benzoyl-substituted hydrazone **8'b** was most active against MRSA, and **9'b** was the most potent against *M. tuberculosis*. The electron-rich pyrrole ring is a privileged heterocycle that mediates hydrogen bonding and interactions with bacterial enzymes and membranes. Overall, activity trends across all derivatives revealed that potency against susceptible strains followed the general order: hydroxamic acids $\approx$ oxadiazoles > hydrazides > thiosemicarbazides > hydrazones > esters. Hydroxamic acids and oxadiazoles demonstrated the most pronounced improvements against multidrug-resistant strains (Fig. 3). It is noteworthy that some synthesized derivatives exhibited selective antibacterial activity against specific bacterial strains rather than broad-spectrum coverage.

2.2.2. Phenotypic mode of action analysis

To gain insight into the mechanisms of action behind the improved activity of the new compounds, we performed phenotypic analysis of the Gram-positive model *Bacillus subtilis* and Gram-negative *E. coli*. Inhibition of DNA gyrase and topoisomerase IV elicits a characteristic and highly specific phenotype, characterized by clear nucleoid condensation into a compact oval structure at mid-cell, typically accompanied by cell elongation due to impaired chromosome segregation, which prevents septum formation and consequently cell division [35]. This phenotype is a reliable *in vivo* marker of topoisomerase inhibition and is not observed with other DNA-targeting agents [36]. It can be visualized with bacterial cytological profiling (BCP), a phenotypic analysis method that is based on a combination of phase-contrast microscopy with diverse fluorescent dyes and protein fusions, allowing the observation of multiple phenotypes in a single assay [37,38]. By choosing different combinations of fluorescent reporters, BCP can inform on various cellular parameters and can thus be modified to suit diverse applications in antibiotic mode of action analysis [39,40].

Here, we chose the derivatives with the most potent activity against *S. aureus* and *E. coli* for phenotypic studies. To assess their effects on Gram-positive bacteria, we used a *B. subtilis* strain expressing a green-fluorescent protein (GFP) fusion to FtsZ and stained it with the membrane dye Nile Red and the DNA dye DAPI, giving insight into cell morphology, cell division, nucleoid packing, and membrane morphology (Fig. 4, summarized in Table 1). Ciprofloxacin was used as positive control for topoisomerase inhibition and the cell wall synthesis inhibitor vancomycin as control for cell envelope defects. Almost all tested compounds showed clear nucleoid packing defects indicative of topoisomerase inhibition, either highly characteristic nucleoid condensation and corresponding anucleic areas (**6e**, **7a**, **8b**, **9e**, **10b**, **10c**, **10d**, **10f**, **11a**, **11c**, **12a**, **12b**, **12f**, **13c**, **13e**) or nucleoid relaxation and fragmentation, which can also be observed with fluoroquinolones (**8'e**, **9a**, **9'f**) [35]. Only compounds **8a**, **8'b**, and **10a** showed mostly regular nucleoid distribution, suggesting that topoisomerases are not their primary target in *B. subtilis*, and only compounds **8'b** and **10a** showed no marked effects on the localization of FtsZ. FtsZ localization is affected indirectly as a downstream consequence of nucleoid packing defects on the nucleoid occlusion and Min systems, which prevent formation of Z-rings over the unsegregated nucleoid and at the cell poles, respectively [41]. Consequently, cells treated with fluoroquinolones can display double Z-rings, often formed close to cell poles or over unsegregated nucleoids, while excessive multi-rings are typically observed with compounds directly interfering with Z-ring polymerization and depolymerization dynamics [22]. Most derivatives tested in this study showed characteristic double rings, polar rings, and rings formed over unsegregated nucleoids, likely due to disrupted coordination of nucleoid organization and cell division. Compound **12f** additionally showed cells with diffuse cytosolic GFP signal, a phenotype that can occur upon cell membrane disturbance such as depolarization [42]. In line with this observation, it also elicited a spotty membrane stain. Several other derivatives showed aberrant membrane stains as well, yet rather forming

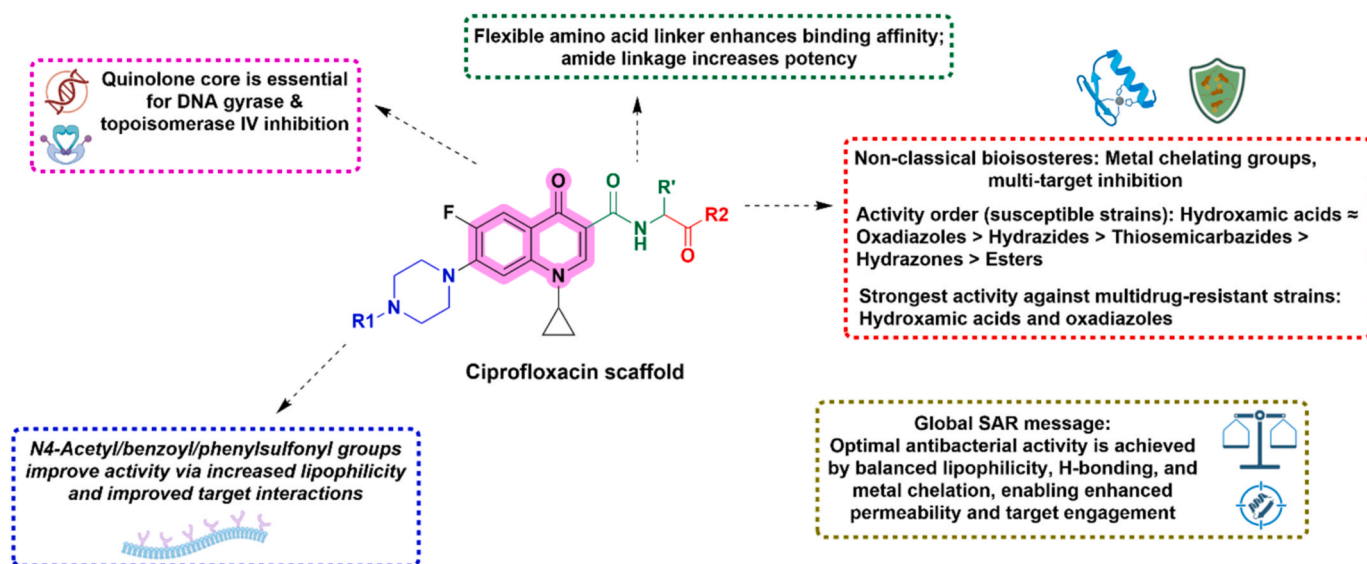


Fig. 3. Structure–activity relationship (SAR) summary of ciprofloxacin derivatives.

larger foci than small spots. This phenotype can occur with both fluoroquinolones and cell envelope-targeting compounds (Fig. 4) and can thus be due to either mechanism [36,39].

To confirm these observations, we followed up on DNA, cell wall, and membrane effects using further bacterial live-cell assays. To this end, we first employed a GFP fusion to RecA (Fig. 5). When topoisomerases are inhibited, the inability of cells to relieve tension in the chromosomal structure results in DNA strand breaks, exposing single-stranded DNA regions due to end resection. RecA is associated with the nucleoid and upon detecting such single-stranded regions, accumulates and forms filaments that mediate homologous DNA repair and induce the SOS response through triggering LexA autocleavage [43]. Fluoroquinolones are strong inducers of RecA foci and filament formation (Fig. 5). Compounds 6e, 8b, 8'e, 9'f, 10a, 10b, 10c, 10d, 10f, 12a, 12b, 12f, 13c, and 13e showed clear RecA foci, confirming an active DNA damage response. It should be noted that RecA accumulation depends on a somewhat intact chromosome structure and cannot be observed upon extensive DNA fragmentation [44], which is a possible explanation for some derivatives with a clear topoisomerase inhibition phenotype not eliciting an active RecA response. Further, RecA activation kinetics can differ between compounds and it is possible that for some compounds the ideal timepoint for observing nucleoid compaction defects (60 min) does not correlate with peak RecA activation [45].

Since we observed membrane foci or spots for compounds 10c, 10d, 10f, 11a, 11c, 12a, 12b, 12f, 13c, and 13e, which can be due to either cell wall synthesis inhibition or membrane depolarization, we tested for effects on cell wall integrity and membrane potential [39]. Peptidoglycan integrity was assessed in *B. subtilis* 168CA using a well-established acetic acid/methanol fixation protocol followed by phase-contrast microscopy [46,47]. In this assay, fixation promotes extrusion of the protoplast through defects in the peptidoglycan layer, appearing as characteristic 'bubbles' on the cell surface in phase contrast microscopy (Fig. 6) [46]. However, none of the tested compounds affected peptidoglycan integrity in these experiments.

We then assessed membrane depolarization with the membrane potentiometric probe 3,3'-dipropylthiadicarbocyanine iodide (DiSC(3) 5) (Fig. S141A). The membrane channel-forming peptide gramicidin served as positive control. All tested derivatives showed a transient, partial depolarization, with the membrane potential approaching untreated control levels after ca. 20 min. This suggests that the compounds transiently interrupt membrane integrity, speculatively during membrane passage into the cytosol, but do not specifically target the cell membrane.

Since fluoroquinolones bind both gyrase and topoisomerase IV, yet with opposite affinity in Gram-positive and Gram-negative bacteria [48], and cell envelope structures differ between these bacteria, we also performed phenotypic mode of action assays in *E. coli*. To this end, we used the wild-type strain W3110 and stained it with DAPI and the membrane dye FM4-64, which, in contrast to Nile red, binds to the outer membrane of Gram-negative bacteria. This BCP assay revealed clear, characteristic topoisomerase inhibition phenotypes for all tested derivatives (Fig. 7, summarized in Table 2). Thereby, it was an interesting observation that compounds belonging to series 6-8 tended to have nucleoid-relaxing and fragmenting effects, while compounds belonging to series 9-13 showed clear compaction phenotypes. Additionally, compounds 9a, 9e, 10d, 12a, 12b, 12f, 13c, and 13e showed reduced FM4-64 staining, which resembled the effects observed with cell envelope-targeting comparator compounds included in the assay (Fig. S141B). The most striking observation, however, was that all compounds elicited distinct cell shape defects culminating in aberrant cell branching in some cells. A similar phenotype has been previously observed for *E. coli* mutants defective in penicillin-binding protein 5 (PBP5/DacA). Interestingly, this branching was traced back to originate from areas of inert peptidoglycan that typically occur at the cell poles, where no new cell wall material is incorporated. Yet, in the $\Delta dacA$ mutant, patches of inert peptidoglycan also occurred in the cell periphery and regions of inert peptidoglycan at its cell poles were observed to be split. Branching occurred at these erroneous patches and split poles and was independent of cell division [49]. This observation suggests that the new compounds may impair the function of PBPs, in particular PBP5. Future studies will have to assess whether this is achieved by a direct interaction or an indirect effect, yet the consistency of the branching phenotype across derivatives points to the latter.

The observation that several derivatives reduced the FM4-64 stain in our BCP assay could further point towards an effect on the outer membrane, as this phenotype is most strongly observed with the outer membrane-targeting lipopeptide polymyxin B and the LpxC inhibitor ACHN-972 (Fig. S141B, Table 2). To test whether the compounds compromise outer membrane integrity, we employed a mupirocin potentiation assay. Mupirocin is a small-molecule antibiotic that targets the isoleucyl-tRNA synthase enzyme. It is able to bind the *E. coli* enzyme but displays weak activity against *E. coli* cells due to its inability to cross the outer membrane. Its activity can be potentiated by sublethal concentrations of outer membrane-impairing agents [36]. However, $0.25\text{--}0.5 \times \text{MIC}$ of our derivatives did not change the activity of mupirocin against *E. coli* W3110 ($64\text{--}128 \mu\text{g/mL}$ for all compounds).

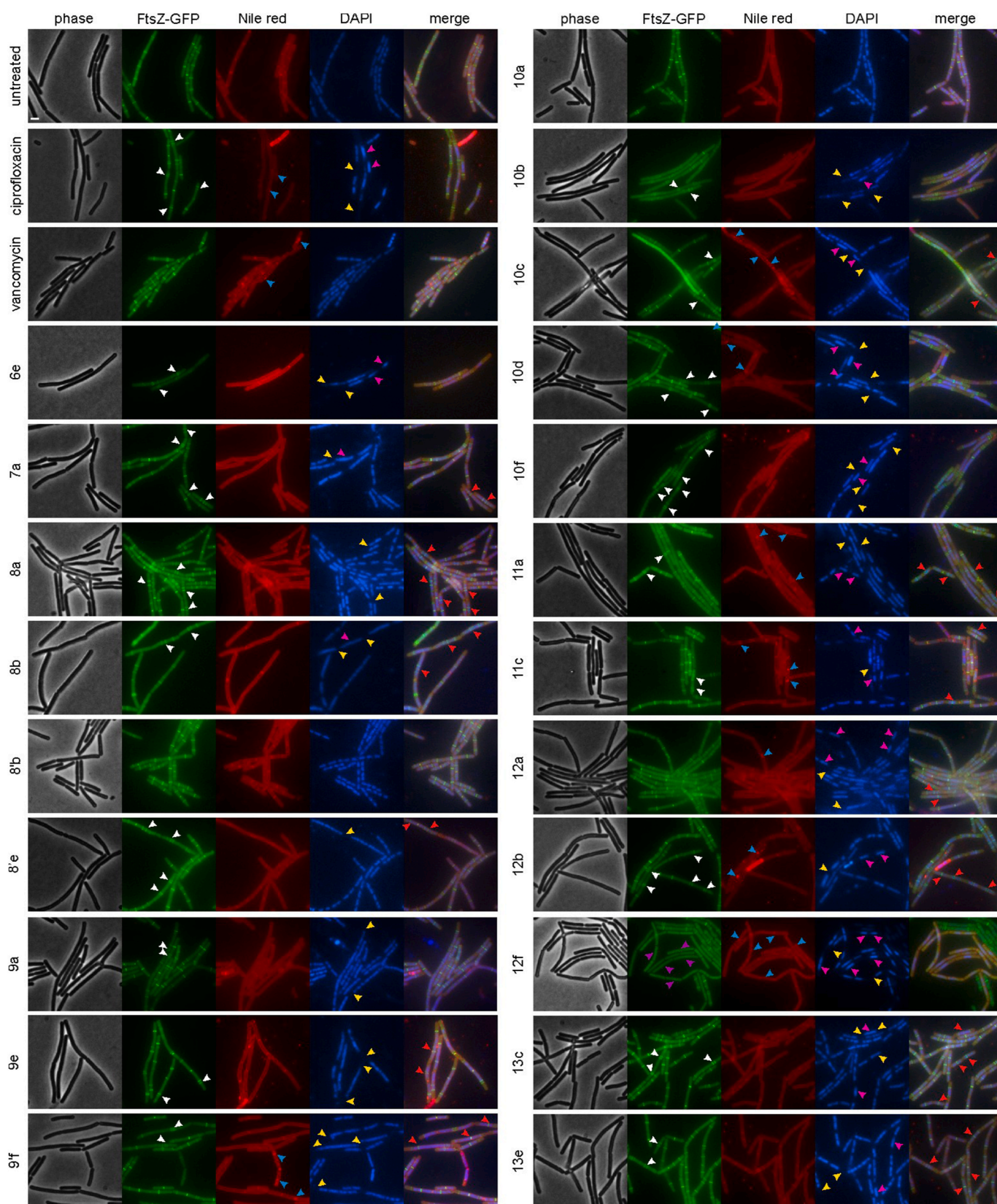


Fig. 4. Bacterial cytological profiling (BCP) of *B. subtilis* 2020 (*Pxyl-ftsZ-gfp*). FtsZ indicates cell division sites, Nile red the cell membrane, and DAPI the nucleoid. Scale bar 2 μ m. White arrows indicate multiple Z rings. Blue arrows indicate membrane foci. Yellow arrows indicate anucleic regions. Pink arrows indicate compacted oval nucleoids. Red arrows indicate Z rings forming over the nucleoid.

Table 1
Summary of phenotypic observations made for *B. subtilis* 2020 (*P_{xyl}-ftsZ-gfp*). CIP: ciprofloxacin, Van: vancomycin, x: effect observed, (x): effect (foci in membrane stain) observed but could be attributed to either cell envelope stress or topoisomerase inhibition.

compound	shape		membrane			DNA					FtsZ					targets	
	normal	elongated	smooth	foci	spotty	regular	condensed	anucleic areas	relaxed	fragmented	regular	double rings	off-center rings	rings over DNA	cytosolic	topoisomerases	cell envelope
untreated	x		x			x					x						
Cip		x		x			x	x				x	x			x	
Van	x			x		x					x						x
6e	x		x				x	x				x	x			x	
7a	x		x				x	x				x	x	x		x	
8a	x		x					x				x		x			
8b		x	x				x	x		x		x		x		x	
8'b	x		x			x					x						
8'e		x	x					x	x	x		x	x	x		x	
9a		x	x					x	x	x		x					
9e		x	x				x	x				x	x	x		x	
9'f		x		x				x	x	x		x	x	x		x	(x)
10a	x		x			x					x						
10b		x	x				x	x				x	x			x	
10c		x		x			x	x				x	x	x		x	(x)
10d		x		x			x	x				x	x			x	(x)
10f		x	x				x	x				x	x			x	
11a		x		x			x	x				x	x	x		x	(x)
11c	x			x			x	x				x	x	x		x	(x)
12a		x		x			x	x				x	x	x		x	(x)
12b		x		x			x	x				x	x	x		x	(x)
12f	x				x		x	x				x	x	x	x	x	x
13c	x			x			x	x				x	x	x		x	
13e	x			x			x	x				x	x	x		x	

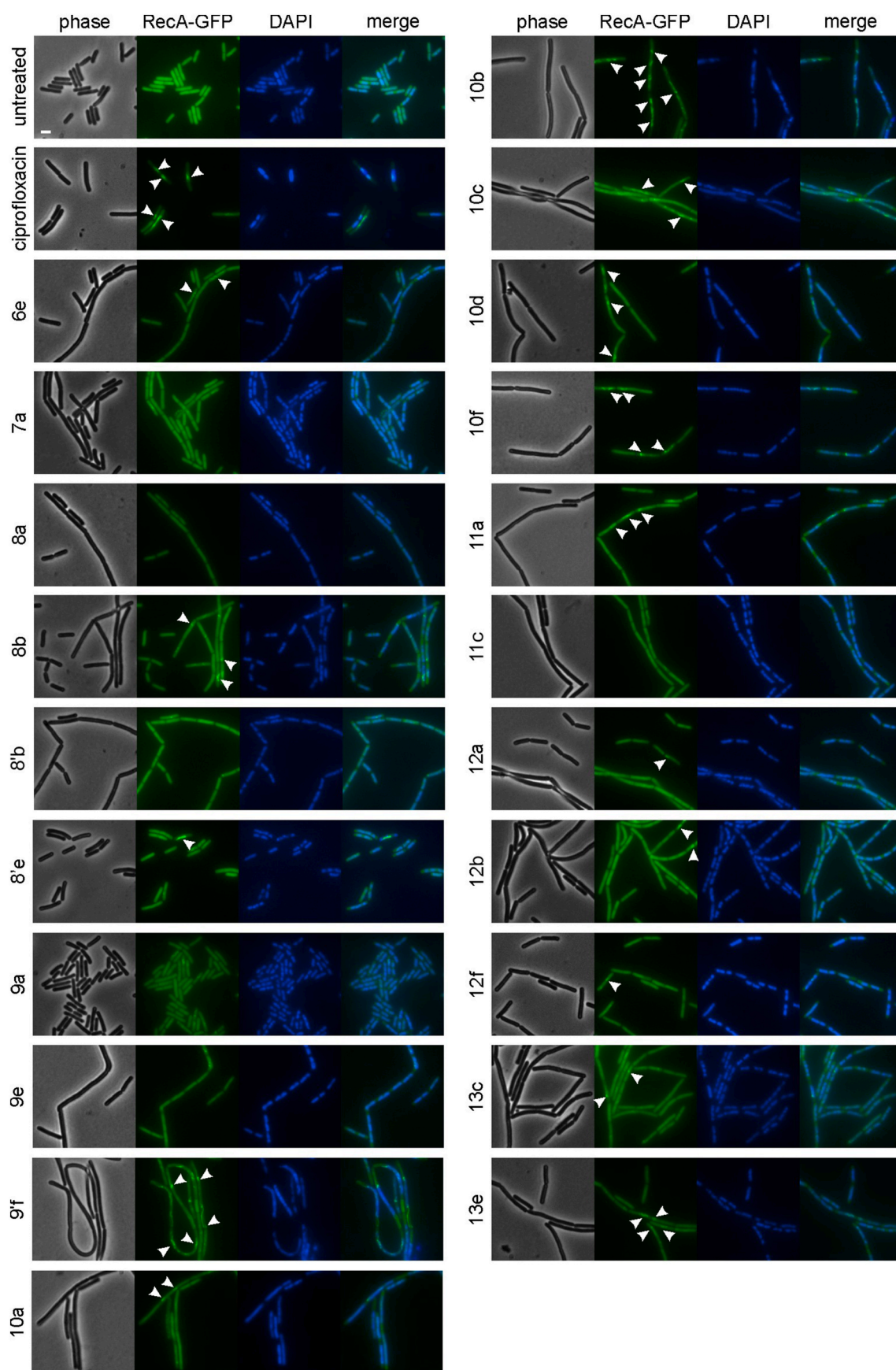


Fig. 5. Localization of RecA in *B. subtilis* UG10 (*Pxyl-recA-mgfp*). RecA is part of the SOS response to DNA damage. It patrols the nucleoid in search of damaged sites and accumulates in foci and elongated structures at single-stranded DNA regions. Scale bar 2 μm. Arrows indicate RecA foci.

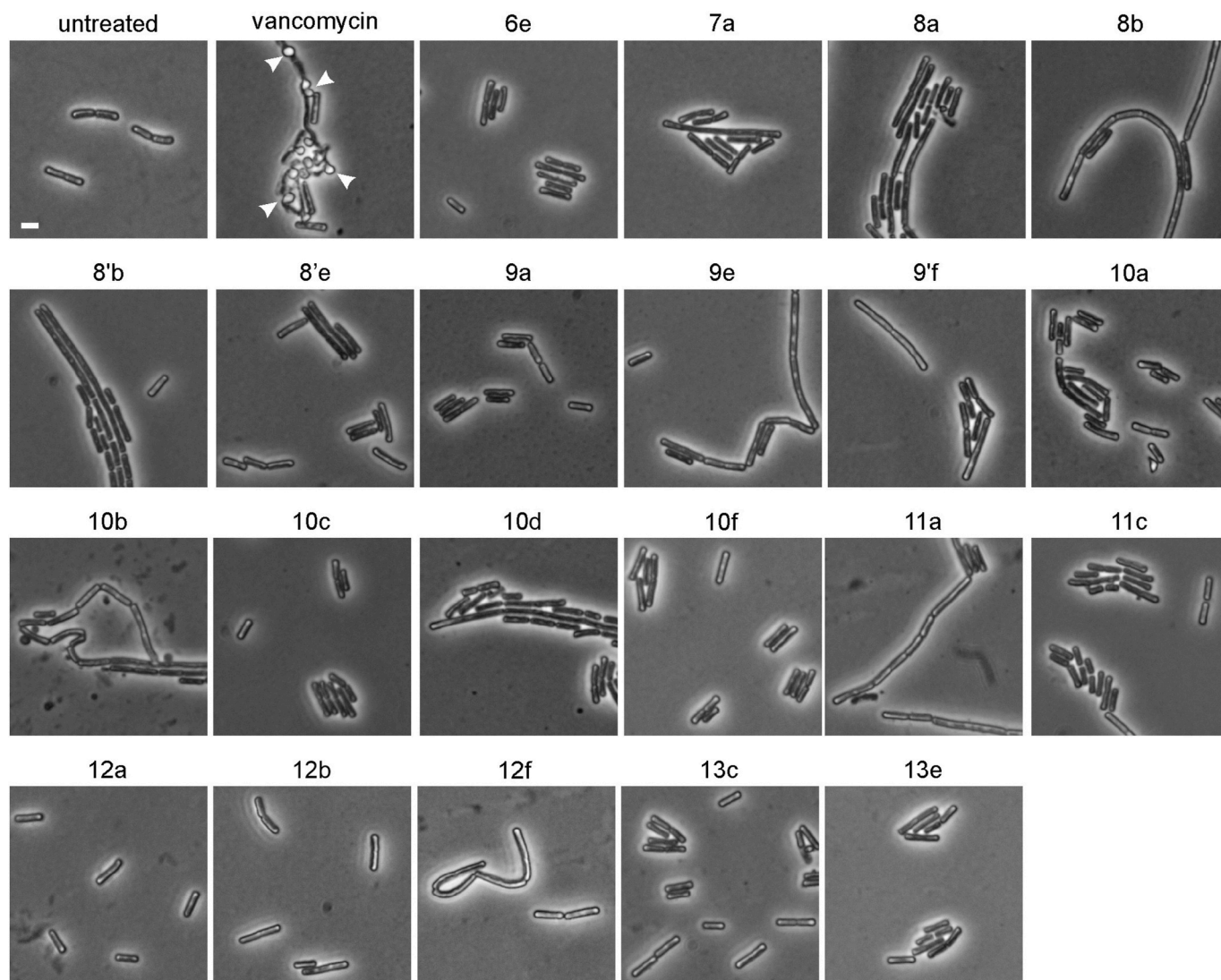


Fig. 6. Acetic acid/methanol fixation of *B. subtilis* 168CA (WT). Autolytic enzymes digest the existing peptidoglycan layer to enable incorporation of new building blocks during cell growth. When cell wall synthesis is impaired, these cell wall breaches are not filled. Fixation in acetic acid/methanol results in the protoplast protruding through holes in the peptidoglycan layer, appearing as bubbles on the cell surface (arrows). Vancomycin serves as positive control. Scale bar 2 μ m.

While this result does not exclude that the outer membrane is affected by the compounds, it is not disturbed to an extent that would allow the passage of mupirocin. To test whether LpxC is targeted *in vivo* and its inhibition plays a significant role in the activity of the compounds, we performed a recently established LpxC overexpression assay [22]. In this assay, the MICs of the test compounds are assessed against an *E. coli* strain expressing LpxC from the arabinose-inducible P_{BAD} promoter in the absence and presence of arabinose [50]. If LpxC is the primary target, MICs will increase at higher arabinose concentrations leading to overexpression of the enzyme, as observed for the positive control ACHN-975 (16-fold MIC increase) [22]. However, no arabinose-dependent change in activity was observed for any of the test compounds.

2.2.3. *In vitro* enzyme assays

The most active compounds against Gram-positive and Gram-negative bacteria were evaluated against representative bacterial targets to validate their proposed mechanism of action. First, we examined the inhibitory activity (IC_{50}) of the compounds against *E. coli* DNA gyrase and *S. aureus* topoisomerase IV by cleavage complex stabilization assay [51,52]. This method provides a direct metric of interference with the enzymes' catalytic cycle. The most active compounds against

Gram-negative strains exhibited notable inhibitory activity against DNA gyrase. They displayed IC_{50} values ranging from 0.18 to 0.90 μ M, representing a 1.1–5.7-fold improvement over ciprofloxacin ($IC_{50} = 1.03 \mu$ M). The observed high affinity for DNA gyrase, likely contributes to their enhanced antibacterial activity against Gram-negative bacteria and their ability to overcome drug resistance (Fig. 8 and Table S4).

For topoisomerase IV, the conventional target in Gram-positive bacteria, the most promising compounds showed considerable inhibitory activity, with IC_{50} values of 1.55–6.62 μ M. These IC_{50} values were 1–4.6 times more potent than ciprofloxacin ($IC_{50} = 7.13 \mu$ M). The results highlight activity against Gram-positive bacteria, including MRSA, could be justified by strong binding affinity for topoisomerase IV. Strong inhibitory activity was driven by structural features such as hydroxamic and oxadiazole thiol groups, as well as N^4 -piperazinyl moieties. These features contribute additional interactions and help fit the compounds optimally within the active sites of both enzymes. It is worth mentioning that a direct correlation between enzymatic inhibition and the *B. subtilis* phenotypic response is not expected. Any observed differences may be due to species-dependent factors such as membrane permeability, efflux activity, and sequence variations in target enzymes that affect intracellular activity. To elucidate selectivity, compounds 10b and 12b were

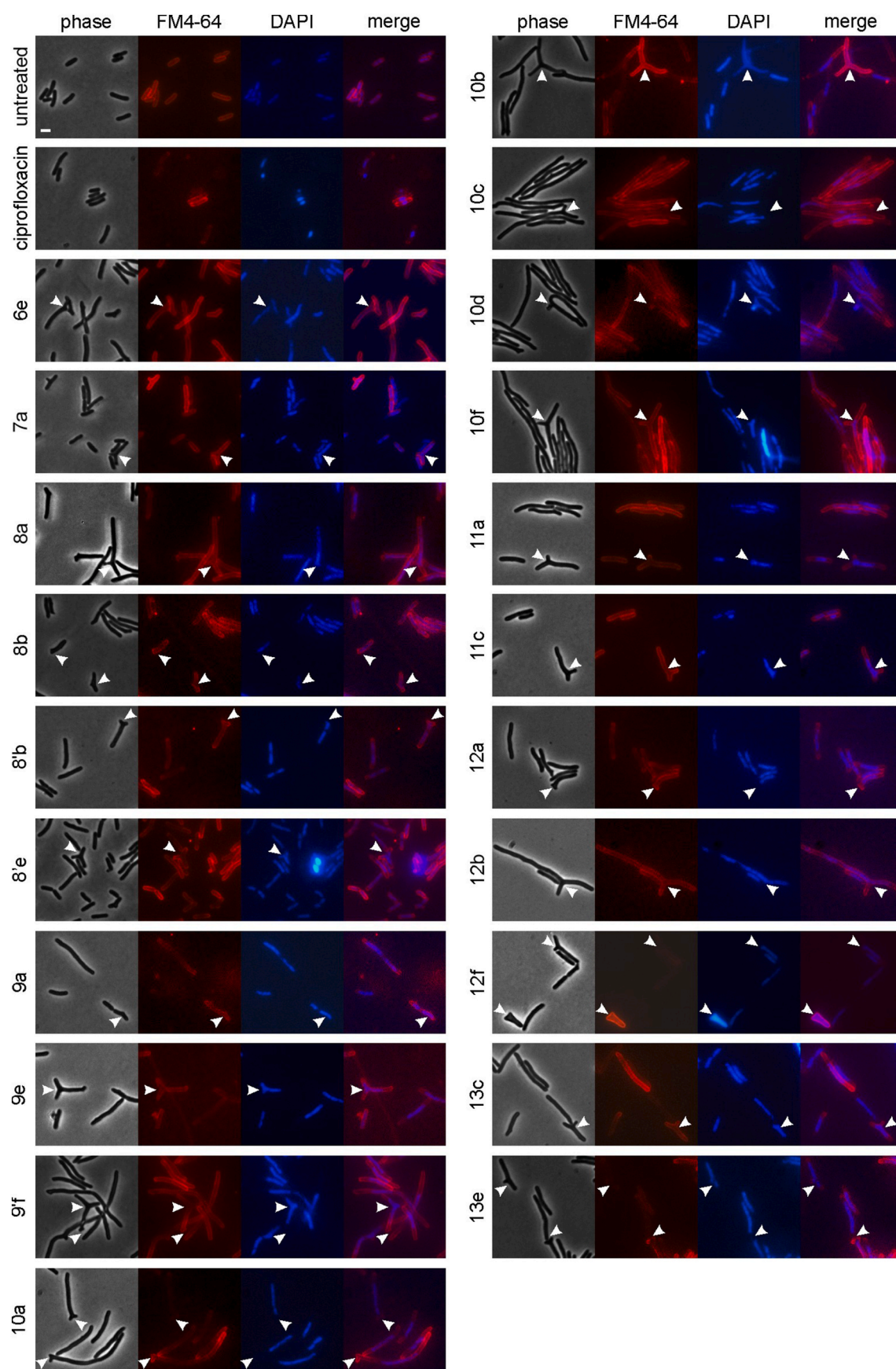
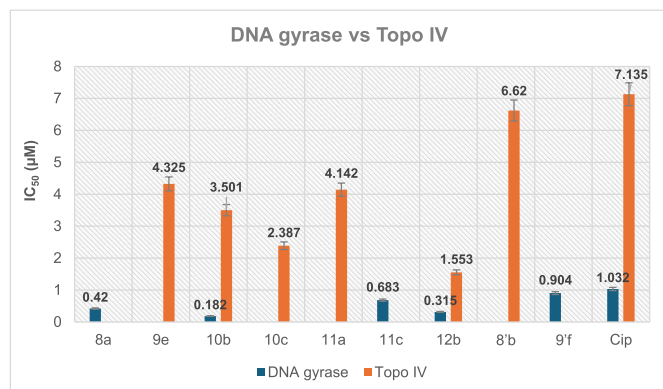


Fig. 7. Bacterial cytological profiling (BCP) of *E. coli* W3110 stained with the membrane dye FM4-64 and the DNA dye DAPI. Arrows indicate aberrant cell branching sites. Scale bar 2 μ m.

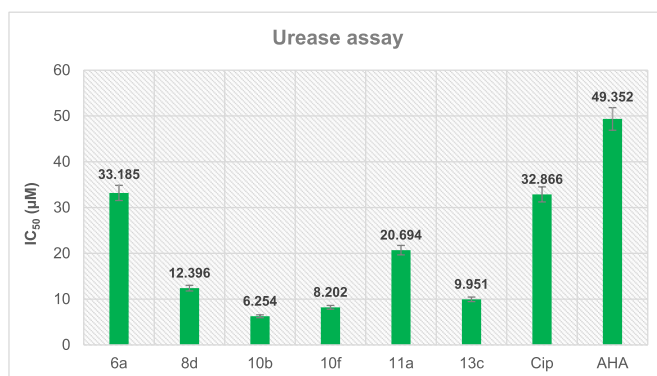
Table 2Summary of phenotypic observations made for *E. coli* W3110. CIP: ciprofloxacin, AMP: ampicillin, CTX: cefotaxime, PolB: polymyxin B, x: effect observed.

compound	shape				membrane		DNA					targets	
	normal	elongated	rounded	branched	smooth	reduced stain	regular	condensed	anucleic areas	relaxed	fragmented	gyrase	cell envelope
Untreated	x				x		x						
Cip		x				x		x	x			x	
Amp		x				x	x						x
CTX		x				x	x						x
PolB			x			x	x						x
ACHN-972			x			x	x						x
6e		x		x						x	x	x	x
7a		x		x						x	x	x	x
8a		x		x						x	x	x	x
8b		x		x						x	x	x	x
8'b		x		x			x	x		x	x	x	x
8'e		x		x			x	x		x	x	x	x
9a		x		x		x	x	x				x	x
9e		x		x		x	x	x				x	x
9'f		x		x			x	x				x	x
10a		x		x			x	x				x	x
10b		x		x			x	x				x	x
10c		x		x			x	x				x	x
10d		x		x		x	x	x				x	x
10f		x		x			x	x				x	x
11a		x		x			x	x				x	x
11c		x		x			x	x				x	x
12a		x		x		x	x	x				x	x
12b		x		x		x	x	x				x	x
12f		x		x		x	x	x				x	x
13c		x		x		x	x	x				x	x
13e		x		x		x	x	x				x	x

**Fig. 8.** IC₅₀ values (μM) of the tested compounds on *E. coli* DNA gyrase and *S. aureus* topoisomerase IV. IC₅₀ values expressed as means ± standard deviations (SD), calculated from triplicate measurements.

evaluated against human topoisomerase II (Table S5). The results demonstrated lower inhibitory activity toward human enzyme, indicating favorable selectivity on bacterial gyrase and a reduced potential for off-target effects (10b 48.05 μM; 12b 71.34 μM; etoposide 19.41 μM).

Next, the urease inhibitory activity of the most potent compounds against *H. pylori* was measured using the established indophenol method [53]. It is a sensitive approach for quantifying ammonia production via the Berthelot reaction [54]. The results were compared to those of acetohydroxamic acid (AHA), an established urease inhibitor and ciprofloxacin (Fig. 9 and Table S6). The tested compounds demonstrated remarkable potency, with IC₅₀ values ranging from 6.25 to 33.2 μM. These values reflect activities 1.6–5.3 times stronger than ciprofloxacin (IC₅₀ 32.9 μM) and 1.5–7.9 times greater than AHA (IC₅₀ 49.4 μM). The potent inhibition aligns with the metal-chelating capacity of the designed molecules, which facilitates effective binding to the

**Fig. 9.** IC₅₀ (μM) of the designed compounds against urease enzyme. IC₅₀ values expressed as means ± standard deviations (SD), calculated from triplicate measurements.

nickel-dependent urease. Hydroxamic derivatives 10b and 10f and the oxadiazole analogue 13c displayed outstanding inhibition, suggesting that these structural features are key contributors to their activity. Overall, the urease inhibition data were consistent with MIC values against *H. pylori*, strengthening the hypothesis that urease inhibition plays a supportive role in the observed antibacterial effects.

Finally, the inhibitory potential against the LpxC enzyme was assessed using a well-established fluorometric assay [55,56]. Compounds exhibiting the strongest activity against Gram-negative strains were chosen for this assay. The known LpxC inhibitors CHIR-090 and BB-78485 were employed as reference standards to validate the assay and enable comparative analysis.

The investigated compounds exhibited promising LpxC inhibition, with IC₅₀ values ranging from 0.052 to 1.18 μM. Importantly, ciprofloxacin, which does not inhibit LpxC, showed negligible activity in this assay (IC₅₀ = 17.9 μM). Some of the designed compounds displayed

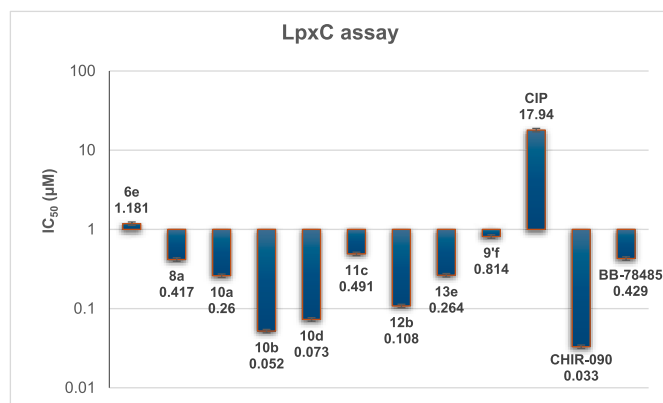


Fig. 10. IC₅₀ (μM) of the designed compounds against LpxC enzyme. IC₅₀ values (mean ± SD) were determined from three independent experiments.

greater inhibitory activity than LpxC inhibitor BB-78485 (IC₅₀ = 0.429 μM), though all remained less potent than CHIR-090 (IC₅₀ = 0.033 μM). Among the synthesized compounds, the most active inhibitors were identified as **10b** and **10d** (hydroxamic acid derivatives) and **12b** (an oxadiazole derivative), displaying IC₅₀ values of 0.052 μM,

0.073 μM, and 0.108 μM, respectively (Fig. 10 and Table S7). Interestingly, our most effective compounds, including compound **12b**, a non-hydroxamate derivative, displayed activity comparable to LpxC inhibitors reported in the literature [57,58]. These results are consistent with improved metal-chelating ability and lipophilicity playing a prominent role in the eminent activity of these compounds.

SAR analysis revealed a clear correlation between the type of zinc-binding group and the inhibitory potency. The compounds followed the general order: hydroxamic acid > oxadiazole > hydrazide > thiosemicarbazide > azomethine > ester. The presence of a hydroxamic acid moiety was associated with the highest activity, consistent with its strong ability to chelate Zn²⁺ ion within the LpxC active site. Oxadiazole derivatives exhibited considerable activity, but slightly lower than hydroxamic acids. This is likely due to less favorable metal-chelation geometry. Compounds bearing hydrazide and thiosemicarbazide groups retained moderate inhibition, suggesting partial coordination and secondary interactions. Although azomethine and ester derivatives were the least active, they were still more potent than ciprofloxacin. This observation underscored that even weakly coordinating groups can contribute to measurable LpxC inhibition when supported by favorable scaffold interactions.

Collectively, these findings revealed that the structural modifications adopted effectively enhanced LpxC inhibitory activity. Importantly, our strategy avoids the common pitfalls associated with the *de novo*

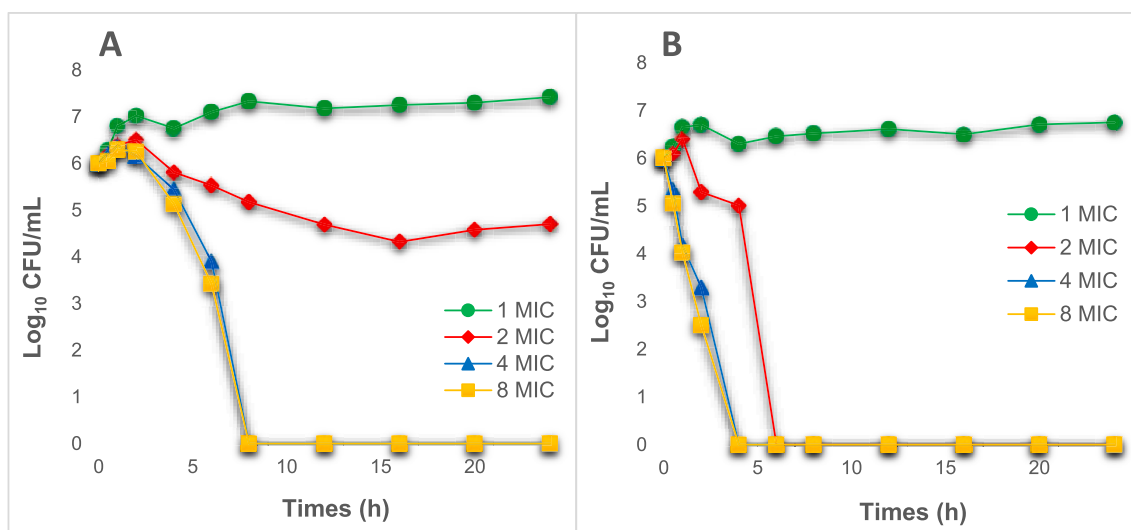


Fig. 11. Bactericidal activity of (A) CIP, and (B) **12b** against *S. aureus*.

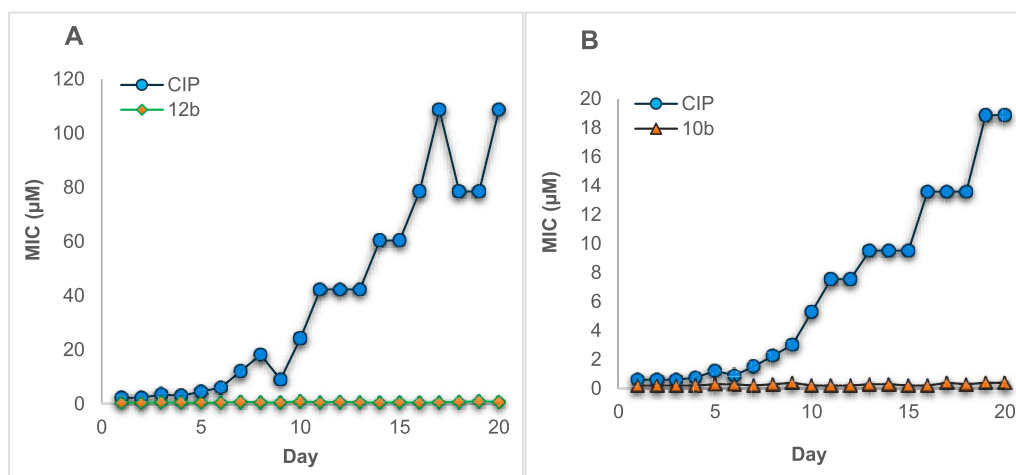


Fig. 12. Development of bacterial resistance to the tested compounds and ciprofloxacin (CIP); (A) *S. aureus*; (B) *E. coli*.

Table 3

IC₅₀ (μM) of selected compounds on SH-SY5Y and WI-38. IC₅₀ values were calculated from triplicate measurements and are expressed as means ± SD.

Compound Code	MW	Cytotoxicity IC ₅₀ (μM)		SI*
		SH-SY5Y	WI-38	
8a	444.47	52.44 ± 2.62	63.76 ± 3.21	10
10b	507.51	49.62 ± 2.41	59.35 ± 3.07	17
12b	548.59	68.15 ± 3.53	80.03 ± 4.29	25
13e	608.65	61.37 ± 2.78	75.19 ± 3.82	15
9f	663.73	57.28 ± 2.86	66.41 ± 3.54	11
Cip	331.3	60.02 ± 2.97	71.26 ± 3.48	-----
Staurosporine	466.54	17.74 ± 0.85	15.09 ± 0.63	-----

SI* (selectivity index): IC₅₀WI-38/MIC of *E. coli* NCTC 13476. Good selectivity indices: SI > 10.

development of LpxC inhibitors. It is based on the rational modification of a well-established scaffold, ciprofloxacin, which already targets DNA gyrase and topoisomerase IV. In this context, LpxC acts as a complementary target, enabling multi-target inhibition rather than reliance on a single mechanism. This multi-target approach increases the likelihood of success and broadens the therapeutic relevance of these compounds against multidrug-resistant Gram-negative bacteria.

In light of the *in vitro* results, it was counterintuitive at first that the *in vivo* LpxC overexpression assay was negative. However, since the compounds also inhibit topoisomerases and possibly PBPs in *E. coli*, it is possible that the loss of one target among several does not cause a measurable loss of activity. This would align with the low resistance development rates observed with compound **10b** (see Fig. 12) and constitute a positive feature of this new compound class.

2.2.4. Time-kill kinetics

The most active derivatives **9e**, **10c**, **11a**, **12b**, **13c**, and **8'b** against *S. aureus* ATCC 6538, were selected to evaluate whether they act as bacteriostatic or bactericidal agents by determining their minimal bactericidal concentrations (MBCs). The compounds exhibited MBC values of 0.7–3.9 μM, demonstrating higher potency than ciprofloxacin

(8.3 μM) (Table S8). Accordingly, the observed MBC/MIC ratios of approximately 1–3 indicate that these compounds are bactericidal under the tested conditions [59].

To assess the bactericidal kinetics of the new compounds, an *in vitro* time-kill assay was performed using compound **12b** as representative. Bactericidal effects were evaluated at four concentrations (1×, 2×, 4×, and 8× MIC), with ciprofloxacin (CIP) serving as a positive control. As shown in Fig. 11, both **12b** and CIP exhibited bacteriostatic activity at 1× MIC. At 2× MIC, compound **12b** achieved rapid bactericidal activity within 6 h, and maintained bactericidal activity throughout 24 h. Conversely, CIP inhibited bacterial growth at 2× MIC with delayed bactericidal activity at higher concentrations. At 4× MIC, **12b** demonstrated rapid bactericidal effects within 4 h, whereas CIP required 8 h to reach comparable activity. Notably, at 8× MIC, the bactericidal activity of **12b** increased further, whereas CIP showed no additional enhancement. A >3-log reduction in colony-forming units (CFUs) was achieved after 2 h of exposure at 8× MIC, while a 2-log reduction was observed at 4× MIC under the same conditions. No detectable viable bacteria were observed after 4 h of exposure at 4× MIC. Importantly, **12b** produced ≥6-log decrease after 24 h. These results reveal that lead compounds such as **12b** display faster bactericidal effects than ciprofloxacin at submaximal concentrations.

One interesting observation was that all concentrations of ciprofloxacin displayed a 4-h delay in killing, while **12b** exhibited a 2-h delay at 2× MIC but effected immediate killing at higher concentrations. This is mechanistically interesting since topoisomerase inhibition alone does not achieve immediate killing. Its bactericidal activity is mediated by progressively accumulating DNA damage during replication, allowing cell growth for several hours until lethal damage is incurred [60]. The observation that **12b** achieves much faster, at higher concentrations near-immediate killing, is thus further evidence for its multimodal mode of action.

2.2.5. Development of bacterial resistance

It is critical to investigate the potential emergence of bacterial resistance towards the newly designed antibacterial candidates.

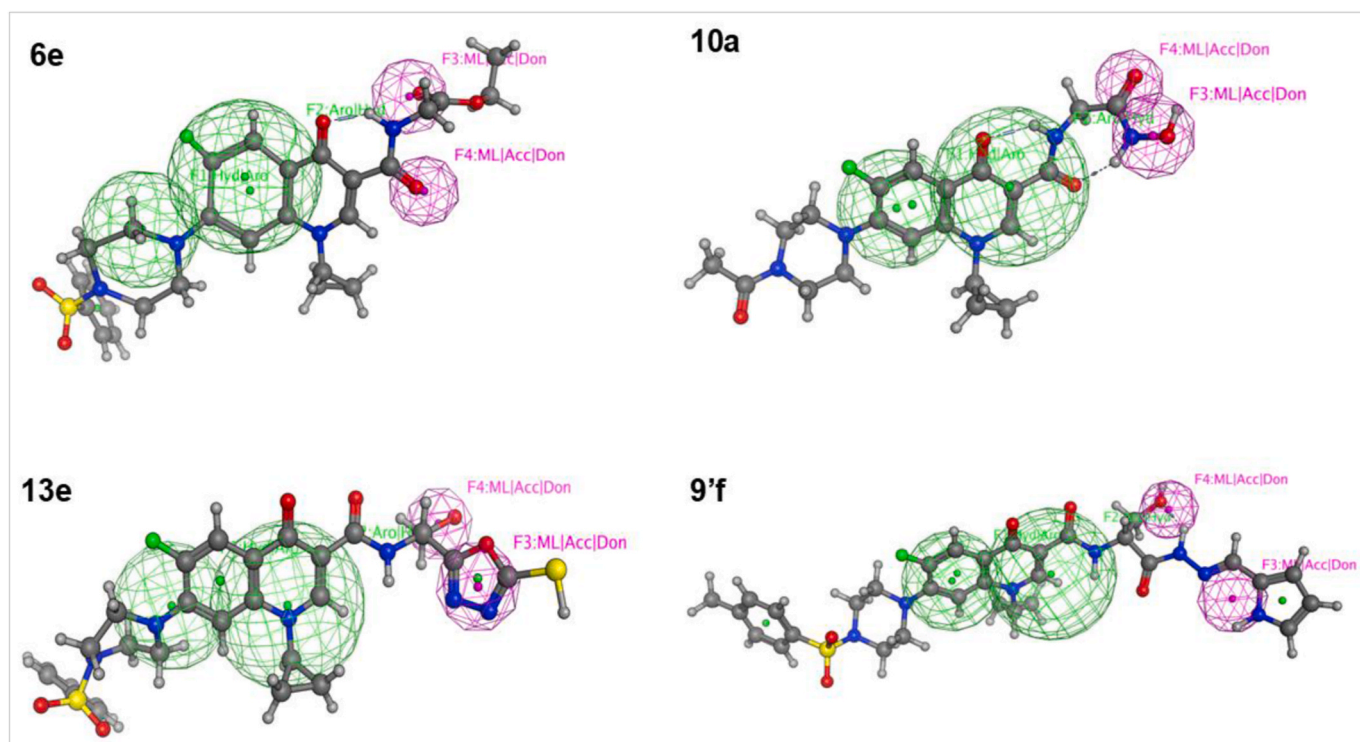


Fig. 13. Overlaying of target compounds **6e**, **9f**, **10a**, and **13e** with the generated pharmacophore query.

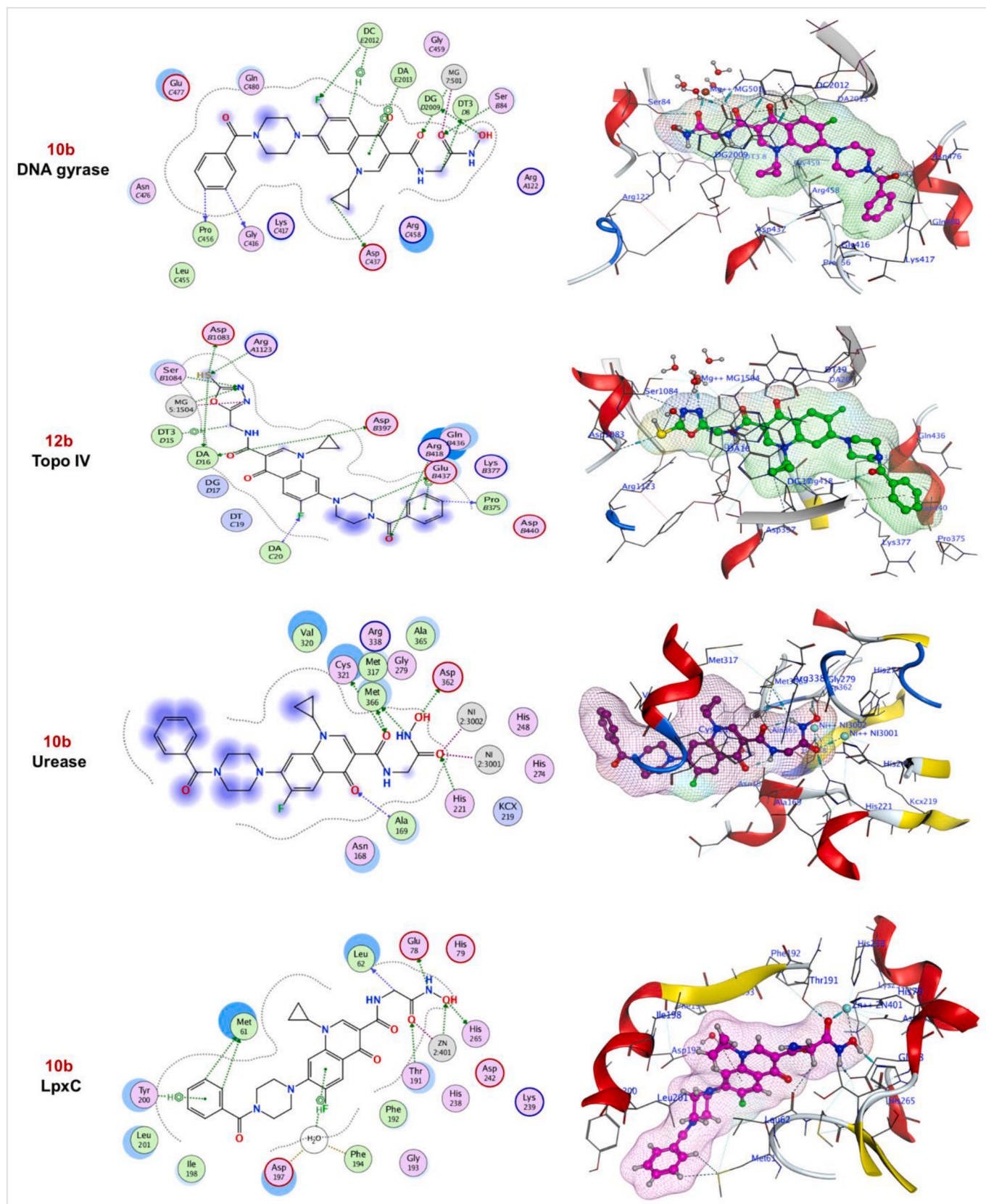


Fig. 14. 2D representation and 3D representation of the interactions of compounds **10b** and **12b** within DNA gyrase, topoisomerase IV, urease and LpxC are shown. H-bonds and metal-ion coordination bonds are depicted in cyan, H- π bonds in red, and van der Waals clashes in orange. The ligand is represented using a ball-and-stick model, with atoms color-coded as follows: magenta/green for carbon, blue for nitrogen, red for oxygen, yellow for sulfur, green for fluorine and white for hydrogen.

Therefore, we evaluated the propensity for bacterial resistance development towards representative lead compound **12b** against *S. aureus* and **10b** against *E. coli*, with CIP used as a reference drug. Compounds were repeatedly exposed to bacteria at their sub-MIC values to allow for resistance development. Resistance was defined as an increase in MIC of more than four-fold compared to the original value. As shown in Fig. 12, no remarkable changes in MIC values of the studied compounds were noticed over 20 days for *S. aureus* or *E. coli*. Conversely, the MICs of CIP increased approximately 48-fold and 32-fold, respectively, during the 20-day period. Collectively, these findings suggest that the tested compounds show minimal potential for bacterial resistance development in the studied strains and time frame, exhibiting only a slight 2–3-fold increase in MIC values.

2.2.6. *In vitro* cytotoxicity

The cytotoxicity of selected lead compounds was evaluated using the MTT assay against human neuroblastoma (SH-SY5Y) cells and normal diploid human fibroblast (WI-38) cells [61,62]. Ciprofloxacin and the apoptosis-inducing kinase inhibitor staurosporine were used as controls (Table 3). All investigated compounds exhibited lower cytotoxicity (>50 μ M) on both cell lines compared with staurosporine (3-fold higher on SH-SY5Y and 4.5-fold higher on WI-38). They showed cytotoxicity levels comparable to ciprofloxacin. The designed compounds demonstrated IC₅₀ values ranging from 49.6 to 68.2 μ M against SH-SY5Y cells and 59.4 to 80.0 μ M against WI-38 cells. The minimal cytotoxic effects observed in both neuronal and normal human cell lines suggest limited nonspecific cytotoxic effects at concentrations exceeding antibacterial MIC values. Notably, the absence of pronounced cytotoxicity in SH-SY5Y cells indicates no significant neuronal cytotoxicity under the tested conditions.

To further assess bacterial selectivity relative to host cells, the selectivity index (SI) was calculated as the ratio of IC₅₀ (WI-38) to MIC against ciprofloxacin-resistant strains. All examined compounds demonstrated favorable selectivity (SI 10 to 25), exceeding the commonly accepted selectivity threshold (SI > 10). This highlights the improved *in vitro* bacterial selectivity and reduced likelihood of host off-target toxicity. Furthermore, *in silico* assessment of cardiotoxicity predicted a low risk of hERG channel inhibition. This provided additional support for favorable safety profiles of the designed derivatives. Taken together, these results indicate that the structural modifications not only enhance antibacterial activity but also improve bacterial selectivity over mammalian cells.

2.3. Molecular modeling studies

We next conducted multi-scale computational validation and evaluation of our designed compounds in correlation with our experimental findings. This investigation integrated pharmacophore analysis, molecular docking, molecular dynamics simulations, and DFT calculations, alongside ADME/Tox assessments. This framework provides comprehensive insights into the compounds' structural, electronic, and pharmacokinetic properties.

2.3.1. Ligand-based pharmacophore search

Ligand-based pharmacophore modeling was performed using MOE to identify and validate our new ciprofloxacin derivatives as LpxC inhibitors. A training set of 46 reported active LpxC inhibitors (IC₅₀ < 500 nM) with diverse chemical structures was used to generate the model. An independent validation set of 18 compounds, including four inactive molecules (IC₅₀ > 500 nM) and ciprofloxacin, was utilized to assess performance (Table S9–10). The analysis produced a pharmacophore model with four features (two hydrophobic regions and two metal ligators), which was validated with 100% sensitivity, specificity, and accuracy. The validation set gave a hit rate of 72.2% (13/18), confirming the reliability of the model. The pharmacophore query revealed that all reported LpxC inhibitors share four common structural

features: two hydrophobic regions essential for occupying the lipophilic tunnel of the LpxC active site, and two metal ligators (hydrogen bond donor–acceptor groups). The metal chelating groups included hydroxamic acid and nonclassical bioisosteres such as 2-(1-hydroxyethyl)imidazole, oxazole, and glycine (Table S11–12 and Fig. S142).

Next, designed compounds were searched against the validated pharmacophore query. The designed molecules fulfilled the required pharmacophoric features (hydrophobic side chain at *N*⁴-piperazine, quinoline nucleus, and a metal-binding group such as hydroxamic, ester, hydrazide, oxadiazole thiol, etc.), with RMSD values of 0.3881–1.1152 Å. Ciprofloxacin derivatives displayed improved alignment scores (–150.2 to –162.8 kcal/mol) compared to ciprofloxacin itself (–81.7 kcal/mol). This highlighted the impact of structural modifications on enhancing LpxC inhibition (Fig. S143–144). Overall, the pharmacophore search supports the design rationale and the potential of the synthesized compounds as LpxC inhibitors. Representative overlays of compounds **6e**, **9f**, **10a**, and **13e** with the pharmacophore query are shown in Fig. 13.

2.3.2. Molecular docking and dynamics simulations

Molecular docking was conducted to get insights into the binding affinity and interaction modes of the most active compounds with target enzymes, complementing experimental assays. In this study, the designed multi-target compounds were docked using MOE, AutoDock 4.2.6, and K_{DEEP}. The bacterial targets included DNA gyrase, topoisomerase IV, urease, and LpxC. The binding affinities and inhibition constants (*K_i*) were determined to validate and support the predicted multi-target inhibitory potential.

All docked compounds demonstrated strong and consistent interactions with the key amino acid residues as the co-crystallized inhibitors, adopting diverse yet favorable binding poses. Remarkably, all compounds achieved binding scores exceeding –8 kcal/mol and displayed higher binding affinities and lower inhibition constants than ciprofloxacin. Among the docked compounds, compound **10b** exhibited high binding affinities toward DNA gyrase (–12.49 kcal/mol), urease (–10.51 kcal/mol), and LpxC (–9.84 kcal/mol). Compound **12b** demonstrated exceptional affinity for topoisomerase IV (–12.80 kcal/mol). Moreover, compounds **10f** and **13c** showed strong binding scores to urease of –10.16 and –10.09 kcal/mol, respectively. Compound **10d** exhibited binding affinity to LpxC (–9.71 kcal/mol), as shown in Fig. 14. Importantly, these binding scores were consistent with the IC₅₀ values observed in the enzyme assays, further supporting their potential as promising multi-target inhibitors (see Supporting Information for detailed docking results, Table S13–16, Fig. S145–173). The docking results were consistent with experimental observations from the metal-chelation studies performed using UV–Vis spectrophotometry with zinc, nickel, and cadmium. These are metals present in the catalytic sites of the target enzymes. The designed compounds demonstrated a reasonable ability to chelate these metals, evidenced by characteristic changes in the UV–Vis spectra, including hypochromic and bathochromic shifts (Table S17 and Fig. S174 and S175). Taken together, the docking and spectrophotometric findings confirm the compounds' potential as effective multi-target inhibitors. It should be noted that *in vitro* metal chelation study does not directly imply intracellular metal chelation. Therefore, the potential multi-target inhibition involving metal chelation remains a mechanistic hypothesis that needs further validation.

Molecular dynamics (MD) simulations (NAMD 3.0) were performed as two independent 100 ns runs to validate the stability and binding affinity of the most active urease and LpxC inhibitors (**8a**, **10b**, **12b**, and **13c**) within their respective active sites (urease: PDB ID 1e9y; LpxC: PDB ID 4isa). The systems were prepared using QwikMD/VMD. Both replicate simulations exhibited comparable RMSD profiles and converged to similar equilibrium states, with minor variations in average RMSD values between runs (0.2–0.6 Å). The results showed that compounds **10b** and **13c** (urease) and **8a**, **10b**, and **12b** (LpxC) maintained stable binding with RMSD values comparable to or lower than those of the

reference inhibitors (AHA and BB-78485). MM-PBSA calculations displayed approximate strong binding affinities ($\Delta G \approx -9.9$ to -11.1 kcal/mol), exceeding those of the standards. Key stabilizing interactions involved His221, Cys321, Ala365, and Asp165 (urease), and Leu62, Glu78, Thr191, Tyr200, and His265 (LpxC). Additionally, distance and contact analyses revealed that the selected compounds maintained closer proximity to key active-site residues and catalytic metal ions. Moreover, they exhibited higher ligand-protein contacts than the reference inhibitors, further confirming their enhanced binding stability throughout the simulations. Protein structural analyses (RMSF, rGyr, SASA) confirmed compact and stable conformations throughout the simulations. These findings support the docking and biological data, reflecting the strong and stable binding of the designed compounds to both targets (see Supporting Information, Fig. S176–186).

2.3.3. Quantum chemical calculations

Quantum chemical calculations were carried out using density functional theory (DFT) at the B3LYP/6-311G(d,p) level. These calculations provided an insight into the molecular geometry, electronic structure, and chemical reactivity of the synthesized ciprofloxacin derivatives. The optimized geometries of all compounds exhibited no imaginary frequencies, confirming their structural stability. Molecular electrostatic potential (MEP) maps revealed that oxygen atoms represent the most electron-rich (nucleophilic) regions. In contrast, the highest positive charges are localized on nitrogen and sulfur atoms, as well as on the hydrogen atoms bonded to them (electrophilic regions), representing potential interaction sites with biological targets. Frontier molecular orbital (FMO) analysis showed moderate HOMO–LUMO energy gaps (5.15–6.59 eV), suggesting favorable charge transfer and good chemical reactivity. The calculated global reactivity descriptors (η , S , χ , μ , and ω) indicated that the designed derivatives possess high softness, enhanced electrophilicity, and strong electron-accepting ability, consistent with their potent biological profiles. Overall, these computational findings correlated with the experimental and docking results. They confirm that the favorable electronic configuration and charge distribution of the new ciprofloxacin derivatives contribute significantly to their promising antibacterial activities. Complete computational methods, parameters, and detailed results are provided in the Supporting Information (Figure S187–189, Table S18).

Afterwards, we explored the relationship between electronic properties and surface charge of the designed compounds. This was achieved by zeta potential measurements under physiological conditions (37 °C, pH 7.4). Zeta potential at physiological pH provides information on drug behavior, stability, absorption, cellular uptake, and interactions with biological targets, all of which influence therapeutic efficacy [63,64]. The ciprofloxacin derivatives exhibited more negative zeta potentials (-9.51 to -15.32 mV) than ciprofloxacin (-6.2 mV) (Table S19). The enhanced negative surface charge could improve stability, membrane interactions and cellular uptake at physiological pH. Electrostatic potential maps generated using MOE further confirmed these findings, showing dominant negative regions (blue) corresponding to high electron density areas. The computational results closely aligned with the experimental zeta potential data (Figure S190).

2.3.4. Prediction of physicochemical properties (drug-likeness) and ADME/Tox profiles

The evaluation of drug-likeness of new compounds is a very crucial step in drug development. Therefore, the physicochemical properties of our compounds were calculated using MOE software and compared with ciprofloxacin. Key descriptors, including molecular weight, logP, logS, TPSA, number of rotatable bonds, hydrogen bond donors/acceptors, and ring count. These descriptors were evaluated against Lipinski's, Veber's, Egan's, and Muegge's rules (Table S20). Most compounds displayed favorable values for TPSA (81.16–150.67 Å²), logP (-0.505 to 3.033), rotatable bonds (4–13), and rings (4–6), complying with the overall criteria of the evaluated rules. The findings suggested good oral

absorption and bioavailability. The compounds exhibited acceptable aqueous solubility (LogS -3.041 to -5.085), suggesting adequate gastrointestinal dissolution. Some compounds showed deviations in one or two parameters, but they adhered to other complementary rules. This compensated for these variations and indicated their potential for good oral bioavailability and drug-likeness.

The pharmacokinetic and toxicological properties of drug candidates were predicted using the freely accessible pkCSM pharmacokinetics web tool (<http://structure.bioc.cam.ac.uk/pkcsml>) [65]. *in silico* ADME analysis indicated favorable pharmacokinetic profiles for the new derivatives, including high intestinal absorption, appropriate distribution characteristics, and low predicted toxicity, comparable to ciprofloxacin, which is already marketed for oral administration (Table S21). Experimental aqueous solubility measurements of representative lead compounds **10b** (LogS -4.07 mol/L) and **12b** (LogS -4.26 mol/L), performed by shake-flask method, showed reasonable agreement with the predicted LogS values (Fig. S191–192, Table S22).

3. Conclusion

Here, we synthesized a series of ciprofloxacin–amino acid derivatives designed as multi-target antibacterial agents. Several of the newly developed compounds exhibited enhanced antibacterial and antimycobacterial activity. The observed improvement in activity is likely related to inhibition of DNA gyrase and topoisomerase IV, along with possible targeting of other enzymes such as urease and LpxC supported by *in vitro* studies. Phenotypic analyses suggested inhibition of topoisomerases and further additional effects on bacterial cell division and transient effects on the cytoplasmic membrane in Gram-positive bacteria and aberrant PBP activity and possible effects on the outer membrane in Gram-negative bacteria that may contribute to the observed antibacterial activity. The most promising candidates displayed bactericidal activity against *S. aureus*, with MBCs of 0.7–3.9 µM and showed a low propensity for resistance development under the tested conditions. Molecular modeling studies supported our rational design and were consistent with the biological findings. Overall, these findings highlight our ciprofloxacin derivatives as promising scaffolds for further mechanistic studies and optimization toward next-generation antibiotics with improved polypharmacological activity.

4. Experimental

Details related to methods, instrumentation, synthesis, and characterization of the compounds are presented in Supporting Information.

CRedit authorship contribution statement

Ahmed M. Kamal El-sagheir: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Margareth Sidarta:** Data curation, Formal analysis, Investigation, Validation. **Alex Bunker:** Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Writing – review & editing. **Michaela Wenzel:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jari Yli-Kauhaluoma:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejmech.2026.119138>.

Data availability

The authors confirm that the data supporting the findings of the study are available within the article.

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