

Efflux Resistance Breakers: A New Technology to Overcome Efflux-Mediated Resistance in Multidrug Resistant Bacteria

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Abstract

We have developed a novel approach to reduce efflux liability in different antibiotic classes, based on advanced structural modelling, identification of “efflux-resistance breaker” (ERB) pharmacophores, and detailed understanding of efflux liability in multidrug resistant (MDR) pathogens. This technology allows development of antimicrobials that are less susceptible to efflux and do not require the use of additional efflux pump inhibitors. These ERB-modified antibiotics maintain high intracellular concentrations resulting in bacterial death, even in the presence of multiple target mutations associated with resistance. The ERB-fluoroquinolones show notably better activity compared to parent fluoroquinolones in a variety of MDR bacteria with up to a 512-fold reduction in MIC (MIC₉₀ 0.03 to 2 µg/mL). The lead compounds, **KSN-L22** and **BL-7** showed MICs of 0.03 to 0.5 µg/mL against MRSA, 0.03 to 2 µg/mL against *Acinetobacter baumannii*, 0.03 to 2 µg/mL against *Escherichia coli* and 0.25 to 4 µg/mL against *Klebsiella pneumoniae* strains. The ERB-fluoroquinolones work by inhibiting both wild type and mutant (GyrA S84L) DNA gyrases (IC₅₀ ~3.8 µg/mL). Both **BL-7** and **KSN-L22** showed *in vivo* efficacy in a thigh infection model with 5-log reduction of bacterial load at both 20 and 50 mg/kg oral dose levels and excellent oral and IV PK/PD profiles. The compounds did not show any toxicity at 1200 mg/kg/day in mice. The off-target toxicity screen did not reveal any issues, including the hERG channel, and the compounds do not induce or inhibit cytochrome p450 enzymes. Use of efflux resistance breaker technology to make the compounds less susceptible to efflux has the potential to revive the use of several classes of current antibiotics.

Introduction

The widespread introduction of antibiotics to the clinical setting was a defining feature of medicine in the 20th century. Though the development of penicillin from natural product to life-saving medication is undoubtedly the best-known story in this regard- a feat for which Alexander Fleming, Howard Florey, and Ernst Chain shared the 1945 Nobel Prize in Physiology or Medicine- the introduction of other antibiotics such as the sulfonamides was similarly instrumental in ushering in the modern antibiotic era. Yet the many advances antibiotics have made possible are now under threat from rising global levels of antimicrobial resistance (AMR). The development and approval of new antibiotic compounds is currently outstripped by the rate of emergence of resistance to existing antimicrobials, a trend that must be reversed if humanity's reliance on antibiotics is to continue long-term¹.

Prokaryotic efflux systems are key mechanisms of antibiotic resistance in a variety of different bacterial species. These pumps have, in the case of quinolone resistance in *Staphylococcus aureus*, been shown to act as mediators of an initial, intermediate resistance phenotype from which higher level resistance mutations can then stem^{2, 3}. Similar observations have been made for quinolone resistance in *E. coli*⁴ and *Streptococcus pneumoniae*⁵, azithromycin and ethambutol resistance in *Mycobacterium* spp.^{6, 7} and metronidazole resistance in *Helicobacter pylori*⁸. Antibiotic development programs have long sought to minimise efflux by selecting candidate compounds that are poor efflux substrates⁹, but recent reports of efflux-mediated resistance to omadacycline¹⁰, eravacycline¹⁰⁻¹² and delafloxacin¹³, three new antibiotics approved by the FDA between 2017 and 2018, indicate that this approach may be untenable long-term. Efflux pump inhibitors (EPIs) have been pursued as adjunct therapies to safeguard approved antibiotics prone to efflux-based resistance, but no EPI has yet achieved market approval¹⁴ and attempts to make antibiotic-EPI conjugates¹⁵⁻¹⁸ have failed to change the direction of a field still predominantly focussed on the combination therapy approach.

In the case of standalone EPIs and poor efflux substrate antibiotics, we opine that since the antibiotic cannot actively prevent its own efflux, it remains fundamentally susceptible to it in each case. Although the antibiotic-EPI conjugate approach seemingly addresses this, such projects have typically involved directly appending known EPIs to known antibiotics, resulting in conjugates with improved efflux inhibitory character but at the expense of reduced antibiotic activity and/or unsuitability for further development¹⁵⁻¹⁸. Being able to develop efflux resistant antimicrobial agents will confer two advantages. Firstly, the cellular concentration of the drug will increase, thereby boosting antimicrobial potency, possibly enough to overcome resistance within the cell. Secondly, this could reduce the likelihood of resistance emergence. There has been a considerable body of research by different

academic groups and pharmaceutical companies, directed towards the development of efflux pump inhibitors (EPIs) which can potentially reverse efflux mediated resistance. The efforts have been largely unsuccessful due to the lack of mechanistic insight and biochemical information despite the recent advances in molecular modelling and computational techniques. The approach to use separate efflux pump inhibitors with antimicrobial agents is also fraught with risks as the antibiotics remain unmodified substrates and can be effluxed by different pumps despite the presence of EPIs. The intracellular concentration of EPIs also needs to be considerably higher to increase the probability of sparing the antimicrobials, but that has led to toxicity which has prevented pre-clinical or clinical development of efflux pump inhibitors. There are additional complexities around matched pharmacokinetics in combination therapy which served as an additional barrier for EPI development.

Herein we report a novel approach capable of both rescuing efflux-afflicted antibiotics, and informing the design of novel efflux-resistant ones through the covalent modification of antibiotic scaffolds with Efflux Resistance Breaker (ERB) fragments to direct the compounds to inhibitor binding pockets within the efflux pumps which makes them less susceptible to efflux. These fragments enable the antibiotics in question to bind target efflux pumps as substrate-inhibitors and block their own efflux from the bacterial cell resulting in high intracellular concentrations of the antibiotic within the bacteria. This results in killing or inhibited growth of the pathogen, even in the presence of multiple target mutations which would generally render the bacteria resistant to antibacterial agents. Along with a direct role in mediating resistance, by reducing the intracellular concentration of the drug within the cell, efflux pumps also contribute to the ability of the cell to develop high level, stable resistance, through other cellular mechanisms. Efflux-resistant antibiotics therefore offer the potential to both increase the cellular concentration of the drug to achieve improved potency, possibly enough to overcome target resistance within the cell, and also reducing the likelihood of resistance emergence. We validated the technology using Fluoroquinolones as the model antibiotics, as the efflux mediated resistance has been well-characterised for this class of antibiotics. This approach has never been employed and differentiates us from previous unsuccessful efforts to develop efflux pump inhibitors.

This new functional class of antibiotics retains the ability to inhibit the bacterial target whilst gaining additional properties in being able to resist efflux by pumps that operate in MDR pathogens, including CDC priority pathogens. We have shown that the ERB-modified fluoroquinolones retain the target specificity for DNA gyrase in both Gram-positive and Gram-negative bacteria, with a significantly lower IC₅₀ against the fluoroquinolone-resistant mutant enzyme, GyrA S84L, than the parent antibiotic, levofloxacin. We have evidence that these compounds also act as efflux-pump inhibitors to potentiate the activity of other antibiotics which are effluxed through the same range of pumps, providing dual activity. We saw significant potentiation of antibiotics known to be substrates of shared efflux pumps

in *Acinetobacter baumannii*, when one of the lead molecules were used at sub-inhibitory concentrations, suggesting that they achieve effective inhibition of efflux pumps, helping to maintain high intracellular concentrations of a variety of efflux pump substrates. Our microbiological data shows that these ERB-fluoroquinolones can overcome high levels of pre-existing FQ-resistance and did not show any adverse safety signals^{12,13} often associated with fluoroquinolones. The *in vitro* DMPK, *in vivo* PK, toxicity and *in vivo* efficacy data of ERB-modified fluoroquinolones suggest this approach is suitable to develop new generation antibacterials that can overcome efflux-mediated resistance.

Results

Identification of Substrate and Inhibitor Binding Pockets within Efflux Pumps

In our effort to develop antimicrobials that can resist efflux, initially we sought to address the differential binding response to substrates and inhibitors through investigating why prokaryotic efflux pumps behave differently in the presence of substrates and inhibitors¹⁹. We have previously reported changes in efflux pumps dynamics in response to substrates and inhibitors that affects the likelihood of efflux of a molecule¹⁹. We studied a large number of efflux pump substrates and inhibitors to determine their preferred binding site within the efflux transporter. To do this, we developed a series of molecular models of key efflux transporters from members of the ESKAPEE pathogens including NorA in *S. aureus*, AdeB in *Acinetobacter baumannii* and MexB in *Pseudomonas aeruginosa*. Models were developed based on a combination of x-ray crystallography data, homology modelling and molecular dynamics simulations. Libraries of known substrates and inhibitors were subsequently docked against each model with the goal of identifying any differences between the two groups.

A clear pattern emerged for all major efflux pumps studied; substrates and inhibitors were predicted to consistently bind within two different, adjacent internal pockets (Figure S1). As exemplified by the MFS-type pump NorA from *S. aureus* and the RND-type pump MexB from *P. aeruginosa*, hydrophobicity maps of pump internal surfaces showed the substrate binding sites to be more hydrophilic than the corresponding inhibitor binding sites. It was thus postulated that the physicochemical properties of a compound determine its binding site within an efflux pump and, consequently, the susceptibility of the compound to efflux. This in turn suggested that efflux-prone antibiotics could be rendered less so by directing them towards the more hydrophobic interior binding surfaces of a target efflux pump which are typically occupied by the efflux pump inhibitors. We have exploited this differential binding response of efflux pumps towards substrate antibiotics and EPIs to chemically modify the antibiotics with small fragments, termed efflux resistance breaker (ERB) fragments, and make them less susceptible to efflux while maintaining their antibacterial activity.

Chemical Modification of Fluoroquinolones to Develop First Generation Efflux Resistant Antibiotics

The second-generation fluoroquinolone drug **ciprofloxacin** was chosen as an archetypal efflux-prone antibiotic on which to test this concept and develop efflux resistant fluoroquinolones. In turn, the NorA efflux pump in *S. aureus* was chosen as a model efflux pump since it has been implicated in the acquisition of fluoroquinolone resistance in *S. aureus*^{2, 20}. Docking against the NorA model predicted **ciprofloxacin** to bind within the NorA substrate pocket with the C7 position of the quinolone core adjacent to the hydrophobic inhibitor pocket (Figure S2a). Molecular modelling experiments suggested that a hydrophobic moiety connected to **ciprofloxacin** at the C7 position *via* a suitable linker would be able to contact the inhibitor binding pocket (Figure S2c) and block efflux of the compound by NorA.

Using the C7 piperazine ring in **ciprofloxacin** as the linker, a naphthalene moiety was identified as a suitable hydrophobic group, with the two connected by a methylene unit; this linker-hydrophobic moiety is hereafter referred to in totality as the *ERB unit*, resulting in compound **ML-77-005** which showed strong interactions with the hydrophobic region of NorA while maintaining a superior *in silico* binding affinity towards DNA gyrase (Table S1). Structurally, **ML-77-005** can be considered as a truncated hybrid of **ciprofloxacin** and **NMP**. **NMP** has been reported to potentiate fluoroquinolones²¹ against the RND-type pumps AcrAB and AcrEF in *E. coli*²¹, thus **ML-77-005** was compared to both **ciprofloxacin** alone and a **ciprofloxacin**/**NMP** combination *in vitro* against different strains of *S. aureus*. While **NMP** only afforded **ciprofloxacin** two-fold potentiation in resistant strains NCTC 13616 and NCTC 13277, **ML-77-005** showed 64-fold improvement over **ciprofloxacin** alone and retained comparable activity in fluoroquinolone-susceptible strain ATCC 9144 (Table 1). Further MIC testing against a panel of 15 *S. aureus* strains, the majority of which possessed point mutations in both *gyrA* and *grrA*, confirmed the superiority of **ML-77-005** (MIC₉₀ 2 µg/mL) *versus* **ciprofloxacin** (MIC₉₀ 128 µg/mL) (Table S2).

To probe the structure-activity relationship of **ML-77-005**, a series of analogues with modifications to the ERB unit were synthesised (Table S3) and tested against an informative panel of *S. aureus* isolates including two fluoroquinolone susceptible strains (ATCC 9144 and 1199), an isogenic mutant of 1199 which has upregulated NorA expression (1199B)²², and two MRSA strains (NCTC 13616 and NCTC 13277) which have both upregulated NorA expression and mutations in the antibiotic target, DNA gyrase. The modifications in the ERB fragments focused on modification of the hydrophobicity of the fragment by introducing different heteroatoms and functional groups that affects the ability of the generated analogues to interact with the hydrophobic residues within the NorA efflux pumps. From this, it was concluded that ERB unit hydrophobicity was a key determinant of ERB-fluoroquinolone activity; compared to **ML-77-005**, analogues lacking a second aromatic ring (**ML-77-046**) or possessing an aromatic heterocycle (**ML-77-052**) showed much reduced activity against fluoroquinolone-resistant *S.*

aureus, while analogues with modifications involving the orientation of the naphthalene moiety (**ML-77-036**) and partial removal of its aromaticity (**ML-77-076**) retained this activity. Based on these results, further analogues of **ML-77-005** were synthesised (Table S3); the activities of each *versus* fluoroquinolone-resistant *S. aureus* further confirmed the importance of ERB unit hydrophobicity, while analogues based on the commercial fluoroquinolones **norfloxacin** (**ML-77-021**), **enoxacin** (**ML-77-025**) and **levofloxacin** (**ML-77-140**) confirmed the wider applicability of the ERB-fluoroquinolone approach. In each case, compounds modified with the ERB approach were found to be significantly more active compared to the unmodified parent antibiotic.

Determination of Efflux Susceptibility of ERB-Modified Ciprofloxacin

To experimentally validate the efflux susceptibility of the ciprofloxacin analogues modified using the ERB approach and determine the origin of the improved activity seen in **ML-77-005**, a reserpine growth assay was performed using *S. aureus* strains NCTC 13616, and the isogenic pair 1199B and 1199 (Figure 1a, b). **Ciprofloxacin** at sub-MIC concentrations was potentiated by the efflux pump inhibitor reserpine in all three strains, evidenced by a shift in the growth curve to the right, indicating, as previously reported²³, that it is an efflux substrate. However, no such potentiation was observed for **ML-77-005**, even in strains with overexpressed NorA, NCTC 13616 and 1199B, inferring that it is not subject to efflux from *S. aureus*. This suggests **ML-77-005** is able to maintain high intracellular concentrations in the presence of significant NorA efflux pump upregulation in fluoroquinolone-resistant *S. aureus* strains and can kill these bacteria with low MIC values (Table S3). This evidence, combined with the MIC results in the isogenic strains with differing NorA expression, strongly show the efflux resistant potential of antibiotics modified with ERB approach compared to unmodified antibiotics.

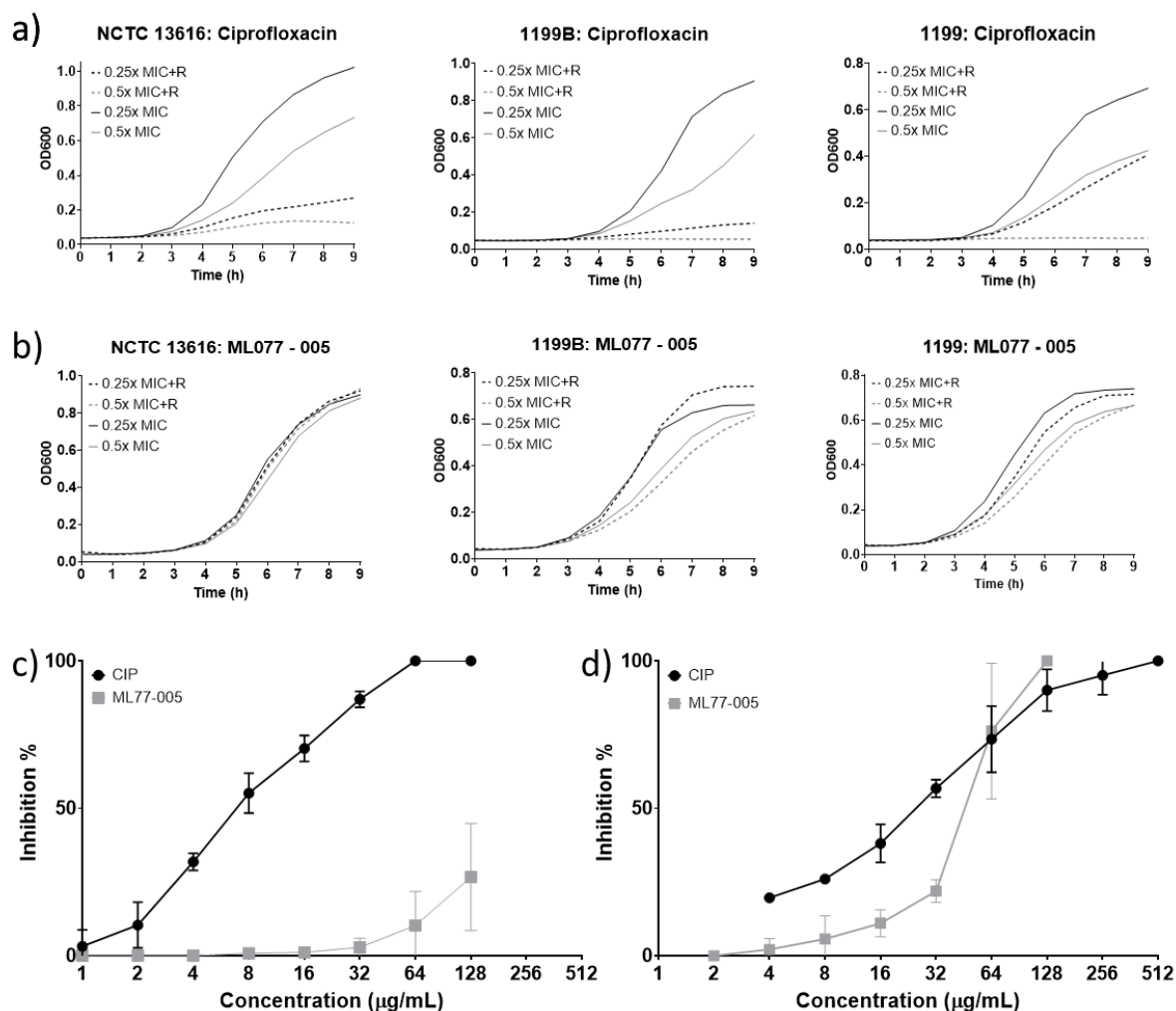


Figure 1: (a-b), Reserpine assay data for **(a) ciprofloxacin** and **(b) ML-77-005** in NorA-overexpressing *S. aureus* strains NCTC 13616 and 1199B and the wild-type strain 1199. The reserpine assay uses reserpine, a natural product and promiscuous competitive efflux pump inhibitor, to determine if an antibiotic is subject to efflux from *S. aureus* or *S. pneumoniae*²⁴. Comparable growth curves for sub-inhibitory concentrations of **ML-77-005** with and without the addition of reserpine implies **ML-77-005** is not subject to efflux by NorA. In contrast, the pronounced growth difference for **ciprofloxacin** in all three strains suggests it is a strong NorA substrate. **(c-d)** Topoisomerase supercoiling assay data for **ciprofloxacin** and **ML-77-005** using both the wild-type (WT) **(c)** and the S84L mutant **(d)** DNA gyrase enzyme. The assay combines purified topoisomerase enzyme with relaxed plasmid DNA, with the enzyme introducing negative supercoils into the DNA unless an inhibitor prevents this; an IC₅₀ can be calculated through comparison of experiments with different inhibitor concentrations. **ML-77-005** has the higher IC₅₀ of the two compounds against both WT and S84L enzymes (>128 and 48.76 μg/mL respectively, compared to 7.53 and 34.69 μg/mL for **ciprofloxacin**), thus improved target inhibition is not the cause of its superior activity in *S. aureus*.

Type II Topoisomerase Inhibitory Activity of ERB-modified Ciprofloxacin ML-77-005

One of the fundamental considerations during the ERB modification of antibiotics was to ensure they maintain on-target activity so that they can still work as an antibiotic while preventing their own efflux.

Commercial antibacterial quinolones work by inhibiting type II topoisomerase enzymes²⁵. A molecular modelling experiment suggested **ML-77-005** binds to the same pocket as **ciprofloxacin** (Figure S3). A cell-free DNA gyrase inhibition assay was used to assess the activity of **ML-77-005** against enzymes from both fluoroquinolone-sensitive (wildtype DNA gyrase) and fluoroquinolone-resistant (GyrA S84L mutant enzyme) *S. aureus* strains (Figure 1c and d). While **ML-77-005** showed reduced inhibition of the wildtype enzyme compared to **ciprofloxacin**, the difference in IC₅₀s of both against the S84L mutant enzyme was statistically insignificant. Notably, while the S84L mutant enzyme IC₅₀ for **ciprofloxacin** (34.69 ± 7.01 µg/mL) was broadly comparable to the IC₅₀ for **ML-77-005** (44.76 ± 3.2 µg/mL), **ML-77-005** was found to be 20 to 64-fold more phenotypically active against fluoroquinolone-resistant strains (n = 11 strains; MICs for **ML-77-005** 2 µg/mL, MICs for **ciprofloxacin** 32 - 128 µg/mL) (Figure 1c, Table S3). Together, these data indicate that **ML-77-005** improves upon **ciprofloxacin** in fluoroquinolone-resistant strains of *S. aureus* through resisting the action of efflux pumps such as NorA rather than by improved inhibition of type II topoisomerase enzymes.

Resistance to First-Generation ERB-modified Ciprofloxacin ML-77-005

Serial passaging experiments with **ML-77-005** were carried out using both fluoroquinolone-sensitive and fluoroquinolone-resistant *S. aureus* strains to generate resistant mutants. Whole genome sequencing of the resulting mutants revealed no efflux-related mutations in either strain of *S. aureus* used; instead, a pair of previously reported target mutations (GyrA D83G²⁶, GrlA S80F²⁷) as well as several off-target mutations were discovered in the mutant ATCC 9144, with no stable mutations found in the mutant NCTC 13616 (Table S4). These data indicate that ERB-fluoroquinolones, while vulnerable to the same resistance mutations as conventional fluoroquinolones, are not affected by any novel mechanisms of resistance. The difficulty in generating highly resistant mutants of *S. aureus* NCTC 13616, already resistant to commercial fluoroquinolones, may also indicate a slowed onset of resistance for an ERB-fluoroquinolone agent *versus* a conventional fluoroquinolone antibiotic.

Activity of ML-77-005 in Wider Gram-Positive Bacteria

The MFS superfamily of efflux pumps is a vast group of transporters found across all kingdoms of life on Earth. In bacteria, MFS-type efflux pumps implicated in antibiotic resistance, such as NorA, are known to share highly conserved amino acid sequences including residues crucial to substrate efflux²⁸. **ML-77-005** was therefore tested in a wider panel of Gram-positive pathogens to probe the limits of its efflux resistance. As with *S. aureus*, **ML-77-005** showed comparable activity to **ciprofloxacin** in fluoroquinolone-sensitive strains of *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Enterococcus faecalis*, and *Enterococcus faecium*, while showing up to 64-fold potentiation in fluoroquinolone-resistant strains of the same (Table S2). **ML-77-005** also had comparable time-kill kinetics to **ciprofloxacin** in *S. aureus* strain NCTC 13616 (Figure S4).

Second Generation ERB-Fluoroquinolones

Despite the promising biological activity profile of **ML-77-005** for treatment of Gram-positive bacterial infections, it showed poor aqueous solubility (<5 μM) and high plasma protein binding (>99%), attributable to the strong hydrophobicity of the naphthalene ring. More importantly, the compound was found to be inactive against WHO and CDC priority Gram-negative bacteria such as *A. baumannii*, (Table S5) making it unsuitable to probe the efflux susceptibility and activity of ERB-modified fluoroquinolones in Gram-negative bacteria. Our analysis of physicochemical properties of ERB-modified fluoroquinolones using SwissADME and ADMET Lab 2.0 identified levofloxacin core as a suitable fluoroquinolone with superior physicochemical properties compared to ERB-modified ciprofloxacin. Molecular modelling studies investigating the interaction of **levofloxacin** with the NorA efflux pump suggested that replacing the piperazine moiety with a pyrrolidine ring and introducing an exocyclic NH_2 group enables greater contact with both the terminal aromatic/heteroaromatic group and the hydrophobic inhibitor-binding pocket of the NorA efflux pump. This provided additional flexibility to the ERB-modified levofloxacin and allowed the terminal pyrimidine ring to form to rotate to ensure optimum contact with the key residues which was not possible with the rigid linear six-membered piperazine ring linked ERB fragment (Figure 2, Figure S5).

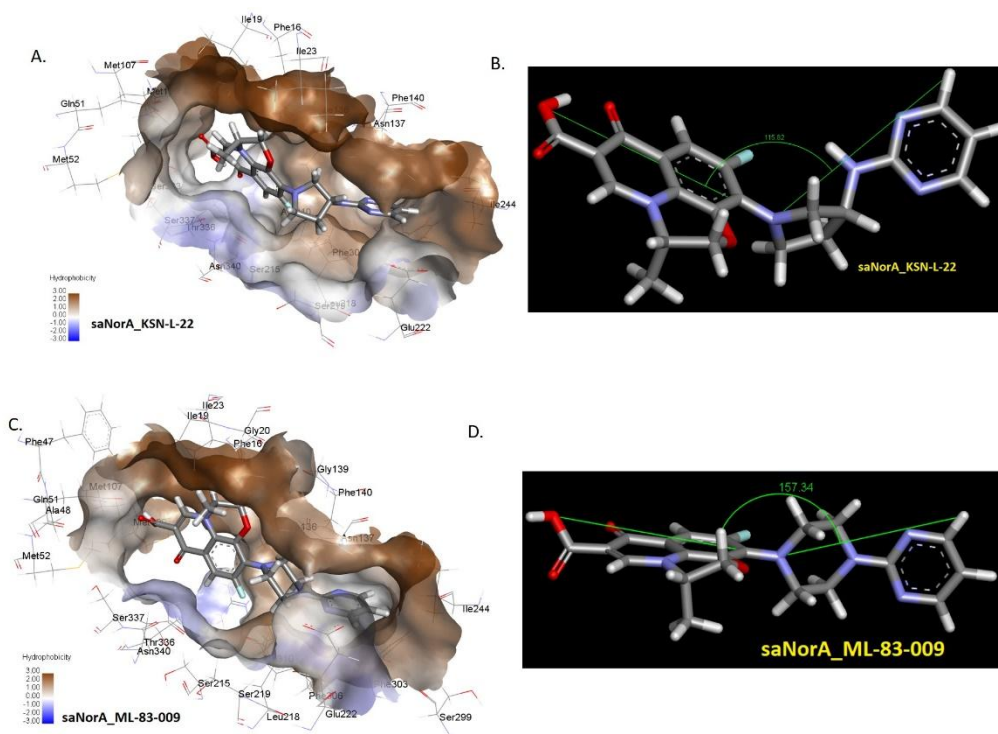


Figure 2: A) KSN-L22 in interaction with the key residues in NorA B) the five membered pyrrolidine with exocyclic amine group provides additional flexibility and curvature, C & D) relatively linear structure of six membered piperazine ring containing **ML-97-030** does not interact efficiently with the key residues.

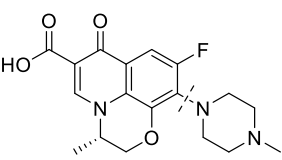
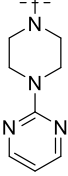
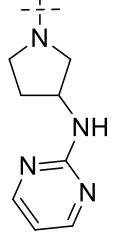
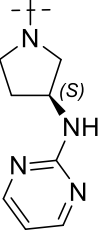
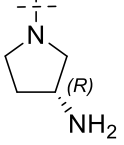
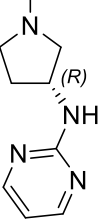
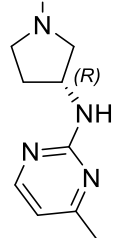
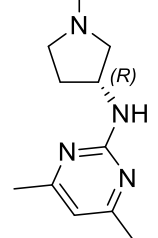
307 To experimentally validate this observation, we synthesized **ML-97-030** and **KSN-L7** (Table 2). The
308 improvement of activity observed for compound **ML-97-030** compared to **levofloxacin** against NorA
309 overexpressing MRSA strains was negligible and when tested in isogenic strains 1199 and 1199B, the
310 MIC for ML-97-030 was fourfold higher in the NorA overexpressing strain, 1199b (Table 2).

Table 1: ML-77-005 improves upon the combination of ciprofloxacin and NMP in ciprofloxacin-resistant strains of *S. aureus*. NMP was used in combination with ciprofloxacin at 50 µg/mL (0.22 mM) and 100 µg/mL (0.44 mM) in line with previous work in *E. coli* by Bohnert and co-workers¹⁴. At MIC concentration in strains NCTC 13616 and NCTC 13277 (64 µg/mL, 0.19 mM), molar ratios of NMP to ciprofloxacin were 1.2 and 2.3, respectively.

		MIC µg/mL			
		Ciprofloxacin	Ciprofloxacin + 50 µg/mL NMP	Ciprofloxacin + 100 µg/mL NMP	ML-77-005
<i>S. aureus</i> strains	ATCC 9144	0.25	1	0.25	0.25
	NCTC 13616	128	64	64	2
	NCTC 13277	128	64	64	2

329 **Table 2.** MIC values for second-generation ERB-fluoroquinolone analogues.

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		Levofloxacin	ML-97-030	KSN-L7	KSN-L19	KSN-L44	KSN-L22	BL-6	BL-7
Structural Features									
Gram-Positive Species									
<i>S. aureus</i> Strains	ATCC 9144	0.125	0.06	0.125	0.25		≤0.03	0.0156	≤0.0039 - 0.0039
	SA-1199	0.125	0.06	0.016	0.125		0.0078		0.016
	SA-1199B	1	0.25	0.03	0.25		0.016		0.016
	NCTC 13616	16	8	2	8	32	0.5-1		0.125-0.25
	NCTC 13277	16	>32	4	8	32	1		0.25-0.5

On the other hand, compound **KSN-L7**, which is a racemic mixture, showed between 4-8-fold potentiation compared to **levofloxacin** against FQ-resistant NCTC 13616 and NCTC 13277 strains, had an MIC only twofold higher in 1199b compared to 1199 and did not demonstrate efflux liability in the reserpine assay (Figure S6). Next, we synthesized pure enantiomers of **KSN-L7** to identify the optical isomer that can form optimum contact with the key residues within the binding pocket while maintaining on-target activity. Compound **KSN-L19**, the *S* isomer, appeared to be less active compared to the *R* isomer **KSN-L22** (Table 2). Compound **KSN-L22** was 16-32-fold more active against fluoroquinolone-resistant strains compared to **levofloxacin** with MICs of 0.5 and 1 µg/mL, while **KSN-L19** showed only 2-4-fold potentiation against fluoroquinolone-resistant strains, suggesting the orientation of the terminal pyrimidine ring plays an important role in reducing efflux susceptibility and activity of the compound. To further probe the role of terminal pyrimidine ring on reducing efflux susceptibility and antibacterial activity, we synthesised a series of analogues of **KSN-L22** incorporating variations on the pyrimidine-2-amine ring by changing the position of N in the pyrimidine ring (**BL-1**), introducing fluorine (**KSN-L34**), monomethyl substitution (**BL-6**), dimethyl substitution (**BL-7**) and finally removing the pyrimidine ring altogether (**KSN-L44**) (Table S6 and Table 2). As expected, removal of the pyrimidine ring altogether (**KSN-L44**) removed the critical contact within the hydrophobic binding pocket of the NorA efflux pump resulting in 32 to 64-fold loss of activity compared to **KSN-L22**. Notably, the 4,6-dimethyl substituted analogue **BL-7** was found to achieve a 2-8-fold improvement in activity in strains of fluoroquinolone-resistant *S. aureus* compared to **KSN-L22**, while other analogues containing pyrimidine ring maintained similar activity against these strains compared to **KSN-L22**. This data suggests that both components of the ERB fragment, the heteroaliphatic ring and the terminal heteroaromatic ring, are important for making the compounds less susceptible to efflux and overcoming efflux mediated resistance. Interestingly, **BL-7** containing dimethyl substitutions that provide additional hydrophobic contact was found to be most active against all strains, which further outlines the importance of targeted hydrophobic contact with the efflux pump in reducing efflux susceptibility. A **moxifloxacin** core-based analogue, **ML-110-014**, containing the same ERB fragment as **BL-7** was synthesized and the compound showed MIC values similar to **KSN-L22** and was found to be 4-8-fold more active than **moxifloxacin** against FQ-resistant strains. This further showed the ability of ERB-approach to reduce efflux liability of fluoroquinolone class of antibiotics.

Determination of Efflux Susceptibility of Second Generation ERB-Fluoroquinolones

The efflux susceptibilities of representative examples of second-generation ERB-fluoroquinolones **KSN-L7**, **KSN-L22**, **BL-7**, and **ML-110-014** were determined using a reserpine inhibition growth assay (Figure 3, Figures S6 and S7) and by testing the compounds in isogenic *S. aureus* strains 1199 and 1199B.

KSN-L7, a racemic mixture, showed a clear reduction in efflux liability compared to levofloxacin, as evidenced by the smaller shift in growth curves in the presence of reserpine (Figure S6). The pure R-isomer, KSN-L22, showed minimal shift, confirming that this isomer is largely resistant to NorA-mediated efflux (Figure 3b). Consistent with these findings, MIC determinations showed only a two-fold difference between 1199 and 1199B for KSN-L22, while BL-7 showed no difference at all, confirming their efflux resistance (Table 2). In contrast, levofloxacin displayed an eight-fold increase in MIC in the NorA-overexpressing strain relative to wild-type.

Notably, BL-7, which incorporates 4,6-dimethyl substitutions providing additional hydrophobic contacts, demonstrated 2- to 8-fold improved potency over KSN-L22 against fluoroquinolone-resistant strains (Table 2 and Figure 3c), supporting the importance of optimised hydrophobic interactions for reducing efflux susceptibility. Furthermore, a moxifloxacin core analogue, ML-110-014, containing the same ERB fragment as BL-7, exhibited MIC values comparable to KSN-L22 and was 4- to 8-fold more active than moxifloxacin against fluoroquinolone-resistant strains (Table S6 and Figure S7).

Together, these results demonstrate excellent alignment between the reserpine growth assay and MIC data and confirm that ERB-modification markedly decreases efflux liability across multiple scaffolds, thereby restoring antibacterial activity against fluoroquinolone-resistant *S. aureus*.

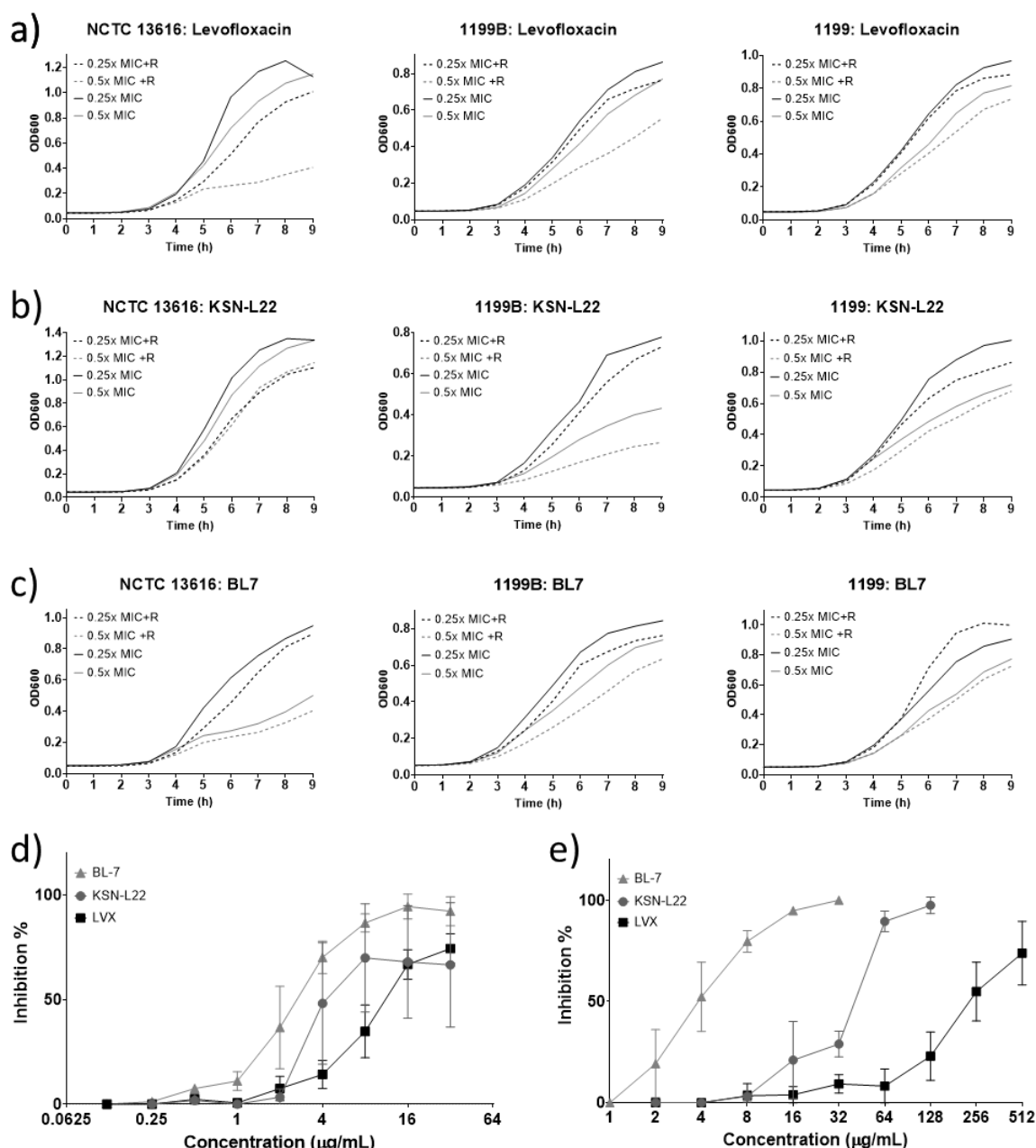


Figure 3: (a-c), Reserpine assay data for a) **Levofloxacin**, b) **KSN-L22** and c) **BL7** in NorA overexpressing strains NCTC 13616 and 1199B and the wild-type strain 1199. Whilst **Levofloxacin** is demonstrated to be a substrate of NorA, these data suggest that neither **KSN-L22** nor **BL7** are substrates for NorA. **(d-e)**, Gyrase inhibition of d) Wild type GyrA and e) S84L mutant GyrA from *S. aureus* demonstrated comparable inhibition of the wild-type enzyme by **KSN-L22**, **BL7** and **Levofloxacin (LVX)**, whilst both **KSN-L22**, and to a greater extent **BL7**, demonstrated significantly better inhibitory activity against the S84L mutant enzyme than **Levofloxacin**.

Accumulation Assay

An accumulation assay was performed to further validate the ERB technology. We have hypothesized that ERB-modified antibiotics, such as **KSN-L22**, are less susceptible to efflux and therefore accumulate more than the parent compound within bacterial cells. This could be the mechanism behind their ability to kill bacteria, even in the presence of multiple target mutations (Table 2). The results showed that the level of accumulation of **levofloxacin** was lower in the NorA over-expressing 1199B strain compared to the wild-type 1199 strain, and this difference was statistically significant ($P < 0.05$) (Figure 4). The data aligned with the MIC observed for **levofloxacin** in these two strains, where an eightfold increase in MIC was noted in the 1199B strain compared to 1199. On the other hand, no significant change in accumulation was observed for **KSN-L22** in the 1199B strain compared to the 1199 strain. Again, these findings were consistent with the MIC data for **KSN-L22**, as no significant changes in MIC were observed in the efflux mutant 1199B strain compared to the wild-type 1199 strain.

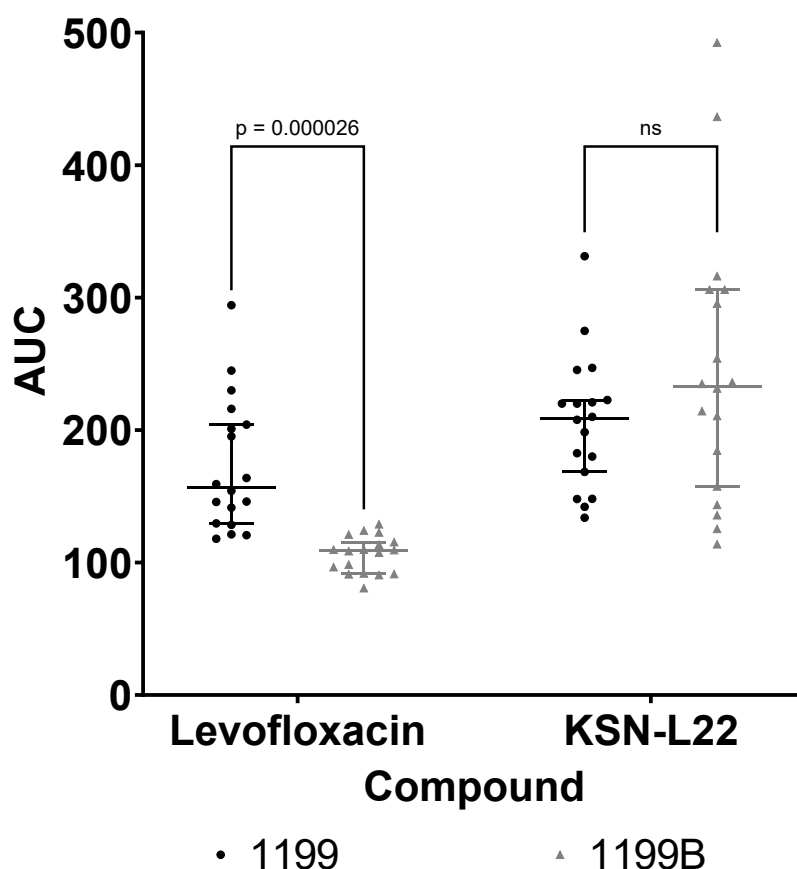
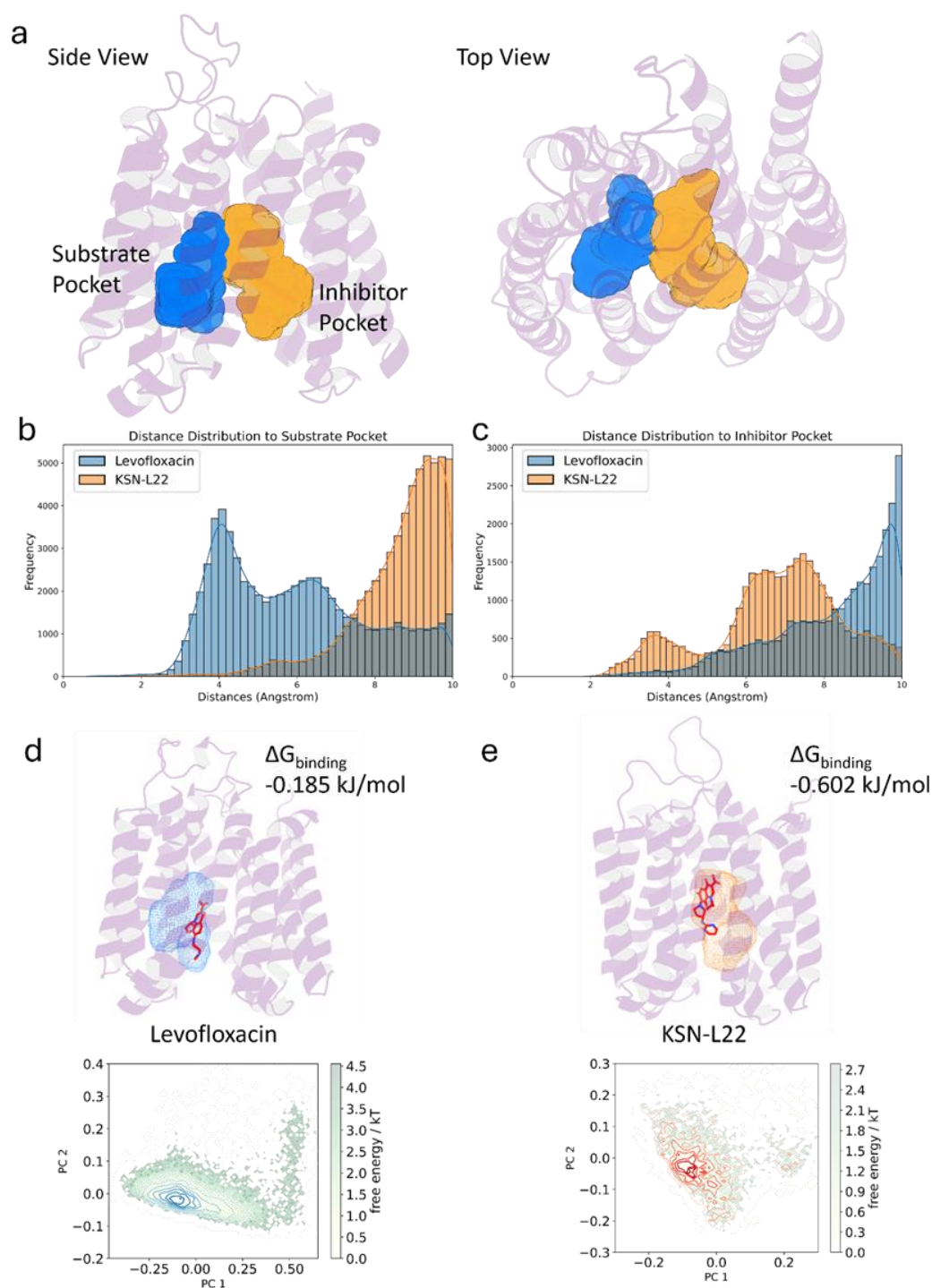


Figure 4: Accumulation assay comparing intracellular antibiotic retention in *S. aureus* 1199 wild-type and 1199B (containing upregulated NorA efflux pump) strains. Multiple unpaired t-tests with Welch correction were applied to determine the significance of the data.

Molecular Dynamics Simulation to Study the Interaction of KSN-L22 and Levofloxacin with NorA efflux pump

419 The molecular dynamics (MD) simulation, along with the Mdpocket analysis, confirmed the existence
 420 of the two adjacent binding pockets within the NorA efflux pump (Figure 5a). The volume of the
 421 inhibitor binding site is 1066 (+/- 182.9) Å³, while the substrate binding site is slightly smaller with a
 422 volume of 903 (+/- 196.2) Å³. To evaluate ligand binding, the distances between the center of mass of
 423 each ligand and both binding pockets are measured (Figure 5b-5c).



424
 425 **Figure 5. a)** Substrate binding hydrophilic pocket (blue) and inhibitor binding hydrophobic pocket (yellow)
 426 **detected by Mdpocket. b,** Distances between COM of the ligand to the COM of the substrate pocket. **c)**

Distances between COM of the ligand to the inhibitor pocket. **d-e**) PCA projection of selected frames, and the position of each ligand inside the protein in the selected stable structure. The binding energy of each system is annotated on the top right of each figure.

The results reveal that **KSN-L22** undergoes a significantly reduced likelihood of interacting with the substrate pocket compared to its parent **levofloxacin**. In contrast, it exhibits a marginally stronger preference for the inhibitor binding pocket. Furthermore, the calculated binding energies suggest that **KSN-L22** exhibits an approximately 3-fold stronger binding affinity to the inhibitor pocket (-0.602 KJ/mol) (Figure 5e) than **levofloxacin** to the substrate pocket (-0.185 KJ/mol) (Figure 5d), reinforcing its inhibitory potential. This provides further evidence that targeting hydrophobic binding pocket within the efflux pump makes ERB-antibiotics like **KSN-L22** less susceptible to efflux.

Type II Topoisomerase Inhibitory Activity of Second Generation ERB-Fluoroquinolones

Evaluation of the inhibitory activities of **levofloxacin**, **KSN-L22**, and **BL-7** against *S. aureus* DNA gyrase in a cell-free environment (Figure 3d, e) revealed a range of IC₅₀ values within the set. The data provide further evidence that ERB-modified fluoroquinolones can maintain on-target activity and the modified compounds work by the same mechanism of action. The activities of all compounds against the wildtype enzyme were broadly comparable (**BL-7** IC₅₀ = 2.52 ± 0.21, **KSN-L22** IC₅₀ = 3.37 ± 0.67, **levofloxacin** IC₅₀ = 8.71 ± 0.95). However, against the mutant S84L enzyme, **BL-7** (S84L IC₅₀ = 3.80 ± 0.42) was approximately seven to ten-fold more active than **KSN-L22** (S84L IC₅₀ = 40.53 ± 3.86) which were themselves approximately five to seven-fold more active against the same enzyme than **levofloxacin** (S84L IC₅₀ = 196.9 ± 36.59). This suggests introduction of the ERB-fragment provides additional contact with the mutant enzyme, leading to improved ability of these compounds to inhibit DNA gyrase in the presence of mutations. This is an additional benefit of the ERB-approach as the interactions with the bacterial target, both mutant and wild-type, can be considered during the design stage of the ERB-fragment and the ERB-modified compound can show improved activity against the mutant target.

Activity of second generation ERB-fluoroquinolones against a large panel of Gram-Positive Bacteria

Lead compounds **KSN-L22** and **BL-7** were evaluated in an extended panel of fluoroquinolone-susceptible and resistant Gram-positive bacteria (Table S7). **BL-7** showed potent activity in a variety of species including *E. faecalis* (MIC₅₀ = 0.125 µg/mL, n = 7 strains), fluoroquinolone-resistant *S. aureus* (MIC₅₀ = x µg/mL, n = 72 strains), *S. epidermidis* (MIC₅₀ ≤ 0.03125 µg/mL, n = 6 strains), *Streptococcus agalactiae* (MIC₅₀ = 0.0625 µg/mL, n = 11 strains), *Streptococcus dysgalactiae* (MIC₅₀ ≤ 0.03125 µg/mL, n = 8 strains), *S. pneumoniae* (MIC₅₀ = 0.0625 µg/mL, n = 9 strains) and *Streptococcus pyogenes* (MIC₅₀ ≤ 0.03125 µg/mL, n = 14 strains). **KSN-L22** also demonstrated a

promising activity profile against *S. agalactiae* (MIC₅₀ = 0.125 µg/mL, n = 10 strains), *S. dysgalactiae* (MIC₅₀ = 0.0625 - 0.125 µg/mL, n = 8 strains) and *S. pyogenes* (MIC₅₀ = 0.0625 µg/mL, n = 12 strains). In general, the ERB-modified compounds showed significantly superior MIC values against fluoroquinolone-resistant bacteria with known up-regulation of efflux pumps containing multiple-target mutations, while commercial fluoroquinolones were either inactive or had much higher MIC values. This data confirms the ability of second generation of ERB-fluoroquinolones to kill a wider spectrum of multidrug-resistant bacteria.

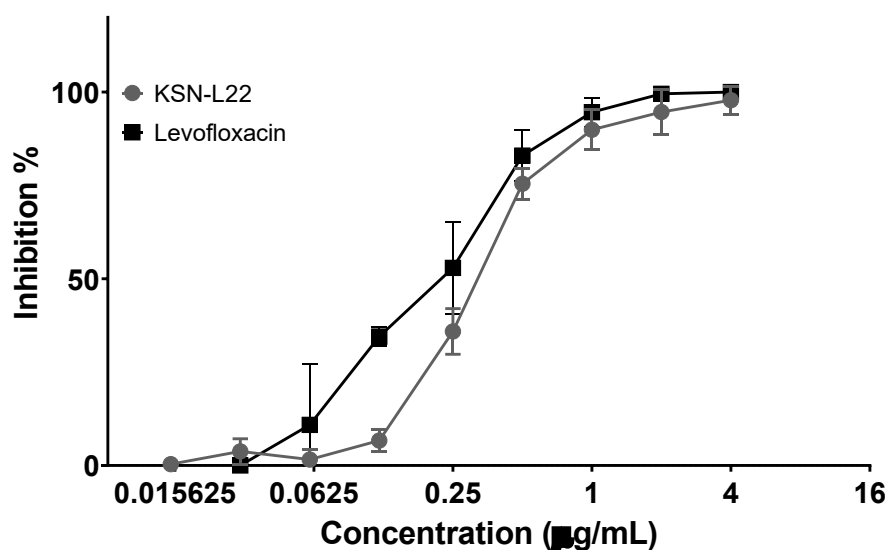
Activity of second generation ERB-fluoroquinolones in Gram-negative Bacteria

Although the focus of the ERB-modifications has been in Gram-positive bacteria with particular focus on NorA efflux pump, sequence alignment of inhibitor binding pockets identified by us revealed a high level of sequence identity and the possibility of developing pan-efflux resistant ERB-modified antibiotics. Additionally, ERB-modification involves targeting more hydrophobic inhibitor binding pockets within the efflux pump which means ERB-modified fluoroquinolones are more hydrophobic compared to the parent antibiotic. Hydrophobic compounds often face additional barriers to permeate across Gram-negative bacterial membrane compared to hydrophilic compounds. Therefore, we tested our second generation ERB-fluoroquinolones **KSN-L22**, **ML-110-014** and **BL-7** in several clinically important Gram-negative species with known up-regulation of RND and MFS efflux pumps (Table S8). In *Acinetobacter baumannii*, a pathogen which utilises a number of complementary efflux systems as part of its antibiotic resistance mechanisms³¹ and for which carbapenem resistance is recognised as an urgent/critical threat by both the WHO³² and CDC³³, all three ERB-fluoroquinolones showed panel-wide activity comparable to that of **levofloxacin** (MIC₅₀ = 4 µg/mL, n = 28 strains) and better than **ciprofloxacin** (MIC₅₀ = 32 µg/mL, n = 28 strains). Of the three leads, **ML-110-114** (MIC₅₀ = 2 µg/mL, n = 28 strains) proved the most active. Time-kill assays for **KSN-L22** and **ML-110-114** showed rapid bactericidal activity in *A. baumannii* strain AYE (Figure S8), establishing both as promising candidates for further development against this pathogen. Lacking an established efflux assay for *A. baumannii* comparable to the reserpine assay used in *S. aureus*, a chequerboard assay comparing the MICs of efflux substrate antibiotics with and without sub-MIC concentrations of **BL-7** was devised; **BL-7** was inferred to be an inhibitor of both AdeB (potentiated known AdeB substrates **ciprofloxacin**, **levofloxacin** and **tobramycin**) and AdeJ (potentiated known AdeJ substrate ceftazidime) but not the AdeG efflux pump (Table 3).

Table 3. *A. baumannii* efflux chequerboard assay. While **BL-7** failed to potentiate chloramphenicol at sub-MIC concentrations, it successfully potentiated the action of ceftazidime and various quinolones and aminoglycosides.

Strain	Efflux Pump	Efflux Substrates	MIC, µg/mL		
			MIC alone	+ BL-7 (0.25 x MIC)	+ BL-7 (0.5 x MIC)
<i>A. baumannii</i> ATCC 17978	AdeABC	Ciprofloxacin	0.25	0.0017-0.188	0.007-0.055
		Levofloxacin	0.125	0.007-0.06	0.009-0.06
		Gentamicin	4	2.13-4.25	0.03-1.75
		Tobramycin	4	1.88-3.76	0.014-0.55
	AdeFGH	Chloramphenicol	64	60	56
	AdeIJK	Ceftazidime	4-16	0.94-3.76	0.003-0.438
	N/a	Colistin	0.25	0.015-0.123	0.0017-0.015
<i>A. baumannii</i> AYE	AdeABC	Ciprofloxacin	64	3.5-15.5	0.188-0.9
		Levofloxacin	4	0.014-1.88	0.012-0.9
		Gentamicin	>1,024	>1,024	60.2 - >1,024
		Tobramycin	256	56-248	0.375-136
	AdeFGH	Chloramphenicol	128	120	224
	AdeIJK	Ceftazidime	>1,024	>1,024	3.76-224
	N/a	Colistin	0.25-0.5	0.015	0.003-0.007

Testing of **KSN-L22** was also conducted in *Neisseria gonorrhoeae*, another CDC Urgent Threat Pathogen³³. In a panel of 75 FQ-resistant clinical isolates, **KSN-L22** demonstrated an MIC₉₀ of 2 µg/mL (Table S8). In *E. coli*, both **KSN-L22** (MIC₅₀ = 0.25-0.5 µg/mL, n = 12 strains) and **ML-110-114** (MIC₅₀ = 0.25 µg/mL, n = 12 strains) were inferior to **ciprofloxacin** (MIC₅₀ ≤ 0.125 µg/mL, n = 12 strains) and **levofloxacin** (MIC₅₀ ≤ 0.06 µg/mL, n = 12 strains). A similar trend was observed in *P. aeruginosa* for **KSN-L22** (MIC₅₀ = 8 µg/mL, n = 9 strains) and **levofloxacin** (MIC₅₀ = 4 µg/mL, n = 9 strains). Comparable IC₅₀s for **levofloxacin** (0.19 ± 0.03 µg/mL) and **KSN-L22** (0.29 ± 0.02 µg/mL) against *E. coli* wildtype DNA gyrase *in vitro* (Figure 6), as well as greater potentiation of **KSN-L22** against *P. aeruginosa* by the membrane permeabiliser polymyxin b nonapeptide (PMBN) (MIC₅₀ = 0.06 µg/mL, n = 7 strains) than **levofloxacin** (MIC₅₀ = 0.125 µg/mL, n = 7 strains), further support the hypothesis that second generation ERB-fluoroquinolones suffer from poor penetration of Gram-negative bacteria due to ERB unit hydrophobicity (Table S8). This suggests optimisation of the ERB-fragments are required to balance the hydrophobicity of the modified antibiotics to ensure permeation across the Gram-negative outer membrane is not compromised as ERB-modification makes the compounds less susceptible to efflux once they successfully enter bacteria. The PMBN data suggests that ERB-modification could overcome efflux mediated resistance in Gram-negative bacteria, but to achieve this, the permeation of the compounds across the Gram-negative outer membrane needs to be carefully considered during the design stage of ERB-modified antibiotics.



Compound	IC ₅₀ (µg/mL)	St. Dev.	P-Value
KSN-L22	0.31	0.01	0.0086
Levofloxacin	0.19	0.03	-

Figure 6. In vitro DNA gyrase inhibition assay using wildtype *E. coli* DNA gyrase demonstrates similar inhibition of the target by KSN-L22 and Levofloxacin.

Resistance to Second-Generation ERB-Fluoroquinolones

To investigate possible resistance mutations against the ERB-fluoroquinolones, serial passaging experiments were conducted in several representative strains of *S. aureus* (Table S9). Whole genome sequencing of the resulting mutants again revealed no efflux-related mutations in the *S. aureus* strains used; instead, previously reported target mutations (GyrA E88K, S84L, ParC E84K, I487L) as well as several off-target mutations were identified.

The mutation frequencies of Gram-positive and Gram-negative bacteria at 2x, 4x and 8x the agar MICs of **KSN-L22** and **BL-7** were determined to assess the likelihood of resistance emergence and further test the hypothesis that ERB-fluoroquinolones bind efflux pumps promiscuously (Table S10). Mutation frequency studies showed that both **KSN-L22** and **BL-7** have lower mutation frequencies than levofloxacin, particularly in *S. aureus* NCTC 13616, where reductions were almost one log and statistically significant ($p = 0.0006$ and 0.0005 , respectively). **BL-7** consistently displayed the lowest frequencies, with no detectable mutants at $\geq 4\times$ MIC in most strains. Similar trends were observed in *E. faecium*, *A. baumannii*, and *E. coli*, suggesting reduced potential for resistance emergence across Gram-positive and Gram-negative species. These results support the hypothesis that ERB-fluoroquinolones engage efflux pump binding pockets, lowering efflux susceptibility and sustaining intracellular drug levels that suppress selection of resistant mutants.

***In vitro* DMPK and toxicity assessment for KSN-L22 and BL-7**

As the overall microbiological profile of **KSN-L22** and **BL-7** against multidrug-resistant Gram-positive bacteria was promising, we decided to perform the *in vitro* and *in vivo* pre-clinical evaluation of the compounds to assess the suitability of these molecules as drug candidates against multidrug-resistant Gram-positive indications. Another important consideration was to determine the effect of ERB-modification of fluoroquinolone scaffold on toxicity and drug-like properties. The off-target toxicity screen using SafetyScreen44 were performed for both **KSN-L22** and **BL-7** with other key biological targets including hERG channel (Table S11, S12). At 10 μ M, **KSN-L22** displayed a clean off-target profile, with only one significant interaction (>50% inhibition) observed at the A₂A receptor (53.6%). Weak to moderate effects (25–50% inhibition) were seen for COX1 (30.3%), kappa opioid receptor (27.4%), and acetylcholinesterase (33.0%), with all other targets showing <25% inhibition and thus not considered significant. **BL-7** exhibited off-target activity with 3-4 receptor, with significant inhibition at the A₂A (82.8%) and M₂ (62.8%) receptors. Weak to moderate inhibition (25–50%) was observed at 5-HT₂A (41.5%), acetylcholinesterase (34.2%), PDE4D2 (31.8%), COX2 (25.9%), and M₁ (25.5%). All remaining targets showed <25% inhibition.

KSN-L22 and **BL-7** were evaluated for their potential to inhibit eight major human cytochrome P450 isoforms using HPLC-UV/VIS and HPLC-MS/MS detection (Table S13). **KSN-L22** exhibited minimal inhibition across most isoforms, with the highest effect observed against CYP3A (midazolam substrate) at 25.2% inhibition at 10 μ M, indicating only weak interaction. All other isoforms showed $\leq$ 13% inhibition, suggesting low risk for clinically relevant drug–drug interactions. **BL-7** displayed a similar low-inhibition profile, with $\leq$ 17% inhibition across CYP1A, CYP2C9, and CYP2C8, and only 10.5% inhibition of CYP3A (midazolam substrate). CYP2B6, CYP2C19, and CYP2D6 inhibition remained <10%. These findings demonstrate that both **KSN-L22** and **BL-7** have low potential to cause significant CYP450-mediated metabolic interactions, supporting their suitability for further preclinical development.

Next, the compounds were evaluated for their toxicity in non-tumour eukaryotic cell line WI38 and in ICR-2 male mice. Both **KSN-L22** and **BL-7** were found to be non-toxic at 100 μ M concentration, highest concentration tested, against WI38. The MTD of **KSN-L22** and **BL-7** was evaluated in male ICR mice following oral administration at escalating doses (100, 200, and 400 mg/kg) (Tables S14 and S15). Clinical observations, body weight, and mortality were monitored for 14 days post-dose. Both compounds were well tolerated at 100, 200 and 400 mg/kg with no mortality, no significant clinical signs of toxicity, and stable body weights. Body weight changes across all groups were within normal physiological variation (<10% loss). Based on these findings, the MTD for both **KSN-L22** and **BL-7** was determined to be $\geq$ 400 mg/kg, supporting their use in subsequent *in vivo* efficacy studies. Taken together, the SafetyScreen44 profiling, CYP450 interaction studies, and MTD data provide reassurance

that ERB modification does not introduce significant off-target liabilities or drug–drug interaction risks. Both KSN-L22 and KSN-BL-7 exhibited clean safety profiles, minimal CYP450 inhibition, and were well tolerated up to ≥ 400 mg/kg in mice. These findings support the use of the ERB approach to develop next-generation fluoroquinolones and potentially extend this strategy to other antibiotic classes.

***In vivo* Pharmacokinetic Studies in Mice**

Both oral and intravenous pharmacokinetic (PK) studies were performed for **KSN-L22** and **BL-7** to determine key PK parameters and assess their suitability for in vivo efficacy studies in mice (Figure S9 and S10). Following intravenous administration at 5 mg/kg, both compounds achieved substantially higher systemic exposure than **levofloxacin**, as reflected by their greater AUC_{0-∞} values (15 728 ng·h/mL for **KSN-L22** and 33 079 ng·h/mL for **BL-7** vs. 9 169 ng·h/mL for levofloxacin). **KSN-L22** exhibited moderate clearance (12.5 mL/min/kg) and a steady-state volume of distribution of 1.4 L/kg, consistent with balanced plasma and tissue distribution, whereas **BL-7** showed low clearance (2.52 mL/min/kg) and low V_{ss} (0.35 L/kg), indicating plasma-restricted disposition. After oral dosing at 20 mg/kg, **KSN-L22** demonstrated improved bioavailability (49.3%) relative to levofloxacin (40.7%), with a higher AUC_{0-∞} and slightly prolonged half-life (5.1 h vs. 5.7 h). **BL-7** showed rapid absorption (T_{max} = 0.5 h), high C_{max} (14 542 ng/mL), and excellent oral bioavailability (82.97%), resulting in markedly enhanced systemic exposure compared with levofloxacin.

***In vivo* Efficacy Studies in Mice**

The efficacy of **KSN-L22** was assessed in a murine thigh infection model using MRSA ATCC 29213 (Figure 7a). Treatment was initiated 2 h post-infection, and animals received **KSN-L22** orally at 50 mg/kg at 2, 8, and 14 h post-infection. **Levofloxacin** was used as a reference under the same regimen. At 26 h, the vehicle group showed a bacterial burden of 7.15 log₁₀ CFU/g. **KSN-L22** treatment reduced bacterial counts to 4.82 log₁₀ CFU/g, corresponding to a 2.33 log₁₀ CFU/g reduction relative to vehicle ($p < 0.0001$, Kruskal–Wallis). Levofloxacin treatment resulted in 4.90 log₁₀ CFU/g, a 2.25 log₁₀ CFU/g reduction relative to vehicle ($p < 0.0001$).

BL-7 was evaluated in a neutropenic murine thigh infection model using MRSA ATCC 33591 (Figure 7b). Mice were immunosuppressed with cyclophosphamide and treated orally with **BL-7** or **levofloxacin** at 50 mg/kg TID at 2, 8, and 14 h post-infection. The vehicle group reached 7.28 log₁₀ CFU/g at 26 h. **BL-7** reduced bacterial counts to 5.68 log₁₀ CFU/g, a 1.60 log₁₀ CFU/g reduction relative to vehicle ($p = 0.0006$), whereas **levofloxacin** reduced counts to 5.58 log₁₀ CFU/g, corresponding to a 1.70 log₁₀ CFU/g reduction relative to vehicle ($p = 0.0001$).

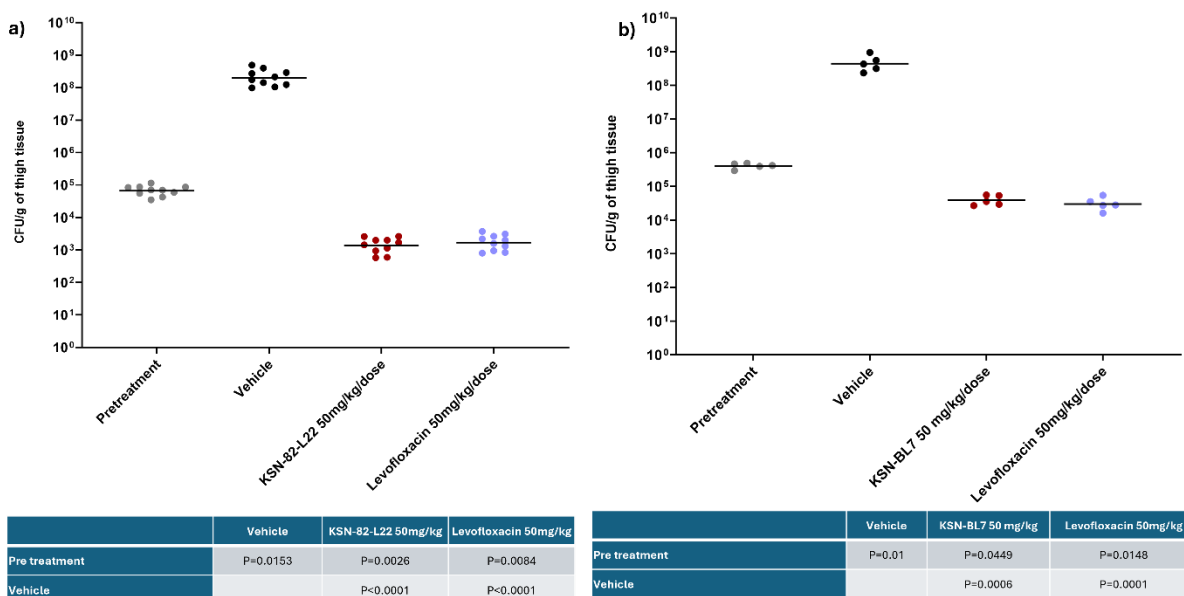


Figure 7: a) *In vivo* efficacy study of **KSN-L22** in MRSA ATCC 29213 and b) *in vivo* efficacy study of **BL-7** in MRSA ATCC 33591 thigh infection models, showing statistically significant reductions in bacterial burdens

Both **KSN-L22** and **BL-7** demonstrated significant reductions in bacterial burden comparable to **levofloxacin** in murine MRSA thigh infection models, supporting their potential as effective *in vivo* antibacterial agents and can be considered promising candidates for further development towards clinical application.

Discussion

Efflux pumps play a central role in bacterial resistance by actively expelling antibiotics, thereby reducing intracellular drug concentrations. By blocking these pumps, either directly or through substrate inhibition, efflux resistance breaker (ERB) technology enhances intracellular antibiotic accumulation and improves potency against resistant strains. This dual-action approach has the potential to mitigate resistance emergence by sustaining intracellular drug levels and reducing the likelihood of resistance mutations.

This study introduces a targeted strategy to counter efflux-mediated resistance through ERB modifications, validated by molecular modelling, biochemical assays, and *in vivo* experiments. The identification of distinct substrate and inhibitor binding pockets within efflux pumps, confirmed by molecular dynamics (MD) simulations, underscores the feasibility of targeting hydrophobic inhibitor sites to reduce efflux susceptibility. By exploiting the differential binding properties of substrates and

inhibitors, ERB technology enhances intracellular antibiotic concentrations while preserving antibacterial efficacy.

One of the most promising attributes of ERB technology is its ability to revitalize existing antibiotics. Fluoroquinolones, a class of antibiotics highly susceptible to efflux, served as a model system for ERB technology. The initial proof of concept was demonstrated using **ML-77-005**, an ERB-modified ciprofloxacin derivative, which exhibited superior activity compared to unmodified **ciprofloxacin** and **ciprofloxacin-NMP** combinations. This enhanced potency was attributed to a significant reduction in efflux susceptibility while maintaining DNA gyrase inhibition. In *Staphylococcus aureus* strains with high-level NorA efflux pump expression, ERB-fluoroquinolones such as **ML-77-005**, **BL-7**, and **KSN-L22** demonstrated reduced efflux susceptibility, leading to increased intracellular drug retention and enhanced antibacterial activity. Structural refinements in second-generation ERB-fluoroquinolones, such as **KSN-L22** and **BL-7**, further validated this approach, with optimized hydrophobic interactions within inhibitor pockets conferring improved activity against fluoroquinolone-resistant strains. MD simulations confirmed that ERB-modified antibiotics exhibit stronger interactions with inhibitor pockets than their parent compounds do with substrate pockets, with a three-fold increase in binding affinity compared to unmodified antibiotics. This mechanistic advantage was reflected in the substantial reduction in minimum inhibitory concentrations (20- to 128-fold lower than unmodified antibiotics), underscoring the potential of ERB technology to counteract multidrug resistance.

Functional validation was provided by intracellular accumulation assays, demonstrating significantly higher retention of **KSN-L22** in efflux-overexpressing strains compared to its parent **levofloxacin**. The observed correlation between increased intracellular accumulation and reduced efflux susceptibility aligned with the superior antibacterial activity of ERB-modified compounds. Additionally, type II topoisomerase inhibition assays confirmed that ERB-modified fluoroquinolones retained their on-target activity, with compounds such as **BL-7** demonstrating enhanced inhibitory potency against mutant enzymes (e.g., S84L mutant). The differential activity between diastereomers, particularly the superior performance of **KSN-L22**, highlights the importance of stereochemistry in optimizing target and efflux pump interactions.

The preclinical evaluation of **KSN-L22** and **BL-7** demonstrated an excellent balance of microbiological potency, pharmacokinetics, and safety. SafetyScreen44 profiling and CYP450 interaction studies confirmed minimal off-target liabilities and low risk for drug–drug interactions, while MTD studies showed both compounds were well tolerated up to ≥ 400 mg/kg with no mortality or significant clinical signs. These findings indicate that ERB modification does not adversely impact the safety profile of fluoroquinolones. Importantly, both compounds exhibited superior systemic exposures compared with levofloxacin following intravenous and oral administration, with **KSN-L22** showing improved

bioavailability and **BL-7** achieving rapid absorption and high C_{max}. Together, these results suggest that **KSN-L22** and **BL-7** possess favourable drug-like properties and robust therapeutic windows, supporting their progression into efficacy studies.

The *in vivo* efficacy of **KSN-L22** and **BL-7** in murine models further support their clinical potential. Both compounds achieved substantially higher systemic exposure than **levofloxacin** following intravenous and oral administration, with **KSN-L22** demonstrating moderate clearance and balanced tissue distribution, and **BL-7** showing low clearance and excellent oral bioavailability. These PK parameters indicate that therapeutically relevant concentrations can be sustained for prolonged periods following oral dosing, an important consideration for clinical translation. Consistent with these profiles, both compounds produced significant, dose-responsive reductions in bacterial burden in murine MRSA thigh infection models. **KSN-L22** achieved a >2 log₁₀ CFU/g reduction relative to vehicle in an immunocompetent model, while **BL-7** demonstrated similar efficacy in a neutropenic model, indicating robust antibacterial activity even in immunocompromised hosts. Together, these data support the advancement of ERB-fluoroquinolones into further preclinical development as promising candidates for the treatment of multidrug-resistant Gram-positive infections. Importantly, the absence of off-target toxicity and the retention of a favourable therapeutic window indicate that ERB modifications do not compromise the safety of fluoroquinolones. However, the diminished activity of certain analogues against Gram-negative bacteria highlights a key challenge: the hydrophobicity of ERB fragments can impede membrane permeation in these organisms. Addressing this limitation through strategic fragment redesign could enhance the applicability of ERB-modified antibiotics.

Unlike traditional strategies that rely on passive structural modifications to improve efflux resistance, ERB technology actively enables antibiotics to bind to inhibitor pockets within efflux pumps, preventing their own efflux. This represents a fundamental shift in antibiotic design, with the additional advantage of potentiating other antibiotics that are efflux substrates. Given that efflux is considered a critical early step in the development of multidrug resistance³⁴ ERB technology addresses a key gap in antimicrobial therapy.

The integration of advanced predictive modelling—including molecular docking, molecular dynamics simulations, and structure-activity relationship analysis—with experimental validation using isogenic strains and biophysical techniques establishes a new framework for antibiotic design. By incorporating desirable properties early in development, ERB technology avoids the challenges and costs associated with modifying antibiotics at later stages. This streamlined discovery process minimizes reliance on trial-and-error approaches and increases the likelihood of identifying clinically viable compounds.

While this study primarily focused on Gram-positive pathogens, preliminary investigations into Gram-negative bacteria (*Acinetobacter baumannii*, *N. gonorrhoeae*) highlight both challenges and opportunities. The hydrophobicity of ERB-modified compounds currently limits their penetration of Gram-negative outer membranes, but strategies such as membrane-permeabilizing agents or tailored ERB fragment designs could address this limitation. The ability of ERB compounds to potentiate other efflux-prone antibiotics also presents opportunities for combination therapy with existing clinical agents.

Current strategies for overcoming antimicrobial resistance (AMR) often rely on modifying existing antibiotics to create derivatives with slightly altered properties. However, these approaches frequently fail to address fundamental resistance mechanisms such as efflux susceptibility. For example, newer generations of fluoroquinolones and tetracyclines have improved target potency but remain vulnerable to the same efflux mechanisms as their predecessors^{34,35}. Consequently, resistance to these derivatives emerges rapidly. Another strategy has been the use of efflux pump inhibitors (EPIs) in combination therapy. However, EPIs have encountered significant challenges, including toxicity, poor pharmacokinetics, and incompatibility with co-administered antibiotics³⁶. Additionally, pharmacokinetic mismatches between EPIs and antibiotics have limited their clinical success³⁷. Unlike these approaches, ERB technology integrates efflux resistance directly into the antibiotic molecule, circumventing the complexities of combination therapy while addressing the root cause of resistance.

Despite its potential, challenges remain. Hydrophobic ERB fragments must be optimized to balance efflux inhibition with membrane permeability in Gram-negative bacteria. Long-term resistance dynamics should also be carefully monitored. Nonetheless, ERB technology offers a transformative approach to antibiotic development by directly addressing a core resistance mechanism. By improving drug-like properties and overcoming efflux-mediated resistance, ERB technology holds promise as a durable and effective solution in the fight against AMR.

Conclusion

Efflux resistance breaker (ERB) technology represents a significant advancement in addressing antibiotic resistance by developing efflux resistant antibiotics. This study demonstrated that ERB modifications enhance intracellular antibiotic accumulation, reduce efflux susceptibility, and maintain antibacterial potency, as validated through molecular modelling, biochemical assays, and *in vivo* studies. The structural refinements in second-generation ERB-fluoroquinolones further support the clinical potential of this strategy, particularly against multidrug-resistant *Staphylococcus aureus* and *Neisseria gonorrhoeae*. Future research should focus on refining ERB modifications to balance hydrophobicity and permeability, expanding its applicability across diverse bacterial pathogens. Given

the growing threat of antimicrobial resistance, ERB technology offers a promising approach to revitalizing existing antibiotics and overcoming a key resistance mechanism, potentially extending the lifespan of current antibiotic therapies while mitigating resistance emergence at its earliest stages.

Supporting Information

The supporting information contains experimental methods, organic synthesis procedures, NMR and HRMS spectra of compounds, additional experimental results, supporting tables, and supporting figures.

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795 **Notes**

796 The authors declare no competing interest.

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