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Original article

Synthesis and evaluation of fluoroquinolone derivatives as substrate-based inhibitors of bacterial efflux pumps

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Abstract

Bacterial efflux pump systems contribute to antimicrobial resistance in pathogenic bacteria. The co-administration of bacterial efflux pump inhibitors with antibiotics is being pursued to overcome efflux-mediated resistance to antibiotics. In this study we investigated a strategy for converting broad-spectrum efflux pump substrates, fluoroquinolone antibacterial agents which are inherently recognized by most efflux transporters, into expanded spectrum efflux pump inhibitors. Employing this strategy against organisms expressing efflux pumps from the MFS, MATE and RND classes of pump systems, we report here the identification of an ofloxacin-based EPI that is a potent inhibitor of MFS (NorA) and MATE (MepA) efflux pumps in *Staphylococcus aureus*. The methods described here outline a process that we feel will be broadly applicable to the systematic identification of bacterial efflux pump inhibitors.

Keywords: Fluoroquinolones; Efflux; Efflux inhibitor; *Staphylococcus aureus*; Antibiotics; NorA

1. Introduction

Survival and growth of bacteria in the presence of antibiotics is the phenotypic expression of drug resistance. Mechanisms of such resistance include the acquisition of target mutations, enzymatic degradation of drug, and a reduction of intracellular drug concentration which can be achieved through decreased membrane permeability or the expression of membrane-based efflux pump systems [1]. The action of such efflux pumps contributes to antimicrobial resistance in pathogenic bacteria [2–4], and can play a role in bacterial pathogenicity [5]. These pump systems can be selective for one class of antimicrobial agent, such as tetracyclines, or relatively nonselective and transport a variety of structurally dissimilar compounds and confer a multidrug resistant (MDR) phenotype [2,4]. As a direct resistance mechanism efflux pumps expel antimicrobial agents once they enter the membrane or cytoplasm, affording subtherapeutic intracellular concentrations. These diminished intracellular concentrations can predispose organisms to the emergence of higher level, adaptive resistance mechanisms [6–11], and have been indicated in reduced postantibiotic effect [12,13]. Intrinsic antimicrobial resistance of certain bacterial species to classes of antibiotics is the result of constitutive expression of drug efflux pumps, thus contributing to the innate multidrug resistance (MDR) nature of some organisms [4,14,15].

Several families of proteins capable of multiple drug extrusion have been described, some of which require ATP hydrolysis for drug transport (ATP binding cassette, or ABC pumps [primary transporters]), and others which require a proton or sodium gradient for drug efflux (major facilitator superfamily [MFS]-, resistance-nodulation-division [RND]-, small multidrug resistance [SMR]-, and multiple drug and toxin extrusion [MATE]-type pumps [secondary transporters]) [2,4,16–18].

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Many of these pumps are capable of extruding multiple structurally unrelated compounds, resulting in an MDR phenotype. Only recently have the structures of components of these pump systems begun to be elucidated [19], affording valuable information for understanding regulation and substrate recognition toward further elucidating the mechanisms of drug recognition and transport [20–23].

Fluoroquinolones (FQs) as antibiotics are extensively used against Gram-positive and Gram-negative bacteria. Gram-positive organisms express FQ efflux systems that utilize the energy of the proton motive force and generally provide modest resistance only to the quinolone-class antibiotics with little or no recognition of other antibacterial agents [1,3,4]. Most notable to the studies here is FQ resistance in *Staphylococcus aureus*, which may be mediated by the *norA*-encoded and *mepA*-encoded FQ efflux pump systems [2,24]. Unlike Gram-positive MDR efflux pump systems that, with respect to antimicrobial agents, almost exclusively recognize FQs, the MDR efflux systems in Gram-negative organisms that efflux FQs also recognize and transport many other antibiotics [2]. Most notable to this study is the MexAB-OprM tripartate efflux system of *Pseudomonas aeruginosa* and the AcrAB efflux system of *Escherichia coli*. These pump systems recognize FQs and impart, depending on levels of expression, efflux-mediated resistance to many FQs [2].

The co-administration of bacterial efflux pump inhibitors (EPIs) with antibiotics is being pursued by a number of academic and commercial laboratories to overcome efflux-mediated resistance to antibiotics [25–32]. These efforts have revealed that inhibiting an efflux pump system responsible for efflux of an antibiotic can reinstate or potentiate activity of that antibiotic [33]. Inhibition of efflux pumps has also been shown to suppress the emergence of higher level resistance mechanisms such as target mutations that occur as a consequence of upregulated efflux pumps [34]. During the last decade dozens of structurally diverse compounds that inhibit various efflux pump systems have been identified, including those from natural sources [35], screening of compound libraries [36], and secondary evaluation of current therapeutics [37]. However, individual EPIs trend toward inhibiting a limited number of efflux systems. This limited spectrum of pump inhibition, presumably due to the number and diversity of pump types, is an obstacle to pump inhibitors finding broad clinical utility in antimicrobial therapy. Strategies to identify single molecules that inhibit multiple classes of efflux systems are needed to advance this field. Co-administration of EPIs that inhibit two different pump types have also shown remarkable synergy when used in combination with certain substrates in Gram-negative organisms [38], further supporting the importance of exploring strategies to identify broad, or at least broader, spectrum pump inhibitors.

In this study we investigated a strategy for the possible conversion of broad-spectrum efflux substrates, FQ antibacterial agents that are inherently recognized by most efflux transporters, into expanded spectrum EPIs by incorporating or integrating structural motifs of EPIs into the FQ structure. Previous work by our group and others has shown that the C-7 position of FQs can accommodate great diversity of structure while often retaining antibacterial activity (Fig. 1) [39,40]. The core structures of ciprofloxacin and ofloxacin were thus chosen for the proof-of-concept study here because properly integrating pump inhibitors and/or structural motifs of pump inhibitors at the C-7 position of these FQs was anticipated to also reveal a number of FQ-based EPIs that retain antibacterial activity.

Many different EPIs have pharmacophore structures that can be attached to, or integrated into, FQ structure. In this study structural features of bisaryl urea-based NorA inhibitors and peptide-based MexAB inhibitors were selected for incorporation at the C-7 position of FQ core structure (Fig. 2). These EPIs were chosen for this initial proof-of-concept study because they are representative of EPIs that target structurally and mechanistically dissimilar pump systems, and because many structural variations of these EPIs have been shown to maintain EPI activity (Fig. 2).

2. Results and discussion

2.1. Synthesis of FQ–EPIs

Conjugation of bisaryl urea-based NorA EPI structure with ciprofloxacin was achieved by attachment of bisaryl urea to the C-7 piperazine of ciprofloxacin (Scheme 1). Phenyl isocyanate (**6**) was coupled with 4-aminobenzylalcohol (**5**) to provide urea **7** [41]. Transformation of the hydroxymethyl group of **7** to a chloromethyl group afforded urea **8**, which was employed in direct alkylation of ciprofloxacin to provide the ciprofloxacin–urea conjugate (**9**) in excellent overall yield.

Conjugation of bisaryl urea-based NorA inhibitor with the core of ofloxacin was achieved by first introducing C-7 amine functionality into the ofloxacin core structure (Scheme 2) [42]. Unlike derivative **9** that retained the C-7 piperazine and thus would be anticipated to retain antimicrobial activity the ofloxacin derivative prepared here (**14**) has the aryl urea attached directly to a C-7 amine group, thus affording very different structure of FQ–EPI conjugate likely at the expense of losing antimicrobial activity. Displacement of the C-7 fluorine on ofloxacin precursor **10** with 4-methoxybenzylamine (**11**) afforded intermediate **12**. Removal of the methoxybenzyl moiety under acidic conditions gave the C-7-amine derivative ofloxacin (**13**) in good yield. Alkylation of amine **13** with chloromethyl urea **8** provided novel ofloxacin-based bisaryl

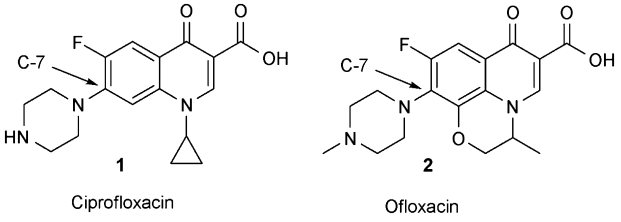


Fig. 1. Fluoroquinolone core structures employed in this work, where C-7 groups of the parent fluoroquinolones ciprofloxacin and ofloxacin (indicated by arrow) are replaced or modified with EPI structural motif.

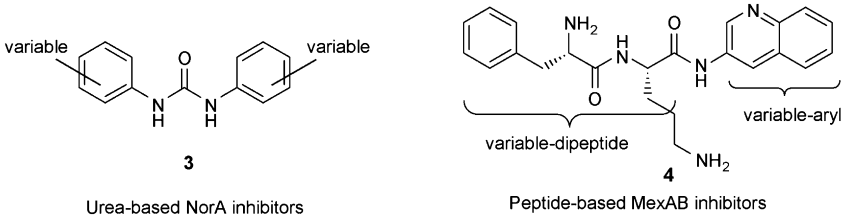


Fig. 2. Representative structures for the two classes of EPIs employed in this study. Structural variations in the aryl rings of bisaryl urea NorA inhibitors and the dipeptide and aryl components of MexAB inhibitors are accommodated, where numerous derivatives retain inhibitory activity against the respective efflux pump systems.

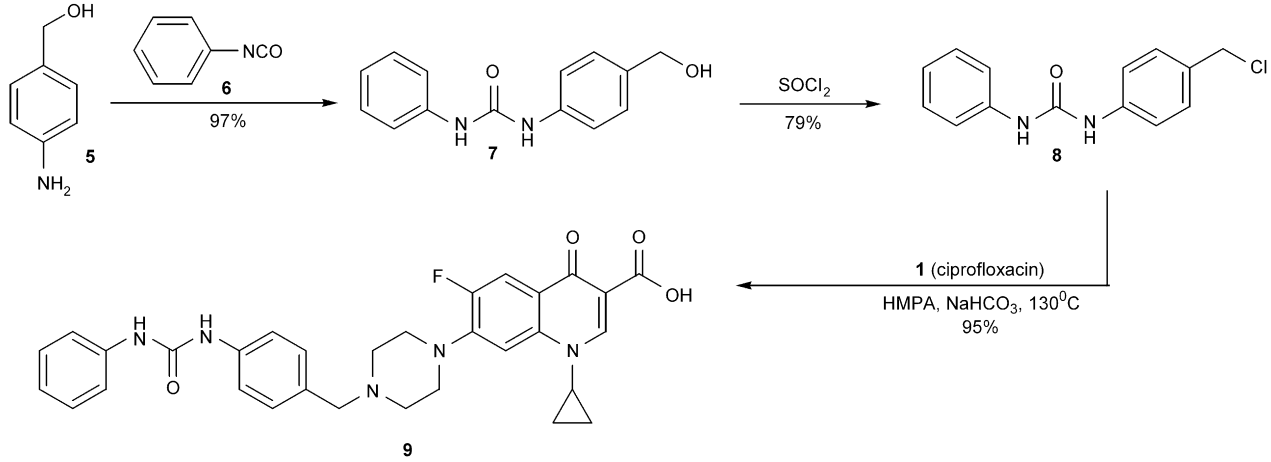
urea conjugate **14** in excellent overall yield (Scheme 2). It is notable that numerous standard conditions to alkylate **13** with chloromethyl-substituted urea **8** unexpectedly failed or gave poor yields, ultimately leading to identification of HMPA as a critical solvent to achieve high yield in this alkylation.

Novel ciprofloxacin derivatives **19** and **24**, bearing dipeptide components of known RND-type efflux pump inhibitors, were synthesized using standard amino acid coupling chemistry to modify the C-7 piperazine of ciprofloxacin (Schemes 3 and 4). Different order of amino acid addition was undertaken because each dipeptide in **19** and **24** has been described in pump inhibitors. Preparation of **19** was achieved by first coupling *N*-Boc protected NHS-activated L-phenylalanine directly to ciprofloxacin (Scheme 3). Removal of Boc protection to give amine **16** was followed by coupling with pentafluorophenyl-actived L-ornithine derivative **17** to give protected dipeptide conjugate **18**. Removal of Fmoc and Boc groups using standard conditions afforded the fully deprotected dipeptide conjugate of ciprofloxacin (**19**).

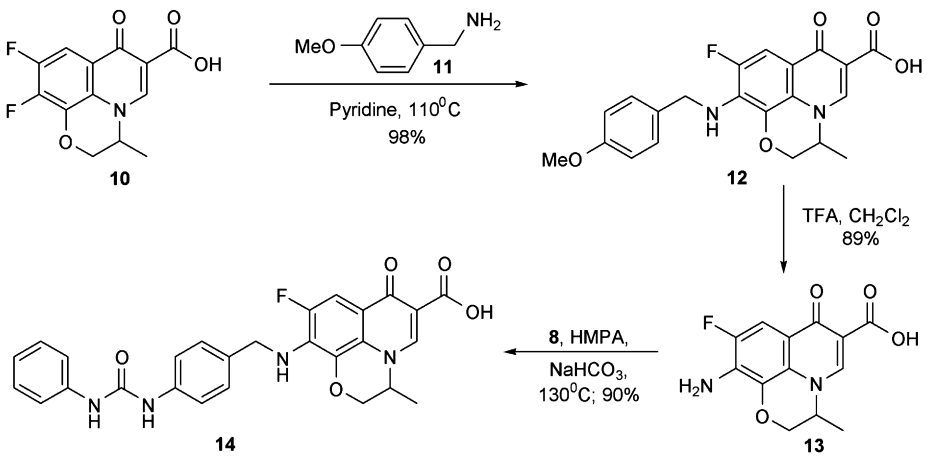
Dipeptide derivative of ciprofloxacin **24** was achieved by first forming amine protected dipeptide **22** (Scheme 4). Carbodiimide-mediated coupling of carboxylic acid **22** with ciprofloxacin followed by amine deprotection steps afforded the target ciprofloxacin-dipeptide derivative **24** in excellent yield. Attempts to similarly couple the dipeptide EPI motif (**22**) directly to the arylamine of C-7-amino-ofloxacin intermediate **13** were unsuccessful, as was direct formation of aryl ureas using aryl isocyanates. While unexpected, this is not surprising because ring electronics that renders the C-7 fluorine readily susceptible to nucleophilic displacement concomitantly diminishes nucleophilicity of the C-7 amine. In our hands this amine group has been refractory to all attempts at acylation-type coupling chemistry employing activated acids and isocyanates.

2.2. Susceptibility testing of FQ–EPIs

Gram-negative organisms utilized in susceptibility testing included *E. coli* AG100 and AG102, which represent parent and *acrAB* overexpressing derivative, respectively [43], and *P. aeruginosa* PAO1, OCR1, and K1536, which represent parent, *mexAB*, and *mexCD* overexpressing derivatives, respectively [44–46]. The *S. aureus* strains utilized all had the SA K1758 background, which is *S. aureus* NCTC 8325-4 in which *norA* has been deleted and replaced with an erythromycin resistance gene [47]. This genetic background was used to eliminate possible confounding effects of the presence of a chromosomal copy of *norA* on subsequent data. Susceptibility testing and determination of MIC values against test organisms was first undertaken to determine which, if any, of the FQ–EPI conjugates retained antimicrobial activity, and to define the concentration range at which the FQ–EPI conjugates could be studied for pump inhibitory activity without killing the test organisms in pump inhibition studies.



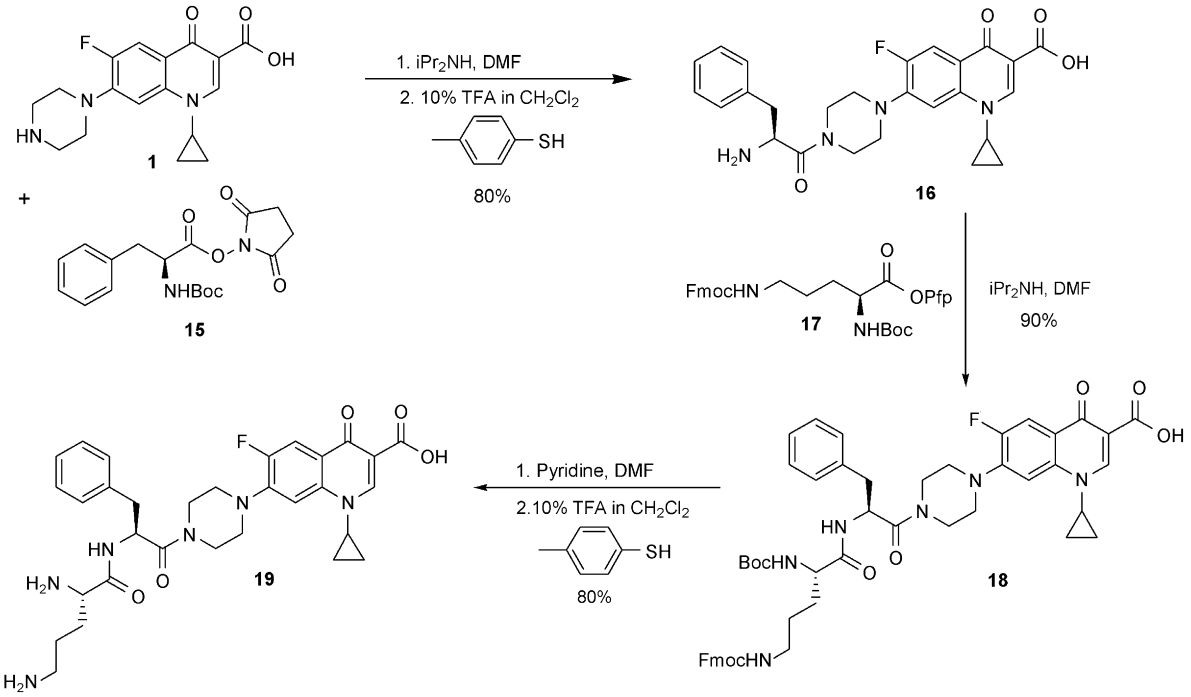
Scheme 1. Synthesis of ciprofloxacin derivative bearing a bisaryl urea EPI at the C-7 position.



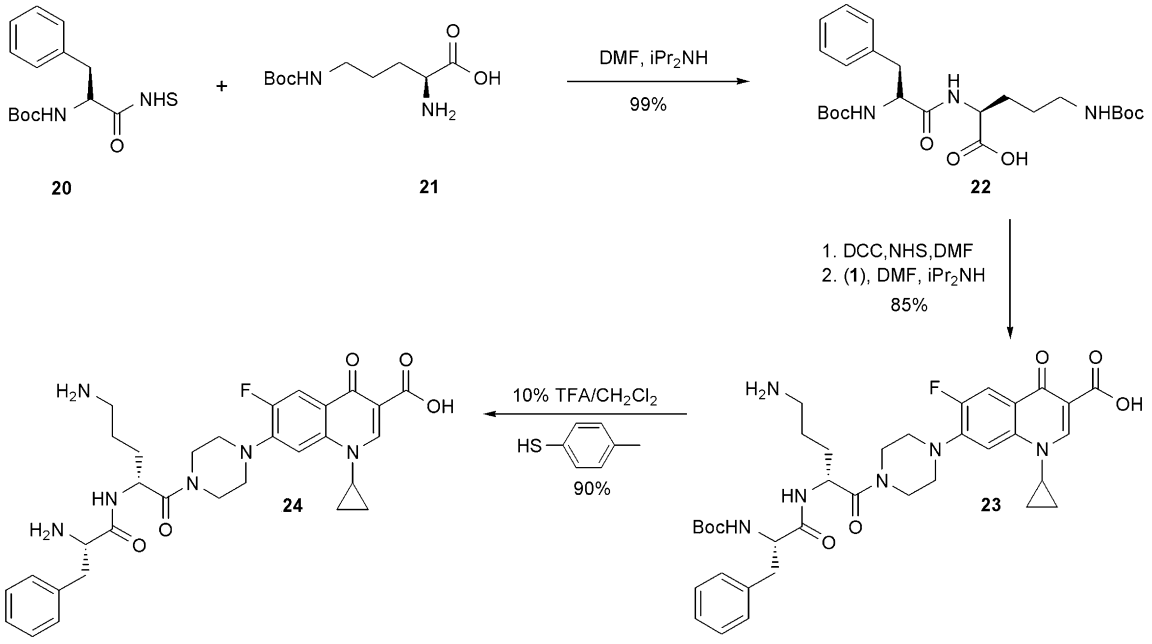
Scheme 2. Synthesis of ofloxacin derivative bearing a bisaryl urea EPI at the C-7 position.

All FQ–EPI compounds had modest (*E. coli*) to minimal (*P. aeruginosa*) antimicrobial activity against Gram-negative test strains; in all cases significantly less activity than the parent fluoroquinolones (Table 1). Unlike the parent compounds few differences in MIC of FQ–EPIs were observed between parent and pump overexpressing strains, thus indicating that these compounds are not substrates, or are much poorer substrates, for the AcrB, MexB, or MexD efflux pumps.

With respect to *S. aureus*, antimicrobial activity of ofloxacin-based EPI **14** and ciprofloxacin-based FQ–EPIs **19** and **24** was poor, whereas bisaryl-containing ciprofloxacin conjugate **9** retained significant activity (Table 1). This result was not unexpected because, of the four FQ–EPI derivatives tested in this proof-of-concept study only **9** retains the C-7 piperazine ring with a basic terminal nitrogen, which is a common requisite feature for most quinolone-class antibiotics. The absence of significant activity of **9** against *P. aeruginosa* and *E. coli* is likely a consequence of reduced membrane permeability due to increased hydrophobic character. The elimination of innate antibacterial activity of **9** against test strains was necessary to assess the potential efflux inhibitory activity of this compound in general, and to specifically determine if equivalent MICs against SA K1758-P and the *mepA* overexpressing strain SA K1758-A were a consequence of inhibiting MepA or simply not being recognized by the efflux pump. To this end, ciprofloxacin-resistant derivatives of *S. aureus* test strains



Scheme 3. Synthesis of ciprofloxacin derivative **19** bearing the L-ornithine–L-phenylalanine C-7 dipeptide.



Scheme 4. Synthesis of ciprofloxacin derivative **24** bearing the L-phenylalanine–L-ornithine C-7 dipeptide.

were created and not only had significantly raised ciprofloxacin MICs (40–160 fold), but were also resistant to more than 100 μg/ml of **9**.

2.3. Inhibition of efflux pumps by FQ–EPIs

Efflux inhibition assays for *P. aeruginosa* utilized strain OCR1, which overexpressed *mexAB* [45]. Concentrations of putative efflux inhibitors **9**, **14**, **19**, and **24** ranged from 0 to 20 μM, representing no more than one third the MIC for any compound. No FQ–EPI achieved significant inhibition of ethidium efflux by MexAB, and RND-type pump system, at any tested concentration.

With respect to *S. aureus*, efflux inhibition studies were focused to evaluation of bisaryl urea conjugates **9** and **14** against the MepA and NorA pump systems. Evaluation of ciprofloxacin-based derivative **9**, which had significant antimicrobial activity against test strains (Table 1), employed derivatives of these strains made resistant to **9**, containing vectors pCU1 and pALC2073 for *norA* and *mepA* expression, respectively. Compound **9** showed no inhibition of ethidium efflux against either the NorA or MepA pumps expressed in these strains. This suggests that **9** being refractory to MepA efflux, and less susceptible to NorA efflux, in susceptibility testing (see Table 1) is likely due to **9** evading the efflux pumps rather than actively inhibiting the pumps. In contrast, ofloxacin-based FQ–EPI **14** was found to be a potent inhibitor of the NorA and MepA efflux pump systems in *S. aureus*. At 0.5 μM, **14** was shown to inhibit NorA-mediated and MepA-mediated efflux of ethidium by 73.6% and 53.4%,

Table 1
Susceptibility data for FQ–EPIs against study strains

Strain	Characteristics	Minimum inhibitory concentration (in μg/ml) of:					
		Ciprofloxacin (**1**)	Ofloxacin (**2**)	**9**	**14**	**19**	**24**
E. coli AG100	Susceptible control for AG102	≤0.019	0.08	5	10	10	10
E. coli AG102	*acrAB* overexpresser	0.04	0.16	10	10	10	10
P. aeruginosa PAO1	Susceptible control for OCR1 and K1536	0.16	1.25	50	50	50	50
P. aeruginosa OCR1	*mexAB* overexpresser	0.78	6.25	50	50	50	50
P. aeruginosa K1536	*mexCD* overexpresser	0.78	6.25	50	100	50	50
SA K1902	Susceptible control for SA K2361	0.16	0.63	0.31	>100	>10	10
SA K2361	*norA* overexpresser	2.5	2.5	1.25	100	>10	>10
SA K1758-P	Susceptible control for SA K1758-A	0.16	0.63	0.63	>100	>10	10
SA K1758-A	*mepA* overexpresser	0.63	1.25	0.63	>100	>10	>10

Table 2
Inhibition of NorA and MepA pump systems by FQ–EPI **14** and the known EPI reserpine

Strain	Concentration of FQ–EPI **14** (μM)			Reserpine (μM)
	0.5	2.0	10	30
S. aureus K2361 (*norA* overexpresser)	73.6%	85.6%	96.4%	81%
S. aureus K1758-A (*mepA* overexpresser)	53.4%	65.9%	84.1%	32%

Data are presented as percent reduction in ethidium efflux.

respectively. Efflux inhibition was dose dependent, in that as the concentration of **14** was increased, greater inhibition was observed (Table 2).

Having identified the novel ofloxacin-based FQ–EPI conjugate **14** as a new, potent inhibitor of NorA (MFS-type pump) and MepA (MATE-type pump) efflux in *S. aureus*, the structural features of **14** responsible for EPI activity were investigated. Comparing EPI activity of **14** with C-7 truncated intermediates **12** and **13** revealed that the C-7 bisaryl urea moiety is critical for pump inhibition (Fig. 3). Similarly, the bisaryl urea alone (**27**), or modified ureas containing the aminomethyl linking group (**25**, **26**) [48], show only approximately 20% inhibition of efflux in the low concentration range employed here. These results demonstrate that potent inhibition of NorA by FQ–EPI **14** is not simply due to the presence of the bisaryl urea attached to the ofloxacin core, but as a consequence of the structure of FQ–EPI **14** as a whole. These results indicate that further design and synthesis of analogs of **14** is warranted, where changes to both the urea moiety and the FQ core structure will likely yield even more potent inhibitors of NorA, and perhaps other *S. aureus* MFS pump-mediated efflux.

3. Conclusions

In this study we tested the hypothesis that broad-spectrum efflux pump substrates might be converted into expanded spectrum EPIs, whereby structural motifs of two different classes of EPIs were incorporated into fluoroquinolone antibiotic structure. From a design standpoint there are dozens of options for linking and/or integrating these two classes of EPIs, as well as other classes of EPIs, with fluoroquinolone core structures. Employing just two classes of EPIs and two fluoroquinolone cores in this proof-of-concept study we demonstrate both successful and unsuccessful outcomes in identifying novel EPIs derived from modification of efflux substrate. Indeed, dipeptide conjugates **19** and **24** did not display EPI activity. This result is perhaps not surprising because SAR of the parent RND pump inhibitors indicates that optimal EPI activity requires the internal amino acid of the dipeptide to be linked directly to an arylamine core (see Fig. 2, structure **4**). Coupling of the EPI dipeptide moiety to C-7-amino quinolone structures, such as the C-7 arylamine analog of the ofloxacin core (Scheme 1, **13**), is one potential approach toward obtaining substrate-based FQ–EPIs based on this design strategy.

Incorporating a bisaryl urea EPI motif at the 7-position of the ofloxacin core afforded the identification of FQ–EPI conjugate **14** as a novel, potent inhibitor of both MFS and MATE efflux pump systems in *S. aureus*. Indeed, FQ–EPI conjugate **14** is one of the most potent inhibitors of the NorA and MepA pump systems reported to date (Table 2), achieving greater than 84% inhibition of efflux at 10 μM, which is a level of inhibition not previously observed with other EPIs against NorA except for compounds such as carbonyl cyanide *m*-chlorophenyl hydrazone that deplete energy by collapsing the

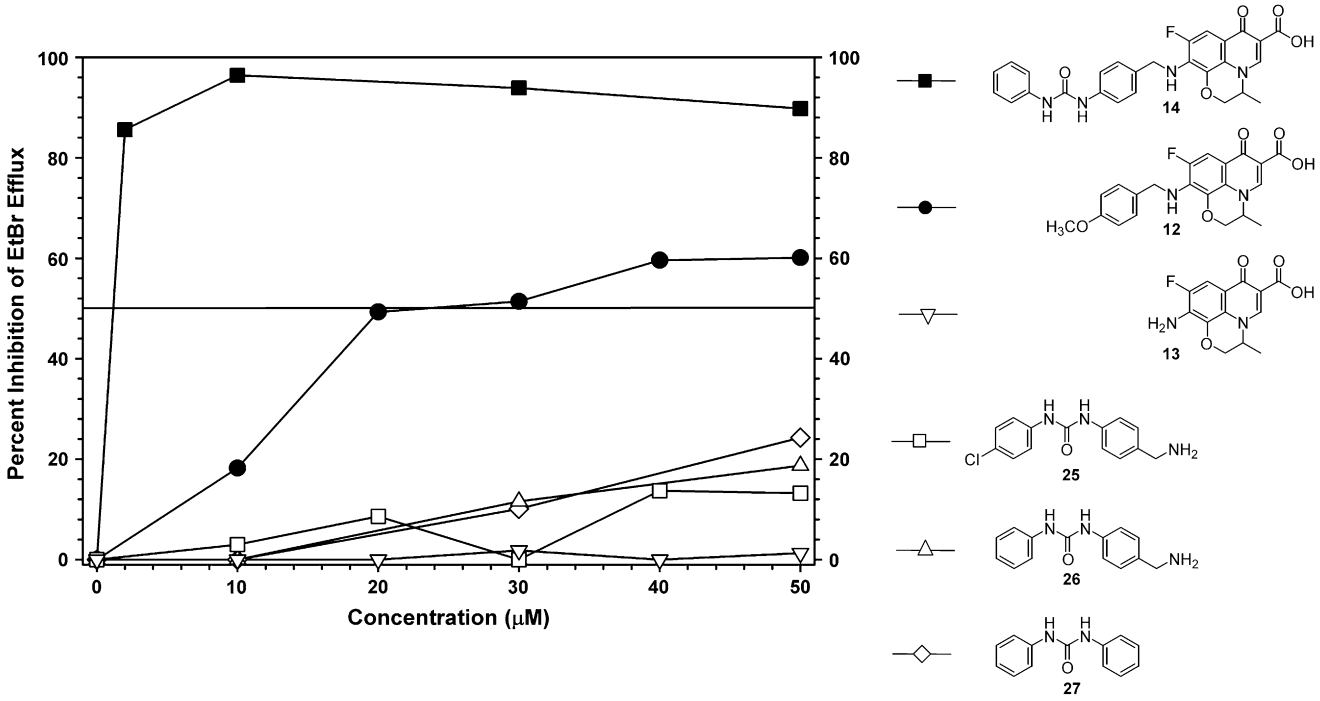


Fig. 3. Inhibition of ethidium efflux from *S. aureus* K2361 (*norA* overexpresser) by FQ–EPI **14** and truncated quinolone and urea components.

transmembrane proton motive force. Further investigation of the structural features of both the quinolone and urea components required for optimal activity of this novel type of EPI is warranted. This study demonstrates that chemical modification of substrates for bacterial efflux pumps is an untapped approach for identifying novel, potent inhibitors of bacterial efflux pump systems, and because broad-spectrum substrates are inherently recognized and gain access to multiple pump systems, may hold the key to future discovery of expanded spectrum EPIs.

4. Experimental

4.1. General synthetic methods

All reactions requiring anhydrous conditions were carried out in oven-dried glassware under an argon atmosphere. Dry solvents were prepared fresh by distillation. All reactants and reagents were of reagent grade or employed as reported. Ciprofloxacin hydrochloride (**1**) and 9,10-difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid (**10**) were from ICN Biomedicals, Inc. and ACROS organics, respectively. Fmoc-Orn(Boc)-OPfp (**17**) was from BACHEM, Boc-Phe-OSu (**15**) from Advanced ChemTech and Boc-L-ornithine (**21**) from Chem-Impex International. Reactions were monitored by thin layer chromatography (TLC) carried out on 0.25 mm silica gel plates (60F254, EM science). Visualization of the TLC plates was by one or more of the following methods: (1) UV light (254 nm), (2) staining solution (1.0 g ceric ammonium sulfate, 24.0 g ammonium molybdate, 31 mL sulfuric acid and 470 mL water) followed by heating, or (3) staining solution (0.3% ninhydrin in *n*-butanol containing 3% acetic acid) followed by heating. Flash chromatography was carried out on silica gel 60 (230–400 mesh, Kieselgel, EM Science). ^{1}H NMR spectra were recorded using QE 300 (General Electric Co.) or Mercury 400 (Varian) spectrometers. Chemical shifts are given in ppm relative to solvent ($CDCl_3$: 7.26, DMSO-d_6: 2.49, acetone-d_6: 2.01, D_2O: 4.80). Additional methods and data for purity analysis of products using analytical HPLC are described in supplementary data.

4.2. Synthesis of FQ–EPIs

4.2.1. N-[4-(Chloromethyl)phenyl]-N′-phenyl-urea (8)

4-Aminobenzylalcohol (**5**) (113 mg, 0.92 mmol) and phenyl isocyanate (**6**) (0.1 mL, 0.92 mmol) were stirred in chloroform (5 mL) at room temperature for 15 min. The resulting white precipitate was filtered, washed with chloroform (15 mL) and dried to give *N*-[4-(hydroxymethyl)phenyl]-*N*′-phenyl-urea **7** (216 mg, 97% yield). Thionyl chloride (0.13 mL, 1.8 mmol) was added dropwise at 30 °C to a suspension of urea **7** (216 mg, 0.89 mmol) in dichloromethane (5 mL). The reaction mixture was stirred at 40 °C for 2.5 h. After completion of the evolution of gas, the reaction was cooled to room temperature, filtered, and solid washed with 50 mL of dichloromethane to give **8** (207 mg, 79% yield). ^{1}H NMR was consistent with that previously reported [41].

4.2.2. 1-Cyclopropyl-6-fluoro-4-oxo-7-{4-[4-(3-phenylureido)-benzyl]-piperazin-1-yl}-1,4-dihydro-quinoline-3-carboxylic acid (9)

Previously prepared *N*-[4-(chloromethyl)phenyl]-*N*′-phenyl-urea **8** (104 mg, 0.4 mmol) was added to a stirred solution of ciprofloxacin hydrochloride (**1**) (66 mg, 0.2 mmol) and sodium bicarbonate (33.6 mg, 0.4 mmol) in HMPA (5 mL). The resulting mixture was stirred at 130 °C for 24 h, at which time no ciprofloxacin remained as determined by TLC (7:1:0.5, ethyl acetate/methanol/water). The reaction mixture was extracted with ethyl acetate, the organic layer was collected, dried over magnesium sulfate, and solvent removed *en vacuo*. The resulting residue was separated by flash chromatography (7:1:0.5, ethyl acetate/methanol/water). Fractions containing **9** were combined, evaporated and dried to afford **9** (105 mg, 95% yield). (**9**) TLC (7:1:0.5 ethyl acetate/methanol/water) $R_f = 0.5$. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): $\delta = 8.65$ (s, 3H), 7.89 (dd, $J = 3.8$, 12 Hz, 1H), 7.56 (d, $J = 7.2$ Hz, 1H), 7.43 (m, 2H), 7.25 (m, 4H), 6.97 (t, $J = 7.0$ Hz, 1H), 3.81 (m, 1H), 3.50 (s, 2H), 2.59 (m, 2H), 1.30 (m, 2H), 1.08 (m, 2H). ^{13}C NMR $(CD_3)_2SO$: $\delta = 7.64$, 35.94, 49.55, 49.60, 52.19, 61.54, 106.52, 106.82, 110.84, 118.18, 121.86, 128.87, 129.53, 131.20, 138.73, 139.27, 139.82, 145.35, 148.07, 152.62, 166.04. ESIMS: calcd for $(M + H^+)$ 556.2315, found 556.2380.

4.2.3. 9-Fluoro-2,3-dihydro-3-methyl-10-[[(4-methoxyphenyl)methyl]amino]-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (12)

A solution of 9,10-difluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid (**10**) (98 mg, 0.35 mmol) and 4-methoxybenzylamine (**11**) (0.14 mL, 1.06 mmol) in pyridine (5 mL) was stirred at 100–110 °C for 48 h. After cooling to room temperature 15 mL of cold water was added and the solution adjusted to pH = 4–5 by addition of acetic acid. The resulting precipitate was collected by filtration and dried under vacuum to give **12** (136 mg, 98% yield). TLC (dichloromethane/methanol 10:1) $R_f = 0.37$. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): $\delta = 15.5$ (s, 1H), 8.85 (s, 1H), 7.49 (d, $J = 12.0$ Hz, 1H), 7.23 (d, $J = 8.4$ Hz, 2H), 6.83 (d, $J = 8.4$ Hz, 2H), 6.64 (m, 1H), 4.86 (m, 1H), 4.55 (d, $J = 9$ Hz, 3H), 4.25 (d, $J = 11.1$ Hz, 1H), 3.68 (s, 3H), 1.40 (d, $J = 7$ Hz, 3H). ^{13}C NMR $(CD_3)_2SO$: $\delta = 18.29$, 47.68, 55.24, 55.42, 68.50, 103.66, 106.46, 114.12, 124.77, 128.64, 131.05, 133.00, 133.13, 146.20, 158.58, 166.77, 176.66. ESIMS: calcd for $(M + H^+)$ 399.1312, found 399.1354.

4.2.4. 10-Amino-9-fluoro-2,3-dihydro-3-methyl-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (13)

Previously prepared **12** (100 mg, 0.25 mmol) was added to a stirred 30% solution of TFA in anhydrous dichloromethane (5 mL) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 5 h, at which time no **12** was

detected by TLC (dichloromethane/methanol, 10:1). The resulting solution was treated with water (10 mL) and adjusted to pH = 9 by the addition of 1 N solution of potassium hydroxide, followed by extraction with dichloromethane (2 × 50 mL). The aqueous layer was collected, and adjusted to pH = 4 by the addition of acetic acid. The resulting precipitate was collected by filtration and dried under vacuum to yield **13** (62 mg, 89% yield). TLC (dichloromethane/methanol 10:1) $R_f = 0.29$. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): $\delta = 15.6$ (s, 1H), 8.87 (s, 1H), 7.53 (d, $J = 12.0$ Hz, 1H), 6.25 (s, 2H), 4.88 (m, 1H), 4.55 (dd, $J = 2.0$, 11.5 Hz, 1H), 4.33 (dd, $J = 2.0$, 11.5 Hz, 1H), 1.44 (d, $J = 7.0$ Hz, 3H). ^{13}C NMR $(CD_3)_2SO$: $\delta = 18.35$, 55.31, 68.37, 102.97, 106.37, 113.57, 124.85, 131.0, 131.68, 131.91, 145.85, 149.34, 166.94, 176.80. ESIMS: calcd for $(M + H^+)$ 279.0736, found 279.0730.

4.2.5. 10-(4-(-3-Phenylureido)-benzylamino)-9-fluoro-3,7-dihydro-3-methyl-7-oxo-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxilic acid (*14*)

Previously prepared *N*-[4-(chloromethyl)phenyl]-*N*′-phenylurea (**8**) (104 mg, 0.4 mmol) was added to a stirred solution of 10-amino-9-fluoro-2,3-dihydro-3-methyl-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid (**13**) (55 mg, 0.2 mmol) in HMPA (5 mL). The resulting mixture was stirred at 130 °C for 24 h, at which time **13** was not detected by TLC (7:1:0.5, ethyl acetate/methanol/water). The reaction mixture was extracted with ethyl acetate, the organic layer was collected, dried over magnesium sulfate, filtered, and solvent removed *en vacuo*. The resulting residue was separated by flash chromatography (7:1:0.5, ethyl acetate/methanol/water). Fractions containing **14** were combined, evaporated and dried to afford 100 mg **14** (90% yield). TLC (7:1:0.5 ethyl acetate/methanol/water) $R_f = 0.5$. ^{1}H NMR (300 MHz, $(CD_3)_2SO$): $\delta = 8.59$ (s, 1H), 8.53 (s, 1H), 7.2–7.4 (m, 7H), 7.2 (t, $J = 7.5$ Hz, 2H), 6.81 (t, $J = 7.5$ Hz, 1H), 5.65 (br s, 2H), 5.04 (d, $J = 4.5$, Hz 2H), 4.56 (m, 1H), 4.3 (dd, $J = 2.0$, 11.0 Hz, 1 H), 4.1 (dd, $J = 2.5$ Hz, 11.0, 1H), 1.25 (d, $J = 7$ Hz, 3H). ^{13}C NMR $(CD_3)_2SO$: $\delta = 18.18$, 54.29, 65.44, 68.49, 97.62, 103.31 ($J_{CF} = 21$ Hz), 108.702, 118.45, 118.55, 118.66, 122.28, 129.17 ($J_{CF} = 21$ Hz), 130.41, 139.83 ($J_{CF} = 8$ Hz), 146.16, 152.96, 165.30. ESIMS: calcd for $(M + Na^+)$; 525.1550, found; 525.1572.

4.2.6. 7-[4-(2-Amino-3-phenyl-propionyl)-piperazin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (*16*)

Diisopropylamine (56 μL, 0.4 mmol) was added to a stirred solution of Boc-L-phenylalanine *N*-hydroxysuccinimide ester (**15**) (79 mg, 0.22 mmol) and ciprofloxacin hydrochloride (**1**) (66 mg, 0.2 mmol) in DMF (3 mL). The solution was stirred for 4 h at room temperature, at which time no **1** was detected by TLC and a single product had formed (TLC 7:1:0.5 ethyl acetate/methanol/water, $R_f = 0.71$). The mixture was concentrated under reduced pressure, dissolved in a minimum volume of methanol and precipitated by addition to diethyl ether. Precipitate was collected by centrifuging and dried under high vacuum. The resulting solid (82 mg) was dissolved in anhydrous dichloromethane (5 mL) and treated with *p*-toluenethiol (17 mg, 0.14 mmol) and trifluoroacetic acid (0.5 mL). After 2 h, the reaction mixture was concentrated under reduced pressure. The resulting residue was washed with hexane (2 × 50 mL), dissolved in minimum volume of methanol and precipitated by addition to ice-cold diethyl ether (50 mL). The resulting precipitate was centrifuged and dried under high vacuum to give **16** (50 mg, 75% yield). TLC (7:1:0.5 ethyl acetate/methanol/water) $R_f = 0.1$. ^{1}H NMR (300 MHz, (CD_3OD)): $\delta = 8.7$ (s, 1H), 7.9 (d, $J = 13.0$ Hz, 1H), 7.2 (m, 8H), 4.6 (m, 1H), 3.2 (m, 4H), 3.3 (m, 2H), 3.0 (m, 7H), 2.3 (m, 1H), 1.2 (m, 2H), 1.0 (m, 2H). ^{13}C NMR (CD_3OD): $\delta = 7.19$, 17.91, 27.28, 35.54, 41.57, 45.10, 51.27, 96.29, 107.48, 126.62, 128.27, 129.34, 129.38, 136.83, 139.33, 148.09, 162.11, 168.20, 170.96. ESIMS: calcd for $(M + H^+)$; 479.2050, found; 479.2081.

4.2.7. 1-Cyclopropyl-7-{4-[2-(2,5-diamino-pentanoylamino)-3-phenyl-propionyl]-piperazin-1-yl}-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (*19*)

To a stirred solution of (**16**) (50 mg, 0.10 mmol) and Fmoc-Orn(Boc)-Pfp (**17**) (68.3 mg, 0.11 mmol) in DMF (5 mL) was added isopropylamine (28 μL. 0.2 mmol). After 0.5 h the resulting mixture was diluted with ethyl acetate (50 mL), transferred to a separatory funnel, and washed with a 5% solution of $NaHCO_3$ (2 × 100 mL) and then brine. The organic layer was collected, dried over Na_2SO_4, filtered and evaporated. The resulting residue (**18**) was subjected to a deprotection step without any further purification. To a solution of **18** in DMF (3 mL) was added piperidine (0.75 mL), and the mixture was stirred at room temperature for 15 h. The reaction mixture was concentrated under the reduced pressure, dissolved in dichloromethane (6 mL), and treated with *p*-toluenethiol (24.8 mg, 0.2 mmol) followed by trifluoroacetic acid (0.6 mL). This mixture was stirred at room temperature for 2 h, diluted with ethyl acetate (5 mL) and evaporated to dryness. The resulting solid was dissolved in a minimum volume of methanol, precipitated by addition to hexanes (150 mL), centrifuged and decanted. The collected precipitate was dissolved in methanol, precipitated with cold diethyl ether, centrifuged, decanted and dried to give **19** in 80% overall yield. ^{1}H NMR (300 MHz, (CD_3OD)): $\delta = 8.76$ (s, 1H), 7.84 (d, $J = 13.0$ Hz, 1H), 7.38 (m, 6H), 5.13 (t, $J = 7.5$ Hz, 1H), 3.44–4.05 (m, 7H), 2.90–3.15 (m, 6H), 2.48 (br s, 1H), 1.85 (m, 4H), 1.43 (m, 2H), 1.23 (m, 2H). ^{13}C NMR (CD_3OD): $\delta = 8.69$, 25.47, 29.74, 37.04, 39.26, 40.17, 43.19, 46.75, 51.37, 56.08, 107.7, 108.34, 112.33 ($J_{CF} = 35$ Hz), 116.51, 120.52 ($J_{CF} = 12$ Hz), 129.05, 130.98, 135.88, 140.91, 146.79 ($J_{CF} = 14$ Hz), 155.36 ($J_{CF} = 243$ Hz), 163.43, 169.62, 170.31, 178.31 ($J_{CF} = 10$ Hz). ESIMS: calcd for $(M + H^+)$; 593.2843, found; 593.2877.

4.2.8. 5-tert-Butoxycarbonylamino-2-(2-tert-butoxycarbonylamino-3-phenyl-propionylamino)-pentanoic acid (*22*)

Boc-protected L-ornithine (**21**) (362 mg, 1.0 mmol), Boc-Phe-OSu (**20**) (232 mg, 1.0 mmol) and diisopropylamine

(140 μL, 1.0 mmol) were combined in DMF (10 mL) and stirred overnight at room temperature. After removing solvent on high vacuum the desired dipeptide (**22**) was obtained in 99% yield (475 mg). ^{1}H NMR (300 MHz, (CD_3OD)): $\delta = 7.28$ (m, 5H), 4.27 (dd, $J = 7.0$ Hz, 7.5, 1H), 4.02 (dd, $J = 5.0$, 7.5 Hz, 1H), 3.16 (m, 2H), 3.03 (m, 1H), 2.84 (m, 1H), 2.68 (m, 2H), 1.49–1.80 (m, 4H), 1.45 (s, 9H), 1.38 (s, 10H). ^{13}C NMR (CD_3OD): $\delta = 24.89$, 25.45, 27.28, 27.37, 38.53, 53.04, 79.05, 126.37, 128.05, 128.98, 129.32, 137.16, 156.19, 156.70, 173.67.

4.2.9. 7-{4-[5-Amino-2-(2-amino-3-phenyl-propionylamino)-pentanoyl]-piperazin-1-yl}-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid (23)

Boc-protected dipeptide L-Phe–L-Orn (**22**) (100 mg, 0.208 mmol) and *N*-hydroxysuccinimide (26.3 mg, 0.23 mmol) were dissolved in DMF (2 mL). To the resulting solution was added *N*,*N*′-dicyclohexylcarbodiimide (51.5 mg, 0.25 mmol) and the mixture stirred for 20 h at room temperature. The resulting mixture of activated acid was treated with diisopropylamine (58.3 μL, 0.42 mmol) followed by addition of ciprofloxacin hydrochloride (**1**) (68.8 mg, 0.208 mmol). After 18 h, 20 mL of ice-cold water was added to the reaction mixture and the product extracted with excess ethyl acetate. The organic layer was dried over Na_2SO_4, filtered, and evaporated to give Boc-protected dipeptide conjugate (**23**) (135 mg, 85% yield) TLC (ethyl acetate/methanol/water, 7:1:0.5) $R_f = 0.67$. Cleavage of Boc groups was achieved by treatment of conjugate **23** (100 mg, 0.12 mmol) with 10% TFA solution in anhydrous dichloromethane (5 mL) in the presence of *p*-toluenethiol (31 mg, 0.25 mmol). After TLC indicated complete reaction, 5 mL of ethyl acetate was added and solvent was removed in vacuum. The resulting residue was washed with hexanes (3 × 50 mL), dissolved in minimum volume of methanol, precipitated by addition to cold diethyl ether (30 mL), centrifuged, decanted and dried to give **24** as a TFA salt (64 mg, 90% yield). TLC (7:1:0.5 ethyl acetate/methanol/water) $R_f = 0.1$. ^{1}H NMR (300 MHz, (CD_3OD)): $\delta = 8.73$ (s, 1H), 7.85 (d, $J = 13.0$, 1H), 7.56 (m, 1H), 7.3 (m, 5H), 4.53 (m, 1H), 4.03 (m, 2H), 3.77 (m, 4H), 3.47 (m, 3H), 3.2 (m, 4H), 1.8 (m, 2H), 1.6 (m, 2H), 1.4 (m, 2H), 1.2 (m, 2H). ^{13}C NMR (CD_3OD): $\delta = 7.17$, 23.86, 27.86, 35.56, 37.45, 38.52, 41.08, 45.14, 49.88, 54.46, 106.08 ($J_{CF} = 2.8$ Hz), 106.77, 111.32 ($J_{CF} = 26$ Hz), 114.90, 118.78, 119.52 ($J_{CF} = 2.4$ Hz), 127.43, 128.70, 129.05, 134.26, 139.29, 145.2 ($J_{CF} = 9$ Hz), 147.99, 153.61 ($J_{CF} = 247$ Hz), 167.23, 168.01, 168.52, 176.89. ESIMS: calcd for ($M + H^+$); 593.2843, found; 593.2898.

4.2.10. General methods employed for preparing bisaryl urea derivatives N-(4-chlorophenyl)-N′-[4-(aminomethyl)phenyl]urea (25) and N-phenyl-N′-[4-(aminomethyl)phenyl]urea (26)

Briefly, aminomethylaniline (196.4 mg, 0.9 mmol) was treated with di-*tert*-butyl dicarbonate (0.09 mL, 0.8 mmol) in a 2:1 THF/water solution (1.5 mL). After 18 h water (20 mL) was added and ethyl acetate extraction followed by evaporation afforded 4-*tert*-butoxycarbonylaminomethyl aniline. A solution of 4-*tert*-butoxycarbonylaminomethyl aniline (198 mg, 0.89 mmol) in chloroform (5 mL) was treated with phenyl isocyanate (81.3 μL, 0.89 mmol, for **25**) or 4-chlorophenyl isocyanate (113.8 μL, 0.89 mmol, for **26**). The resulting precipitates were filtered, washed with chloroform and dried to give the Boc-protected ureas in near quantitative yield. Treatment of each with a 10% solution of TFA in anhydrous dichloromethane, followed by separation as reported [48], afforded **25** and **26**.

4.3. Susceptibility testing and evaluation of efflux inhibition

4.3.1. Antimicrobial susceptibility testing

Antimicrobial activities of test compounds were determined using a standard broth microdilution approach following CLSI guidelines [49]. MIC data were used to determine appropriate concentrations to employ in efflux inhibition assays to avoid possible confounding effects of inhibition of bacterial growth.

4.3.2. Construction of plasmids

Plasmids were constructed to allow overexpression of *norA* or the gene for the newly-described MATE family multidrug transporter MepA in the SA K1758 background. Plasmid vectors included pCU1 and pALC2073 for *norA* and *mepA* expression, respectively [50,51]. Using PCR *norA* and its promoter were amplified and then cloned into pCU1, producing pK364. Likewise the *mepA* coding region was amplified and then cloned into pALC2073, producing pK436 and placing the gene under the control of the *xyl*/*tetO* tetracycline inducible promoter of pALC2073 [24]. Plasmids were electroporated into SA K1758 producing SA K1902 (empty pCU1), K2361 (pK364), SA K1758-P (empty pALC2073), and SA K1758-A (pK436). Prior to MIC testing all *S. aureus* strains were grown overnight in the presence of 10 μg/ml chloramphenicol to maintain plasmids, and tetracycline (50 ng/ml) was included in experiments involving strains containing the pALC2073 backbone to induce high-level expression of *mepA* from pK436.

4.3.3. Creation of ciprofloxacin-resistant derivatives of S. aureus

To accurately assess the efflux inhibitory potential of compounds that have good antimicrobial activity such as **9**, it was necessary to separate the antibacterial and possible efflux inhibitory effects. SA K1758 was passed repeatedly on ciprofloxacin gradient plates to obtain a mutant with a significantly raised MIC [52]. Plasmids pCU1, pALC2073, pK364, and pK436 were electroporated into this background to produce versions of SA K1902, SA K2361, SA K1758-P and SA K1758-A having ciprofloxacin MICs increased to 6.25, 100, 6.25, and 100 μg/ml, respectively. Compared to derivatives containing empty plasmids, the further 16-fold increase in ciprofloxacin MIC observed for both the *norA* and *mepA*

overexpressing, ciprofloxacin-resistant strains is indicative of avid efflux pump overexpression.

4.3.4. Efflux inhibition assay

The loss of ethidium cation from *S. aureus* cells was determined fluorometrically as previously described [53]. Experiments were performed in duplicate, and the results were expressed as mean total efflux over a 5 min time course. The effect of inclusion of increasing concentrations of inhibitors was determined and total efflux under these circumstances was compared to that in the absence of inhibitors to arrive at the percent reduction in efflux.

For *P. aeruginosa* organisms were grown overnight in LB broth, diluted 25-fold in fresh, pre-warmed LB broth and subjected to vigorous agitation (300 rpm) at 37 °C until the optical density at 600 nm (OD_{600}) reached 1.2–1.5. Cells were then pelleted, washed and resuspended in ice-cold uptake buffer (100 mM NaCl, 50 mM sodium phosphate [pH 7.2], 0.1% glycerol) to an OD_{600} of approximately 1.2. The organism suspension was held on ice until use. At this point, organisms were diluted with ice-cold uptake buffer to an OD_{600} of 0.2. Organisms then were warmed to 30 °C for 10 min in a shaking water bath (300 rpm), at which point ethidium bromide was added (final concentration, 10 μM). Aliquots (1 ml) were removed at frequent intervals for determination of fluorescence ($\lambda_{ex} = 530$, $\lambda_{em} = 600$). Intracellular ethidium achieved steady state 10 min after its addition (data not shown). At this point various concentrations of inhibitors were added in some experiments and incubation continued for an additional 10 min. The difference in fluorescence at 20 min between experiments without and with inhibitors was determined to arrive at a percent reduction in efflux. Carbonyl cyanide *m*-chlorophenyl hydrazone (250 μM) added at the 10 min time point in parallel experiments served as a positive control.

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Appendix. Supplementary data

^{1}H NMR spectra and purity analysis for FQ–EPI conjugates evaluated in microbiological studies. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.01.042.

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