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Eradicating uropathogenic *Escherichia coli* biofilms with a ciprofloxacin–dinitroxide conjugate†

Anthony D. Verderosa,^{ab} Jessica Harris,^a Rabeb Dhouib,^b
Makrina Totsika ^{*b} and Kathryn E. Fairfull-Smith ^{*a}

Urinary tract infections (UTIs) are amongst the most common and prevalent infectious diseases worldwide, with uropathogenic *Escherichia coli* (UPEC) reported as the main causative pathogen. Fluoroquinolone antibiotics are commonly used to treat UTIs but for infections involving UPEC biofilms, which are commonly associated with catheter use and recurrent episodes, ciprofloxacin is often ineffective. Here we report the development of a ciprofloxacin–dinitroxide (CDN) conjugate with potent UPEC biofilm-eradication activity. CDN **11** exhibited a 2-fold increase in potency over the parent antibiotic ciprofloxacin against UPEC biofilms. Moreover, CDN **11** resulted in almost complete UPEC biofilm cell eradication (99.7%) at concentrations as low as 12.5 μ M, and significantly potentiated ciprofloxacin's biofilm-eradication activity against UPEC upon co-administration. The biofilm-eradication activity of CDN **11** highlights the potential of nitroxide functionalized antibiotics as a promising strategy for the treatment of biofilm-related UTIs.

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1. Introduction

Urinary tract infections (UTIs) affect millions of individuals worldwide and equate to billions of dollars in health care expenditure each year.^{1,2} Acute cystitis is the most common clinical syndrome of UTI, and women are at the highest risk with nearly 50% suffering from a UTI at some point in their lifetime.³ Men are less susceptible to UTIs, but their risk increases with age, and once infected they are more likely to experience a more severe UTI syndrome, such as urosepsis or pyelonephritis.⁴ The recurrence of UTIs is also a major issue as up to 30% of women with acute infection will suffer a recurrent episode within the first four months.³ UTIs also frequently occur in hospitalized patients, especially among patients with indwelling catheters, diabetes, and bladder cancer.^{5–7} Consequently, UTIs are ranked among the most prevalent infectious diseases worldwide.⁸ *Escherichia coli* is the primary causative pathogen associated with UTIs and is present in approximately 80% of all diagnosed cases.³ *E. coli* is a commensal inhabitant of the gastrointestinal tract and

performs an important role in maintaining the stability of the luminal microbial flora and normal intestinal homeostasis.⁹ As a species, *E. coli* is highly diverse at the genomic level, with strains clustering in four main phylogroups (A, B1, B2 and D). Strains from different phylogroups have distinct gene content, phenotypic and ecological characteristics, which allow them to adapt to different niches. Strains belonging to phylogroup B2 and D are responsible for most extra-intestinal infections, including UTIs in which case they are referred to as uropathogenic *E. coli* (UPEC).

UPEC have a variety of virulence factors that contribute to pathogenicity and allow them to form biofilms during UTI. Biofilms are communities of surface-associated bacteria encased in a protective extracellular polymeric substance (EPS) and can form on abiotic surfaces, such as urinary catheters, or on host tissues and inside cells, such as UPEC intracellular bacterial communities (IBCs) within luminal bladder epithelial cells. The ability of UPEC to form biofilms is a major contributor to UTI severity, persistence, and recurrence.^{10–12} UPEC biofilms render treatment with current antimicrobials difficult as they are often extremely tolerant to conventional antibiotics.¹³ Treatment of uncomplicated acute UTIs normally involves administering a short course of antibiotics, and one of the most potent and commonly administered antibiotic therapies for UTIs is ciprofloxacin.¹⁴ However, catheter-associated and recurrent UTIs, which typically involve UPEC biofilm formation, are refractory to even the highly potent antibiotic ciprofloxacin.^{15,16} The high tolerance of biofilm-residing cells to antibiotics can involve many factors; however, an antibiotic's inability to

^a Queensland University of Technology, School of Chemistry, Physics and Mechanical Engineering, 2 George St, Brisbane, Queensland 4001, Australia. E-mail: k.fairfull-smith@qut.edu.au

^b Queensland University of Technology, School of Biomedical Sciences, Institute of Health and Biomedical Innovation, 300 Herston Rd, Brisbane, Queensland 4006, Australia. E-mail: makrina.totsika@qut.edu.au

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penetrate and diffuse evenly throughout the EPS of the biofilm is considered to be a major contributing factor.¹⁷ Consequently, strategies which involve dispersing biofilm-residing cells have become an important area of anti-biofilm research.

Biofilm dispersal agents facilitate biofilm cell detachment and subsequently trigger a transition from the sessile quiescent state (antibiotic tolerant) to the motile free-swimming state (antibiotic susceptible). Consequently, a variety of biofilm dispersal agents have been discovered (for example, autoinducers,¹⁸ nitric oxide,¹⁹ nitroxides,²⁰ and amino acids²¹). We have previously shown that nitroxides (long-lived stable free radical species) can inhibit or disperse *Pseudomonas aeruginosa* and *E. coli* biofilm-residing cells returning them to an antimicrobial susceptible state.^{20,22,23} While biofilm dispersal agents are indeed a promising approach to biofilm remediation, they are not without limitations. As most dispersal agents are inherently non-antimicrobial and biofilm dispersal is a known method of pathogen translocation, which can seed infection in new areas, the use of these agents must be co-administered with an antimicrobial agent to prevent the spread of infection. However, co-administration does not guarantee that both agents will be present at the target site in the required concentrations. Consequently, methods for circumventing this shortcoming, such as tethering the dispersal agent to the antibiotic,^{24–26} and/or developing new antimicrobial agents that can directly target biofilm residing cells (for example, halogenated phenazines (HPs),²⁷ quaternary ammonium compounds (QACs),²⁸ and antimicrobial peptides (AMPs)²⁹) have become attractive approaches to biofilm remediation. While these agents have demonstrated potent biofilm-eradication capabilities, none to date have been designed to target UPEC biofilms.

As we have previously established that the nitroxide 4-carboxy-2,2,6,6-tetramethylpiperidin-1-yloxy (CTEMPO) can disperse enterohemorrhagic *E. coli* (EHEC) biofilms and that a combined treatment (CTEMPO/ciprofloxacin) improves the efficacy of ciprofloxacin against EHEC biofilms,²² we were curious to investigate whether combining the anti-biofilm activity of the nitroxide CTEMPO with the antimicrobial activity of ciprofloxacin would produce an agent with potent UPEC biofilm-eradication capabilities. Furthermore, as the nitroxide and the antibiotic are potentially more effective at different concentrations, we also sought to examine whether the number of nitroxides per conjugate was directly related to the biofilm-eradication efficacy of the resulting compound. Thus, herein we report a synthetic strategy to tether multiple nitroxides to ciprofloxacin and reveal the potent UPEC biofilm-eradication capabilities of the resulting derivative.

2. Results and discussion

2.1. Design and synthesis of ciprofloxacin–nitroxide(s) conjugates

In order to test the anti-biofilm potency of ciprofloxacin conjugated to multiple nitroxide units, we developed a simple and effective synthetic strategy for the sequential addition of one, two or more nitroxides to a single ciprofloxacin moiety.

Our approach involved utilizing an amino acid as the linker between the nitroxide(s) and ciprofloxacin, as amino acids have been previously shown to exhibit effective anti-biofilm properties.²¹ More specifically, the amino acid lysine has demonstrated potent *E. coli* antimicrobial activity and biofilm disruption capabilities when coupled to other anti-biofilm agents.³⁰ Thus, incorporating a lysine linker between the nitroxide(s) and ciprofloxacin was hypothesized to enhance the *E. coli* biofilm-eradication potential of our conjugates. Amide formation between the carboxylic acid of lysine and the secondary amine of the piperazine ring of ciprofloxacin (a site on ciprofloxacin previously identified as a useful handle for functionalization and known to conserve Gram-negative efficacy)^{24,25} provided a compound with two primary amines (α -amine and ϵ -amine, see Scheme 1, compound 1 for labeling) for further functionalization. We chose to use the ϵ -amino group of lysine for nitroxide functionalization and the α -amino group of lysine for peptide propagation. Selective deprotection of these two primary amines allows for one, two or any number of nitroxide(s) to be added to the ciprofloxacin moiety, thus enabling the ratio of nitroxide to antibiotic to be easily tuned for different pathogens.

Following this approach, the synthetic route to ciprofloxacin–mononitroxide (CMN) **10** and ciprofloxacin–dinitroxide (CDN) **11** (Scheme 1) began by protecting the carboxylic acid of ciprofloxacin as an ethyl ester to give **2**. Amidation between the free carboxylic acid of Boc-Lys(Cbz)-OH **1** and the secondary amine of the piperazine ring of **2** using EDC, HOBt, and *i*-Pr₂NEt in DCM generated the fully protected conjugate **3** in high yield (80%). The protecting groups of **3** were chosen to allow for the selective deprotection to either grow the peptide chain (Boc-deprotection of the α -amino group) or add nitroxide functionality (Cbz-deprotection of the ϵ -amino group). Selective deprotection of the Boc-protected α -amine utilizing TFA in DCM produced **4** in quantitative yield. Alternatively, deprotection of the Cbz-protected ϵ -amine *via* Pd/C in methanol gave **5** in excellent yield (95%). Amidation between the deprotected α -amine of **4** and the carboxylic acid of **1** employing the above conditions afforded **6** in high yield (90%). Deprotection of the two Cbz-protected ϵ -amines of **6** produced **7** in high yield (91%). Subsequent amidation (as described above) of CTEMPO with **5** or **7** produced **8** or **9** in good yields (80% and 85% respectively). Final deprotection of **8** and **9** by base mediated ester hydrolysis followed by Boc-deprotection of the α -amine afforded the desired CMN derivative **10** and the CDN derivative **11** in high yields (84% and 87%, respectively).

In addition to CMN **10** and CDN **11**, their corresponding methoxyamine derivatives **14** and **15** were also sought to assess the effect of the nitroxide moiety on biofilm-eradication. CTEMPO was reacted with iron(II) sulfate heptahydrate, hydrogen peroxide, and DMSO to produce methoxyamine derivative 1-methoxy-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (CTEMPOME). Amidation of CTEMPOME with **5** or **7**, followed by base mediated ester hydrolysis and Boc-deprotection, as described previously, afforded ciprofloxacin–

Scheme 1 Synthetic route to CMN **10**, and CDN **11**, and their corresponding methoxyamines CMM **14**, and CDM **15**. Reagents and conditions: (a) EDC, HOBT, *i*-Pr₂NEt, DCM, R.T., O/N; (b) Pd/C, MeOH, H₂, R.T., 1.5 h; (c) TFA, DCM, R.T., 1 h; (d) 2 M NaOH, MeOH, 50 °C, 5 h.

monomethoxyamines (CMM) derivative **14** or ciprofloxacin-dimethoxyamine (CDM) derivative **15** in excellent yields (92% and 99%, respectively). We have demonstrated the use of sequential peptide propagation as a simple, high yielding, and

effective method for producing multiple sites for further functionalization of the ciprofloxacin motif. While we have chosen to utilize these additional sites for nitroxide functionalization their utility is unlimited.

2.2. Ciprofloxacin–nitroxide(s) conjugates display antibacterial activity

Our earlier work has established that nitroxides can disperse *P. aeruginosa* and *E. coli* biofilms.^{20,22} Based on these properties, we previously produced several nitroxide functionalized antibiotics with demonstrated potent biofilm-eradication activity against *P. aeruginosa*.^{24,25} In this work, we specifically designed our new conjugates to target UPEC biofilms. We first sought to determine the antibacterial activity of our conjugate compounds **10**, **11**, **14**, and **15** against the reference cystitis isolate UTI89³¹ using minimum inhibitory concentration (MIC) assays. Both CMN **10** and CDN **11** demonstrated antibacterial activity against UTI89 (albeit less potent than ciprofloxacin), while the nitroxide CTEMPO alone had no activity and the methoxyamine derivatives CMM **14** and CDM **15** had significantly higher MICs compared to their respective nitroxide-containing conjugates (Table 1). These findings suggest that the presence of nitroxide(s) enhances the antibacterial activity of CMN **10** and CDN **11**, however the nitroxide–ciprofloxacin conjugates do not have improved MICs against planktonic UPEC compared to the parent antibiotic.

We also considered whether the mode of action of CDN **11** against planktonic UPEC UTI89 cells followed the standard fluoroquinolone pathway (interfering with DNA gyrase and topoisomerase IV).³² As both CDN **11** (free radical nitroxide) and CDM **15** (methoxyamine) exhibited planktonic cell antimicrobial activity, and nitroxides had no direct effect against UPEC UTI89 cells (planktonic or biofilm; Table 1), the presence of the nitroxide would be predicted not to contribute to the antimicrobial activity of CDN **11**. However, the nitroxides in CDN **11** did appear to improve the conjugate's antimicrobial activity compared to the methoxyamine derivative CDM **15**, suggesting that the presence of the free radical nitroxide in CDN **11** enhances antimicrobial activity indirectly. We hypothesize that the presence of the free radical (or one of its redox states) facilitates the conjugate's cell entry allowing access to the nucleoid of the cell where the fluoroquinolone-based compound can act *via* its usual mode of action.

Table 1 Measured MIC and MBEC values for CMN **10**, CDN **11**, CMM **14**, CDM **15**, CTEMPO, and ciprofloxacin against UPEC strain UTI89

Compound	MIC ^a (μM)	MBEC ^b (μM)
CMN 10	≤1.95	>1000 ^c
CDN 11	≤10.5	≤400
CMM 14	≤30.6	>1000 ^c
CDM 15	≤81.6	>650 ^c
CTEMPO	>1000 ^c	>1000 ^c
Ciprofloxacin	≤0.045	≤800
Ciprofloxacin & CTEMPO (1 : 2)	≤0.045	≤800

^a Determined *via* broth microdilution method in accordance with CLSI standard. ^b Determined using CBD. ^c Highest concentration tested. All values were obtained from a minimum of two individual experiments each conducted with three different UTI89 cultures and three technical replicates per culture.

2.3. The ciprofloxacin–dinitroxide conjugate CDN **11** has enhanced antibacterial activity against UPEC biofilms

We have previously shown that nitroxides can disperse *E. coli* and *P. aeruginosa* biofilms.²² Here we hypothesized that, despite their higher MIC, our nitroxide–ciprofloxacin conjugates would have enhanced activity against UPEC biofilms. To test this tenet, we utilized the Calgary Biofilm Device (CBD) to establish mature UTI89 biofilms, which were then treated with different concentrations of ciprofloxacin or our ciprofloxacin–nitroxide conjugate compounds **10**, **11**, **14**, and **15**, to establish their minimum biofilm eradication concentration (MBEC; minimum concentration required to eradicate 99.9% of viable cells). Ciprofloxacin acted as a baseline for our compounds **10**, **11**, **14**, and **15**, and also served as a device standard allowing our results to be directly compared to those reported by previous studies using the same biofilm formation device (as fold improvement over standard). UTI89 biofilms were highly tolerant to ciprofloxacin, requiring 800 μM of the antibiotic for complete eradication (Table 1). Methoxyamine derivatives CMM **14** and CDM **15** exhibited no improvement over ciprofloxacin against UPEC biofilms (MBEC > 1000 μM and > 650 μM, respectively). Surprisingly, CMN **10** was also unable to completely eradicate UPEC biofilms even at the maximum concentration tested (1000 μM, *i.e.* MBEC > 1000 μM), despite having the lowest MIC against planktonic UTI89 cells (Table 1). Conversely, CDN **11** demonstrated substantial biofilm-eradication activity, with complete eradication occurring at only 400 μM (MBEC ≤ 400 μM), a full 2-fold improvement over the parent compound ciprofloxacin.

In addition, CDN **11** exhibited at least a 3 log reduction in viable biofilm-associated cells (calculated as colony forming units (CFUs) recovered from treated biofilms) compared to untreated controls at concentrations as low as 12.5 μM (Fig. 1). While CDN **11** eradicated 99.7% of biofilm-residing cells at low concentrations (12.5 μM), complete eradication (99.9%) required substantially higher concentrations, a finding which is consistent with the presence of persister cells within the biofilm. Persister cells are specialized quiescent survivor cells, which are highly tolerant to antimicrobials and normally comprise approximately 1% of the biofilm formation.^{33,34} Importantly, despite CDN **11** having a higher MIC against planktonic UPEC than CMN **10** (5-fold) or ciprofloxacin (>200-fold), it was at least twice as potent against biofilm-residing cells than any of the other agents tested, including ciprofloxacin. This finding exemplifies that MICs are not an accurate indication of the biofilm-eradication potential of new antimicrobial agents and that MBEC values need to be determined alongside MICs. Furthermore, our results suggest that nitroxides may have some effect on persister cells, which could either be direct by reversing persister cells to a drug susceptible state, or indirect by dispersing the protective EPS and biofilm cell layers surrounding persister cells that shield them from antimicrobials within the biofilm structure.

Fig. 1 UPEC biofilm eradication by dinitroxide-ciprofloxacin conjugate CDN 11. Mature UPEC UTI89 biofilms were treated with CDN 11 at a concentration range of 6.25–400 μM for 24 hours (control represents biofilms treated with DMSO only (no antibiotic)). Cells were recovered from biofilms and viable CFU mL^{-1} were enumerated by serial dilution and plating on LB agar. Dot plots show data from 5 biological repeats each with 2 technical replicates. Horizontal lines with error bars show group means $\pm$ SD.

2.4. Tethering nitroxides to ciprofloxacin is necessary for enhancing the antibiotic's activity against UPEC biofilms

As CMN 10 was found to be less active against both planktonic and biofilm-residing UPEC cells than ciprofloxacin, it appeared that the addition of only one nitroxide to the ciprofloxacin moiety was insufficient to improve the biofilm-eradication properties of the resulting compound towards this strain. Hence, the more potent biofilm-eradication activ-

ity of CDN 11 must be attributed to the presence of two nitroxide moieties. As nitroxides alone have no inherent antimicrobial activity against UPEC UTI89, the activity of CDN 11 likely arises from a dual-action effect, where the nitroxides promote biofilm cell dispersal and ciprofloxacin then eradicates the dispersed cells. Consequently, we considered whether having the nitroxides directly linked to the ciprofloxacin moiety was imperative to the compound's biofilm-eradication activity. To assess this, we conducted synergistic treatment of mature UPEC biofilms, which utilized a 1 : 2 molar ratio of ciprofloxacin and CTEMPO (Table 1). Interestingly, co-administering ciprofloxacin and CTEMPO in this ratio did not improve the biofilm-eradication activity of ciprofloxacin against UPEC biofilms, a finding which confirms the fundamental importance of tethering the nitroxides to the antibiotic moiety in order to enhance their biofilm-eradication activity.

2.5. A ciprofloxacin–dinitroxide conjugate induces UPEC cell filamentation during eradication of biofilm-residing cells

To further confirm the biofilm-eradication activity of CDN 11 against UPEC biofilms, we conducted fluorescence microscopy (FL) of UPEC biofilms treated with different concentrations of CDN 11 for 24 hours and differentially stained for live/dead cell determination (Fig. 2). As expected, biofilms treated with CDN 11 at the MBEC concentration (400 μM) were extremely small and contained entirely dead cells (Fig. 2B). Biofilms were progressively larger and contained an increasing number of viable cells as the treatment concentration of CDN 11 diminished (Fig. 2C–H). Furthermore, the majority (>99%) of cell eradication was observed at a much

Fig. 2 Fluorescence micrographs of mature UPEC UTI89 biofilms treated with CDN 11 at (A) 0 μM , carrier (DMSO) control biofilm; (B) 400 μM (MBEC value); (C) 200 μM ; (D) 100 μM ; (E) 50 μM ; (F) 25 μM ; (G) 12.5 μM ; (H) 6.25 μM , and then stained with SYTO9 for live cells (green) and propidium iodide for dead cells (red). (A–H) Are representative images taken across 3 biological replicates per condition. Scale bars are 5 μm .

Table 2 DNC 11 potentiation of ciprofloxacin activity against UPEC biofilms

CDN 11 (μM)	Ciprofloxacin (μM)	$\text{Log}_{10}(\text{CFU})^a$	Log reduction ^b	Decrease in CFU (%)
0	400	2.99	—	—
20	380	1.35	1.64	98
40	360	1.82	1.17	93
80	320	2.69	0.3	50

^a Calculated by taking the $\text{log}_{10}(\text{CFU})$, where CFU represents the number of remaining viable UTI89 cells in a biofilm after treatment.

^b Calculated by subtracting $\text{log}_{10}(\text{CFU})$ of co-administration biofilm treatment from ciprofloxacin only (400 μM). Values represent the mean of at least three biological replicates, each with three technical replicates.

lower concentration (12.5 μM) than the concentration required for complete eradication (99.9%) (400 μM), a result which is consistent with our viable CFU findings (Fig. 1). Interestingly, biofilms treated with 25 μM (Fig. 2F), and to a lesser extent with 12.5 μM (Fig. 2G), displayed some level of cell filamentation. Cell filamentation is a mode of bacterial growth that results in cell elongation and is considered a survival strategy conferring antibiotic tolerance to *E. coli* after exposure to ciprofloxacin (and other antibiotics).⁸ Despite the altered cell morphology, the biofilm's susceptibility to CDN 11 appeared to be unaffected as evidenced by the low number of viable CFU recovered from biofilms treated at sub-MBEC concentrations of CDN 11 (Fig. 1).

2.6. A ciprofloxacin–dinitrooxide conjugate potentiates the UPEC biofilm eradication activity of ciprofloxacin

As we observed significant biofilm-eradication even at sub-MBEC concentrations of CDN 11, we tested whether this conjugate could potentiate the efficacy of ciprofloxacin against UPEC biofilms. We therefore examined the effect of different concentrations of CDN 11 (20, 40, and 80 μM) on UPEC biofilms in combination treatment with ciprofloxacin (320–380 μM) (Table 2). Co-administration of CDN 11 at 20 μM with ciprofloxacin at 380 μM (total combined concentration 400 μM) resulted in a 98% decrease (1.6-log reduction) in live CFU recovered from biofilms after treatment compared to treatment at the equivalent concentration of ciprofloxacin alone (400 μM). Importantly, CDN 11 potentiated the efficacy of ciprofloxacin against UPEC biofilms, demonstrating a 2-fold increase in potency for ciprofloxacin (MBEC drop from ≤ 800 to ≤ 400 μM).

2.7. A ciprofloxacin–dinitrooxide conjugate that is active against UPEC biofilms is not toxic to human bladder epithelial cells

As CDN 11 is active against UPEC biofilms, it is a promising candidate for the treatment of biofilm-related UTIs. To further examine the compounds suitability for future therapeutic development, we evaluated its toxicity against human bladder epithelial cells using the lactate dehydrogenase (LDH) assay. CDN 11 exhibited no measurable toxicity to T24 cells after 24 hours of treatment even at the maximum concentration tested (500 μM , $\text{IC}_{50} > 500$ μM).

3. Conclusions

The synthetic strategy reported herein provides a simple and high yielding methodology for the sequential addition of nitroxides to the ciprofloxacin scaffold. The application of this methodology has allowed us to broaden the biofilm-eradication spectrum of activity for antibiotic-nitroxide hybrids against UPEC biofilms. We have shown that tethering two nitroxides to a single ciprofloxacin moiety can significantly improve the biofilm-eradication capabilities of the resulting compound against UPEC biofilms. CDN 11 was at least twice as potent as CMN 10 (single nitroxide containing conjugate) or the parent antibiotic ciprofloxacin against UPEC biofilms (MBEC assays), despite being far less active against planktonic cells of the same strain (MIC assays).

Furthermore, the biofilm-eradication capabilities of CDN 11 at low concentrations were impressive, with over 99% eradication of UPEC biofilms occurring at only 12.5 μM . This work clearly demonstrates the advantages of tethering more than one nitroxide to a single ciprofloxacin moiety. In addition, CDN 11 was found to potentiate the efficacy of ciprofloxacin against UPEC biofilms. Co-treatment of UPEC biofilms with CDN 11 (20 μM) and ciprofloxacin (380 μM) improved biofilm eradication by 98% compared to ciprofloxacin alone (administered at the equivalent concentration (400 μM)), indicating that CDN 11 is not only a potent biofilm-eradication agent but can also be exploited for potentiating the efficacy of ciprofloxacin against biofilm-related infections. Nitroxide functionalized antibiotics are thus promising biofilm-eradication agents with the potential to be developed into urgently needed antibacterials for the treatment of biofilm-related UTIs. Our work further demonstrates that synthetic strategies aimed at improving the biofilm-eradication efficacy of existing antibiotics is a powerful approach for biofilm remediation.

4. Experimental section

4.1. Chemistry general information

Materials. Synthetic reactions of an air-sensitive nature were carried out under an atmosphere of ultra-high purity argon. Anhydrous DCM was obtained from the solvent purification system, Pure Solv™ Micro, by Innovative Technologies. All other reagents were purchased from commercial suppliers and used without further purification. Ciprofloxacin,

4-carboxy-2,2,6,6-tetramethylpiperidin-1-yloxy (CTEMPO), N^6 -((benzyloxy)carbonyl)- N^2 -(*tert*-butoxycarbonyl)-L-lysine (Boc-L-Lys-(Cbz)-OH) (1) were purchased from Sigma-Aldrich. 1-Methoxy-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (CTEMPOMe) was prepared in house by previously documented procedures.²⁵

Methods and instrumentation. All ^1H NMR spectra were recorded at 600 MHz on a Bruker Avance 600 instrument. All ^{13}C NMR spectra were recorded at 150 MHz on a Bruker Avance 600 instrument. Spectra were obtained in the following solvents: CDCl_3 (reference peaks: ^1H NMR: 7.26 ppm; ^{13}C NMR: 77.19 ppm), CD_3OD (reference peaks: ^1H NMR: 3.31 ppm; ^{13}C NMR: 49.00 ppm). All NMR experiments were performed at room temperature. Chemical shift values (δ) are reported in parts per million (ppm) for all ^1H NMR and ^{13}C NMR spectral assignments. ^1H NMR spectroscopy multiplicities are reported as: s = singlet, br. s = broad singlet, d = doublet, dd = doublet of doublets, m = multiplet. Coupling constants are reported in Hz. All spectra are presented using MestReNova 11.0. The purity of all final compounds was determined to be 95% or higher using HPLC analysis. Column chromatography was performed using LC60A 40–63 Micron DAVISIL silica gel. Thin-layer chromatography (TLC) was performed on Merck Silica Gel 60 F254 plates. TLC plates were visualized under a UV lamp (254 nm) and/or by development with phosphomolybdic acid (PMA). Melting points were measured with a Variable Temperature Apparatus by the capillary method and are uncorrected.

LCMS equipment and method. Samples were separated by HPLC (Dionex Ultimate 3000) on a Phenomenex Luna C18 column (250 mm $\times$ 2.0 mm $\times$ 5 μm) held at 40 $^\circ\text{C}$. Mobile phase A was 20% ACN, and mobile phase B was 90% ACN, both containing 10 mM ammonium acetate, flowing at 0.2 mL min^{-1} . The gradient commenced at 57% B for 3 minutes, increasing to 100% B over 7 minutes, and holding at 100% B for a further 5 minutes, before returning to initial conditions for 5 minutes. Post-column, the eluent was split ($\sim 9:1$) for both UV and MS detection. High-resolution mass spectra were acquired on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization source, operating in the positive ion mode with a mass resolution of 120 000 (FWHM at m/z 400).

4.2. Synthesis of ciprofloxacin-nitroxide(s) conjugates

General procedure for amidation 3, 6, 8, 9, 12, 13. The carboxylic acid (1.1 equiv.), HOBt (1.4 equiv.), and $i\text{-Pr}_2\text{NEt}$ (3 equiv.) were all sequentially added to a solution of amine (1 equiv.) in anhydrous dichloromethane (50 mL) under an atmosphere of argon at 0 $^\circ\text{C}$ (ice-water bath). The resulting solution was stirred to 10 minutes at 0 $^\circ\text{C}$ before EDC (1.3 equiv.) was added and the reaction stirred at room temperature overnight. The reaction mixture was washed with water (3 $\times$ 100 mL) then brine (3 $\times$ 100 mL), before being dried over sodium sulfate and the solvent was removed *in vacuo* to afford a crude solid product.

Purification was achieved *via* column chromatography (SiO_2 , chloroform 98%, methanol 2%).

General procedure for Boc-deprotection 4, and step 1 or 2 of 10, 11, 14, 15. Anhydrous trifluoroacetic acid (10 mL) was added dropwise to a solution of Boc-protected amine (1 equiv.) in anhydrous dichloromethane (10 mL) under an atmosphere of argon at 0 $^\circ\text{C}$, then stirred for 1 hour at room temperature. The reaction mixture was first diluted with additional dichloromethane (30 mL), then quenched with saturated sodium hydrogen carbonate (50 mL). The organic phase was separated and the solvent removed *in vacuo* to afford the deprotected product. Purification, if required, was achieved *via* column chromatography (SiO_2 , chloroform 98%, methanol 2%).

General procedure for Cbz-deprotection 5, 7. Palladium on carbon (10% wt) (20 mol%) was added to a solution of Cbz-protected amine (1 equiv.) in anhydrous methanol under an atmosphere of H_2 (1 atm). The mixture was stirred for 4 hours at room temperature then filtered. The solvent was removed *in vacuo* to yield the deprotected product. Purification, if required, was achieved *via* column chromatography (SiO_2 , chloroform 98%, methanol 2%).

General procedure for ester hydrolysis step 1 or 2 of 10, 11, 14, 15. 2 M aqueous sodium hydroxide (7 equiv.) was added to a solution of the specific ethyl ester (1equiv) in methanol (30 mL), and the resulting solution was stirred at 50 $^\circ\text{C}$ for 5 hours. The reaction mixture was cooled to room temperature and diluted with deionized water (50 mL). The pH was adjusted to ~ 6 using 2 M aqueous hydrochloric acid and the mixture extracted with dichloromethane (3 $\times$ 20 mL). The combined organic extracts were dried over anhydrous sodium sulfate, and the solvent was removed *in vacuo* to afford the pure solid product.

Ethyl-7-(4-(N^6 -((benzyloxy)carbonyl)- N^2 -(*tert*-butoxycarbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (3). Reagents: (2) (2.0 g, 5.56 mmol, 1 equiv.), Boc-L-Lys(Cbz)-OH (1) (2.3 g, 6.12 mmol, 1.1 equiv.), HOBt (1.05 g, 7.78 mmol, 1.4 equiv.), EDC (1.4 g, 7.22 mmol, 1.3 equiv.), $i\text{-Pr}_2\text{NEt}$ (2.9 mL, 16.7 mmol, 3 equiv.). Product: Light yellow powder (3.2 g, 80%) ^1H NMR (600 MHz, CDCl_3): δ = 8.50 (s, 1H, $\text{NCH}=\text{C}$), 8.03 (d, J = 13.0 Hz, 1H, Ar-H), 7.35–7.27 (m, 5H, Ar-H), 7.25 (s, 1H, Ar-H), 5.43 (d, J = 8.6 Hz, 1H, C(O)NHCH), 5.05 (s, 2H, ArCH_2), 4.91 (m, 1H, C(O)NHCH_2), 4.63 (td, J = 8.5, 4.5, 1H, C(O)NHCH), 4.37 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 3.94 and 3.69 (2 $\times$ m, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.77 (m, 2H, $\text{NCH}_2\text{CH}_2\text{N}$), 3.40 (m, 1H, $\text{C}=\text{CHNCH}$), 3.33–3.09 (m, 4H, $\text{NCH}_2\text{CH}_2\text{N}$, and 2H, C(O)NHCH_2), 1.71 and 1.54 (2 $\times$ m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.56 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.44 (m, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.42 (s, 9H, 3 $\times$ CCH_3), 1.39 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 1.31 (m, 2H, NCHCH_2), 1.12 (m, 2H, NCHCH_2). ^{13}C NMR (150 MHz, CDCl_3): δ = 173.2, 170.9, 165.9, 156.6, 155.8, 154.3, 152.6, 148.4, 144.0, 138.1, 136.7, 128.7, 128.2, 128.1, 123.8, 113.7, 113.6, 110.7, 105.4, 80.0, 66.7, 61.1, 50.4, 49.9, 49.8, 45.6, 42.0, 40.8, 34.7, 33.3, 29.6, 28.5, 22.4, 14.6, 8.3. MP: 99.5–102.8 $^\circ\text{C}$. HRMS (ESI): m/z calculated for $\text{C}_{38}\text{H}_{48}\text{FN}_5\text{O}_8 + \text{H}^+$ [$\text{M} + \text{H}^+$]: 722.3565; found 722.3588. LC-MS: R_t = 8.31 min; area 100%.

Ethyl-7-(4-(N⁶-((benzyloxy)carbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (4). Reagents: (3) (1.0 g, 1.39 mmol, 1 equiv.). Product: Light yellow powder (864 mg, >99%). ¹H NMR (600 MHz, CDCl₃): δ = 8.52 (s, 1H, NCH=C), 8.07 (d, *J* = 13.0 Hz, 1H, Ar-H), 7.37–7.28 (m, 5H, Ar-H), 7.28–7.26 (s, 1H, Ar-H), 5.06 (s, 2H, ArCH₂), 4.86 (m, 1H, C(O)NHCH₂), 4.38 (q, *J* = 7.1 Hz, 2H, OCH₂CH₃), 3.86 (m, 1H, C(O)NHCH), 3.86 and 3.69 (2 × m, 2H, NCH₂CH₂N), 3.69 (m, 2H, NCH₂CH₂N), 3.40 (m, 1H, C=CHNCH), 3.30–3.13 (m, 4H, NCH₂CH₂N, and 2H, C(O)NHCH₂), 1.59–1.41 (br, m, 6H, NHCH₂CH₂CH₂CH₂CH), 1.40 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.31 (m, 2H, NCHCH₂), 1.12 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CDCl₃): δ = 174.2, 173.2, 166.0, 156.6, 154.3, 152.6, 148.4, 144.1, 144.0, 138.1, 136.7, 128.7, 128.3, 128.1, 123.9, 123.8, 113.8, 113.7, 110.8, 105.3, 66.7, 61.1, 51.1, 49.8, 45.3, 42.0, 40.8, 35.3, 34.6, 30.0, 22.9, 14.6, 8.3. MP: 103.5–105.7 °C. HRMS (ESI): *m/z* calculated for C₃₃H₄₀FN₅O₆ + H⁺ [*M* + H⁺]: 622.3049; found 622.3059. LC-MS: *R*_t = 3.63 min; area 100%.

Ethyl-7-(4-((tert-butoxycarbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (5). Reagents: (3) (910 mg, 1.26 mmol, 1 equiv.), Pd/C 10% wt (296 mg, 0.252 mmol, 20 mol%). Product: Yellow solid (703 mg, 95%). ¹H NMR (600 MHz, CDCl₃): δ = 8.45 (br, s, 2H, CH₂NH₂), 8.36 (s, 1H, NCH=C), 7.73 (d, *J* = 13.0 Hz, 1H, Ar-H), 7.25 (s, 1H, Ar-H), 5.73 (d, *J* = 8.3 Hz, 1H, C(O)NHCH), 4.64 (d, *J* = 7.4 Hz, 1H, C(O)NHCH), 4.33 (q, *J* = 7.1 Hz, 2H, OCH₂CH₃), 3.94–3.70 (br, m, 4H, NCH₂CH₂N), 3.49 (m, 1H, C=CHNCH), 3.38–3.16 (br, m, 4H, NCH₂CH₂N), 3.07 (m, 2H, CH₂NH₂), 1.90 (m, 2H, NH₂CH₂CH₂CH₂CH₂CH), 1.78 and 1.60 (2 × m, 2H, NH₂CH₂CH₂CH₂CH₂CH), 1.55 (m, 2H, NH₂CH₂CH₂CH₂CH₂CH), 1.42 (s, 9H, 3 × CCH₃), 1.37 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.37 (m, 2H, NCHCH₂), 1.07 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CDCl₃): δ = 173.2, 170.9, 165.0, 155.8, 154.1, 152.5, 148.1, 144.1, 138.0, 122.7, 112.8, 112.7, 109.4, 105.8, 80.0, 60.9, 50.3, 50.0, 45.7, 42.1, 39.6, 35.1, 33.0, 28.5, 27.1, 22.4, 14.6, 8.4. MP: 120.1–122.4 °C. HRMS (ESI): *m/z* calculated for C₃₀H₄₂FN₅O₆ + H⁺ [*M* + H⁺]: 588.3210; found 588.3218. LC-MS: *R*_t = 3.20 min; area 100%.

Ethyl-7-(4-(N⁶-((benzyloxy)carbonyl)-N²-(N⁶-((benzyloxy)carbonyl)-N²-(tert-butoxycarbonyl)-L-lysyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (6). Reagents: (4) (430 mg, 0.69 mmol, 1 equiv.), Boc-Lys(Cbz)-OH (1) (290 mg, 0.76 mmol, 1.1 equiv.), HOBt (131 mg, 0.97 mmol, 1.4 equiv.), EDC (172 mg, 0.90 mmol, 1.3 equiv.), i-Pr₂NEt (0.4 mL, 2.07 mmol, 3 equiv.). Product: Light yellow powder (676 mg, 99%). ¹H NMR (600 MHz, CDCl₃): δ = 8.51 (s, 1H, NCH=C), 8.05 (d, *J* = 13.0 Hz, 1H, Ar-H), 7.36–7.27 (m, 10H, Ar-H), 7.25 (s, 1H, Ar-H), 6.96 (d, *J* = 8.3 Hz, 1H, C(O)CHNHC(O)CH), 5.26 (d, *J* = 7.6 Hz, 1H, C(O)NH), 5.16–5.08 (br, m, 2H, 2 × C(O)NHCH₂), 5.07 (s, 4H, 2 × ArCH₂), 4.91 (m, 1H, C(O)NHCH), 4.37 (q, *J* = 7.1 Hz, 2H, OCH₂CH₃), 4.08 (m, 1H, NC(O)CH), 3.90 and 3.67 (2 × m, 2H, NCH₂CH₂N), 3.77 and 3.67 (2 × m, 2H, NCH₂CH₂N), 3.40 (m, 1H, C=CHNCH), 3.30–3.05 (m, 8H, NCH₂CH₂N, and 2 × C(O)NHCH₂), 1.76 and 1.63 (2 × m, 4H,

(CH₃)₃OC(O)NHCHCH₂ and NC(O)CHCH₂), 1.50 (m, 4H, (CH₃)₃OC(O)NHCHCH₂CH₂CH₂ and NC(O)CHCH₂CH₂CH₂), 1.41 (s, 9H, 3 × CCH₃), 1.39 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.36 (m, 4H, (CH₃)₃OC(O)NHCHCH₂CH₂CH₂ and NC(O)CHCH₂CH₂CH₂), 1.31 (m, 2H, NCHCH₂), 1.11 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CDCl₃): δ = 173.2, 172.1, 170.1, 165.9, 156.8, 156.7, 155.8, 154.3, 152.6, 148.3, 144.0, 143.9, 138.1, 136.7, 128.6, 128.2, 128.1, 123.8, 113.7, 113.6, 110.7, 105.4, 80.2, 66.7, 61.0, 54.7, 50.2, 49.9, 48.6, 45.5, 42.1, 40.5, 40.4, 34.6, 32.3, 31.9, 29.5, 29.4, 28.4, 22.5, 22.1, 14.6, 8.3. MP: 100.3–102.8 °C. HRMS (ESI): *m/z* calculated for C₅₂H₆₆FN₇O₁₁ + H⁺ [*M* + H⁺]: 984.4883; found 984.4879. LC-MS: *R*_t = 10.74 min; area 100%.

Ethyl-7-(4-((tert-butoxycarbonyl)-L-lysyl-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (7). Reagents: (6) (400 mg, 0.41 mmol, 1 equiv.), Pd/C 10% wt (87 mg, 0.082 mmol, 20-mol%). Product: Light yellow solid (266 mg, 91%). ¹H NMR (600 MHz, CD₃OD): δ = 8.66 (s, 1H, NCH=C), 8.05 (d, *J* = 13.3 Hz, 1H, Ar-H), 7.25 (s, 1H, Ar-H), 4.91 (m, 1H, C(O)NHCH), 4.33 (q, *J* = 7.1 Hz, 2H, OCH₂CH₃), 4.06 (m, 1H, NC(O)CH), 3.90, 3.80, 3.71, 3.66, 3.38, and 3.26 (6 × m, 8H, 2 × NCH₂CH₂N), 3.41 (m, 1H, C=CHNCH), 2.94 (m, 4H, 2 × NH₂CH₂), 1.89–1.60 and 1.58–1.46 (2 × m, 12H, 2 × C(O)CHCH₂CH₂CH₂), 1.44 (s, 9H, 3 × CCH₃), 1.37 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.36 (m, 2H, NCHCH₂), 1.16 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CD₃OD): δ = 175.4, 174.9, 172.0, 166.1, 157.8, 155.6, 154.0, 145.8, 139.9, 123.7, 123.6, 113.2, 113.1, 110.5, 107.5, 80.7, 61.7, 55.7, 50.2, 49.8, 49.6, 46.7, 43.2, 40.6, 40.5, 36.3, 32.6, 32.4, 28.7, 28.3, 28.1, 23.9, 23.6, 14.7, 8.6. MP: 69.2–70.0 °C. HRMS (ESI): *m/z* calculated for C₃₆H₅₄FN₇O₇ + H⁺ [*M* + H⁺]: 716.4147; found 716.4146. LC-MS: *R*_t = 3.67 min; area 99.07%.

Ethyl-7-(4-(N²-(tert-butoxycarbonyl)-N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (8). Reagents: (5) (200 mg, 0.34 mmol, 1 equiv.), CTEMPO (75 mg, 0.37 mmol, 1.1 equiv.), HOBt (65 mg, 0.48 mmol, 1.4 equiv.), EDC (85 mg, 0.44 mmol, 1.3 equiv.), i-Pr₂NEt (0.2 mL, 1.02 mmol, 3 equiv.). Product: Orange solid (209 mg, 80%). ¹H NMR (600 MHz, CDCl₃): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 8.55 (s, 1H, NCH=C), 8.10 (d, *J* = 12.0 Hz, 1H, Ar-H), 7.29 (s, 1H, Ar-H), 5.45 (s, 1H, C(O)NHCH), 4.66 (s, 1H, C(O)NHCH), 4.40 (q, *J* = 7.0 Hz, 2H, OCH₂CH₃), 4.01, 3.83, 3.71, and 3.35 (4 × m, 8H, 2 × NCH₂CH₂N), 3.42 (m, 1H, C=CHNCH), 3.07 (m, 2H, CH₂NH₂), 1.83–1.70 and 1.69–1.55 (2 × br, s, 6H, NH₂CH₂CH₂CH₂CH₂), 1.46 (s, 9H, 3 × CCH₃), 1.42 (t, *J* = 7.1 Hz, 3H, OCH₂CH₃), 1.35 (s, 2H, NCHCH₂), 1.16 (s, 2H, NCHCH₂). ¹³C NMR (150 MHz, CDCl₃): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 173.0, 170.6, 165.8, 155.6, 152.4, 148.2, 137.9, 113.6, 110.7, 105.1, 79.9, 60.9, 50.3, 49.8, 49.6, 45.4, 42.0, 34.6, 33.0, 29.2, 28.6, 22.4, 14.4, 8.3. MP: 130.8–133.2 °C. HRMS (ESI): *m/z* calculated for C₄₀H₅₈FN₆O₈ + H⁺ [*M* + H⁺]: 770.4378; found 770.4371. LC-MS: *R*_t = 5.07 min; area 97.85%. EPR: *g* = 1.9954, *a*_N = 1.6012 mT.

Ethyl-7-(4-(N²-(N²-(tert-butoxycarbonyl)-N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-L-lysyl)-N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (9). Reagents: (7) (130 mg, 0.18 mmol, 1 equiv.), CTEMPO (80 mg, 0.40 mmol, 2.2 equiv.), HOBt (68 mg, 0.50 mmol, 2.8 equiv.), EDC (90 mg, 0.47 mmol, 2.6 equiv.), i-Pr₂NEt (0.2 mL, 1.08 mmol, 6 equiv.). Product: Orange solid (164 mg, 85%). ¹H NMR (600 MHz, CDCl₃): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 8.56 (s, 1H, NCH=C), 8.10 (d, J = 13.0 Hz, 1H, Ar-H), 7.31 (s, 1H, Ar-H), 7.06 (m, 1H, CHC(O)CHNHCH₂), 5.27 (s, 1H, C(O)NH), 4.98 (m, 1H, C(O)NHCH), 4.40 (q, J = 7.1 Hz, 2H, OCH₂CH₃), 4.00 (m, 1H, NC(O)CH), 3.85 and 3.71 (2 × m, 4H, NCH₂CH₂N), 3.42 (m, 1H, C=CHNCH), 3.30–3.05 (m, 8H, NCH₂CH₂N, and 2 × C(O)NHCH₂), 1.90 and 1.66 (2 × m, 4H, (CH₃)₃OC(O)NHCHCH₂ and NC(O)CHCH₂), 1.55 (m, 4H, (CH₃)₃OC(O)NHCHCH₂CH₂CH₂ and NC(O)CHCH₂CH₂CH₂), 1.42 (t, J = 7.1 Hz, 3H, OCH₂CH₃), 1.36 (m, 4H, (CH₃)₃OC(O)NHCHCH₂CH₂CH₂ and NC(O)CHCH₂CH₂CH₂), 1.27 (s, 9H, 3 × CCH₃), 1.27 (m, 2H, NCHCH₂), 1.18 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CDCl₃): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 172.9, 171.9, 169.7, 165.6, 148.1, 146.9, 137.8, 124.3, 123.8, 110.5, 60.9, 48.4, 34.3, 31.7, 31.3, 30.1, 30.0, 29.5, 29.2, 22.5, 14.3, 13.9, 8.6. MP: 135.5–138.0 °C. HRMS (ESI): m/z calculated for C₅₆H₈₆FN₉O₁₁ + H⁺ [M + H⁺]: 1080.6509; found 1080.6547. LC-MS: R_t = 4.66 min; area 97.30%. EPR: g = 1.9956, a_N = 1.5813 mT.

1-Cyclopropyl-6-fluoro-7-(4-(N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (10). Reagents: Step 1 (Boc-deprotection): (8) (150 mg, 0.19 mmol, 1 equiv.); step 2 (ester hydrolysis) Crude product from step 1 (~121 mg, 0.19 mmol, 1 equiv.). Product: Orange solid (97 mg, 84% over two steps). ¹H NMR (600 MHz, CD₃OD): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 8.77 (s, 1H, NCH=C), 7.94 (s, 1H, Ar-H), 7.61 (d, J = 6.9 Hz, 1H, Ar-H), 4.05–3.68 (m, 6H, NCH₂CH₂N and CHC(O)NHCH₂), 3.51–3.28 (m, 4H, NCH₂CH₂N), 3.25 (m, 1H, C=CHNCH), 2.39 (tt, J = 15.1, 5.0 Hz, 1H, CHC(O)NHCH₂), 1.73–1.40, (3 × s, 8H, NHCH₂CH₂CH₂CH₂CH), 1.41 (m, 2H, NCHCH₂), 1.23 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CD₃OD): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 176.5, 174.9, 170.2, 151.0, 149.3, 146.7, 140.7, 112.8, 107.6, 105.1, 79.7, 79.5, 79.3, 57.3, 51.3, 50.8, 46.4, 43.1, 40.6, 38.5, 37.1, 35.4, 33.2, 30.4, 23.6, 8.9. MP: 145.5–147.4 °C. HRMS (ESI): m/z calculated for C₃₃H₄₆FN₆O₆ + H⁺ [M + H⁺]: 642.3541; found 642.3542. LC-MS: R_t = 3.15 min; area 100%. EPR: g = 1.9956, a_N = 1.5862 mT.

1-Cyclopropyl-6-fluoro-7-(4-(N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-N²-(N⁶-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)-L-lysyl)-L-lysyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (11). Reagents: Step 1 (Boc-deprotection): (9) (120 mg, 0.11 mmol, 1 equiv.); Step 2 (ester

hydrolysis) Crude product from step 1 (~108 mg, 0.11 mmol, 1 equiv.). Product: Orange solid (91 mg, 87% over two steps). ¹H NMR (600 MHz, CD₃OD): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 8.79 (s, 1H, NCH=C), 7.95 (s, 1H, Ar-H), 7.62 (s, 1H, Ar-H), 4.02–3.73 (3 × br, m, 4H, NCH₂CH₂N), 3.54 (m, 1H, C=CHNCH), 3.52–3.34 and 3.28–3.19 (3 × br, m, 6H, NCH₂CH₂N and CHC(O)NHCH₂), 1.86, 1.75, 1.58, 1.44 (4 × m, 10H, NHCH₂CH₂CH₂CH₂CH and 2 × ONCCH₂), 1.25 (m, 2H, NCHCH₂), 1.20 (m, 2H, NCHCH₂). ¹³C NMR (150 MHz, CD₃OD): (*note compound is a free-radical, some signals appear broadened, and other signals are missing) δ = 178.4, 174.2, 172.3, 169.6, 149.4, 140.8, 138.3, 127.4, 122.3, 108.6, 107.7, 50.3, 48.5, 40.9, 37.4, 35.6, 32.7, 31.8, 30.4, 24.0, 9.2. MP: 130.4–132.6 °C. HRMS (ESI): m/z calculated for C₄₉H₇₄FN₉O₉ + H⁺ [M + H⁺]: 952.5672; found 952.5652. LC-MS: R_t = 3.14 min; area 95.58%. EPR: g = 1.9956, a_N = 1.5911 mT.

Ethyl-7-(4-(N²-(tert-butoxycarbonyl)-N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (12). Reagents: (5) (117 mg, 0.20 mmol, 1 equiv.), CTEMPOMe (48 mg, 0.22 mmol, 1.1 equiv.), HOBt (38 mg, 0.28 mmol, 1.4 equiv.), EDC (50 mg, 0.26 mmol, 1.3 equiv.), i-Pr₂NEt (0.1 mL, 0.60 mmol, 3 equiv.). Product: Light beige intractable solid (116 mg, 87%). ¹H NMR (600 MHz, CDCl₃): δ = 8.54 (s, 1H, NCH=C), 8.08 (d, J = 13.0 Hz, 1H, Ar-H), 7.29 (d, J = 6.9 Hz, 1H, Ar-H), 5.57 (t, J = 5.9 Hz, 1H, CHC(O)NHCH₂), 5.42 (d, J = 8.6 Hz, 1H, C(O)NHCH), 4.63 (td, J = 8.4, 4.7 Hz, 1H, C(O)NHCH), 4.39 (q, J = 7.1 Hz, 2H, OCH₂CH₃), 3.96–3.69 (3 × br, m, 4H, NCH₂CH₂N), 3.59 (s, 3H, NOCH₃), 3.44 (m, 1H, C=CHNCH), 3.35–3.16 (br, m, 6H, NCH₂CH₂N and CHC(O)NHCH₂), 2.38 (tt, J = 15.1, 5.0 Hz, 1H, CHC(O)NHCH₂), 1.74, 1.64, 1.60–1.49, 1.40 (4 × m, 10H, NHCH₂CH₂CH₂CH₂CH and 2 × ONCCH₂), 1.44 (s, 9H, 3 × OCCH₃), 1.40 (t, J = 7.1 Hz, 3H, OCH₂CH₃), 1.34 (m, 2H, NCHCH₂), 1.19 (2 × s, 6H, 2 × CCH₃), 1.13 (m, 2H, NCHCH₂), 1.09 (2 × s, 6H, 2 × CCH₃). ¹³C NMR (150 MHz, CDCl₃): δ = 175.1, 173.2, 107.9, 166.0, 155.8, 154.3, 152.7, 148.4, 144.1, 144.0, 138.1, 123.9, 113.9, 113.7, 110.8, 105.4, 80.0, 65.6, 61.1, 59.4, 50.4, 50.0, 49.9, 45.6, 42.7, 42.6, 42.1, 39.1, 36.7, 34.7, 33.2, 30.0, 29.4, 28.5, 22.6, 20.5, 14.6, 8.4. HRMS (ESI): m/z calculated for C₄₁H₆₁FN₆O₈ + H⁺ [M + H⁺]: 785.4613; found 785.4626. LC-MS: R_t = 9.73 min; area 100%.

Ethyl-7-(4-(N²-(N²-(tert-butoxycarbonyl)-N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-L-lysyl)-N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (13). Reagents: (7) (129 mg, 0.18 mmol, 1 equiv.), CTEMPOMe (86 mg, 0.40 mmol, 2.2 equiv.), HOBt (68 mg, 0.50 mmol, 2.8 equiv.), EDC (90 mg, 0.47 mmol, 2.6 equiv.), i-Pr₂NEt (0.2 mL, 1.08 mmol, 6 equiv.). Product: Pale yellow intractable solid (187 mg, 94%). ¹H NMR (600 MHz, CDCl₃): δ = 8.53 (s, 1H, NCH=C), 8.05 (d, J = 12.9 Hz, 1H, Ar-H), 7.25 (d, J = 6.9 Hz, 1H, Ar-H), 7.00 (d, J = 8.1 Hz, 1H, COC(O)NHCH), 5.90 (t, J = 5.8 Hz, 1H, CH₂CHC(O)NHCH₂), 5.79 (t, J = 5.8 Hz, 1H, CH₂CHC(O)NHCH₂), 5.32 (d, J = 7.2 Hz, 1H, C(O)NH), 4.91 (td,

$J = 8.2, 4.5$ Hz, 1H, C(O)NHCH), 4.38 (q, $J = 7.1$ Hz, 2H, OCH₂CH₃), 4.05 (m, 1H, NC(O)CH), 3.94, 3.82, 3.70, 3.66, and 3.33–3.26 (5 × m, 8H, 2 × NCH₂CH₂N), 3.45 (m, 1H, C=CHNCH), 3.24–3.15 (m, 4H, CHC(O)NHCH₂), 2.42 (tt, $J = 12.8, 3.7$ Hz, 1H, CHC(O)NHCH₂), 1.80, 1.65, 1.58, 1.52, 1.41–1.36 (5 × m, 20H, 2 × NHCH₂CH₂CH₂CH₂CH and 4 × ONCCH₂), 1.44 (s, 9H, 3 × OCCH₃), 1.40 (t, $J = 7.1$ Hz, 3H, OCH₂CH₃), 1.32 (m, 2H, NCHCH₂), 1.22–1.16 (4 × s, 12H, 2 × CCH₃), 1.13 (m, 2H, NCHCH₂), 1.09 (4 × s, 12H, 2 × CCH₃). ¹³C NMR (150 MHz, CDCl₃): $\delta = 175.5, 175.3, 175.2, 172.1, 170.0, 166.0, 155.9, 152.7, 148.4, 138.1, 123.9, 113.8, 113.7, 110.8, 105.5, 80.2, 65.6, 61.1, 59.4, 55.0, 50.2, 50.0, 48.6, 45.6, 42.7, 42.6, 42.5, 42.1, 38.9, 38.7, 36.6, 36.5, 34.7, 33.0, 32.4, 31.8, 29.8, 29.4, 29.3, 28.5, 22.7, 22.3, 20.5, 14.6, 8.4$. HRMS (ESI): m/z calculated for C₅₈H₉₂FN₉O₁₁ + H⁺ [M + H⁺]: 1110.6979; found 1110.7000. LC-MS: $R_t = 13.09$ min; area 98.49%.

1-Cyclopropyl-6-fluoro-7-(4-(N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-L-lysyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (14). Reagents: Step 1 (ester hydrolysis): (12) (110 mg, 0.14 mmol, 1 equiv.); Step 2 (Boc-deprotection) Crude product from step 1 (~90 mg, 0.14 mmol, 1 equiv.). Product: White solid (85 mg, 92% over two steps). ¹H NMR (600 MHz, CDCl₃): $\delta = 8.77$ (s, 1H, NCH=C), 8.05 (d, $J = 13.0$ Hz, 1H, Ar-H), 7.40 (d, $J = 6.9$ Hz, 1H, Ar-H), 5.67 (t, $J = 5.9$ Hz, 1H, CHC(O)NHCH₂), 3.96–3.69 (3 × br, m, 4H, NCH₂CH₂N), 3.59 (s, 3H, NOCH₃), 3.57 (m, 1H, C=CHNCH), 3.42–3.19 (3 × br, m, 6H, NCH₂CH₂N and CHC(O)NHCH₂), 2.39 (tt, $J = 15.1, 5.0$ Hz, 1H, CHC(O)NHCH₂), 1.74, 1.63–1.42 (3 × m, 10H, NHCH₂CH₂CH₂CH₂CH and 2 × ONCCH₂), 1.25 (m, 2H, NCHCH₂), 1.20 (m, 2H, NCHCH₂), 1.19 (2 × s, 6H, 2 × CCH₃), 1.09 (2 × s, 6H, 2 × CCH₃). ¹³C NMR (150 MHz, CDCl₃): $\delta = 177.3, 175.4, 167.0, 154.6, 152.9, 147.7, 145.5, 145.4, 139.2, 120.7, 112.9, 112.8, 108.5, 105.6, 100.3, 65.6, 59.4, 51.0, 50.2, 49.8, 45.2, 42.7, 42.6, 42.0, 38.8, 36.7, 35.5, 34.3, 33.0, 31.1, 29.8, 29.6, 22.7, 20.4, 8.4$. MP: 79.5–82.3 °C. HRMS (ESI): m/z calculated for C₃₄H₄₉FN₆O₆ + H⁺ [M + H⁺]: 657.3776; found 657.3778. LC-MS: $R_t = 3.83$ min; area 97.57%.

1-Cyclopropyl-6-fluoro-7-(4-(N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-N²-(N⁶-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)-L-lysyl)-L-lysyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (15). Reagents: Step 1 (ester hydrolysis): (13) (135 mg, 0.12 mmol, 1 equiv.); Step 2 (Boc-deprotection) Crude product from step 1 (~123 mg, 0.12 mmol, 1 equiv.). Product: Light yellow intractable solid (117 mg, 99% over two steps). ¹H NMR (600 MHz, CD₃OD): $\delta = 8.76$ (s, 1H, NCH=C), 8.94 (d, $J = 12.9$ Hz, 1H, Ar-H), 7.61 (s, 1H, Ar-H), 4.89 (m, 1H, C(O)NHCH), 3.93, 3.87, 3.78, 3.46, 3.39, 3.23–3.08 (6 × m, 8H, 2 × NCH₂CH₂N and NC(O)CH), 3.60 and 3.58 (2 × s, 6H, NOCH₃), 3.45 (m, 1H, C=CHNCH), 3.24–3.15 (m, 4H, CHC(O)NHCH₂), 2.56 (m, 1H, CHC(O)NHCH₂), 1.82, 1.73, 1.62, 1.52, 1.51, 1.40 (5 × m, 20H, 2 × NHCH₂CH₂CH₂CH₂CH and 4 × ONCCH₂), 1.31 (m, 2H, NCHCH₂), 1.24–1.14 (4 × s, 12H, 2 × CCH₃), 1.16 (m, 2H, NCHCH₂), 1.09 (4 × s, 12H, 2 × CCH₃). ¹³C NMR (150 MHz, CD₃OD): $\delta = 177.9, 172.4, 163.1, 162.9, 155.8, 154.1, 149.3, 121.2, 119.2, 117.3, 115.3, 107.6, 65.9, 60.4, 55.5, 50.3, 50.0, 49.8, 49.6, 46.7, 46.5, 43.6, 43.5, 43.2, 40.1, 39.8, 37.2, 37.1$,

36.7, 35.5, 33.4, 32.6, 30.3, 30.2, 24.0, 23.9, 20.7, 8.6. HRMS (ESI): m/z calculated for C₅₁H₈₀FN₉O₉ + H⁺ [M + H⁺]: 982.6141; found 982.6136. LC-MS: $R_t = 7.34$ min; area 97.86%.

4.3. Biology general information

Bacterial strain, culture conditions, and human cell culture. *Escherichia coli* UTI89 a reference cystitis isolate of serotype O18:K1:H7³¹ was grown routinely in lysogeny broth (LB) medium with shaking (200 rpm) at 37 °C. Minimum inhibitory concentration (MIC) assays were conducted in Mueller Hinton (MH) medium (OXOID, Thermo Fisher), biofilms were grown in LB medium, and biofilm challenges (antimicrobial susceptibility testing) were performed in MH medium. Human bladder epithelial cell line, T24 (ATCC® HTB-4™) was purchased from the American Type Culture Collection (Manassas, VA, USA) and was cultured in McCoy's 5A modified medium (Life Technologies, Gibco, Australia) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Gibco, Australia) at 37 °C in a humidified atmosphere of 5% CO₂ until 90% confluency was reached.

4.4. Antibacterial assays

***E. coli* UTI89 MIC susceptibility assay for compounds 10, 11, 14, 15, CTEMPO, and ciprofloxacin.** The MIC for each compound 10, 11, 14, 15, CTEMPO, and ciprofloxacin were determined by the broth microdilution method, in accordance with the 2015 (M07-A10) Clinical and Laboratory Standards Institute (CLSI). In a 96-well plate, twelve two-fold serial dilutions of each compound were prepared to a final volume of 100 µL in MH medium. At the initial time of inoculation, each well was inoculated with 5 × 10⁵ bacterial cells, which had been prepared from fresh overnight cultures in MH. The MIC for a compound is defined as the lowest concentration of an agent that prevented visible bacterial growth after 18 hours of static incubation at 37 °C. The symbol ≤ indicates the precise MIC value lies between the concentration reported and the 2-fold dilution below. MIC values were also confirmed by spectrophotometric analysis at OD_{600nm} in a BMG Spectrostar plate reader. Compounds 10, 11, 14, and 15 were tested between the concentration range of 500 to 0.12 µM, while the antibiotic ciprofloxacin was tested between the concentration range of 12 to 0.006 µM, and CTEMPO was tested between the concentration range of 1000 to 1.95 µM. Working solutions of compounds 10, 11, 14, and 15, and ciprofloxacin were prepared in MH medium that had been inoculated with bacteria at 5 × 10⁶ CFU mL⁻¹. Negative controls containing DMSO at the highest concentration required to produce a 500 µM final concentration for compounds 10, 11, 14, and 15, were also prepared and serially diluted (12 dilutions total) in the same method as the antimicrobial agents. MIC values for compounds 10, 11, 14, and 15, CTEMPO and ciprofloxacin were obtained from at least 2 independent experiments, each consisting of at least 3

biological replicates and at least 2 technical replicates of each biological replicate.

***E. coli* UTI89 MBEC susceptibility assay for compounds 10, 11, 14, 15, CTEMPO, and ciprofloxacin.** Biofilms were grown using the Calgary Biofilm Device (CBD) purchased from Innovotech Inc. (Canada) and used unmodified. The device consists of a two-part reaction vessel. The top component contains 96 identical pegs protruding down from the lid, which fits into a standard flat bottom 96-well plate (bottom component). Biofilm cultivation was achieved following a previously documented methodology.³⁵ Overnight cultures of the specific bacterial species prepared in LB, were diluted to $\sim 10^6$ CFU mL⁻¹ via spectrophotometry (OD_{600nm}), in LB medium. The enclosed flat bottom 96-well plate was inoculated with $\sim 10^5$ CFU (150 μ L) UTI89 in each well. The peg lid was returned to the inoculated microtiter plate, and the complete CBD was incubated at 150 rpm, 37 °C, and 95% relative humidity for 24 hours. Establishment of mature biofilms at this stage of the assay was determined by removing at least three individual pegs from the device, placing them in fresh LB media and sonicating for 30 minutes at <20 °C, which sufficiently disrupts biofilms and dislodges cells into the recovery media (LB). Recovered cells were enumerated by serial dilution and plating on LB agar. For treatment of established biofilms, the CBD lid containing 24 hour biofilms was rinsed for 60 seconds in PBS (96-well plate, 200 μ L in each well) to remove loosely adherent planktonic cells before being transferred to a new flat bottom 96-well plate (challenge plate), which contained 2-fold serial dilutions of compounds 10, 11, 14, and 15 (concentration range between 1000 to 6.25 μ M) or CTEMPO (concentration range between 1000 to 6.25 μ M) or ciprofloxacin (concentration range between 800 to 6.25 μ M) or ciprofloxacin/CTEMPO (final concentration 400 μ M; specific concentration ratios 400: 0, 380: 20, 360: 40, 320: 80) in MH medium (total volume 200 μ L per well). The complete CBD was then incubated at 120 rpm, 37 °C, and 95% relative humidity for 24 hours. The lid was removed from the challenge plate and rinsed twice for 60 seconds in PBS (96-well plate, 200 μ L in each well). The rinsed CBD lid, with attached pegs containing the treated biofilms, was either transferred to a new 96-well plate containing fresh LB recovery media (for live CFU enumeration by plating) or pegs were removed for staining and microscopic analysis. To assist the transfer of any remaining viable cells into the recovery media, the device was sonicated for 30 minutes (<20 °C). The peg lid was then discarded, and 50 μ L from each well was serially diluted and spotted on LB agar plates for CFU enumeration. The remainder recovery plate was then covered and incubated at 37 °C, and 95% relative humidity for 24 hours. MBEC values were determined as the lowest concentration that resulted in zero bacterial growth after 24 hours (99.9% eradication compared to controls). The symbol $\leq$ indicates the precise MBEC value lies between the concentration reported and the 2-fold dilution below.

4.5. Biofilm visualization by fluorescence microscopy

Live/dead staining (fluorescence microscopy) of *E. coli* UTI89 biofilms treated with CDN 11. Live/dead cell staining of all *E. coli* UTI89 biofilms was performed with the LIVE/DEAD™ BacLight™ Bacterial Viability kit L7007 (Life Technologies, Australia) as per manufacturer's instructions. The kit utilizes the two nucleic acid stains SYTO9 and propidium iodide (PI). SYTO9 is a membrane-permeable, green-fluorescing stain, with excitation and emission maxima of 480 nm and 500 nm, respectively. PI is a membrane-impermeable, red-fluorescing stain, only capable of entering membrane-compromised cells, with excitation and emission maxima of 530 nm and 620 nm, respectively.

Biofilms were grown and treated as documented above with the following modifications. Treated biofilms were first rinsed for 30 seconds in saline (0.9%), before being incubated in the dark at 30 °C for 25 minutes, with SYTO9 and PI (200 μ L). The final applied concentration of SYTO9 and PI was 3.35 μ M and 20 μ M, respectively. Stained biofilms on pegs were then rinsed for 30 seconds in saline (0.9%) and mounted using ProLong® Diamond Antifade Mountant (Life Technologies, Australia) or saline (0.9%). Visualization of stained pegs was conducted no more than 1 hour after staining and mounting using a Zeiss Axio Vert.A1 FL-LED.

4.6. Cytotoxicity assay

LDH release assay for cytotoxicity assessment of CDN 11. The cytotoxicity of CDN 11 against human T24 urinary bladder epithelial cells was examined utilizing the standard Pierce™ LDH cytotoxicity assay kit (Life Technologies, Australia) and assays were performed according to the manufacturer's instructions. Briefly, T24 cells were seeded at a density of 7500 cells/100 μ L in a 96 well tissue culture plate and after 24 hours incubation at 37 °C in a humidified atmosphere of 5% CO₂. The stock solution of CDN 11 (in DMSO) was then diluted in PBS and added to the T24 cells to give the final concentration of 500 μ M. The treated samples were incubated for 24 hours under the same conditions as above. Lysis buffer 10 $\times$ was used for maximum LDH release (positive control), and cells treated with DMSO/PBS (4.5% DMSO final concentration) or sterile water served as negative controls. After 24 hours 50 μ L of the supernatant was transferred into a new 96-well plate, mixed with 50 μ L of the reaction mixture (LDH assay kit) and incubated at room temperature (protected from light) for 30 minutes before the stop solution (50 μ L) was added. The plate was then centrifuged (1000 $\times$ g) for 5 minutes to remove air bubbles, and the absorbance at 490 and 680 nm was measured using a Spectrostar plate reader (BMG).

Abbreviations

AMP	Antimicrobial peptides
Boc	<i>tert</i> -Butyloxycarbonyl
CBD	Calgary biofilm device

Cbz	Carboxybenzyl
CFU	Colony forming units
Cipro	Ciprofloxacin
CMM	Ciprofloxacin–monomethoxyamine
CMN	Ciprofloxacin–mononitroxide
CDM	Ciprofloxacin–dimethoxyamine
CDN	Ciprofloxacin–dinitroxide
CTEMPO	4-Carboxy-2,2,6,6-tetramethylpiperidin-1-yloxy
CTEMPOMe	1-Methoxy-2,2,6,6-tetramethylpiperidine-4-carboxylic acid
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPS	Extracellular polymeric substance
EPR	Electron paramagnetic resonance
HOBT	Hydroxybenzotriazole
HP	Halogenated phenazines
IBC	Intracellular bacterial communities
MBEC	Minimum biofilm eradication concentration
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance
Pd/C	Palladium on carbon
i-Pr ₂ Net	N,N-Diisopropylethylamine
QAC	Quaternary ammonium compounds
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UPEC	Uropathogenic <i>Escherichia coli</i>
UTI	Urinary tract infections

Conflicts of interest

There are no conflicts to declare.

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References

- 1 B. Foxman, R. Barlow, H. D'Arcy, B. Gillespie and J. D. Sobel, *Ann. Epidemiol.*, 2000, **10**, 509–515.
- 2 E. H. Kennedy, M. T. Greene and S. Saint, *J. Hosp. Med.*, 2013, **8**, 519–522.
- 3 B. Foxman, *Infect. Dis. Clin. North Am.*, 2014, **28**, 1–13.
- 4 B. Foxman, K. L. Klemstine and P. D. Brown, *Ann. Epidemiol.*, 2003, **13**, 144–150.
- 5 R. Kucheria, P. Dasgupta, S. Sacks, M. Khan and N. Sheerin, *Postgrad. Med. J.*, 2005, **81**, 83–86.
- 6 A. Stapleton, *Am. J. Med.*, 2002, **113**, 80–84.
- 7 S. H. Vermeulen, N. Hanum, A. J. Grotenhuis, G. Castaño-Vinyals, A. G. van der Heijden, K. K. Aben, I. U. Mysorekar and L. A. Kiemeny, *Br. J. Cancer*, 2015, **112**, 594–600.
- 8 B. Foxman, *Am. J. Public Health*, 1990, **80**, 331–333.
- 9 F. Yan and D. B. Polk, *Curr. Opin. Gastroenterol.*, 2004, **20**, 565–571.
- 10 B. M. Zalewska-Piatek, S. I. Wilkanowicz, R. J. Piatek and J. W. Kur, *Pol. J. Microbiol.*, 2009, **58**, 223–229.
- 11 J. W. Costerton, Z. Lewandowski, D. E. Caldwell, D. R. Korber and H. M. Lappin-Scott, *Annu. Rev. Microbiol.*, 1995, **49**, 711–745.
- 12 T. J. Hannan, M. Totsika, K. J. Mansfield, K. H. Moore, M. A. Schembri and S. J. Hultgren, *FEMS Microbiol. Rev.*, 2012, **36**, 616–648.
- 13 S. Mittal, M. Sharma and U. Chaudhary, *Pathog. Global Health*, 2015, **109**, 26–29.
- 14 J. M. McCarty, G. Richard, W. Huck, R. M. Tucker, R. L. Tosiello, M. Shan, A. Heyd and R. M. Echols, *Am. J. Med.*, 1999, **106**, 292–299.
- 15 M. G. Blango and M. A. Mulvey, *Antimicrob. Agents Chemother.*, 2010, **54**, 1855–1863.
- 16 A. Ito, A. Taniuchi, T. May, K. Kawata and S. Okabe, *Appl. Environ. Microbiol.*, 2009, **75**, 4093–4100.
- 17 K. Lewis, *Antimicrob. Agents Chemother.*, 2001, **45**, 999–1007.
- 18 J. B. Kaplan, *J. Dent. Res.*, 2010, **89**, 205–218.
- 19 N. Barraud, D. J. Hassett, S.-H. Hwang, S. A. Rice, S. Kjelleberg and J. S. Webb, *J. Bacteriol.*, 2006, **188**, 7344–7353.
- 20 C. de la Fuente-Núñez, F. Reffuveille, K. E. Fairfull-Smith and R. E. W. Hancock, *Antimicrob. Agents Chemother.*, 2013, **57**, 4877–4881.
- 21 C. J. Sanchez, K. S. Akers, D. R. Romano, R. L. Woodbury, S. K. Hardy, C. K. Murray and J. C. Wenke, *Antimicrob. Agents Chemother.*, 2014, **58**, 4353–4361.
- 22 F. Reffuveille, C. de la Fuente-Núñez, R. E. W. Hancock and K. E. Fairfull-Smith, *Pathog. Dis.*, 2015, **73**, ftv016.
- 23 N. R. B. Boase, M. D. T. Torres, N. L. Fletcher, C. de la Fuente-Núñez and K. E. Fairfull-Smith, *Polym. Chem.*, 2018, **9**, 5308–5318.
- 24 A. D. Verderosa, S. C. Mansour, C. de la Fuente-Núñez, R. E. W. Hancock and K. E. Fairfull-Smith, *Molecules*, 2016, **21**, 841.
- 25 A. D. Verderosa, C. de la Fuente-Núñez, S. C. Mansour, J. Cao, T. K. Lu, R. E. W. Hancock and K. E. Fairfull-Smith, *Eur. J. Med. Chem.*, 2017, **138**, 590–601.
- 26 N. Barraud, B. G. Kardak, N. R. Yepuri, R. P. Howlin, J. S. Webb, S. N. Faust, S. Kjelleberg, S. A. Rice and M. J. Kelso, *Angew. Chem., Int. Ed.*, 2012, **51**, 9057–9060.

- 27 A. T. Garrison, Y. Abouelhassan, V. M. Norwood, D. Kallifidas, F. Bai, M. T. Nguyen, M. Rolfe, G. M. Burch, S. Jin, H. Luesch and R. W. Huigens, *J. Med. Chem.*, 2016, **59**, 3808–3825.
- 28 M. C. Jennings, L. E. Ator, T. J. Paniak, K. P. Minbiole and W. M. Wuest, *ChemBioChem*, 2014, **15**, 2211–2215.
- 29 G. H. De Zoysa, A. J. Cameron, V. V. Hegde, S. Raghothama and V. Sarojini, *J. Med. Chem.*, 2015, **58**, 625–639.
- 30 M. M. Konai and J. Haldar, *ACS Infect. Dis.*, 2015, **1**, 469–478.
- 31 M. A. Mulvey, J. D. Schilling and S. J. Hultgren, *Infect. Immun.*, 2001, **69**, 4572–4579.
- 32 D. C. Hooper, *Clin. Infect. Dis.*, 2001, **32**, S9–S15.
- 33 I. Keren, N. Kaldalu, A. Spoering, Y. Wang and K. Lewis, *FEMS Microbiol. Lett.*, 2004, **230**, 13–18.
- 34 T. K. Wood, S. J. Knabel and B. W. Kwan, *Appl. Environ. Microbiol.*, 2013, **79**, 7116–7121.
- 35 J. J. Harrison, C. A. Stremick, R. J. Turner, N. D. Allan, M. E. Olson and H. Ceri, *Nat. Protoc.*, 2010, **5**, 1236–1254.