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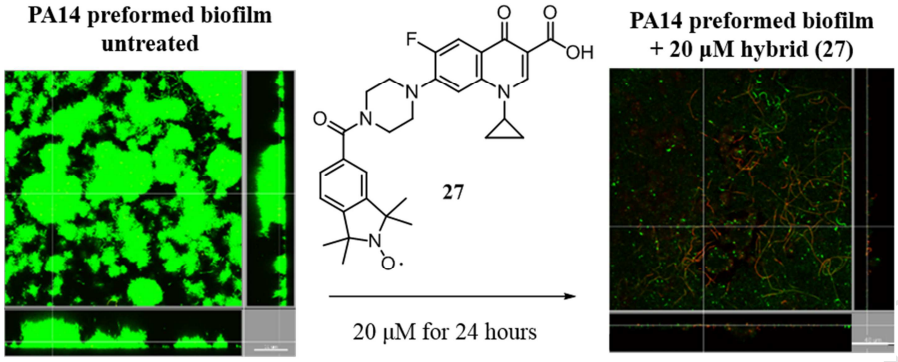
Graphical Abstract



Ciprofloxacin-Nitroxide Hybrids with Potential for Biofilm Control

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Author Contributions

All authors contributed to the writing of this manuscript and have given approval to the final version of the manuscript.

ABSTRACT

As bacterial biofilms display extreme tolerance to conventional antibiotic treatments, it has become imperative to develop new antibacterial strategies with alternative mechanisms of action. Herein, we report the synthesis of a series of ciprofloxacin-nitroxide conjugates and their corresponding methoxyamine derivatives in high yield. This was achieved by linking various nitroxides or methoxyamines to the secondary amine of the piperazine ring of ciprofloxacin using amide bond coupling. Biological evaluation of the prepared compounds on preformed *P. aeruginosa* biofilms in flow cells revealed substantial dispersal with ciprofloxacin-nitroxide hybrid **25**, and virtually complete killing and removal (94%) of established biofilms in the presence of ciprofloxacin-nitroxide hybrid **27**. Compounds **25**-**28** were shown to be non-toxic in both human embryonic kidney 293 (HEK 293) cells and human muscle rhabdomyosarcoma (RD) cells at concentrations up to 40 μM. Significantly, these hybrids demonstrate the potential of antimicrobial-nitroxide agents to overcome the resistance of biofilms to antimicrobials via stimulation of biofilm dispersal or through direct cell killing.

KEYWORDS

radical; antibiotic; biofilm; nitroxide; ciprofloxacin

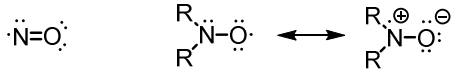
1. INTRODUCTION

The attachment of bacteria to surfaces, and their subsequent ability to aggregate into colonies called biofilms, is a significant problem in healthcare systems around the world [1-3]. It has been estimated that biofilms are involved in around 80% of all microbial infections in humans [4], including those associated with medical devices [5] and chronic wounds [6]. While a variety of effective antimicrobial strategies exist for the treatment of planktonic bacteria, these approaches are rarely effective against biofilms [7, 8], which have been reported to be up to one thousand times more resistant to antibiotic therapies [4, 9, 10]. Accordingly, there is an urgent need to develop novel strategies for the treatment of established biofilms.

It is now well recognized that bacteria reside primarily in biofilms but can revert to planktonic lifestyle by modulating the expression of specific genes [11]. Thus, one approach to target bacteria in biofilms has involved the development of small molecules with the ability to inhibit and/or disperse bacterial biofilms through non-microbicidal mechanisms [12, 13]. Nitric oxide (NO) has been identified to play a central role in biofilm formation and dispersal [14-16] across a range of biofilm-forming species [17]. When used at low, non-toxic concentrations (in the pM to low nM range), nitric oxide is capable of dispersing a pre-formed biofilm by triggering the transitions of cells to the motile, planktonic state [15, 17]. Mechanistically, this effect has been correlated with a decrease in the intracellular levels of the secondary messenger cyclic di-GMP, which is involved in biofilm development [11, 18].

The controlled delivery of nitric oxide to biological systems is challenging as it is an extremely reactive gas with a short half-life of 0.1-5 seconds [19]. Efforts to circumvent the problems associated with nitric oxide delivery have included the synthesis of NO-donor molecules [20], and extensive reviews on the dispersal activity of NO-donor in bacterial biofilms have been written recently [21]. Utilizing the NO-donor concept, a variety of anti-biofilm compounds have been developed [22]. However, as NO-donor molecules are also often inherently unstable [23], the use of nitroxides as an alternative for biofilm dispersal have recently been examined.

Nitroxides are stable free radical species that possess a disubstituted nitrogen atom linked to a univalent oxygen atom [24]. Both nitroxides and nitric oxide are structurally similar, as both species possess an unpaired electron, which is delocalized over the nitrogen-oxygen bond (Figure 1). Furthermore, the biological effects of nitroxides can be rationalized by their nitric oxide-mimetic properties, with both compounds known to be efficient scavengers of protein-derived radicals [25]. In contrast to gaseous nitric oxide, nitroxides have the advantage in that they are typically air-stable crystalline solids.



Nitric oxide **Nitroxide**

Figure 1. The structure of nitric oxide and the general structure of a nitroxide.

Previously, we have demonstrated that nitroxides can act in a similar manner to nitric oxide and disperse *Pseudomonas aeruginosa* biofilms generated in flow cell chambers [26]. When applied at 20 μM concentrations, nitroxides were able to both inhibit *P. aeruginosa* biofilm formation and trigger the dispersal of established *P. aeruginosa* biofilms. The dispersal ability of nitroxides has also been documented by others using the less-sensitive crystal violet staining assay at higher concentrations (in the 5 mM range) [27, 28]. Nitroxides have also recently been shown to enhance the anti-bacterial activity of silver nanoparticles when coupled together to give a nitroxide-coated silver nanoparticle [29]. In addition to demonstrating the inhibiting and dispersal capabilities of nitroxides, we have also reported the potential for biofilm removal when the biofilm dispersing properties of nitroxides are utilized in combination with an antibiotic (ciprofloxacin) [30]. The results of this study indicate that the well-known resistance of biofilms to antimicrobial treatments could be alleviated by employing the dispersal ability of nitroxides. Furthermore, we have recently shown that combining a nitroxide and an antibiotic within a single molecule is an effective approach to eradicate mature *P. aeruginosa* biofilms [31]. These results demonstrate that the covalent tethering of the antibiotic to the nitroxide positions the antibiotic near the site of nitroxide-induced biofilm dispersal, and thereby allows the antibiotic to act directly on the newly dispersed cells before they resume their preferred biofilm mode of growth. In fact, ciprofloxacin-nitroxide hybrid **1** (Figure 2), which bears the TEMPO nitroxide moiety, was shown to both induce *P. aeruginosa* biofilm dispersal and subsequently eradicate the resulting dispersed cells (up to 95% removal of mature biofilms at 40 uM was observed) [31].

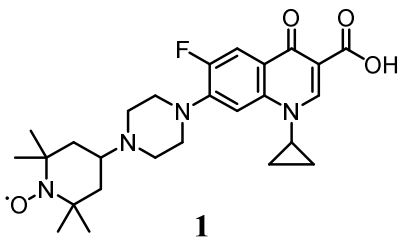


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Figure 2. Ciprofloxacin-nitroxide hybrid **1**.

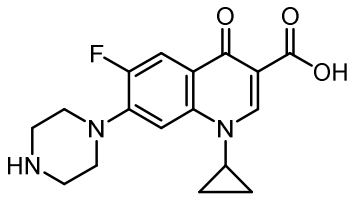
In our present study, we explored the synthesis of ciprofloxacin-nitroxide conjugate molecules joined via the secondary amine of the piperazine ring of ciprofloxacin using an amide linkage. The rationale behind this approach was that the amide functionality may allow access to ciprofloxacin-nitroxide conjugates which have improved organic (DMSO) solubility to aid in compound delivery into aqueous biological systems compared to their tertiary amine linked analogues [31]. Furthermore, the use of an amide bond linkage between the two moieties expands the variety of carboxylic acid-bearing cyclic nitroxides that can be tethered to the secondary amine of ciprofloxacin allowing for the effects of nitroxide ring size on anti-biofilm activity to be explored.

Herein, we report the design and synthesis of the second generation of ciprofloxacin-nitroxide hybrid molecules together with their biological evaluation as anti-biofilm agents for the treatment of existing *P. aeruginosa* biofilms.

2. RESULTS AND DISCUSSION

2.1. Chemistry

In line with our previous strategy to generate ciprofloxacin-nitroxide conjugates, we again chose to exploit the secondary amine of the piperazine ring at the 7-position of the fluoroquinolone based antibiotic ciprofloxacin **2** (Figure 3) as a useful handle where synthetic transformations could be performed without significantly altering the antimicrobial properties of ciprofloxacin [31].



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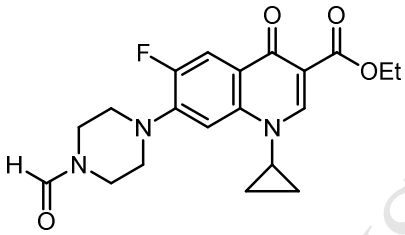

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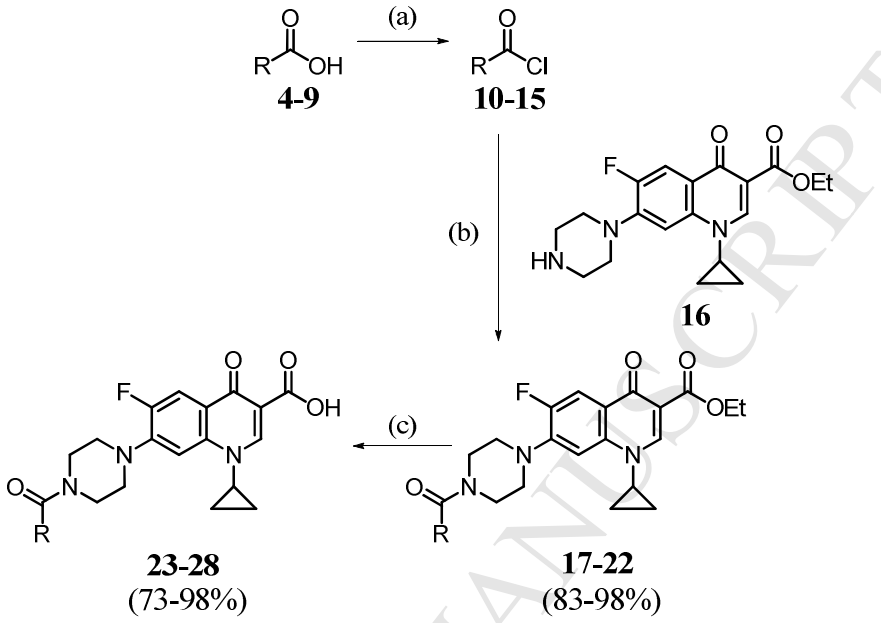
Figure 3. Ciprofloxacin **2** and *N*-formyl ciprofloxacin derivative **3**.

To generate our second generation of ciprofloxacin-nitroxide hybrids, we tethered nitroxides to the secondary amine of the piperazine ring of ciprofloxacin **2** using amide bond coupling. The commercially available cyclic nitroxides 4-carboxy-2,2,6,6-tetramethylpiperidin-1-yloxyl (CTEMPO) **6** and 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (CPROXYL) **4** as well as the more rigid isoindoline nitroxide 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) **8** [32] were selected as the nitroxide coupling partners as these systems are resistant to degradation through disproportionation (due to the presence of bis(*tert*-alkyl) groups on the carbon atoms α to the nitroxide) [33] and both the piperidine- and isoindoline-based systems have previously demonstrated dispersal activities in bacterial biofilms [26].

The amide coupling methodology was first optimized using CTEMPO **6**. To begin the synthesis, the carboxylic acid of ciprofloxacin **2** was protected as an ethyl ester using previously documented procedures to give **16** [34]. The carboxylic acid of CTEMPO **6** was activated by conversion to the corresponding acid chloride **12** with thionyl chloride and then immediately reacted with a 1,4-dioxane solution of the protected ciprofloxacin **16** under basic conditions. After heating at 60^{o}C for 1 hour, no starting material **16** remained (TLC analysis) and the desired ciprofloxacin-nitroxide hybrid **19** was isolated in moderate yield (59%). A second product, determined to be formamide derivative **3** (Figure 3) by 2D NMR spectroscopy and mass

spectrometry, was also formed in the reaction (41% yield). *N*-Formylation of the piperazine residue of ciprofloxacin has been previously reported to occur in the presence formic acid [35] or DMF [36] but as neither of these reagents were used directly in the amide synthesis of **19**, it was reasoned the formyl ciprofloxacin **3** may have arisen from an impurity in the commercially acquired 1,4-dioxane. This theory was confirmed when a 1,4-dioxane solution of protected ciprofloxacin **16** produced the *N*-formylated analogue **3** after heating at 60^{o}C for 1 hour. The use of an alternative solvent (DCM) in place of 1,4-dioxane provided a facile solution to avoid formation of this side product. Reaction of the protected ciprofloxacin **16** and acid chloride **12** in DCM in the presence of *N*,*N*-diisopropylethylamine (Scheme 1) gave the desired ciprofloxacin-nitroxide hybrid **19** in excellent yield (94%) after stirring at room temperature for 1 hour. These optimized conditions were then employed to generate conjugate compounds **17** and **21** in isolated yields of 87% and 98% respectively. Final deprotection of ethyl esters **17**, **19** and **21** via base mediated hydrolysis furnished ciprofloxacin-nitroxide hybrids **23**, **25** and **27** in excellent yield (73-98%). The free carboxylic acid of the fluoroquinolone core is important for antimicrobial activity as it binds, through magnesium, to the bacterial enzyme DNA gyrase [37, 38].

Scheme 1. Synthetic route to ciprofloxacin-nitroxide hybrids **23**, **25** and **27** and their corresponding methoxyamines **24**, **26** and **28**.[a]



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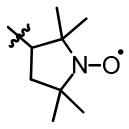
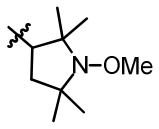
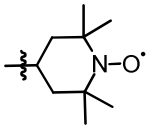
R Group			
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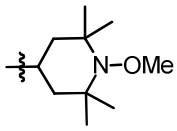
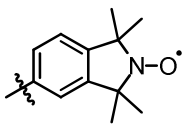
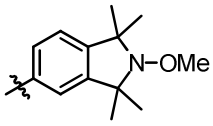
R Group			

[a]*Reagents and conditions:* (a) $SOCl_2$, toluene, pyridine, 0 ºC → rt, 1 h; (b) *i*-Pr_2NEt, DCM, rt, 1 h; (c) 2 M NaOH, MeOH, 50 ºC, 5 h.

In addition to the generation of three novel ciprofloxacin-nitroxide hybrid compounds **23**, **25** and **27**, methoxyamine derivatives **24**, **26** and **28** were also desired as control compounds to enable a direct comparison of the biofilm dispersal effect of the nitroxide moiety. The methoxyamine functionality was introduced to the carboxy-functionalized nitroxides **4**, **6** and **8** at the beginning of the synthetic sequence such that intermediates could be well characterized by

NMR spectroscopy (nitroxides are paramagnetic and typically display significantly broadened NMR signals). Utilising well-known Fenton chemistry [39], the nitroxides **4**, **6** and **8** were treated with methyl radicals generated from hydrogen peroxide, iron(II) sulphate heptahydrate and DMSO [40] to furnish methoxyamines **5**, **7** and **9** in excellent yield (88-92%). Amide coupling via the corresponding acid chlorides **11**, **13** and **15** using the methodology documented above gave the protected ciprofloxacin-methoxyamine conjugates **18**, **20** and **22** in high yield (83-97%). Subsequent ethyl ester deprotection using base mediated hydrolysis afforded the desired ciprofloxacin-methoxyamines **24**, **26** and **28** in high yield (80-85%).

2.2. Biological Evaluation

Our previous studies have indicated that pre-formed *P. aeruginosa* biofilms can be dispersed upon treatment with nitroxides [26]. Furthermore, we have documented the ability of the nitroxide CTEMPO **6** to almost completely remove mature *P. aeruginosa* and *E. coli* biofilms when used in combination with the antibiotic ciprofloxacin **2** in a flow cell assay [30]. Here, we employed a similar approach involving pre-formed *P. aeruginosa* biofilms grown in flow cell chambers to evaluate the dispersal and biofilm removal properties of the prepared ciprofloxacin-nitroxide compounds **23-28**. *P. aeruginosa* biofilms were formed in flow cell chambers for 48 h and then treated with 20 μM solutions of the hybrid compounds **23-28** (dissolved in DMSO and delivered into BM2 minimal medium supplemented with 0.4% of glucose) for 24 h. This specific concentration was chosen as it was previously established to be the most effective concentration for nitroxide-mediated biofilm dispersal [26]. Visualization of the resulting biofilms using the Live/Dead BacLight bacterial viability kit coupled with confocal microscopy provided the images shown in Figures 4, 5 and 6. From these images, we calculated the percentages of biofilm biomass removed by comparing the amount of biomass remaining from 3-day-old untreated biofilms relative to the remaining adhered biomass from flow cells treated with compounds **23-28**). We also calculated the percentage of dead cells present in each biofilm sample to assess the overall biofilm removal ability of the hybrid compounds. These results are displayed in Table 1.

The results obtained from the hybrid compounds bearing the TEMPO unit (**25** and **26**) at concentrations of 20 μM were examined first. The ciprofloxacin-nitroxide **25** displayed biofilm removal potential (37%) without major killing of biofilm cells (6%) (Table 1 and Figure 4b). Intriguingly, the corresponding methoxyamine **26** also exhibited some observable removal

activity (17%) (Figure 4c), although it was more modest than compound **25**. The dispersal of mature *P. aeruginosa* biofilms by alkoxyamines has been previously observed by others for an ethoxyamine derivative in a crystal violet biofilm assay, however in this previous study, the corresponding nitroxide compound was still more effective at inducing dispersal [27].

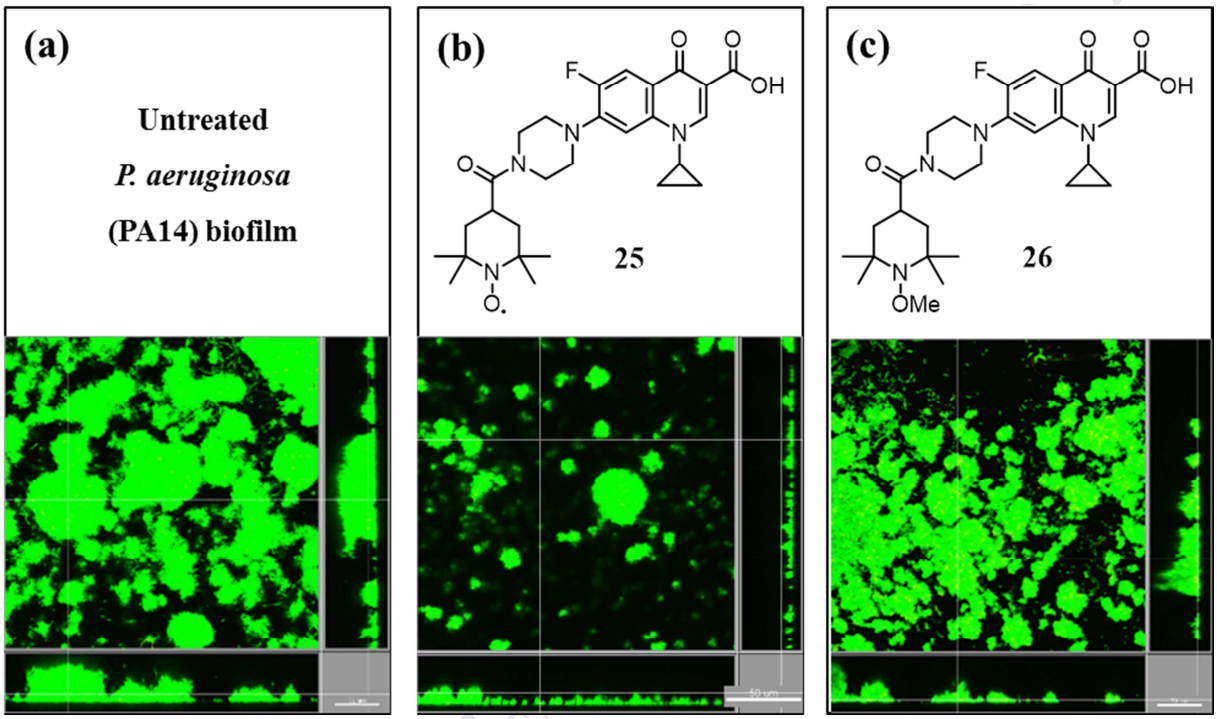


Figure 4. Confocal laser scanning microscopy images of 2-day-old pre-formed *P. aeruginosa* PA14 biofilms grown in a flow cell at 37°C, treated with (b) 20 µM of **25** and (c) 20 µM of **26** for 24 hours and then visualized with SYTO-9 (stains live cells green) and propidium iodide (stains dead cells red). Panel (a) shows an untreated *P. aeruginosa* PA14 biofilm after 3 days. At least two replicates were performed per condition. The scale bars represent 40 µm in length for images (a) and (c), and 50 µm for image (b). Each panel also shows the *xy*, *yz* and *xz* dimensions.

Next we analyzed the flow cell assay results from the ciprofloxacin-nitroxide compounds bearing PROXYL moieties (**23** and **24**). Both the nitroxide **23** and the methoxyamine **24** (Figures 5b and 5c), appeared to remove mature *P. aeruginosa* biofilms at 20 µM. However, the degree of removal was greater for the nitroxide **23** (67% biofilm removal) than the corresponding methoxyamine **24** (24% biofilm removal), and in particular **23,** unlike **24,** appeared to have greater dispersal potential (dispersing the large dense bacterial aggregates within the flow cell (Figures 5b and 5c). The ciprofloxacin-PROXYL analogue **23** was also tested at 10 µM in the

flow cell assay against mature *P. aeruginosa* biofilms. Some dispersal effects along with a 50% reduction in biofilm mass was observed (Figure 5a), however, the anti-biofilm activity of compound **23** was more pronounced at 20 µM (67%, Table 1).

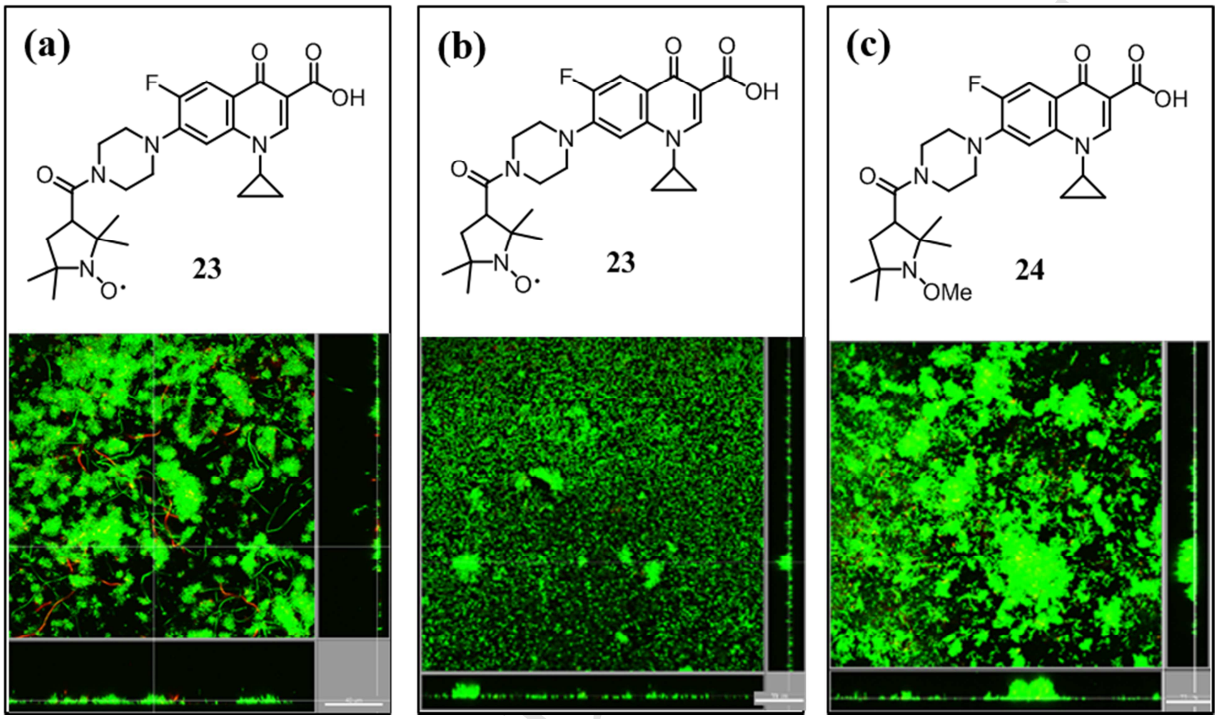


Figure 5. Confocal laser scanning microscopy images of 2-day-old pre-formed *P. aeruginosa* PA14 biofilms grown in a flow cell at 37°C, treated with (a) 10 µM of **23**, (b) 20 µM of **23** and (c) 20 µM of **24** for 24 hours and then visualized with SYTO-9 (stains live cells green) and propidium iodide (stains dead cells red). At least two replicates were performed per condition. The scale bars represent 40 µm in length for images (a)-(c). Each panel also shows the *xy*, *yz* and *xz* dimensions.

Lastly, the activity of the conjugate molecules bearing isoindoline moieties (**27** and **28**) were examined against mature *P. aeruginosa* biofilms in flow cell chambers. In the presence of 20 µM of ciprofloxacin-nitroxide hybrid **27**, a substantial reduction in the total biofilm biovolume (85%) occurred (Figure 6a) and 60% of the remaining biofilm biomass was composed of dead cells. This represents an improvement over hybrid compound **1** (our previously most active hybrid conjugate), which reduced total biofilm biovolume by 80% with 50% of the remaining biofilm biomass containing dead cells [31]. Furthermore, compound **27** was also found to be substantially more effective at treating *P. aeruginosa*-based biofilms than the parent antibiotic, ciprofloxacin (use at its MIC value), which only reduced the total biofilm biovolume by 7% with very few dead cells (8%) detected in the remaining biofilm biomass [30].

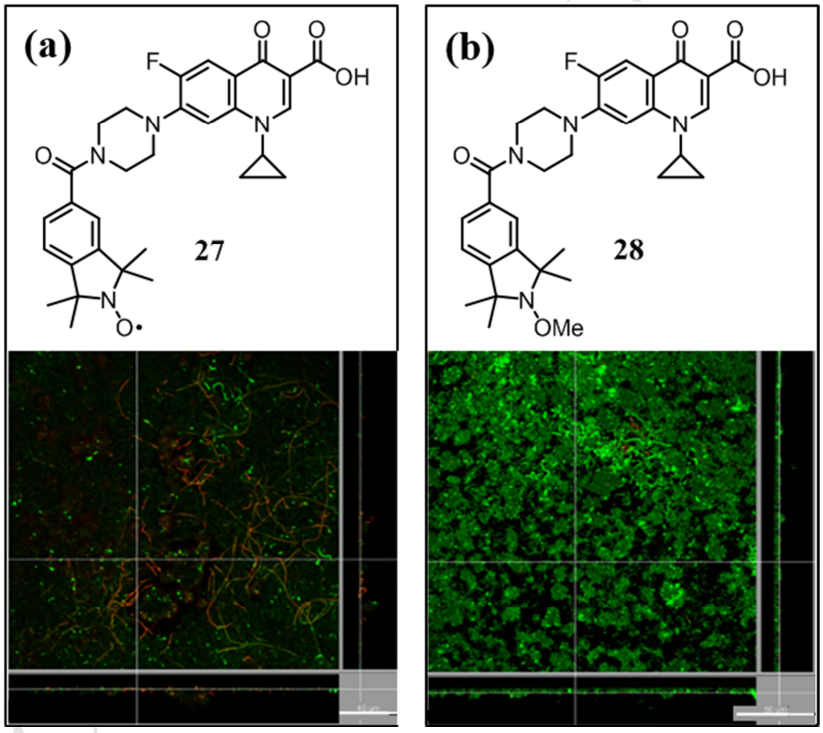


Figure 6. Confocal laser scanning microscopy images of 2-day-old pre-formed *P. aeruginosa* PA14 biofilms grown in a flow cell at 37°C, treated with (a) 20 µM of **27** and (b) 20 µM of **28** for 24 hours and then visualized with SYTO-9 (stains live cells green) and propidium iodide (stains dead cells red). Compound **27** led to cell filamentation, presumably via the active ciprofloxacin moiety. At least two replicates were performed per condition. The scale bars represent 40 µm in length for images (a) and (b). Each panel also shows the *xy*, *yz* and *xz* dimensions.

Table 1. Total live *P. aeruginosa* PA14 biofilm biomass eradication and biofilm dead cell values for 2-day-old biofilms treated with compounds **23**-**28** at 20 μM relative to the biomass and dead cell values of untreated 3-day-old biofilms.

Compound	Remaining Biomass (%)	Removed Biomass (%)	Dead Cells in Remaining Biomass (%)	Total Live Biofilm Biomass Eradication (%)[a]
Control PA14	--	--	4	--
23	33	67	0	67
24	76	24	9	31
25	63	37	6	41
26	83	17	16	30
27	15	85	60	94
28	47	53	2	54

[a]Calculated by adding the amount of dead cells remaining in the biofilm biomass to the initially removed biomass (*i.e.*, compound **27** had initially 85% removed biomass but as 60% of the remaining 15% of biomass was dead, the total live biofilm biomass eradication was 94%).

The observed filamentation and cell death were typical effects in the presence of ciprofloxacin [41]. Thus, compound **27** led to virtually complete removal of mature biofilms formed by *P. aeruginosa*, and treated cells often exhibited filamentous phenotypes (Figure 6a). Interestingly, the corresponding methoxyamine **28**, by comparison, was also able to reduce biofilm cell density by 53% at the same concentration (Table 1) but fewer cells were actually killed (2%) (Figure 6b). Of the three ciprofloxacin-nitroxide types examined (**23**, **25** and **27**), the most effective biofilm removing agent was the isoindoline analogue **27**. The TEMPO analogue **25** was the least active.

The minimal inhibitory concentrations (MIC) of each prepared compound were also measured using the broth microdilution method [42, 43]. The results shown in Table 2, revealed that the prepared compounds **23-28** exhibited minimal antibacterial activity, especially compared to free ciprofloxacin. The most effective compound with antimicrobial activity against planktonic bacteria was compound **23**, where no bacterial growth was observed at 160 μM. The obtained values are, however, substantially higher than that of ciprofloxacin **2** alone which gave a previously reported MIC of 0.5 μM.[48] Thus, the addition of nitroxide units to the piperazine ring of ciprofloxacin through amide bonds has resulted in decreased efficacy of the antibiotic. However, despite the substantially higher MIC of compounds **23-28**, ciprofloxacin alone has been previously shown to be ineffective at biofilm removal [30], whereas the ciprofloxacin-

nitroxide compounds **23**, **25** and **27** prepared in this work display significant anti-biofilm activities. This is evident with our most active compound (**27**), which at 20 µM (10.95 µg/mL) resulted in substantial removal (85%) of existing *P. aeruginosa* biofilms (Table 1).

Table 2. MIC values of ciprofloxacin-nitroxide hybrids **17**-**28** against *P. aeruginosa* PA14 planktonic bacteria.

Compound	MIC (µM)
23	160
24	311
25	312
26	>303
27	292 (160)[a]
28	>284 (>160)[a]
Ciprofloxacin **2**	0.5[b]

[a]More accurate value determined from mechanism of action studies via CFU/mL counts (Figure 7).
[b]Previously reported value measured using the same procedure.[41]

Additional killing assays of planktonic *P. aeruginosa* PA14 cultures showed that compound **27** was more effective at killing *P. aeruginosa* PA14 planktonic cells compared with compound **28**, and led to complete killing of bacterial cultures at the higher concentration used (see supporting information), thus correlating with its increased ability to directly eradicate and kill biofilm cells (Table 1). The lower dose required for biofilm killing in flow cell assays compared to planktonic killing assays is not surprising, as it has been observed before for similar and other compounds [41].

Next, we aimed to investigate mechanistic insights responsible for the biofilm inhibitory activity of specific ciprofloxacin-nitroxide hybrid compounds (Figure 7). We chose to examine the most active and least active nitroxides (**27** and **25**) from the flow cell assays and their corresponding methoxyamines (**28** and **26**). We leveraged our viable dispersal cell assay[30] consisting of performing CFU counts from the effluent of flow cell chambers upon nitroxide treatment. This system allows precise monitoring of viable cell counts over time post-treatment. We found that compounds **25** and **26** stimulated dispersion of bacteria from biofilms over time cf. the untreated group (Figure 7a), with compound **25** resulting in more dispersed bacteria as compared to **26** (Figure 7a). As such, we believe that the increased biofilm removal activity observed for **25** in comparison with **26** can at least partly be attributed to the increased dispersal activity of compound **25** (Table 1). On the other hand, no obvious increase in bacterial dispersal

was observed in treatments using **27** or **28** when compared to untreated controls (Figure 7b). This may imply that dispersed bacteria are immediately removed or, more likely, that **27** and **28** do not trigger biofilm dispersal but act through direct cell killing. The minimal viable dispersed bacteria reported in Figure 7b suggests that dispersal is not part of the mechanism of action of these compounds. However both **27** and **28** are capable of removing biofilms (Table 1), which indicates they may kill biofilm cells as a major mechanism of action, particularly in the case of **27** (Table 1). It is also likely that the compounds killed the dispersed cell population released from biofilms, as both **27** and **28** are capable of killing planktonic bacteria (see supporting information).

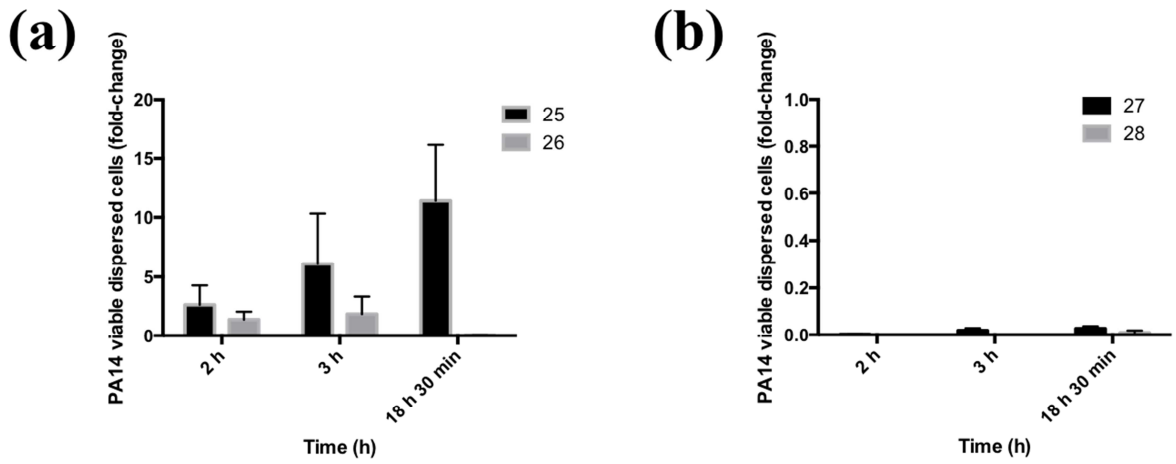


Figure 7. Mechanism of action studies. (a) Compound **25** stimulated cell dispersal from biofilms. Viable biofilm dispersal cell assay done using *P. aeruginosa* PA14 biofilms, which were grown in flow cells for 2 days and subsequently treated with 20 μM of compounds **25** and **26**. Dispersed cells were collected from the effluent of the flow cell chambers upon treatment with the compounds at the specified times. Collected bacteria were then plated for CFU counts on Pseudomonas Isolation Agar (PIA) plates and incubated at 37°C. The next day, bacteria were counted (CFU/mL) and fold-change differences in cell counts were calculated in dispersed cells of treated samples compared to those from untreated *P. aeruginosa* PA14 samples. Note: the fold-change of PA14 viable dispersed cells from compound **26** at 18 h 30 min was virtually 0. **(b)** Compound **27** kills planktonic bacteria and does not induce cell dispersal from biofilms. Biofilm dispersal cell assay was performed as described in (a) except treating biofilms with 20 μM of compounds **27** and **28**.

As biofilms are associated with a wide range of infections in humans, the cytotoxicity of a selection of the prepared compounds was examined in two different human cell lines using the lactate dehydrogenase (LDH) cytotoxicity assay. Compounds **25**-**28** were shown to be non-toxic

in both human embryonic kidney 293 (HEK 293) cells and human muscle rhabdomyosarcoma (RD) cells at concentrations from 5 µM to 40 µM (Figure 8). The addition of small quantities of DMSO as the solvent was also shown to have no significant effect on cell viability (see supporting information).

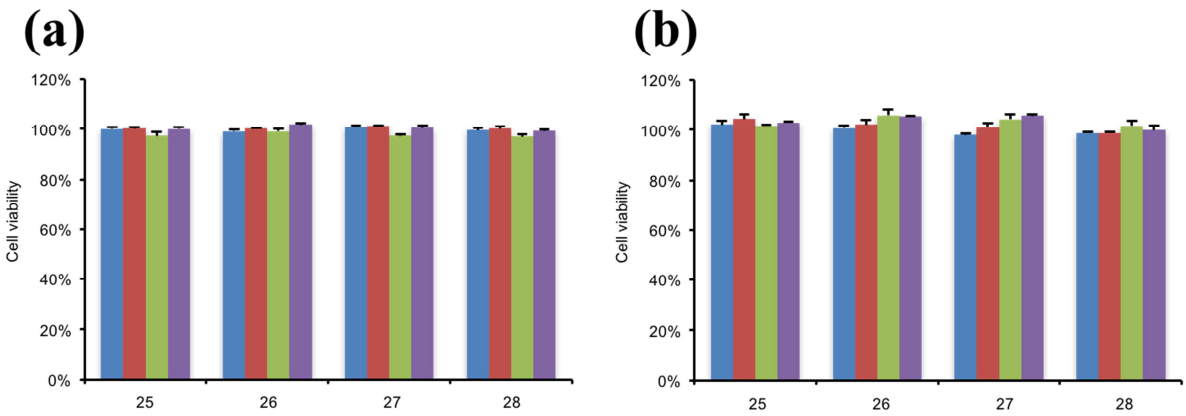


Figure 8. Cell viability studies for compounds **25-28** as a function of concentration (blue = 5 µM, red = 10 µM, green = 20 µM, purple = 40 µM): (a) HEK 293 cells, (b) RD cells.

3. CONCLUSION

Three ethyl ester protected ciprofloxacin-nitroxide hybrid compounds **17**, **19** and **21** and their methoxyamine analogues **18**, **20** and **22** were prepared using amide bond coupling in high to excellent yield (83-98%) from the corresponding acid chloride functionalized nitroxides **10**, **12** and **14** or methoxyamines **11**, **13** and **15** and the ethyl ester protected ciprofloxacin **16**. Deprotection of the amide-linked ethyl ester analogues **17-22** with base gave the corresponding ciprofloxacin-nitroxides **23**, **25** and **27** and their methoxyamines **24**, **26** and **28** in good to high yield (73-98 %). The synthesized compounds **23-28** exhibited modest antibacterial activities with MIC values ranging from 160 to >300 µM. In killing assays, both **27** and **28** exhibited anti-planktonic activity with **27** showing increased killing. These results suggest that the nitroxide moiety is key to the effectiveness of analogue **27** against planktonic cells. Evaluation of the prepared compounds **23-28** for anti-biofilm activity against mature *P. aeruginosa* biofilms was

performed in a flow cell assay. Several of the hybrid compounds were found to have the desired dual-action effect against established biofilms. For instance, treatment with compounds **25** and **26** substantially increased dispersal of bacteria from biofilms over time, particularly in the case of **25**, which likely reflected the biofilm removal results (37% for **25** and 17% for **26**) with no major biofilm cell killing events obtained (6% for **25** and 16% for **26**). On the other hand, treatment with compound **27** did not result in increased cell dispersal from biofilms but led to the highest biofilm removal (85%) and biofilm cell-killing activity (60%) of any of the compounds tested and also exerted toxicity towards planktonic cells. In addition, the corresponding methoxyamine **28** also did not stimulate dispersal but disrupted biofilms less than **27** (53%) without killing biofilm cells (1.5% dead cells). From these experiments, we conclude that the ability of both **27** and **28** to repress biofilms is independent of dispersal events. We propose that these compounds likely kill biofilm cells at lower concentrations (~20 μM). The cytotoxicity of a selection of the prepared compounds (**25**-**28**) was also examined in both human embryonic kidney 293 (HEK 293) cells and human muscle rhabdomyosarcoma (RD) cells and found to be non-toxic at concentrations up to 40 μM.

The results presented here demonstrate that the combination of an antibiotic and a nitroxide within a single molecule is an effective approach to facilitate the efficient control of mature biofilms via stimulation of biofilm dispersion or through direct cell killing, and thereby help overcome the resistance of biofilms to antimicrobials.

4. EXPERIMENTAL SECTION

4.1. General Procedures

Reactions of an air-sensitive nature were carried out under an atmosphere of ultra-high purity argon. Where anhydrous THF, DMF, DCM or acetonitrile are documented, these solvents were obtained from the solvent purification system, pure solv micro by Innovative Technologies. Anhydrous toluene was dried by storage over sodium wire. Triethylamine and *i*-Pr_2NEt were stored over potassium hydroxide. All other reagents were purchased from commercial suppliers and used without further purification. All ^1H NMR spectra were recorded at either 400 or 600 MHz on either a Varian Inova 400, a Bruker Avance 400 or a Bruker Avance 600 instrument. All

^{13}C NMR spectra were recorded at either 100 or 150 on either a Varian Inova 400, a Bruker Avance 400 or a Bruker Avance 600 instrument. Samples were prepared in $CDCl_3$, unless otherwise stated, using oven dried glassware. ^{1}H NMR spectra in $CDCl_3$ were referenced to the solvent peak at 7.27 ppm. ^{13}C NMR spectra run in $CDCl_3$ were referenced to the solvent peak at 77.2 ppm. Coupling constants are reported in Hz. High-resolution ESI mass spectra were obtained with an Agilent Q-TOF LC high-resolution mass spectrometer, which utilized electrospray ionization in positive ion mode. The mass-selective detector was optimized by using calibration standards with reference masses at *m/z* 121.050873 and 922.009798. Fourier transform infrared (FTIR) spectra were recorded on a Nicolet 870 Nexus Fourier Transform Infrared Spectrometer equipped with a DTGS TEC detector and an ATR objective. Melting points were measured with a Variable Temperature Apparatus by the capillary method and are uncorrected. Analytical HPLC was carried out on an Agilent Technologies HP 1100 Series HPLC system using an Agilent C18 column (4.6 × 250 mm, 5 μm) or an Agilent Zorbax RX-SIL column (4.6 × 250 mm, 5 μm) with a flow rate of 1 mL/min. The purity of all final compounds was determined to be 95 % or higher using HPLC analysis or qNMR techniques. EPR spectra were obtained with the aid of a miniscope MS 400 Magnettech EPR spectrometer. 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 visualised under a UV lamp (254 nm), and/or by development with phosphomolybdic acid (PMA).

4.2. Materials

5-Carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl **8** and ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate **16** were prepared according to known procedures [32, 34].

4.3. Interference compounds

Nitroxides are recognized quenchers of fluorescent molecules and this effect can occur through space with optimal separation distances in the range of 0.5-2 nm [44]. Thus, there is potential in the biofilm flow cell assay for nitroxides to quench the fluorescence of the SYTO-9 and propidium iodide dyes used during the confocal microscopy analysis if the nitroxides and

fluorophores are localized within the same region of the bacterial cell. However, as the fluorescence arising from the nitroxide containing compounds **17**, **19**, **21**, **23**, **25** and **27** was similar to the fluorescence emitted by the methoxyamine control compounds **18**, **20**, **22**, **24**, **26** and **28**, the potential effect of nitroxide-induced fluorescence quenching on the obtained biological results is minimal.

4.4. Biofilm dispersal flow cell assays

P. aeruginosa PA14 biofilms were pre-formed at 37°C over 48 h in flow chambers using previously established techniques [26]. The biofilms were then exposed for 24 hours to 10 or 20 μM solutions of ciprofloxacin-nitroxide hybrid compounds **17-28** resuspended in DMSO in the flow cell chambers with channel dimensions of 1 × 4 × 40 mm. Flow chambers were inoculated with 400 μL of an overnight *P. aeruginosa* PA14 culture diluted to an OD_{600} of ~0.05. Next, chambers were left without flow for 2 h, after which medium was pumped through the system at a constant rate of 2.4 mL/h. Staining and visualisation of the resulting biofilms was performed using the Live/Dead BacLight bacterial viability kit and a confocal laser scanning microscope (Olympus, Fluoview FV1000). Three-dimensional reconstructions and residue biofilm biovolume calculations were achieved using Imaris software.

4.5. Bacterial killing experiments

Killing experiments involved performing 1:100 dilutions of overnight cultures of *P. aeruginosa* PA14 in the abscense or presence of increasing concentrations of nitroxides (0-160 μM). After 24 h of treatment, 10-fold serial dilutions were performed, bacteria were plated on LB agar plates and allowed to grow overnight at 37 °C after which colony forming unit (CFU) counts were recorded.

4.6. MIC assays

The MIC assays were performed using the broth microdilution method [42, 43] in sterile 96-well polypropylene microtiter plates. Nitroxides were added to the plate as solutions in DMSO at the desired concentrations, and the bacteria were inoculated at a final concentration of 5×10^5 CFU/mL per well. The plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of compound at which no growth was observed.

4.7. Cytotoxicity assays

Human embryonic kidney 293 (HEK 293) cells (ATCC, VA) and human muscle rhabdomyosarcoma (RD) cells (ATCC, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, MA) supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher, MA) at 37°C in 5% CO_2. The day before treatment, 50,000 HEK 293 cells or 20,000 RD cells were seeded into each well in 96-well plates. The compounds were dispersed in DMSO at the concentration of 5 mM. Different concentrations of compounds **25**-**28** were added into the wells for another 24 hours, and 0.8% of DMSO was added in all wells to eliminate the effects of DMSO. The release of the lactate dehydrogenase (LDH) was then measured, following the manual of Pierce LDH Cytotoxicity Assay Kit (Thermofisher, MA). Untreated cells (live cells) and cells treated with lysis buffer for 3 hours (dead cells) were used as the reference for normalization. All experiments were performed in triplicate.

*4.8. General procedure for the synthesis of methoxyamine derivatives (**5**), (**7**) and (**9**)*

Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$, 2.5 equiv) was added to a solution of nitroxide compound (1 equiv) in DMSO. The mixture was then cooled to 0 ºC and 35 % aqueous hydrogen peroxide (4 equiv) was added in a dropwise manner. The resulting mixture was stirred at 0 ºC for 10 minutes and then at room temperature for an additional 1.5 hours. The reaction mixture was diluted with deionized water (40 mL) and adjusted to pH ~3 using aqueous hydrochloric acid (2 M) before being extracted with diethyl ether (3 × 20 mL). The combined organic extracts were washed with deionized water (200 mL) and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* to yield the desired methoxyamine product.

*4.8.1. 1-Methoxy-2,2,5,5-tetramethylpyrrolidine-3-carboxylic acid (**5**)*

Reagents: CPROXYL **4** (120 mg, 0.64 mmol, 1 equiv), $FeSO_4.7H_2O$ (448 mg, 1.61 mmol, 2.5 equiv), 35 % aqueous H_2O_2 (0.23 mL, 2.56 mmol, 4 equiv) and DMSO (2.5 mL). Data for **5**: white solid (117 mg, 0.58 mmol, 91 %); mp 40-41 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3100-2500 (w, br, O-H, COOH) and 1703 (s, C=O, COOH). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.63 (s, 1 H, NO$\underline{CH_3}$), 2.76 (s, 1 H, C(O)$\underline{CH}$), 2.10 (s, 1 H, C(O)CH$\underline{CH_2}$), 1.74 (dd, *J* = 12.8, 7.8 Hz, 1 H, C(O)CH$\underline{CH_2}$), 1.35 (s, 3 H, $\underline{CH_3}$), 1.23 (s, 1 H, $\underline{CH_3}$), 1.19 (s, 1 H, $\underline{CH_3}$), 1.09 (s, 1 H, $\underline{CH_3}$). ^{13}C

NMR (150 MHz, $CDCl_3$) δ = 178.7, 65.0, 61.2, 48.3, 38.6, 33.6, 28.9, 25.8, 16.3. HRMS (ESI): *m/z* calcd for $C_{10}H_{19}NO_3 + H^+$ [$M+H^+$]: 202.1428. Found 202.1428. Absolute quantitative NMR: 98.8% pure.

*4.8.2. 1-Methoxy-2,2,6,6-tetramethylpiperidine-4-carboxylic acid (**7**).*

Reagents: CTEMPO **6** (120 mg, 0.60 mmol, 1 equiv), $FeSO_4.7H_2O$ (448 mg, 1.61 mmol, 2.7 equiv), 35 % aqueous H_2O_2 (0.23 mL, 2.56 mmol, 4.3 equiv) and DMSO (2.5 mL). Data for **7**: clear white solid (113 mg, 0.52 mmol, 88 %); mp 97-99 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3100-2500 (w, br, O-H, COOH) and 1693 (s, C=O, COOH). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.62 (s, 3 H, NO$\underline{CH_3}$), 2.66 (t, *J* = 12.7 Hz, 1 H, $\underline{CH}$), 1.75 (d, *J* = 11.8 Hz, 2 H, $\underline{CH_2}$), 1.66 (t, *J* = 12.9 Hz, 2 H, $\underline{CH_2}$), 1.23 (s, 6 H, 2 × $\underline{CH_3}$), 1.12 (s, 6 H, $\underline{CH_3}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 180.7, 65.7, 59.4, 41.7, 32.9, 20.3. HRMS (ESI): *m/z* calcd for $C_{11}H_{21}NO_3 + H^+$ [$M+H^+$]: 216.1583. Found 216.1587. Absolute quantitative NMR: 97.0% pure.

*4.8.3. 2-Methoxy-1,1,3,3-tetramethylisoindoline-5-carboxylic acid (**9**)*

Reagents: CTMIO **8** (100 mg, 0.43 mmol, 1 equiv), $FeSO_4.7H_2O$ (300 mg, 1.08 mmol, 2.5 equiv), 35 % aqueous H_2O_2 (0.2 mL, 2.50 mmol, 5.8 equiv) and DMSO (2.5 mL). Data for **9**: white solid (99 mg, 0.40 mmol, 93 %); mp 181-183 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3100-2500 (w, br, O-H, COOH) and 1679 (s, C=O, COOH). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.03 (dd, *J* = 7.9, 1.6 Hz, 1 H, Ar-H), 7.86 (d, *J* = 1.2 Hz, 1 H, Ar-H), 7.20 (d, *J* = 7.9 Hz, 1 H, Ar-H), 3.80 (s, 3 H, NO$\underline{CH_3}$), 1.47 (br s, 12 H, 4 × $\underline{CH_3}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 172.1, 151.8, 146.0, 129.8, 128.7, 123.8, 121.9, 67.5, 67.2, 65.7, 42.9, 30.2, 25.1. HRMS (ESI): *m/z* calcd for $C_{14}H_{19}NO_3 + H^+$ [$M+H^+$]: 250.1443. Found 250.1440. HPLC analysis: retention time = 2.992 min; peak area, 95.26 %; eluent A, Methanol; eluent B, H_2O; isocratic (80:20) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

*4.9. General procedure for the synthesis of amide coupled compounds (**17-22**)*

Pyridine (2 equiv) was added to a solution of carboxylic acid (1 equiv) in anhydrous toluene under an atmosphere of argon. The resulting solution was cooled to 0 ºC in an ice-water bath and thionyl chloride (1.5 equiv) was added dropwise. The solution was stirred at room temperature for 1 hour. The solvent was then removed *in vacuo* and the resulting residue taken up in anhydrous dichloromethane (10 mL). This crude product was added to a stirring solution of the amine bearing compound (1.2 equiv) and *i*-Pr_2NEt (2 equiv) dissolved in anhydrous dichloromethane under an atmosphere of argon. The resulting mixture then stirred at room temperature for 1 hour before water was added to the mixture. The organic phase was separated

and the aqueous phase was re-extracted with dichloromethane (3 × 20 mL). The combined extracts were dried over anhydrous sodium sulfate and the solvent removed *in vacuo* to afford a crude solid product. Purification was achieved via column chromatography (SiO_2, gradient elution: 100 % chloroform to 95 % chloroform, 5 % methanol).

*4.9.1. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(2,2,5,5-tetramethyl-1-oxy-pyrrolidine-3-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**17**)*

Reagents for acid chloride formation: CPROXYL **4** (100 mg, 0.54 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 2.2 equiv). Reagents for amide coupling: **16** (230 mg, 0.65 mmol, 1.2 equiv), *i*-Pr_2NEt (0.2 mL, 1.08 mmol, 2 equiv) and anhydrous dichloromethane (5 mL). Data for **17**: yellow solid (248 mg, 0.47 mmol, 87 %); mp 118-120 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1688 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing*) δ = ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.59 (s, 1 H, NCH=C), 8.11 (d, *J* = 10.2 Hz, 1 H, Ar-H), 7.36 (s, 1 H, Ar-H), 4.46 (q, *J* = 6.3 Hz, 2 H, OCH_2CH_3), 4.01 (s, 2 H, 2 × NCH_2), 3.51 (s, 2 H, 2 × NCH_2), 3.36 (s, 2 H, 2 × NCH_2), 3.26 (m, 1 H, C=CHNCH), 1.48 (t, *J* = 5.91 Hz, 3 H, OCH_2CH_3), 1.42 (s, 2 H, $NCHCH_2$), 1.23 (s, 2 H, $NCHCH_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 171.7, 164.3, 152.8, 151.2, 147.0, 142.4, 136.7, 122.4, 112.4, 112.2, 109.3, 103.9, 59.7, 51.0, 48.4, 40.0, 33.5, 13.2, 7.1. HRMS (ESI): *m/z* calcd for $C_{28}H_{36}FN_4O_5$ + H^+ [M+H^+]: 528.2758. Found 528.2753. HPLC analysis: retention time = 5.481 min; peak area, 99.52 %; eluent A, Methanol; eluent B, H_2O; isocratic (70:30) over 25 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9975, a_N = 1.4898 mT.

*4.9.2. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(1-methoxy-2,2,5,5-tetramethylpyrrolidine-3-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**18**)*

Reagents for acid chloride formation: **5** (120 mg, 0.60 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 2 equiv). Reagents amide coupling: **16** (276 mg, 0.77 mmol, 1.2 equiv), *i*-Pr_2NEt (0.2 mL, 1.08 mmol, 1.8 equiv) and anhydrous dichloromethane (5 mL). Data for **18**: pale yellow solid (317 mg, 0.58 mmol, 97 %); mp 132-134 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1689 (m, C=O, amide).^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.50 (s, 1 H, NCH=C), 8.01 (d, *J* = 13.1 Hz, 1 H, Ar-H), 7.25 (d, *J* = 7.0 Hz, 1 H, Ar-H), 4.37

(q, *J* = 7.1 Hz, 2 H, O$\underline{CH_2}CH_3$), 3.89 (s, 2 H, N$\underline{CH_2}$), 3.83 (s, 2 H, N$\underline{CH_2}$), 3.66 (s, 3 H, NO$\underline{CH_3}$), 3.43 (m, 1 H, C=CHN$\underline{CH}$), 3.23 (d, *J* = 4.9 Hz, 2 H, 2 × N$\underline{CH_2}$), 3.22 (m, 2 H, 2 × N$\underline{CH_2}$), 3.10 (br, s, 1 H, NC(O)$\underline{CH}$), 2.38 (br, s, 1 H, NC(O)$\underline{CH_2}$), 1.60 (dd, *J* = 12.6, 7.4 Hz, 1 H, NC(O)CH$\underline{CH_2}$), 1.40 (t, *J* = 7.1 Hz, 3 H, OCH$_2\underline{CH_3}$), 1.33 (s, 2 H, NCH$\underline{CH_2}$), 1.31 (s, 3 H, $\underline{CH_3}$), 1.26 (s, 3 H, CH_3), (br, s, 3 H, $\underline{CH_3}$), 1.14 (dd, *J* = 8.9, 5.9 Hz, 2 H, NCH$\underline{CH_2}$), 1.06 (br, s, 3 H, $\underline{CH_3}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 173.1, 165.8, 154.6, 152.2, 148.3, 144.1, 144.0, 138.1, 123.7, 123.6, 113.7, 113.4, 110.6, 105.2, 65.0, 61.0, 50.9, 49.9, 46.4, 42.1, 34.7, 14.6, 8.3. HRMS (ESI): *m/z* calcd for $C_{29}H_{39}FN_4O_5$ + H^+ [M+H^+]: 543.2991. Found 543.2990. HPLC analysis: retention time = 3.286 min; peak area, 99.70 %; eluent A, Methanol; over 25 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

*4.9.3. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**19**)*

Reagents for acid chloride formation: CTEMPO **6** (100 mg, 0.50 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 2.4 equiv). Reagents amide coupling: **16** (216 mg, 0.60 mmol, 1.2 equiv), *i*-Pr_2NEt (0.3 mL, 1.0 mmol, 2 equiv) and anhydrous dichloromethane (5 mL). Data for **19**: light orange powder (255 mg, 0.47 mmol, 94 %); mp 241 ºC decomposed. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1690 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing*) δ = 8.57 (s, 1 H, N$\underline{CH}$=C), 8.12 (d, *J* = 12.1 Hz, 1 H, $\underline{Ar\text{-}H}$), 7.33 (br, s, 1 H, $\underline{Ar\text{-}H}$), 4.43 (q, *J* = 7.0 Hz, 2 H, O$\underline{CH_2}CH_3$), 3.94 (br, s, 4 H, 2 × N$\underline{CH_2}$), 3.46 (br, s, 2 H, N$\underline{CH_2}$), 3.40 (br, s, 1 H, N$\underline{CH}$), 3.32 (br, s, 2 H, N$\underline{CH_2}$), 1.60-1.48 (br, s, 12 H, $\underline{CH_3}$), 1.45 (t, *J* = 7.0 Hz, 3 H, OCH$_2\underline{CH_3}$), 1.37 (br, s, 2 H, NCH$\underline{CH_2}$), and 1.19 (br, s, 2 H, NCH$\underline{CH_2}$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 172.7, 165.4, 153.8, 152.1, 147.9, 143.5, 123.4, 113.4, 110.4, 104.7, 60.7, 50.4, 49.1, 44.7, 42.1, 34.2, 14.1, 7.9. HRMS (ESI): *m/z* calcd for $C_{29}H_{38}FN_4O_5$ + H^+ [M+H^+]: 542.2905. Found 542.2902. HPLC analysis: retention time = 6.206 min; peak area, 99.90 %; eluent A, Methanol; eluent B, H_2O; isocratic (80:20) over 25 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9989, a_N = 1.5751 mT.

*4.9.4. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**20**)*

Reagents for acid chloride formation: **7** (100 mg, 0.46 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 2.6 equiv). Reagents for amide coupling: **16** (216 mg, 0.60 mmol, 1.3 equiv), *i*-Pr_2NEt (0.3 mL, 1.0 mmol, 2.2 equiv) and anhydrous dichloromethane (5 mL). Data for **20**: off-white powder (231 mg, 0.41 mmol, 90 %); mp 218-219 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1645 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.50 (s, 1 H, NCH=C), 8.02 (d, *J* = 13.1 Hz, 1 H, Ar-H), 7.26 (d, *J* = 7.0 Hz, 1 H, Ar-H), 4.38 (q, *J* = 7.1 Hz, 2 H, OCH_2CH_3), 3.84 (br, s, 2 H, NCH_2), 3.71 (br, s, 2 H, NCH_2) 3.62 (s, 3 H, NOCH_3), 3.42 (m, 1 H, C=CHNCH), 3.30 (br, s, 2 H, NCH_2), 3.21 (br, s, 2 H, NCH_2), 2.87 (m, 1 H, NC(O)CH), 1.80 (m, 2 H, CHCH_2), 1.51 (m, 2 H, CHCH_2), 1.40 (t, *J* = 7.1 Hz, 3 H, OCH_2CH_3), 1.33 (q, *J* = 6.6 Hz, 2 H, NCHCH_2), 1.23 (s, 6 H, 2 × CH_3), 1.15 (s, 6 H, 2 × CH_3), and 1.14 (m, 2 H, NCHCH_2). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 173.7, 173.2, 154.3, 152.6, 148.4, 144.2, 144.1, 138.1, 123.7, 113.7, 110.7, 106.2, 66.7, 61.1, 59.5, 50.9, 49.7, 45.5, 42.3, 41.6, 34.7, 33.0, 31.5, 20.6, 14.6, 8.3. HRMS (ESI): *m/z* calcd for $C_{30}H_{41}FN_4O_5$ + Na^+ [M+Na^+]: 579.2933. Found 579.2933. HPLC analysis: retention time = 3.355 min; peak area, 99.73 %; eluent A, Methanol; over 25 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

*4.9.5. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**21**)*

Reagents for acid chloride formation: **9** (120 mg, 0.51 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 2.4 equiv). Reagents for amide coupling: **16** (218 mg, 0.61 mmol, 1.2 equiv), *i*-Pr_2NEt (0.3 mL, 1.0 mmol, 2 equiv) and anhydrous dichloromethane (5 mL). Data for **21**: light yellow powder (286 mg, 0.50 mmol, 98 %); mp 126-128 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1688 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing)* δ = 8.57 (s, 1 H, NCH=C), 8.09 (d, *J* = 12.3 Hz, 1 H, Ar-H), 7.34 (br, s, 1 H, Ar-H), 4.44 (q, *J* = 6.5 Hz, 2 H, OCH_2CH_3), 4.12 (br, s, 2 H, NCH_2), 3.85 (br, s, 2 H, NCH_2), 3.47 (s, 1 H, C=CHNCH), 3.39 (br, s, 4 H, 2 × NCH_2), 2.87 (m, 1 H, NC(O)CH), 1.56 (br, s, 12 H, 4 × CH_3), 1.46 (t, *J* = 6.8 Hz, 3 H, OCH_2CH_3), 1.38 (br, s, 2 H, NCHCH_2), 1.20 (s, 2 H, NCHCH_2).

^{13}C NMR (150 MHz, $CDCl_3$) δ = 172.2, 164.9, 153.4, 151.7, 147.5, 143.2, 137.2, 125.4, 122.9, 112.7, 109.9, 104.4, 60.2, 33.9, 13.7, 7.5. HRMS (ESI): *m/z* calcd for $C_{32}H_{36}FN_4O_5$ + H^+ [M+H^+]: 576.2717. Found 576.2713. HPLC analysis: retention time = 2.867 min; peak area, 99.90 %; eluent A, Methanol; over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9981, a_N = 1.4793 mT.

*4.9.6. Ethyl 1-cyclopropyl-6-fluoro-7-(4-(2-methoxy-1,1,3,3-tetramethylisoindoline-5-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**22**)*

Reagents for acid chloride formation: **9** (90 mg, 0.36 mmol, 1 equiv), anhydrous toluene (5 mL) and pyridine (0.1 mL, 1.20 mmol, 3.3 equiv). Reagents for amide coupling: **16** (155 mg, 0.43 mmol, 1.2 equiv), *i*-Pr_2NEt (0.2 mL, 0.7 mmol, 2 equiv) and anhydrous dichloromethane (5 mL). Data for **22**: white foamy solid (178 mg, 0.30 mmol, 83 %); mp 124-126 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide) and 1689 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.53 (s, 1 H, NCH=C), 8.05 (d, *J* = 13.0 Hz, 1 H, Ar-H), 7.31 (dd, *J* = 7.7, 1.3 Hz, 1 H, Ar-H), 7.28 (d, *J* = 7.0 Hz, 1 H, Ar-H), 7.20 (s, 1 H, Ar-H), 7.15 (d, *J* = 7.7 Hz, 1 H, Ar-H), 4.39 (q, *J* = 7.1 Hz, 2 H, OCH_2CH_3), 4.01 (br, s, 2 H, NCH_2), 3.79 (s, 3 H, $NOCH_3$), 3.71 (br, s, 2 H, NCH_2), 3.42 (ddd, *J* = 10.8, 7.1, 3.9 Hz, 1 H, C=CHNCH), 3.30 (br, s, 2 H, NCH_2), 3.24 (br, s, 2 H, NCH_2), 1.69 (s, 6 H, 2 × CH_3), 1.45 (br, s, 6 H, 2 × CH_3), 1.41 (t, *J* = 7.1 Hz, 3 H, OCH_2CH_3), 1.33 (q, *J* = 6.6 Hz, 2 H, $NCHCH_2$), 1.15 (q, *J* = 6.5 Hz, 2 H, $NCHCH_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 173.3, 171.0, 166.6, 154.4, 152.7, 148.4, 147.7, 146.1, 144.3, 138.2, 134.4, 126.4, 123.9, 123.8, 121.9, 120.9, 113.8, 113.7, 110.8, 105.3, 67.3, 65.7, 61.1, 34.7, 14.6, 8.4. HRMS (ESI): *m/z* calculated for $C_{33}H_{39}FN_4O_5$ + H^+ [M+H^+]: 591.2956. Found 591.2955. HPLC analysis: retention time = 3.268 min; peak area, 99.61 %; eluent A, Methanol; over 25 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

*4.10. General procedure for ester hydrolysis (compounds **23-28**)*

2 M aqueous sodium hydroxide (7 equiv) was added to a solution of the specific ethyl ester (1 equiv) in HPLC grade methanol and the resulting solution was stirred at 50 ºC 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 × 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.

*4.10.1. 1-Cyclopropyl-6-fluoro-7-(4-(2,2,5,5-tetramethyl-1-oxy-pyrrolidine-3-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**23**)*

Reagents: **17** (57 mg, 0.10 mmol, 1 equiv), 2 M aqueous NaOH (0.35 mL, 0.70 mmol, 7 equiv) and HPLC grade methanol (2.5 mL). Data for **23**: pale yellow powder (47 mg, 0.09 mmol, 87 %); mp 229 ºC decomposes. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1649 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing*) δ = 14.87 (s, 1 H, CO<u>OH</u>), 8.81 (s, 1 H, N<u>CH</u>=C), 8.10 (d, *J* = 11.7 Hz, 1 H, <u>Ar-H</u>), 7.41 (br, s 1 H, <u>Ar-H</u>), 3.98 (br, s, 4 H, 2 × N<u>CH_2</u>), 3.57 (br, s, 1 H, C=CHN<u>CH</u>), 3.27 (m, 4 H, 2 x N<u>CH_2</u>), 1.44 (m, 2 H, NCH<u>CH_2</u>), 1.26 (br, d, *J* = 17.3, 2 H, NCH<u>CH_2</u>). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 177.1, 166.8, 147.7, 139.0, 108.4, 105.2, 49.4, 35.3, 8.4. HRMS (ESI): *m/z* calcd for $C_{26}H_{32}FN_4O_5$ + H^+ [M+H^+]: 500.2440. Found 500.2440. HPLC analysis: retention time = 4.392 min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9992, a_N = 1.5024 mT.

*4.10.2. 1-Cyclopropyl-6-fluoro-7-(4-(1-methoxy-2,2,5,5-tetramethylpyrrolidine-3-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**24**)*

Reagents: **18** (78 mg, 0.15 mmol, 1 equiv), 2 M aqueous NaOH (0.5 mL, 1.05 mmol, 7 equiv) and HPLC grade methanol (3 mL). Data for **24**: pale yellow powder (60 mg, 0.12 mmol, 80 %); mp 196-198 ºC. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1626 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 14.90 (s, 1 H, CO<u>OH</u>), 8.76 (s, 1 H, N<u>CH</u>=C), 8.03 (d, *J* = 12.4 Hz, 1 H, <u>Ar-H</u>), 7.37 (d, *J* = 5.1 Hz, 1 H, <u>Ar-H</u>), 3.92 (s, 2 H, N<u>CH_2</u>), 3.86 (s, 2 H, N<u>CH_2</u>), 3.62 (s, 3 H, NO<u>CH_3</u>), 3.56 (br, s, 1 H, C=CHN<u>CH</u>), 3.38 (s, 2 H, N<u>CH_2</u>), 3.31 (s, 2 H, N<u>CH_2</u>), 3.11 (br, s, 1 H, NC(O)<u>CH</u>), 2.39 (br, s, 1 H, NC(O)<u>CH_2</u>), 1.61 (dd, *J* = 16.9, 11.7 Hz, 1 H, NC(O)CH<u>CH_2</u>), 1.41 (s, 3 H, CH_3), 1.33 (s, 2 H, NCH<u>CH_2</u>), 1.27 (s, 3 H, <u>CH_3</u>), 1.26 (s, 3 H, CH_3), 1.22 (s, 2 H, NCH<u>CH_2</u>), 1.07 (br, s, 3 H, <u>CH_3</u>). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 177.0, 166.8, 154.9, 152.4, 147.6, 145.5, 145.4, 139.1, 120.2, 120.1, 112.6, 112.3, 108.1, 105.2, 65.0, 50.5, 49.6, 46.2, 41.9, 35.5, 29.8, 8.4. HRMS (ESI): *m/z* calcd for $C_{27}H_{35}FN_4O_5$ + H^+ [M+H^+]: 515.2651. Found 515.2651. HPLC analysis: retention time = 3.711

min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

4.10.3. 1-Cyclopropyl-6-fluoro-7-(4-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (25)

Reagents: **19** (84 mg, 0.16 mmol, 1 equiv), 2 M aqueous NaOH (0.6 mL, 1.12 mmol, 7 equiv) and HPLC grade methanol (3 mL). Data for **25**: orange powder (78 mg, 0.15 mmol, 98 %); mp 245 ºC decomposes. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1624 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing*) δ = 14.92 (s, 1 H, CO<u>OH</u>), 8.78 (s, 1 H, N<u>CH</u>=C), 8.06 (d, *J* = 10.9 Hz, 1 H, <u>Ar-H</u>), 7.45 (br, s 1 H, <u>Ar-H</u>), 4.00 (br, s, 4 H, 2 × N<u>CH_2</u>), 3.63 (s, 2 H, N<u>CH_2</u>), 3.54 (s, 1 H, N<u>CH</u>), 3.44 (s, 2 H, N<u>CH_2</u>), 1.49 (br, s, 2 H, NCH<u>CH_2</u>), and 1.29 (br, s, 2 H, NCH<u>CH_2</u>). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 176.0, 165.8, 153.4, 151.8, 146.6, 144.3, 138.0, 119.3, 111.7, 111.6, 107.2, 104.1, 49.4, 48.5, 44.0, 40.5, 34.6, 7.6. HRMS (ESI): *m/z* calcd for $C_{27}H_{34}FN_4O_5$ + H^+ [M+H^+]: 514.2575. Found 514.2587. HPLC analysis: retention time = 4.283 min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9995, a_N = 1.6108 mT.

*4.10.4. 1-Cyclopropyl-6-fluoro-7-(4-(1-methoxy-2,2,6,6-tetramethylpiperidine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**26**)*

Reagents: **20**, (78 mg, 0.14 mmol, 1 equiv), 2 M aqueous NaOH (0.4 mL, 1.00 mmol, 7 equiv) and HPLC grade methanol (3 mL). Data for **26**: White powder (61 mg, 0.12 mmol, 82 %); mp 259 ºC decomposes. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1627 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) δ = 14.89 (s, 1 H, CO<u>OH</u>), 8.77 (s, 1 H, N<u>CH</u>=C), 8.04 (d, *J* = 12.9 Hz, 1 H, <u>Ar-H</u>), 7.38 (d, *J* = 6.9 Hz, 1 H, <u>Ar-H</u>), 3.87 (br, s, 2 H, N<u>CH_2</u>), 3.75 (br, s, 2 H, N<u>CH_2</u>) 3.63 (s, 3 H, NO<u>CH_3</u>), 3.56 (m, 1 H, C=CHN<u>CH</u>), 3.40 (br, s, 2 H, N<u>CH_2</u>), 3.30 (br, s, 2 H, N<u>CH_2</u>), 2.88 (t, *J* = 12.5 Hz, 1 H, NC(O)<u>CH</u>), 1.82 (s, 2 H, CH<u>CH_2</u>), 1.52 (d, *J* = 12.9 Hz, 2 H, CH<u>CH_2</u>), 1.41 (d, *J* = 6.5 Hz, 2 H, NCH<u>CH_2</u>), 1.24 (s, 6 H, 2 x <u>CH_3</u>), 1.22 (m, 2 H, NCH<u>CH_2</u>), 1.16 (s, 6 H, 2 × <u>CH_3</u>). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 177.2, 177.1, 166.9, 155.0, 152.5, 147.7, 145.5, 139.2, 120.4, 112.8, 112.5, 108.3, 105.3, 65.8, 59.6, 49.5, 45.3, 41.4, 35.5, 31.5, 20.7, 8.4. HRMS (ESI): *m/z* calcd for

$C_{28}H_{37}FN_4O_5$ + H^+ [M+H^+]: 529.2807. Found 529.2806. HPLC analysis: retention time = 4.187 min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

*4.10.5. 1-Cyclopropyl-6-fluoro-7-(4-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**27**)*

Reagents: **21** (80 mg, 0.14 mmol, 1 equiv), 2 M aqueous NaOH (0.4 mL, 1.00 mmol, 7 equiv) and HPLC grade methanol (3 mL). Data for **27**: bright yellow powder (55 mg, 0.10 mmol, 73 %); mp 258-259 ºC decomposes. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1626 (m, C=O, amide). ^{1}H NMR (600 MHz, $CDCl_3$) (**note compound is a free-radical, some signals appear broadened and other signals are missing*) δ = 14.91 (s, 1 H, CO<u>OH</u>), 8.80 (s, 1 H, N<u>CH</u>=C), 8.06 (d, *J* = 12.0 Hz, 1 H, <u>Ar-H</u>), 7.43 (s, 1 H, <u>Ar-H</u>), 4.12 (br, s, 2 H, N<u>CH_2</u>), 3.87 (br, s, 2 H, N<u>CH_2</u>), 3.59 (s, 1 H, C=CHN<u>CH</u>), 3.44 (br, s, 4 H, 2 × N<u>CH_2</u>), 1.29 (s, 2 H, NCH<u>CH_2</u>), 1.27 (s, 2 H, NCH<u>CH_2</u>). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 176.6, 166.3, 154.0, 152.4, 147.2, 144.9, 138.5, 120.0, 112.3, 112.2, 107.8, 104.7, 35.0, 7.9. HRMS (ESI): *m/z* calcd for $C_{30}H_{32}FN_4O_5$ + H^+ [M+H^+]: 548.2409. Found 548.2410. HPLC analysis: retention time = 4.295 min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt. EPR: g = 1.9982, a_N = 1.4833 mT.

*4.10.6. 1-Cyclopropyl-6-fluoro-7-(4-(2-methoxy-1,1,3,3-tetramethylisoindoline-5-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**28**)*

Reagents: **22**, (70 mg, 0.12 mmol, 1 eq), 2 M aqueous NaOH (0.4 mL, 1.00 mmol, 8.3 equiv) and HPLC grade methanol (3 mL). Data for **28**: light yellow powder (57 mg, 0.10 mmol, 85 %); mp 301-302 ºC decomposes. IR (ATR) ν_{max} (cm^{-1}) = 3500-3200 (w, br, N-H, amide), 3100-2500 (w, br, O-H, COOH) and 1623 (m, C=O, amide). ^{1}H NMR (600 MHz, CD_2Cl_2) δ = 14.94 (s, 1 H, CO<u>OH</u>), 8.81 (s, 1 H, N<u>CH</u>=C), 8.08 (d, *J* = 13.0 Hz, 1 H, <u>Ar-H</u>), 7.46 (d, *J* = 7.1 Hz, 1 H, <u>Ar-H</u>), 7.38 (dd, *J* = 7.7, 1.4 Hz, 1 H, <u>Ar-H</u>), 7.26 (s, 1 H, <u>Ar-H</u>), 7.23 (d, *J* = 7.8 Hz, 1 H, <u>Ar-H</u>), 4.02 (br, s, 2 H, N<u>CH_2</u>), 3.93 (s, 3 H, NO<u>CH_3</u>), 3.75 (br, s, 2 H, N<u>CH_2</u>), 3.59 (m, 1 H, C=CHN<u>CH</u>), 3.37 (br, s,4 H, 2 × N<u>CH_2</u>), 1.57 (br, s, 12 H, 4 × <u>CH_3</u>), 1.43 (br, s, 2 H, 2 × NCH<u>CH_2</u>), 1.23 (m, 2 H, NCH<u>CH_2</u>). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 177.8, 170.9, 167.1,

148.3, 148.0, 146.4, 139.7, 135.0, 129.9, 126.8, 124.0, 122.3, 122.2, 121.2, 112.9, 112.7, 108.8, 106.2, 67.6, 65.9, 36.0, 8.7. HRMS (ESI): *m/z* calcd for $C_{31}H_{35}FN_4O_5$ + H^+ [M+H^+]: 563.2633. Found 563.2637. HPLC analysis: retention time = 3.868 min; peak area, 99.90 %; eluent A, DCM; eluent B, THF; isocratic (70:30) over 20 min with a flow rate of 1 mL min^{-1} and detected at 254 nm; column temperature, rt.

SUPPORTING INFORMATION

^{1}H NMR and ^{13}C NMR spectra, HPLC chromatograms and EPR spectra for all novel compounds. Plots showing planktonic cell killing for **27** and **28**, and cell viability studies for various DMSO concentrations.

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HIGHLIGHTS

- Amide-linked ciprofloxacin-nitroxide conjugates were synthesized in high yield.
- Hybrid **25** caused dispersal of *P. aeruginosa* biofilms.
- Hybrid **27** caused virtually complete killing and removal of *P. aeruginosa* biofilms.
- Compounds **25**-**28** were shown to be non-toxic in two human cell lines (up to 40 µM).