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Research paper

# Ciprofloxacin-nitroxide hybrids with potential for biofilm control

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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.



## 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

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[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 s [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 (Fig. 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.

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** (Fig. 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 μM was observed) [31].

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

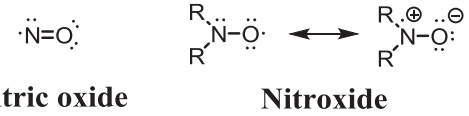
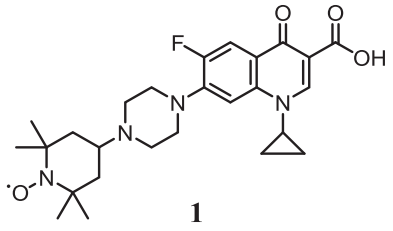



**Fig. 1.** The structure of nitric oxide and the general structure of a nitroxide.



**1**

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

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.

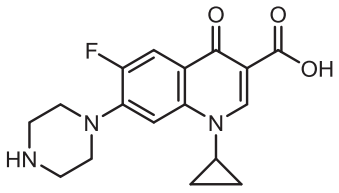
Herein, we report the design and synthesis of the second generation of ciprofloxacin-nitroxide hybrid molecules together with their biological evaluation as anti-biofilm agents for the treatment of existing *P. aeruginosa* biofilms.

## 2. Results and discussion

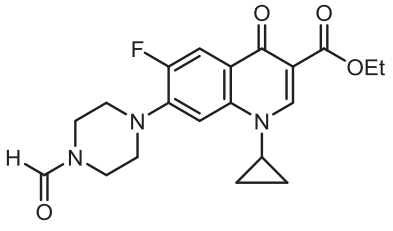
### 2.1. Chemistry

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** (Fig. 3) as a useful handle where synthetic transformations could be performed without significantly altering the antimicrobial properties of ciprofloxacin [31].

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-



**2**



**3**

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