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Short Communication

SHORT COMMUNICATION

Potentiation of ciprofloxacin action against Gram-negative bacterial biofilms by a nitroxide

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One sentence summary: A nitric oxide analog capable of potentiating ciprofloxacin action against Gram-negative bacterial biofilms.
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ABSTRACT

We previously showed that soluble nitroxides (nitric oxide analogues) mimicked the well-established ability of nitric oxide to cause biofilm dispersal and further showed that these compounds could prevent biofilm formation. Here, we investigated the effect of the nitroxide carboxy-TEMPO in combination with sub μg/ml concentrations of ciprofloxacin on pre-formed flow cell biofilms formed by Gram-negative bacteria. Combination therapy led to substantial eradication of existing biofilms formed by *Pseudomonas aeruginosa* PA14 (99.3%) and *Escherichia coli* O157 (93%).

Keywords: biofilm; nitroxide; ciprofloxacin

MAIN TEXT

Biofilms are multicellular structures composed of bacterial subpopulations embedded in a complex extracellular matrix mainly consisting of polysaccharides (de la Fuente-Núñez *et al.* 2013b). Many species of bacteria are capable of forming biofilms, both in nature and in clinical settings, particularly during infections due to implant surgery. This bacterial behavior shows high antibiotic resistance and is a major issue in the clinic. Indeed, no antimicrobial agent has been developed that efficiently targets bacterial biofilms. At the late stages of the biofilm developmental cycle, cells that disperse from mature biofilms can then differentiate back to the planktonic state, which enables bacteria to undergo swimming motility, and potentially forming new biofilms in a new infection site (McDougald *et al.* 2011). A variety of different signals can induce dispersal including nitric oxide (NO) (Barraud *et al.* 2006, 2009; Schreiber *et al.* 2011). Indeed a low non-toxic concentration of NO can turn biofilm cells into free-swimming cells which thereby increases their susceptibility of bacteria to antimicrobials (Barraud *et al.* 2006; McDougald *et al.* 2011). As the delivery of gaseous nitric oxide can be challenging, an alternative strategy involving the use of nitroxides (sterically hindered versions of nitric oxide) was recently investigated. Nitroxides were shown to both inhibit *Pseudomonas aeruginosa* bacterial biofilm formation and disperse pre-existing biofilms (de la Fuente-Núñez *et al.* 2013a). Nitroxides (aminoxyls) are persistent and stable free-radical species containing a univalent oxygen atom bound to a disubstituted nitrogen atom (Likhtenshtein *et al.* 2008). As the structures of both nitric oxide and nitroxides contain an unpaired electron which is

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delocalized over the nitrogen–oxygen bond, nitroxides can be considered sterically hindered versions of nitric oxide. Furthermore, the biological effects of nitroxides can often be explained by their nitric oxide-mimetic properties, with both compounds acting as efficient scavengers of protein-derived radicals and possessing superoxide dismutase mimetic properties (Lam *et al.* 2008) Many nitroxides, however, have the additional benefit of existing as crystalline solids at room temperature and are therefore much easier to handle and deliver than gaseous nitric oxide. In this work, we aimed to investigate whether nitroxides could be used to reduce the well-known (de la Fuente-Núñez *et al.* 2013b) resistance of biofilms to antibiotic treatment.

The nitroxide carboxy-TEMPO (4-carboxy-2,2,6,6-tetramethylpiperidine 1-oxyl, Fig. 1 top panel), was obtained commercially (382 000, Sigma-Aldrich) and prepared as previously described (Fairfull-Smith, Brackmann and Bottle 2009). We recently showed that the relative levels of NO play an important role in *P. aeruginosa* biofilm physiology, as NO production was shown to be necessary for biofilm formation and an excess of NO (or nitroxide) induced biofilm dispersion in pre-formed biofilms (de la Fuente-Núñez *et al.* 2013a). Our previous study identified carboxy-TEMPO as the optimal biofilm-dispersing mimetic among the different nitroxides tested, since it prevented biofilm formation and led to dispersal of pre-existing biofilms of *P. aeruginosa* PA14 (de la Fuente-Núñez *et al.* 2013a). We reasoned that a biofilm dispersant might make biofilms more susceptible to antibiotic inhibition. Therefore, we investigated the effect, on 2-day-old *P. aeruginosa* PA14 (Breidenstein, de la Fuente-Núñez and Hancock 2011) and *Escherichia coli* O157 (Chase-Topping *et al.* 2008) biofilms, of the combination of sub-inhibitory levels of carboxy-TEMPO with the conventional antibiotic ciprofloxacin.

To evaluate the dispersal ability of the nitroxide compound, biofilms were grown in BM2 minimal medium supplemented with 0.4% of glucose for 48 h in a flow cell system, as previously described (de la Fuente-Núñez *et al.* 2013a). Flow cell chambers were inoculated by injecting 400 μL of an overnight culture diluted to an OD_{600} of 0.05. After inoculation, chambers were left without flow for 2 h to enable adherence, after which medium was pumped through the system at a constant rate of 0.5 rpm (2.4 ml/h). At the end of the second day, biofilms were exposed

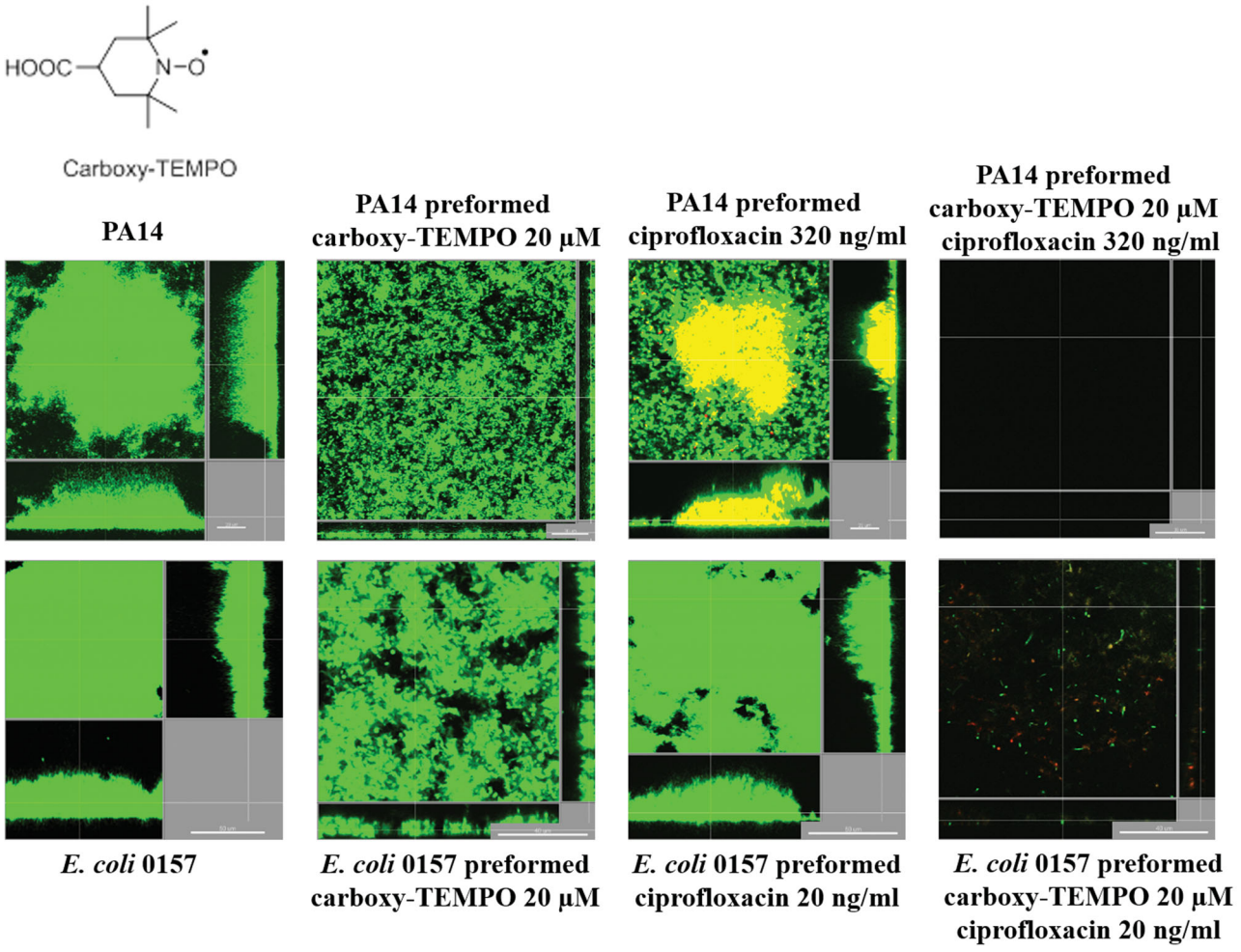

Figure 1. Potentiation of ciprofloxacin action against Gram-negative bacterial biofilms by the nitroxide carboxy-TEMPO. Bacteria were grown as biofilms in a flow cell system. Treatments [ciprofloxacin (320 ng/ml), nitroxide (20 μM) or both] were added after 2 days of biofilm growth for a subsequent 24 h. After 3 days, bacteria were stained green with the all bacteria stain Syto-9 and red with the dead-bacteria stain propidium iodide (merge shows as yellow to red) prior to confocal imaging. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz and xz dimensions). These studies reflect at least three replicates with similar outcomes. Scale bars are 20 μM in all PA14 samples, except the far-right image (PA14 treated with both carboxy-TEMPO and ciprofloxacin), which is 30 μM. Scale bars in *E. coli* 0157 images correspond to 40 μM with the exception of *E. coli* 0157 untreated and the sample treated with ciprofloxacin 20 ng/ml, which are 50 μM.

to 20 μM of compound carboxy-TEMPO for 24 h. Staining and visualization of the resulting biofilms were performed using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR) and a confocal laser scanning microscope (Olympus, Fluoview FV1000) as previously described (de la Fuente-Núñez *et al.* 2013a).

Pseudomonas aeruginosa PA14 and *E. coli* O157 biofilms formed in flow cell chambers appeared thick and well-structured with multiple microcolonies. Since dispersed cells are planktonic, we assumed we would see synergy of nitroxides with bactericidal antibiotics, despite their generally poor activity versus biofilms; however, in preliminary 96-well plate synergy assays, we only observed promising results with the antibiotic ciprofloxacin. The fluoroquinolone ciprofloxacin, which is commonly used to treat *P. aeruginosa* infections, is an antibiotic acting on cell division by inhibition of DNA gyrases. We treated pre-formed biofilms grown in flow cells with concentrations corresponding to the minimal inhibition concentration (MIC) of ciprofloxacin for *P. aeruginosa* PA14 (320 ng/ml) and *E. coli* O157 (20 ng/ml). Some dead cells (stained yellow) were observed for the treated *P. aeruginosa* biofilm although the thickness was the same (Fig. 1 top panel), while ciprofloxacin treated biofilms formed by *E. coli* remained almost identical to untreated biofilms (Fig. 1 bottom panel). In contrast, carboxy-TEMPO exhibited stand-alone activity in causing dispersal of pre-existing biofilms in both Gram-negative bacteria, consistent with our previous work on *Pseudomonas* (de la Fuente-Núñez *et al.* 2013a). Indeed, thinner biofilms were observed, thus indicating that biofilm thickness was decreased compared to the untreated controls (Fig. 1). By using Imaris software (Bitplane AG) to calculate the biofilm biovolume (μM^3), it was shown that after treatment with carboxy-TEMPO, the total biofilm biovolume of *P. aeruginosa* biofilms treated with 20 μM of carboxy-TEMPO showed a decrease of 60% in biofilm biovolume compared to the untreated controls ($P < 0.05$) (Table 1). We found that this effect was, at least in part, due to increased dispersal of bacteria from biofilms upon treatment with carboxy-TEMPO (Fig. S2, Supporting Information). To assay for dispersed cells from *P. aeruginosa* biofilms, aliquots of 30-min flow rate effluent cells were collected at the designated times (0, 3, 6 and 24 h) and centrifuged, the pellet was then resuspended in 1 ml of LB and serially diluted 10-fold, and 100-μl portions from serial dilutions of these aliquots were plated onto LB agar plates. The plates were incubated at 37°C overnight, and colony counts were performed to obtain the numbers of CFU/ml at each time point. The experiment was repeated at least twice. On the other hand, *E. coli* biofilms were reduced to 29% cf. the biofilm biovolume of untreated biofilms (Table 1). However, due to variability among samples, these results were not significant ($P > 0.05$). Interestingly, carboxy-TEMPO did not trigger cell death in either *P. aeruginosa* or *E. coli* biofilms (Fig. 1). Conversely, at the concentrations tested, we observed no antibiofilm effect of carboxy-TEMPO on *Staphylococcus aureus* MRSA biofilms (Fig. S1, Supporting Information) thus suggesting that the activity of this nitroxide might be limited to Gram-negative organisms.

Table 1. Total residual biofilm biovolume of treated 2-day-old biofilms relative to biovolume of untreated biofilms (%). Bacteria were grown as biofilms in a flow cell system. Treatments [ciprofloxacin (320 ng/ml), nitroxide (20 μM) or both] were added after 2 days of biofilm growth for a subsequent 24 h. Calculations were done using Imaris software. These studies reflect the average of at least three replicates with very similar outcomes.

	TEMPO	Ciprofloxacin + TEMPO
P. aeruginosa PA14	40% ($P < 0.05$)	0.7% ($P < 0.01$)
E. coli O157	29% ($P > 0.05$)	13% ($P < 0.05$)

Next, we investigated the activity of carboxy-TEMPO in combination with ciprofloxacin on pre-formed biofilms. Two-day-old biofilms were exposed to the combined treatment of ciprofloxacin (at its MIC for each bacterial species) and carboxy-TEMPO (20 μM) for 24 h. Examination of the flow cells using confocal microscopy at the end of the experiment revealed that biofilm thickness and the total number of attached cells had been substantially decreased (Fig. 1). In fact, on average, the total *P. aeruginosa* biofilm biovolume in samples treated with carboxy-TEMPO plus ciprofloxacin was decreased by 99.3% compared to untreated biofilms (Table 1), cf. treatment with carboxy-TEMPO alone resulted in a 60% decrease in total biofilm biovolume compared to untreated samples (Table 1). For *E. coli* biofilms, carboxy-TEMPO combined with ciprofloxacin reduced the total biofilm biovolume (by 87% relative to control) and 47±6% of the remaining 13% of biofilm biovolume was composed of dead cells, compared to >2% in the untreated samples (Table 1), indicating an overall decrease of 93% in live cells. Thus, the combination of nitroxide plus ciprofloxacin led to an almost complete eradication of mature biofilms formed by *P. aeruginosa* and *E. coli*.

In conclusion, the nitroxide carboxy-TEMPO not only caused dispersal of mature biofilms but also enhanced ciprofloxacin activity in eradicating biofilms formed by Gram-negative pathogens.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSPD online.

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Conflict of interest. None declared.

REFERENCES

Barraud N, Hassett DJ, Hwang SH, *et al.* Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol* 2006;**188**:7344–53.

Barraud N, Storey MV, Moore ZP, *et al.* Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. *Microb Biotechnol* 2009;**2**:370–8.

Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. Pseudomonas *aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011;**19**:419–26.

Chase-Topping M, Gally D, Low C, *et al.* Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat Rev Microbiol* 2008;**6**:904–12.

de la Fuente-Núñez C, Reffuveille F, Fairfull-Smith KE, *et al.* The effect of nitroxides on swarming motility and biofilms, multicellular behaviors in *Pseudomonas aeruginosa*. *Antimicrob Agents Ch* 2013a;**57**:4877–81.

de la Fuente-Núñez C, Reffuveille F, Fernández L, *et al*. Bacterial biofilm development as a multicelular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Opin Microbiol* 2013b;**16**:580–92.

Fairfull-Smith KE, Brackmann F, Bottle SE. The synthesis of novel isoindolinenitroxides bearing water solubilising functionality. *Eur J Org Chem* 2009;**12**:1902–15.

Lam MA, Pattison DI, Bottle SE, *et al*. Nitric oxide and nitroxides can act as efficient scavengers of protein-derived free radicals. *Chem Res Toxicol* 2008;**21**:2111–9.

Likhtenshtein G, Yamauchi J, Nakatsuji S, *et al*. *Nitroxides: Applications in Chemistry, Biomedicine, and Materials Science*. Weinheim: Wiley-VCH, 2008.

McDougald D, Rice SA, Barraud N, *et al*. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* 2011;**10**:39–50.

Schreiber F, Beutler M, Enning D, *et al*. The role of nitric-oxide-synthase-derived nitric oxide in multicellular traits of *Bacillus subtilis* 3610: biofilm formation, swarming, and dispersal. *BMC Microbiol* 2011;**11**:111.