AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Sodium Nitrite Inhibits Killing of *Pseudomonas aeruginosa* Biofilms by Ciprofloxacin

Anna C. Zemke,[a] Brian R. Kocak,[b] Jennifer M. Bomberger[b]

Division of Pulmonary, Allergy and Critical Care Medicine[a] and Department of Microbiology and Molecular Genetics,[b] University of Pittsburgh, Pittsburgh, Pennsylvania, USA

ABSTRACT Sodium nitrite inhibits bacterial respiration and is in development as an antimicrobial for chronic bacterial infections associated with cystic fibrosis. The goal of the current study was to investigate the interaction between nitrite and ciprofloxacin. Using liquid culture killing assays and a biotic biofilm model, we observed that nitrite induces tolerance of ciprofloxacin.

KEYWORDS *Pseudomonas aeruginosa*, biofilm, ciprofloxacin, fluoroquinolone, nitrite, nitrosative stress

Received 26 February 2016 **Returned for modification** 1 April 2016 **Accepted** 22 October 2016

Accepted manuscript posted online 31 October 2016

Citation Zemke AC, Kocak BR, Bomberger JM. 2017. Sodium nitrite inhibits killing of *Pseudomonas aeruginosa* biofilms by ciprofloxacin. Antimicrob Agents Chemother 61:e00448-16. https://doi.org/10.1128/AAC.00448-16.

Address correspondence to Jennifer M. Bomberger, jbomb@pitt.edu.

Cystic fibrosis (CF) is marked by chronic airway infections that ultimately lead to death from respiratory failure (1). *Pseudomonas aeruginosa* is the most common infecting organism in adults with CF, and new antibiotic treatments are needed because of the high innate and acquired resistance of the organism (2). Sodium nitrite (NIT) is in early-phase human subject study as a nebulized antimicrobial agent in CF. NIT has broad antimicrobial activity, including activity against the CF pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia* complex spp., and *Achromobacter* spp. (3–6). NIT is metabolized to nitric oxide (NO) by both bacteria and the host; thus, NIT can be thought of as a sustained release formulation of NO (7, 8). NIT and NO act as bacterial respiratory poisons by inhibiting core metabolism and oxygen uptake (3, 9). The purpose of this study was to determine if there is an interaction between NIT and ciprofloxacin (CIP). We found that NIT induced tolerance of CIP in *P. aeruginosa* in biofilms grown on the surface of airway epithelial cells and in liquid culture.

To study the interaction between NIT and CIP, we used a biotic biofilm model where green fluorescent protein (GFP)-expressing *P. aeruginosa* strain PAO1 biofilms were grown on the surface of the CF airway epithelial cell line CFBE41o-. For live-cell imaging experiments, cocultures were grown in perfusion chambers, as described in reference 3. Cocultures were grown for 4.5 h and then treated with 1 μg/ml CIP and 15 mM NIT for 90 min. Biofilm biomass was quantified using Nikon Elements software. A static adaptation of this model was also used in which biofilms were grown on the apical surface of polarized CFBE41o- cells grown at the air-liquid interface (3). Static biofilms were treated with 1 μg/ml CIP or 75 mM NIT (the calculated concentration obtained by nebulization) or NIT plus CIP (the methods employed are detailed in reference 3). For liquid culture experiments, strains were diluted 1:100 in LB (pH 6.5), grown aerobically for 2 h to return bacteria to the log phase, and then treated as described below. Bacterial counts were obtained through serial dilution plating.

Control biofilms grown in perfusion chambers showed robust growth by 6 h (Fig. 1A). NIT (15 mM) decreased biomass by 83% (Fig. 1B), while CIP (1 μg/ml) decreased biomass by 57% (Fig. 1D). Bacterial filamentation was seen on exposure to CIP, as has been previously reported (10). The combination of NIT plus CIP blocked the decreases in biomass caused by both NIT and CIP individually (Fig. 1E), with the resulting biomass being indistinguishable from the control. Biofilm biomass quantification is shown in Fig.

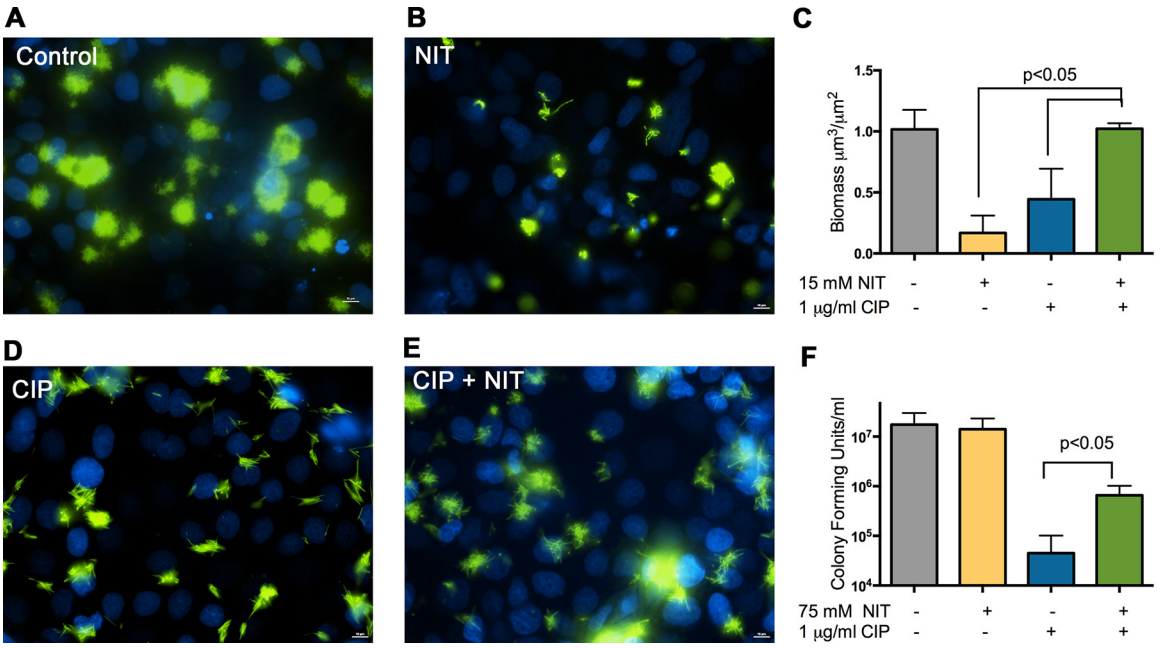

FIG 1 PAO1 biofilms (A) were grown on the surface of CFBE41o- cells for 4.5 h and then treated with 15 mM NIT (B) or 1 μg/ml CIP (D) or both NIT and CIP (E) for 90 min. Hoechst 33342-stained airway cell nuclei are in blue. GFP-expressing PAO1 cells are in green. (C) Biofilm biomass quantification. $P < 0.05$ for control versus CIP and control versus NIT (one-way analysis of variance [ANOVA] followed by Tukey's test); for control versus CIP plus NIT, the results were nonsignificant (n.s.). (F) PAO1 biofilms were grown on the surface of polarized CFBE41o- cells for 6 h and then treated for 90 min as indicated. The remaining bacteria were quantified by serial dilution. NIT plus CIP caused a 1.5 log decrease in killing compared to CIP alone. $P < 0.05$ (one-way ANOVA followed by Tukey's test).

1C. In the static model where biofilms were grown on airway cells without perfusion, 1 μg/ml CIP killed at >2 logs. However, in the presence of NIT, CIP killing was decreased to 1 log (Fig. 1F).

In aerobic liquid culture, NIT completely inhibited growth of *P. aeruginosa* at concentrations of 10 to 20 mM (Fig. 2A; previously described in references 3, 5, and 11). To determine if NIT would induce CIP tolerance in liquid culture, log-phase aerobic cultures of *P. aeruginosa* PAO1 were treated with NIT (from 0 to 15 mM) and CIP (from 0.1 μg/ml to 1.6 μg/ml) and then counted by serial dilution. At the MIC of CIP (0.1 μg/ml), the addition of NIT decreased killing in a dose-dependent fashion, with an effect ceiling at 15 mM. A statistically significant decrease in killing was seen with subinhibitory (7.5 mM) NIT (Fig. 2B). At 0.4 μg/ml, CIP was bactericidal, with 0.8 μg/ml killing bacteria to below the 10^2 CFU/ml detection limit in this assay (Fig. 2C). The addition of 15 mM NIT induced CIP tolerance at levels of up to 1.6 μg/ml CIP (Fig. 2C). To exclude the possibility of a permanent phenotypic change in MIC, the CIP susceptibility of PAO1 exposed to NIT was determined by disk diffusion assay and compared to that of unexposed controls. The zone of inhibition was unchanged by prior exposure to NIT (Fig. 2D). These data are consistent with the conclusion that NIT induces tolerance of CIP in liquid culture. NIT inhibits bacterial respiration (3, 9). We have previously shown that respiratory inhibition by NIT does not require NO as an intermediate (3). The addition of the stoichiometric NO scavenger CPTIO did not block NIT-induced CIP tolerance (Fig. 2E), although it did largely restore growth (Fig. 2F). These data suggest that respiratory inhibition, even without complete growth arrest, leads to CIP tolerance.

NIT treatment induced CIP tolerance in aerated liquid culture and, more importantly, in two host-associated biofilm models where biofilms were grown on airway epithelial cells. NIT may induce CIP tolerance by impairing respiration. In both liquid culture and biofilms, bacterial cellular respiration is linked to antibiotic efficacy. Loss of catabolite repression control (*crc*) leads to the generation of biofilms with a higher basal energetic state and thus sensitizes a larger part of the biofilm to killing by CIP (12). More recently, Lobritz et al. found accelerated respiration just before cell death caused by a panel of

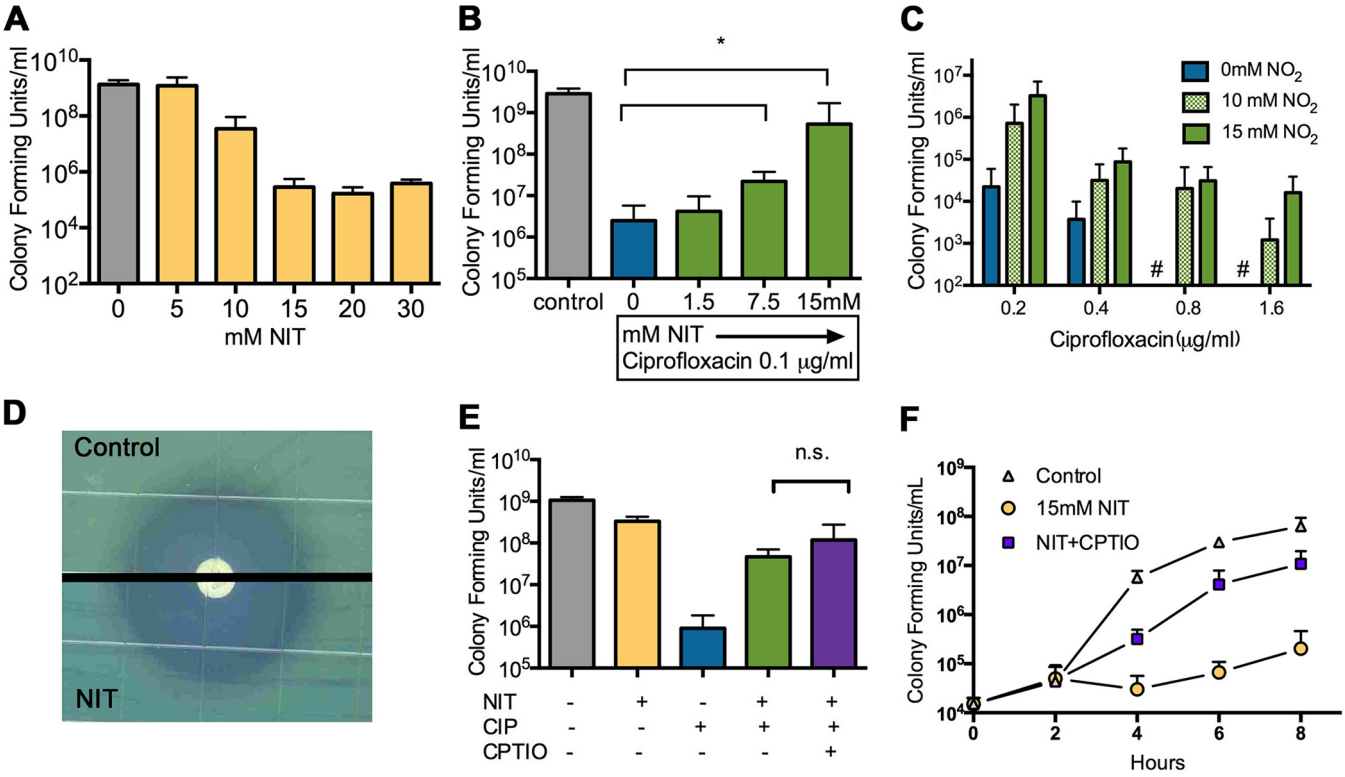

FIG 2 (A) Log-phase, aerated liquid cultures of PAO1 were treated with NIT for 6 h. Growth was arrested with 15 mM NIT. (B) Log-phase, aerated cultures of PAO1 were treated for 5.5 h with 0.1 μg/ml CIP and increasing NIT concentrations. $P < 0.05$ (one-way ANOVA followed by Tukey's test for 7.5 mM and 15 mM NIT). (C) Log-phase, aerated cultures of PAO1 were treated with NIT and CIP for 20 h. #, below assay detection limit of 10^2 CFU/ml. (D) The levels of susceptibility determined by disk diffusion were identical for bacteria exposed to 15 mM NIT (bottom) and control bacteria (top). $n = 3$; a representative image is shown. (E) Addition of 1.5 mM CPTIO did not block NIT-mediated CIP tolerance in liquid, aerobic cultures of PA14 (using experimental conditions identical to those described for panel A). (F) In aerobic culture, scavenging of NO mediated by 1.5 mM CPTIO attenuated 15 mM nitrite-induced growth arrest. The experimental conditions were identical to those described for panel A. Bacteria were counted by serial dilution.

bactericidal antibiotics that included norfloxacin. Bacteriostatic antibiotics, excluding rifampin, cause a decrease in respiratory rate that is dominant over respiratory acceleration linked with bactericidal antibiotics (13). We believe that we are seeing another manifestation of this phenomenon with NIT-induced CIP tolerance; however, our data are correlative, and other explanations are possible.

In summary, NIT induces CIP tolerance in biofilms grown on human airway epithelial cells. Beyond nitrite's development as a potential antibiotic, nebulized nitrite (AIR001 formulation) is in trials as a treatment for pulmonary hypertension and for heart failure with a preserved ejection fraction. Thus, understanding potential drug-drug interactions is critical for safe design of future human studies.

ACKNOWLEDGMENTS

We thank Lauren Lashua, Abiola Ogunsola, and Jeffrey Melvin for excellent technical assistance and advice on the manuscript.

J.M.B. received funding from Breathe Pennsylvania, NIH R00HL098342 and P30DK072506. A.C.Z. received funding from the Cystic Fibrosis Foundation (ZEMKE14D0 and NIH 5T32HL007563-27). B.R.K. received a Breckenridge Fellowship from the University of Pittsburgh.

REFERENCES

1. Paranjape SM, Mogayzel PJ. 2014. Cystic fibrosis. Pediatr Rev 35:194–205. https://doi.org/10.1542/pir.35-5-194.
2. Cystic Fibrosis Foundation. 2015. Patient registry: annual data report 2015. https://www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf.
3. Zemke AC, Shiva S, Burns JL, Moskowitz SM, Pilewski JM, Gladwin MT, Bomberger JM. 18 September 2014. Nitrite modulates bacterial antibiotic susceptibility and biofilm formation in association with airway epithelial cells. Free Radic Biol Med https://doi.org/10.1016/j.freeradbiomed.2014.08.011.

4. Major TA, Panmanee W, Mortensen JE, Gray LD, Hoglen N, Hassett DJ. 2010. Sodium nitrite-mediated killing of the major cystic fibrosis pathogens Pseudomonas aeruginosa, Staphylococcus aureus, and Burkholderia cepacia under anaerobic planktonic and biofilm conditions. Antimicrob Agents Chemother 54:4671–4677. https://doi.org/10.1128/AAC.00379-10.
5. Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, Karabulut AC, Hennigan RF, Hwang SH, Buettner G, Schurr MJ, Mortensen JE, Burns JL, Speert D, Boucher RC, Hassett DJ. 2006. Anaerobic killing of mucoid Pseudomonas aeruginosa by acidified nitrite derivatives under cystic fibrosis airway conditions. J Clin Invest 116:436–446. https://doi.org/10.1172/JCI24684.
6. Schlag S, Nerz C, Birkenstock TA, Altenberend F, Gotz F. 2007. Inhibition of staphylococcal biofilm formation by nitrite. J Bacteriol 189:7911–7919. https://doi.org/10.1128/JB.00598-07.
7. Kim-Shapiro DB, Gladwin MT. 6 December 2013. Mechanisms of nitrite bioactivation. Nitric Oxide https://doi.org/10.1016/j.niox.2013.11.002.
8. Arai H. 2011. Regulation and function of versatile aerobic and anaerobic respiratory metabolism in Pseudomonas aeruginosa. Front Microbiol 2:103. https://doi.org/10.3389/fmicb.2011.00103.
9. Rowe JJ, Yarborough JM, Rake JB, Eagon RG. 1979. Nitrite inhibition of aerobic bacteria. Curr Microbiol 2:51–54. https://doi.org/10.1007/BF02601735.
10. Nonejuie P, Burkart M, Pogliano K, Pogliano J. 2013. Bacterial cytological profiling rapidly identifies the cellular pathways targeted by antibacterial molecules. Proc Natl Acad Sci U S A 110:16169–16174. https://doi.org/10.1073/pnas.1311066110.
11. Williams DR, Rowe JJ, Romero P, Eagon RG. 1978. Denitrifying Pseudomonas aeruginosa: some parameters of growth and active transport. Appl Environ Microbiol 36:257–263.
12. Zhang L, Chiang W-C, Gao Q, Givskov M, Tolker-Nielsen T, Yang L, Zhang G. 2012. The catabolite repression control protein Crc plays a role in the development of antimicrobial-tolerant subpopulations in Pseudomonas aeruginosa biofilms. Microbiology 158:3014–3019. https://doi.org/10.1099/mic.0.061192-0.
13. Lobritz MA, Belenky P, Porter CBM, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl Acad Sci U S A 112:8173–8180. https://doi.org/10.1073/pnas.1509743112.