Inhibition of Biofilm Formation by Esomeprazole in *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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*Staphylococcus aureus* and *Pseudomonas aeruginosa* are common nosocomial pathogens responsible for biofilm-associated infections. Proton pump inhibitors (PPI), such as esomeprazole, may have novel antimicrobial properties. The objective of this study was to assess whether esomeprazole prevents sessile bacterial growth and biofilm formation and whether it may have synergistic killing effects with standard antibiotics. The antibiofilm activity of esomeprazole at 0.25 mM was tested against two strains each of *S. aureus* and *P. aeruginosa*. Bacterial biofilms were prepared using a commercially available 96-peg-plate Calgary biofilm device. Sessile bacterial CFU counts and biomass were assessed during 72 hours of esomeprazole exposure. The killing activities after an additional 24 hours of vancomycin (against *S. aureus*) and meropenem (against *P. aeruginosa*) treatment with or without preexposure to esomeprazole were also assessed by CFU and biomass analyses. *P. aeruginosa* and *S. aureus* strains exposed to esomeprazole displayed decreased sessile bacterial growth and biomass (*P* < 0.001, each parameter). After 72 h of exposure, there was a 1-log10 decrease in the CFU/ml of esomeprazole-exposed *P. aeruginosa* and *S. aureus* strains compared to controls (*P* < 0.001). After 72 h of exposure, measured absorbance was 100% greater in *P. aeruginosa* control strains than in esomeprazole-exposed strains (*P* < 0.001). Increased killing and decreased biomass were observed for esomeprazole-treated bacteria compared to untreated controls exposed to conventional antibiotics (*P* < 0.001, each parameter). Reduced biofilm growth after 24 h was visibly apparent by light micrographs for *P. aeruginosa* and *S. aureus* isolates exposed to esomeprazole compared to untreated controls. In conclusion, esomeprazole demonstrated an antibiofilm effect against biofilm-producing *S. aureus* and *P. aeruginosa*.

**Materials and Methods**

- **Bacterial strains and culture conditions.** Laboratory strains of mucoid *P. aeruginosa* (ATCC 700888) and mucoid *S. aureus* (ATCC 29213) as well as two clinical bloodstream isolates of *P. aeruginosa* and *S. aureus* (one each) obtained from a central venous catheter were used for all experiments. All isolates were stored in Cryocare vials (Key Scientific Products, Round Rock, TX) at −80°C. Fresh isolates were subcultured at least twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 35°C prior to each investigation. An inoculum of approximately 10⁶ CFU/ml was used in every experiment. The inoculum was prepared from an overnight culture grown in broth, diluted accordingly based on the absorbance at 630 nm and verified subsequently by quantitative culture by direct agar plating onto Mueller-Hinton (MH) plates.

- **Materials.** Bacterial biofilms were prepared using a commercially available biofilm reactor consisting of 96 independent pegs mounted on the inside lid of a 96-well microtiter plate (Calgary biofilm device [CBD]; Innovotech, Inc.) (1). Single-strain biofilms were grown by incubating at 37°C the CBD peg lids in microtiter...
plates containing an inoculum of either *P. aeruginosa* or *S. aureus* (10^6 cells/ml) for up to 72 h in a heated, shaking incubator (Shake N Bake hybridization oven; Boekel Scientific, Feasterville, PA) with or without the addition of esomeprazole at 0.25 mM. All experiments were performed at least in triplicate.

**PPI biofilm prevention: experimental plan.** In one series of experiments, pegs were removed at 2 h, 4 h, 6 h, 24 h, 48 h, and 72 h by using sterile forceps, washed for 1 min using 200 μl of 0.9% saline, placed into 200 μl of recovery medium (MH broth), and sonicated on high for 8 min to remove adherent bacteria. Serial dilutions of the bacterial suspension were prepared in saline (0.9% NaCl), directly plated on MH agar plates, and counted after overnight incubation at 37°C. To assess the quantitative determination of biomass formation, a colorimetric assay adapted from the method of O’Toole et al. was used (11). Additional pegs were removed at 2 h, 4 h, 6 h, 24 h, 48 h, and 72 h by using sterile forceps and washed for 1 min using 200 μl of 0.9% saline. Pegs were then placed into a solution of 0.1% crystal violet for 15 min. Pegs were then removed using sterile forceps and washed for an additional minute using 200 μl sterile of the 0.9% saline to remove nonstained crystal violet. The attached dye was reelected into MH broth with 95% ethanol, and the absorbance at 580 nm was determined using a spectrophotometer (PowerWave x Select; Bio-Tek Instruments, Inc., Winooski, VT). Uninoculated medium was used as a negative control and served as the blank for all absorbance readings.

**PPI adjunctive therapy for biofilm-embedded bacteria.** In another series of experiments, *S. aureus* and *P. aeruginosa* biofilms were grown for 72 hours in the CBD as described above with or without the addition of esomeprazole at 0.25 mM. After this time, sessile bacteria were challenged with either vancomycin (*S. aureus* biofilms) or meropenem (*P. aeruginosa* biofilms) by transferring the CBD peg lids to a 96-well antibiotic challenge plate. CFU on the CBD pegs were assessed by quantitative culture, and biomass after 24 h of antibiotics exposure was assessed as described above.

**Microscopic analysis.** Microscopic analysis of the biofilms was performed using microfluidic channels controlled by an airtight interface between inflow and outflow ports connected to a pneumatic continuous displacement pump at a shear flow rate of 0.8 dyne/cm^2 (BioFlux Controller, Fluxion Biosciences, South San Francisco, CA). The viewing angle allowed for the visualization of biofilm growth by using an inverted light microscope (EVOS; Advanced Microscopy Group, Bothell, WA). Biofilms of *S. aureus* and *P. aeruginosa* were visualized at ×100 after 24 h growth with or without the addition of esomeprazole at 0.25 mM.

**RESULTS**

Esomeprazole-treated bacteria displayed decreased sessile bacterial growth and biomass. *P. aeruginosa* and *S. aureus* strains exposed to esomeprazole displayed decreased sessile bacterial growth and biomass (Fig. 1) (*P* < 0.001, each). After 72 h of...
exposure, the log_{10} CFU/ml of *P. aeruginosa* in control strains was 8.5 ± 0.03 CFU/ml (mean ± standard deviation), compared to 7.5 ± 0.07 CFU/ml in esomeprazole-exposed strains (*P* < 0.001). After 72 h of exposure, the log_{10} CFU/ml of *S. aureus* in control strains was 8.9 ± 0.18 CFU/ml, compared to 7.7 ± 0.11 CFU/ml in esomeprazole-exposed strains (*P* < 0.001).

Measured absorbance was significantly higher at all time points sampled for *P. aeruginosa* strains (average optical density [OD], 0.49) than for *S. aureus* strains (average OD, 0.22). After 72 h of exposure, measured absorbance was 100% greater in *P. aeruginosa* control strains (OD, 0.62 ± 0.011) than for esomeprazole-exposed strains (OD, 0.35 ± 0.024; *P* < 0.001). However, there was no significant difference in absorbance after 72 h of exposure between *S. aureus* control strains (OD, 0.23 ± 0.02) and esomeprazole-exposed strains (OD, 0.21 ± 0.010; *P* > 0.05).

**FIG 2** Effects of esomeprazole, as adjunctive therapy with standard antibiotics, on CFU and biomass for *P. aeruginosa* and *S. aureus*. Significantly decreased CFU/ml counts of sessile *P. aeruginosa* and *S. aureus* were observed for isolates exposed to esomeprazole at 0.25 mM and antibiotics, compared to cells exposed to antibiotics alone.

Increased killing of sessile bacteria and decreased biomass were observed in esomeprazole-treated bacteria given conventional antibiotics. Increased killing and decreased biomass were observed for esomeprazole-treated bacteria compared to untreated controls exposed to conventional antibiotics (Fig. 2; *P* < 0.001, each). After 24 h of exposure, the *P. aeruginosa* CFU/ml decreased from 9.0 ± 0.059 CFU/ml in controls to 8.2 ± 0.51 in meropenem-exposed isolates, to 7.6 ± 0.27 in esomeprazole-treated patients, to 6.6 ± 0.13 in isolates exposed to meropenem and esomeprazole (*P* < 0.001). After 24 h of exposure, the *S. aureus* CFU/ml decreased from 9.1 ± 0.14 CFU/ml in controls to 6.9 ± 0.48 in vancomycin-exposed isolates, to 6.5 ± 0.24 in esomeprazole-treated patients, to 5.0 ± 1.6 in isolates exposed to vancomycin and esomeprazole (*P* < 0.001).

After 24 h of exposure, *P. aeruginosa* absorbance decreased from 0.64 ± 0.029 in controls to 0.46 ± 0.083 in meropenem-exposed isolates, to 0.43 ± 0.052 in esomeprazole-treated patients, to 0.33 ± 0.041 in isolates exposed to meropenem and esomeprazole (*P* < 0.001). After 24 h of exposure, the *S. aureus* absorbance decreased from 0.54 ± 0.038 in controls to 0.48 ± 0.042 in vancomycin-exposed isolates, to 0.44 ± 0.042 in esomeprazole-treated patients, to 0.25 ± 0.0058 in isolates exposed to vancomycin and esomeprazole (*P* < 0.001).

**Light micrographs of *P. aeruginosa* and *S. aureus* exposed to 24 h of esomeprazole compared to controls.** Light micrographs of *P. aeruginosa* and *S. aureus* cells exposed to 24 h of esomeprazole compared to controls are shown in Fig. 3. Reduced biofilm growth after 24 h was visibly apparent in the *P. aeruginosa* and *S. aureus* isolates exposed to esomeprazole compared to the untreated controls.

**DISCUSSION**

Biofilm-related infections are an important cause of health care-associated infections. Despite significant research involving antibiotic-impregnated catheters, other coated catheters, and other novel techniques, biofilm-related infections are important causes of device-related and catheter-related infections (2). Biofilm-embedded bacteria are challenging to treat, as they display tolerance to antibiotic killing activity and the host immune system (6, 8). Traditional antibiotics were developed to kill planktonic bacteria and often have a limited effect on the killing of sessile bacteria encased within a biofilm. In addition, antimicrobial resistance development is common in sessile
bacteria. For these reasons, there is an urgent need to develop nonantimicrobial treatment strategies to prevent or treat biofilm-associated infections (7).

In this study, we demonstrated that the PPI esomeprazole displays an antibiofilm effect, as demonstrated by decreased biomass and morphological antibiofilm changes associated with the addition of esomeprazole against two common biofilm-producing health care-associated pathogens. This study builds on the findings of Nguyen et al., who investigated the use of benzimidazole PPI against biofilm-embedded Streptococcus mutans (10). In that study, lansoprazole or omeprazole was used to test the antibiofilm effect of S. mutans in a number of different in vitro models. Lansoprazole at 0.1 mM markedly inhibited biofilm glycolysis. The study concluded that benzimidazole PPI may be useful against oral biofilm-producing bacteria. This study extends these previous findings by demonstrating an antibiofilm effect in the health care-associated pathogens P. aeruginosa and S. aureus.

Sambanthamoorthy et al. recently identified a novel benzimidazole that inhibits bacterial biofilm formation (14). In their study, a small-molecule screen identified a benzimidazole that prevented biofilm formation in multiple bacterial pathogens, including S. aureus and P. aeruginosa. Using a colorimetric assay similar to our study, a greater-than-50% decrease in biomass was observed in isolates preexposed to the benzimidazoles identified in the screening procedure. Using a catheter infection model, coating the surface of the catheter with ABC-1, the most potent antibiofilm benzimidazole, significantly reduced biofilm formation. Interestingly, those authors described in their study that omeprazole, the parent compound of esomeprazole, did not possess significant antibiofilm activity under the conditions examined. Whether esomeprazole has antibiofilm properties independent of omeprazole or whether the test conditions of these two experiments differed will require further study. In our study, the colorimetric assay for S. aureus did not indicate a decrease in biomass with the addition of esomeprazole. However, the killing activity of S. aureus was significantly increased with the addition of esomeprazole to meropenem. Morphological changes were apparent in S. aureus exposed to esomeprazole. These discrepancies highlight the importance of multiple experiments to assess antibiofilm properties.

Two interesting hypotheses can be tested in future studies on the basis of these results. Despite the ability to coat intravascular catheters with antibiotics, many patients still experience catheter-associated bloodstream infections (13). A direct study arising from these results here could investigate the use of esomeprazole as a lock solution as an adjunct prevention measure with antimicrobial-coated catheters. The use of proton pump inhibitors has also been implicated as a risk factor for Clostridium difficile infection (3). It is possible that inhibition of biofilm formation in the gut by commensal bacteria may enhance the ability of C. difficile to penetrate to colonic epithelial cells and cause active disease. Both of these hypotheses will require further study.

This study has many limitations. We chose to use fixed, physiologically relevant concentrations of vancomycin, meropenem, and esomeprazole. Future dose-response studies will be required to assess if greater killing or a decrease in biomass is possible with different dosing regimens. These studies will also be required to be repeated in other relevant in vitro/in vivo models to confirm these results.

In conclusion, the addition of esomeprazole significantly decreased biomass and enhanced killing of P. aeruginosa and S. aureus by standard antimicrobial agents. Future research should focus on the novel benefits and toxicities associated with these findings.

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REFERENCES


