Antibiofilm activities of certain biocides in *Pseudomonas aeruginosa*

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**ABSTRACT**

**Background and objectives:** *Pseudomonas aeruginosa* is an opportunistic pathogen that can produce biofilm. Biofilm is a complex, three-dimensional structure in which microorganisms are attached to a surface and embedded in a matrix made of extracellular polymers. Due to high resistance to antimicrobial agents, biofilms create difficulties in various situations in healthcare. In this study, antibiofilm activities of some biocides in *P. aeruginosa* were studied.

**Materials and methods:** The biofilm production ability of *P. aeruginosa* strain 214 (a clinical isolate) was determined in the presence of six biocides including ethylene diamine tetra acetic acid (EDTA), silver nitrate (AgNO₃), bismuth ethanedithiol (BisEDT), bismuth dimercaprol (BisBAL), bismuth-2-mercaptoethanol (BisMEO) and bismuth propanedithiol (BisPDT) using the modified microtiter plate method. Bactericidal activity of the biocides against biofilm and planktonic cells was investigated. In this study, permeation of biocides through alginate layer was evaluated with a sandwich cup method.

**Results:** The results demonstrated that in the presence of bismuth thiols, biofilm production in MIC and sub MIC concentrations was considerably inhibited. Bismuth thiols had lower antibiofilm bactericidal activity than EDTA and silver nitrate. One possible mechanism of biofilm resistance is exopolysaccharide production which prevents the access of antimicrobial agents to cells inside the biofilm. Bismuth thiols could not penetrate, while EDTA and silver nitrate had high penetration rate.

**Conclusions:** Due to the frequent use of silver nitrate and EDTA in various applications, low efficacy in the inhibition of biofilm production, unstudied toxicity of BTs for humans and high efficacy in the inhibition of biofilm production, it is suggested that combinatory effect of BTs with silver nitrate or EDTA on biofilms and biofilm production be investigated.

**Keywords:** *P. aeruginosa*, Biofilm inhibition, Biocides, Bactericidal activity.

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**INTRODUCTION**

Biofilms consist of groups of bacteria attached to surfaces and encased in a hydrated polymeric matrix. Bacterial biofilms are abundant in the environment and are responsible for several human bacterial infections (1). In such a state, microorganisms are highly resistant to antimicrobial treatment. According to an estimate by the National Institute of Health (NIH, USA), about 80% of all microbial infections are caused by biofilms (2). Possible explanations for the increased resistance of biofilm bacteria include limited diffusion of antimicrobial agents through the biofilm matrix (such as the alginate layer in *P. aeruginosa*), enzyme-mediated resistance, level of metabolic activity within the biofilm, genetic adaptations and other possible mechanisms (3). Mechanisms of biofilm resistance to antimicrobial compounds vary depending on the bacteria present in the biofilm and the drug or biocide being applied (4).

One of the best studied models for biofilm formation is the bacterium *P. aeruginosa*, (1, 5). *P. aeruginosa* is one of the most important opportunistic human pathogens (6), which causes many types of infections, including biofilm-associated chronic lung infections in cystic fibrosis patients (1). There is also an increasing awareness of the important role of *P. aeruginosa* biofilms in the contamination of medical biomaterials such as catheters and prostheses (6).

In this study, we investigated the effects of biocides including diamine tetra acetic acid (EDTA), silver nitrate (AgNO₃), bismuth ethanedithiol (BisEDT), bismuth dimercaprol (BisBAL), bismuth-2-mercaptoethanol (BisMEO) and bismuth propanedithiol (BisPDT) on biofilm production and bactericidal activity on biofilm and planktonic cells of *P. aeruginosa*.

**MATERIALS AND METHODS**

**Bacterial strain.** *P. aeruginosa* strain 214, a clinical strain isolated in a previous study (7), was used in
this study. MIC (minimal inhibitory concentration) of EDTA, silver nitrate, BisEDT, BisPDT, BisMEO and BisBAL were determined according to CLSI microdilution methods.

**Biocides.** Aqueous solution of EDTA and silver nitrate were used in this study. Four bismuth thiol (BTs) included BisEDT, BisPDT, BisMEO and BisBAL. BTs solutions were prepared in propylene glycol using Bismuth nitrate and commercially prepared thiols in 2:1 ratio.

**Inhibition of biofilm production.** A modified micro titer plate test was used to determine inhibition of biofilm production (8). Briefly, bacteria were grown on tryptic soy agar plus 0.2% glucose after which they were re-suspended in tryptic soy broth plus 0.2% glucose and the optical density of suspension at 650 nm (OD650) was adjusted to 0.1. Then, 180 μl of the bacterial suspension were inoculated in six parallel wells of a 96-well plate. Appropriate volume of each biocide (in various concentrations) was added to microwell plate wells. Positive control wells had 200 μl bacterial suspension and negative control wells contained only 200 μl tryptic soy broth plus 0.2% glucose.

After incubation for 24 h at 37°C, the content of each well was aspirated, and each well washed with sterile physiological saline to remove all non-adherent cells. Attached bacteria were fixed with absolute methanol for 10 min. Later, the plates were stained for 20 min with crystal violet (1% W/V). Excess stain was washed off and the plates were rinsed with tap water. After the plates were air dried, the dye bound to biofilm formation was removed with isopropanol. Positive control wells had 200 μl tryptic soy broth plus 0.2% glucose.

The relative inhibition of biofilm (expressed as mean percentage) was determined as follows:

\[
\text{Percent inhibition} = 100 - \left( \frac{\text{OD}_{650} \text{ of biocide well}}{\text{OD}_{650} \text{ of positive control well}} \right) \times 100
\]

Statistical analysis was performed with one way analysis of variance. P values of 0.05 and less were considered statistically significant.

**Bactericidal activity of biocides.** To test the bactericidal activity of the biocides against biofilm and planktonic cells, urinary catheter pieces of 1.5 cm in length were inoculated with the strain, as previously described (7). Briefly, bacteria grown on tryptic soy agar with 5% glucose were re-suspended in saline adjusted to 0.5 McFarland turbidity. 200 μl of this suspension and catheter pieces were added to 19.8 ml tryptic soy broth with 5% glucose and incubated for 6 days at 37°C after which the catheter pieces were washed with PBS. 1 ml of this PBS (planktonic cells) or each catheter piece (biofilm cells) was transferred to each tube with Muller Hinton Broth (MHB) containing a given biocide for 24 h at 37°C. After 24 h, catheter pieces were transferred to 1 ml PBS, vortexed for 5 min. after which the suspensions were diluted, plated on nutrient agar and viable cells were counted after incubation for 24 h at 37°C. For planktonic cells, 1 ml of MHB containing biocide was diluted and plated on nutrient agar. The number of surviving bacteria was determined in a similar manner for the biofilm cells.

**Alginate extraction.** Alginate was extracted from *P. aeruginosa* 214 as previously described (9). Briefly, a modified Mian’s medium was inoculated with the test strain and incubated at 37°C for 72 h. Bacterial cultures were stirred with a magnetic bar for 3 to 5 h, and the cells were removed by centrifugation for 1 h at 17,700 g at 4°C. Crude alginate was precipitated from the supernatant by the addition of cold absolute ethanol to a final concentration of 80% (v/v). The precipitate was collected by centrifugation at 3,000 g for 30 min, washed twice in 80% (v/v) ethanol and once in 96% (v/v) ethanol, and lyophilized.

**Determination of alginate layer permeability to biocides.** Sandwich cup method was used for the determination of alginate layer permeability to biocides (6). A loop phase culture of *P. aeruginosa* 214 grown in nutrient broth at 37°C and adjusted to an optical density of 0.6 at 600 nm was used. After a culture plate insert with a 0.4-mm-pore-size filter (12 mm in diameter; Bedford, MA) was centered in a 100-mm diameter petri dish, 20 ml of nutrient agar at 47°C was mixed with 50 μl of the bacterial suspension and poured into the dish. Following the hardening of agar, 200 ml of either heat-solubilized 1% Noble agar or purified 214 alginate preparation was placed into the insert and allowed to solidify. Finally, 50 μl of either BTs (60 μM), EDTA (60 mM) or silver nitrate (0.3 mM) was added to the insert. Plates were then incubated overnight at 37°C. Zone sizes representing inhibition of bacterial growth were measured and the radius (after the contribution of the insert was subtracted) of the zone detected with Noble agar, was defined as representing 100% penetration of biocides. Inhibition of biocide diffusion through alginate layer
Table 1: Percent inhibition of Biofilm production.

<table>
<thead>
<tr>
<th>Biocides</th>
<th>1/8×MIC</th>
<th>1/4×MIC</th>
<th>1/2×MIC</th>
<th>1×MIC</th>
<th>2×MIC</th>
<th>4×MIC</th>
<th>8×MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>86±0.088</td>
<td>90±0.045</td>
<td>76±0.829</td>
<td>86±0.089</td>
<td>84±0.084</td>
<td>92±0.118</td>
<td>93±0.071</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>23±0.193</td>
<td>20±0.215</td>
<td>25±0.313</td>
<td>24±0.114</td>
<td>24±0.349</td>
<td>10±0.206</td>
<td>96±0.032</td>
</tr>
<tr>
<td>BisBAL</td>
<td>99±0.005</td>
<td>99±0.005</td>
<td>98±0.023</td>
<td>98±0.025</td>
<td>98±0.017</td>
<td>98±0.028</td>
<td>98±0.035</td>
</tr>
<tr>
<td>BisEDT</td>
<td>99±0.006</td>
<td>98±0.032</td>
<td>98±0.042</td>
<td>100±0.000</td>
<td>100±0.000</td>
<td>99±0.006</td>
<td>100±0.000</td>
</tr>
<tr>
<td>BisPDT</td>
<td>98±0.011</td>
<td>98±0.013</td>
<td>99±0.011</td>
<td>99±0.014</td>
<td>98±0.021</td>
<td>98±0.014</td>
<td>99±0.008</td>
</tr>
<tr>
<td>BisMEO</td>
<td>98±0.013</td>
<td>98±0.016</td>
<td>98±0.015</td>
<td>97±0.027</td>
<td>98±0.010</td>
<td>98±0.003</td>
<td>98±0.021</td>
</tr>
</tbody>
</table>

MIC: Minimal Inhibitory Concentration was determined as follow:

\[
\text{Inhibition rate} = 100 \times \left(1 - \frac{\text{radius with alginate}}{\text{radius with Noble agar}}\right)
\]

\[
\text{Penetration rate} = 100 \times \text{Inhibition rate}
\]

Since BTs did not show the inhibitory zone sizes of bacterial growth, even when higher concentrations were subsequently used, the inhibitory zones were not observed.

RESULTS

Biofilm production inhibition. MICs of EDTA, silver nitrate, BisBAL, BisEDT, BisPDT and BisMEO were determined to be 6mM, 27.7μM, 1.56μM, 0.78μM, 1.56μM and 1.56μM, respectively.

As showed in Table 1, at MIC and sub-MIC concentrations, BTs significantly inhibited biofilm production (P<0.05). At 1×MIC, BTs inhibited biofilm production at least 97%, whereas EDTA and silver nitrate showed low inhibition (Table 1). EDTA and silver nitrate did not have significant sub-inhibitory effect on biofilm formation.

Bactericidal activity of biocides. Table 2 shows the bactericidal action of EDTA, silver nitrate, BisEDT, BisPDT, BisMEO and BisBAL on planktonic and biofilm cells of *P. aeruginosa* 214. In addition to considerable differences between bactericidal concentrations of BTs and silver nitrate for planktonic cells and biofilm cells, biofilm cells were more resistant to these biocides. Biofilm cells were less resistant to EDTA.

Permeability of alginate layer to the biocides. Table 3 shows the rate of biocide diffusion through agar containing 1% alginate. A value of 100% was assigned to the permeation rate of biocides through Noble agar without alginate. When alginate was added at 1% to the agar, EDTA and silver nitrate could permeate the alginate layer but BTs could not permeate it (0% penetration rate) and showed an inhibition rate of 100%.

DISCUSSION

Biofilm are known for their recalcitrance to antimicrobial treatment. Due to the high resistance of biofilms to current antimicrobial treatments, scientists have focused their research on effective strategies to remove existing biofilms or to inhibit biofilm formation such as designing antimicrobial surfaces, physical treatments and application of new effective biocides (10).

In this study, the activities of six biocides including EDTA, silver nitrate, BisEDT, BisPDT, BisMEO and BisBAL on planktonic and biofilm production were assessed.
BisBAL on biofilm production inhibition were investigated. It was observed that EDTA and silver nitrate did not considerably inhibit biofilm production at MIC concentration. BTs had strong inhibitory effect on biofilm production and at sub-MIC concentrations, biofilm production was inhibited significantly. In agreement with these results, Domenico et al. reported that BTs inhibited biofilm production in tested Staphylococci at sub-MIC concentrations (11). Our results show that these biocides have a high potential to inhibit biofilm formation on surfaces. Domenico et al. showed that catheters impregnated with BisBAL overnight inhibited biofilm production for 40 days (11).

However, BTs did not show a significant ability to remove existing biofilms and higher concentrations of the solutions were required to kill biofilm cells completely as compared to planktonic cells. Our results indicate that P. aeruginosa biofilm showed a higher resistance to BTs (16 to 128 fold) than planktonic cells (Table 2).

The results also indicate that 30 h treatment of P. aeruginosa biofilm with BisBAL was not sufficient to kill attached cells but was enough to reduce polysaccharide production consequent to which cells became susceptible to the compound. One possible explanation of this phenomenon is that BTs might be adsorbed by biofilms (10). In this study, the sandwich cup method results (Table 3) showed that all four BTs were completely inhibited by alginate (100%). Since BTs have cationic structure (like aminoglycosides), exopolysaccharide presence is suggested as one possible mechanism for resistance to BTs.

As with BTs, biofilms were more resistant to silver nitrate than planktonic cells. However, silver nitrate could penetrate the alginate layer (87.5%). It is therefore postulated that exopolysaccharide has a limited role in the high resistance of biofilm and other possible resistant mechanisms such as high expression of efflux pumps and enzymes and exchange of plasmids in biofilms may have a role in this resistance.

In contrast to BTs and silver nitrate, EDTA showed high bactericidal activity against biofilm cells because bactericidal concentration of EDTA for biofilm cells was just 2 times higher than bactericidal concentration for planktonic cells (Table 2). These results are supported by other studies in which biofilm cells also show low resistance to EDTA (1, 2).

Our results indicate that EDTA could penetrate alginate layer easily (78%). Therefore, the reason for biofilm resistance to EDTA may not be exopolysaccharide presence and other resistant mechanisms suggested for biofilm could be responsible. It is hence suggested that in emergency situations and in locations that catheters and indwelling devices are infected with biofilms which cannot be replaced (such as mechanical heart valves), combinations of EDTA and antibiotics could be used.

Due to the low toxicity and common use of silver nitrate and EDTA in various applications and low efficacy in the inhibition of biofilm production, and lack of studies on toxicity of BTs for humans and high efficacy in the inhibition of biofilm production, it is suggested that combinatory effect of BTs with silver nitrate or EDTA on biofilm production could be investigated.

REFERENCES


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