Activity of amine oxide against biofilms of *Streptococcus mutans*: a potential biocide for oral care formulations

Sébastien Fraud¹, Jean-Yves Maillard¹*, Michael A. Kaminski² and Geoffrey W. Hanlon¹

¹School of Pharmacy and Biomolecular Sciences, Brighton University, Brighton BN2 4GJ, UK; ²Procter & Gamble, Cincinnati, OH, USA

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Aims: To assess the potential bactericidal activity of amine oxide (C₁₀-C₁₆-alkyldimethyl N-oxides) against *Streptococcus mutans* grown as planktonic suspension and as biofilm on hydroxyapatite discs, and its ability to control acidification of the media.

Methods: Amine oxide bacteriostasis was investigated using the Bioscreen C Microbiological Growth Analyser, while a standard suspension test was used to determine its bactericidal efficacy. In addition, the lethal activity of amine oxide was studied against sedimentation biofilms of *S. mutans* on hydroxyapatite (HA) discs and resuspended biofilms. Several parameters were considered such as the surfactant concentration, pH, the starting inoculum and the maturity of the biofilm.

Results: Amine oxide was bacteriostatic against planktonic *S. mutans* at a low concentration (0.006% v/v) and highly bactericidal against *S. mutans* in suspension or in a mature biofilm on hydroxyapatite, although the concentration required to achieve the latter effect was four times higher. The activity of amine oxide against biofilms depended upon its concentration and the age of the biofilm. In addition, amine oxide pre-treatment of the HA discs did not affect the growth of the biofilm. Finally, amine oxide did not prevent the acidification of the medium, although lower pHs had a potentiating effect on amine oxide activity.

Conclusion: Amine oxide showed high potential for controlling early biofilms caused by periodontal bacteria. Further investigations should be carried out, particularly on the potential toxicity of amine oxide and its efficacy in complex formulations for oral care products.

Keywords: biocides, dental plaque, killing kinetics, biofilms

Introduction

Chemical biocides are potentially a valuable complement to mechanical plaque control for the prevention of dental caries and periodontal diseases.¹⁻³ Among the biocides used in this context, biguanides (e.g. chlorhexidine, heptidine), phenolic compounds such as triclosan, povidone iodine, and quaternary ammonium compounds have been shown to inhibit biofilm development and maturation, and bacterial metabolism.¹⁻³⁻⁵

The oral environment constitutes a complex microcosm, although the ability of certain bacteria to attach and colonize the tooth surface is important in the development of caries and periodontal diseases.⁶ *Streptococcus mutans* is among a group of microorganisms that may contribute to the early formation of dental plaque and is considered to be the principal aetiological agent of dental caries.⁷⁻¹⁰ Its ability to synthesize polysaccharides and endodextran from sucrose, its capacity to produce acid and to survive in an acidic environment allow it to predominate in dental plaque and to induce caries.⁷

Although some studies on plaque control have been performed in situ, most have concerned the determination of minimum inhibitory concentrations (MIC), and only a few have investigated the efficacy of biocides against microbial biofilm, which would represent better the state of microorganisms associated with caries and periodontal diseases.¹¹ This is particularly pertinent since bacteria grown as biofilm have been shown to have a decreased susceptibility to many antimicrobials.¹²,¹³ In addition, the relatively recent development of oral biofilm models is of significant benefit for studying the efficiency of anti-plaque agents.¹

The bacteriostatic and bactericidal activity of amine oxide (C₁₀-C₁₆-alkyldimethyl N-oxides) has recently been reported together with its effect on several microorganisms.¹⁴ This has prompted...
Amine oxide activity against biofilms

Further investigations into the possible role of amine oxide in the elimination or control of bacteria, particularly those related to the oral environment. In this study, we have assessed the antibacterial activity of amine oxide (C10-C16-alkyldimethyl N-oxides) against S. mutans grown as a planktonic suspension and as a biofilm on hydroxyapatite (HA) discs in a saliva-based medium.

Materials and methods

Bacteriostatic and bactericidal effect of amine oxide

The effect of amine oxide (C10-C16-alkyldimethyl N-oxides; Procter & Gamble, Cincinnati, OH, USA) on growth of Streptococcus mutans NCTC 10449 was investigated using a Bioscreen C Microbiological Growth Analyser (ThermoLabsystems, Basingstoke, UK). A 100-well plate was prepared as follows: 40 μL of bacterial culture (10^8 cfu/mL) was added to 200 μL of double strength Tryptone Soya Broth (TSB; Oxoid) and amine oxide and sterile deionized water were then added to give a range of biocide concentrations in single strength broth. The plate was incubated at 37°C for 24 h and the A550 of each well recorded every 10 min after 60 s of shaking.

In addition, a modified quantitative suspension test15 was used to investigate the inactivation kinetics of S. mutans exposed to amine oxide. Briefly, a 1 mL aliquot of test suspension (10^8 cfu/mL) was added to 9 mL of the appropriate concentration of biocide solution at 20°C. After the required contact time, an aliquot was neutralized in Tween/lecithin for 10 min, and viable bacteria counted using a modified Miles-Misra drop counting method.15

The efficacy of the neutralizer to quench the activity of amine oxide was investigated before testing with a method described by Langsrud and Sundheim.16

Biofilms of S. mutans

Biofilms were grown on sintered circular ceramic hydroxyapatite (HA) discs (Clarkson Chromatography Products Inc, South Williamsport, PA, USA). All volunteers gave signed consent to the saliva collection protocol. A non-antimicrobial toothpaste was provided for daily use. Volunteers were asked not to have food 1 h prior to, and during, saliva collection. Teeth were cleaned with water for 2 min prior to saliva collection and volunteers were provided with sugar-free chewing gum (Orbit) for saliva production. Saliva specimens were stored in ice before processing. After collection, samples were pooled, centrifuged (30 min, 4°C, 27 000g) and the supernatant pasteurized (60°C, 30 min). After a second centrifugation, aliquots of supernatant were stored at –80°C until required for use. Prior to use, saliva was pre-filtered through a 5 μm (Fisher Scientific, Loughborough, UK) and then a 0.45 μm filter.

HA discs were placed in a 24-well polystyrene cell culture plate (Fisher, Loughborough, UK) and incubated for 4 h at 37°C with 500 μL of sterilized saliva under agitation (120 rpm). After formation of a pellicle, saliva was removed and replaced by 800 μL of TSB + 0.15% (w/v) sucrose and 800 μL of sterile saliva. Each well was inoculated with a suspension of S. mutans (200 μL of 10^8 cells/mL) and plates were incubated aerobically at 37°C for 1, 16.5, 40.5, 64.5, or 88.5 h. The saliva-based medium was renewed after 16.5 h and thereafter at 24 h intervals. The pH of the medium after 16.5 h and 24 h incubation was measured using a microelectrode (Lazarlab, Los Angeles, CA, USA). At the end of the appropriate incubation time, HA discs were washed twice to remove loosely adherent bacteria. To harvest adherent cells, each disc was vortexed vigorously in 1 mL of diluent for 2 min. Following this, the fluids containing the detached cells were vortexed for 1 min with glass beads to disaggregate clumps of cells. Viable counts were carried out as before.

In addition, the effect of the starting inoculum on biofilm formation was investigated. The same protocol as described above was used except that different starting inoculum concentrations were used (200 μL of 10^6, 10^5, 10^4 cells/mL in diluent).

Activity of amine oxide against S. mutans biofilm

HA discs supporting S. mutans biofilm of different ages (see above) were washed and transferred to new wells containing 2 mL of the appropriate concentration of amine oxide. The surviving bacteria were resuspended as described above and viable counts were carried out as before. In addition, the activity of amine oxide was examined against resuspended S. mutans biofilm cells. Biofilm of S. mutans grown on HA discs for different lengths of time were harvested and the cells resuspended and exposed to amine oxide (1.1% v/v) for 2 min. After exposure, the neutralizer was added and left for 10 min before viable counts were performed. The efficacy of amine oxide neutralization was also investigated.

Effect of amine oxide pre-treatment of HA discs on biofilm formation

HA discs were pre-treated with amine oxide (1.1% v/v) for 5 min with or without saliva. After pre-exposure to amine oxide, the discs were immersed in saliva for 4 h prior to S. mutans inoculation (200 μL; 10^8 cfu/mL). Biofilms were grown for 8 h, before the cells were recovered and enumerated as described above.

Effect of amine oxide on acidification

An S. mutans suspension (1 mL; 10^8 cfu/mL) was added to a flask containing 49 mL of TSB supplemented with 0.15% (w/v) sucrose with or without amine oxide at different concentrations. Flasks were incubated at 37°C for 24 h under constant agitation (120 rpm) and the pH of the medium recorded every 10 min using an electrode probe.

Effect of pH on the activity of amine oxide

The effect of different pH solutions on the activity of amine oxide against S. mutans was measured using the quantitative suspension test described above. The pH of solutions was measured after the addition of 1 mL of S. mutans (10^8 cfu/mL) and 0.018% (v/v) amine oxide, and readjusted accordingly.

Results

Effect of amine oxide concentrations on bacterial growth

A range of amine oxide concentrations (0.002–8% v/v) was tested against S. mutans. Concentrations above 0.006% (v/v) inhibited completely the growth of the microorganism (Figure 1). Amine oxide at a concentration of 0.002% (v/v) produced a similar growth curve to the control wells, whereas 0.004% (v/v) affected the exponential growth rate and produced a slightly lower final absorbance reading.

Inactivation kinetics of S. mutans treated with different amine oxide concentrations

The inactivation kinetics of a range of amine oxide concentrations are presented in Table 1. The choice of the concentrations investigated was dictated by the need to find a concentration that was not
rapidly bactericidal, notably for the experiments investigating variations in pH. Amine oxide concentrations of 0.018% (v/v) and 0.004% (v/v) in particular were chosen for this purpose (see below), and in addition, amine oxide concentrations of 0.275% (v/v) and 1.1% (v/v) were investigated. Amine oxide was shown to be highly bactericidal at concentrations of 0.275% (v/v) and above (Table 1) against planktonic \textit{S. mutans}. In addition, using a protocol described by Langsrud and Sundheim,\textsuperscript{16} it was observed that the neutralizer used (i.e. 30 g/L Tween and 3 g/L lecithin) quenched effectively amine oxide at a concentration of at least 1.1% (v/v) (data not shown).

\textbf{Biofilm of \textit{S. mutans} and HA discs}

The protocol used for growing \textit{S. mutans} biofilm on HA discs was shown to be highly reproducible as measured by the low variability between separate results. The variability in cell number recovered from the biofilm for each time point was small (data not shown). In addition, after 16 h incubation, no further increase in cell number was measured (i.e. $10^8$ cfu/mL recovered cells; data not shown). \textit{S. mutans} biofilms were very uniform covering the entire surface of the HA discs, and although the microorganisms appeared densely packed and consistent with biofilm observation, there was no observable evidence of an exopolymer matrix (data not shown).

After 16.5 h and 40.5 h incubation times at 37°C, the pHs of media were 5.00–0.10 and 4.92–0.04, respectively. The recovery protocol used was satisfactory as very few cells were seen remaining attached to the surface of the HA discs, when investigated using a scanning electron microscope (data not shown). It was also noticed that when a lower starting inoculum was used (10$^3$ and 10$^4$ cfu/mL), the maximum number of cells recovered from biofilms (i.e. $10^8$ cfu/mL) was reached after 40 h incubation (Figure 2).

\textbf{Effect of amine oxide on \textit{S. mutans} biofilms and on cells resuspended from biofilms}

Amine oxide (0.275% v/v) reduced the bacterial count by over 5 log$\text{_{10}}$ within 5 min contact against 16 h biofilms of \textit{S. mutans} (Table 2). However, its efficacy decreased rapidly with mature biofilms (i.e. >40 h) against which little or no activity was observed. Likewise, a 2 min exposure to 1.1% (v/v) amine oxide completely eliminated viable cells within biofilms at an early stage of development (16 h) (Table 3), however, the same concentration of amine oxide was inactive against mature (i.e. >40 h) biofilms. Cells resuspended from these biofilms were inactivated more readily, although the activity of amine oxide decreased

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Concentration (%)$^a$ & 10 min & 30 min & 60 min \\
\hline
0.5 & >5 & >5 & >5 \\
0.275 & >5 & >5 & >5 \\
0.02 & 2.25 & 4.46 & >5 \\
0.018 & 2.43 & >5 & >5 \\
0.01 & 0.50 & 0.69 & 0.48 \\
0.005 & 0.55 & 0.56 & 0.47 \\
0.0025 & 0.43 & 0.51 & 0.42 \\
\hline
\end{tabular}
\caption{Effect of amine oxide on planktonic \textit{S. mutans} using a quantitative suspension test}
\end{table}

\textsuperscript{a}pH 7 (unadjusted).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Effect of low concentrations of amine oxide (AO) on the growth of \textit{S. mutans} at 37°C (average of six replicates ± SD for control TSB, and three replicates ± SD for amine oxide testing).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Effect of inoculum size on the growth of the biofilm per time point per disc.}
\end{figure}
according to the age of the biofilm from which the cells were resuspended (Table 3), i.e. cells from a 64 h biofilm were more resilient than those from a 40 h biofilm. It was interesting to observe that these resuspended cells lost their resistant phenotype after an overnight subculture in TSB. A 5 log₁₀ reduction after 2 min exposure to 1.1% (v/v) amine oxide was then observed using a quantitative suspension test (data not shown).

Effect of amine oxide pre-treatment of HA discs on biofilm formation

Several pre-treatment conditions were tested to assess the effect of saliva, incubation time and neutralizer and the effect of pre-exposure of amine oxide (1.1% v/v) on the growth of S. mutans biofilm (Table 4). HA discs pre-exposed to the surfactants in a variety of conditions did not affect the growth of the biofilm (i.e. the number of cells recovered from the biofilm after 8 h incubation).

Effect of amine oxide on acidification

To investigate the effect of amine oxide on the acidification of the medium, it was important to choose a biocide concentration that would allow observed changes in growth pattern, i.e. not a bactericidal concentration. Therefore, from Figure 1, a concentration of 0.004% (v/v) amine oxide was used. It was observed that amine oxide (0.004% v/v) did not affect the acidification ($P < 0.001$; Student’s $t$-test) of the medium (Figure 3). In addition, the decrease in pH could be correlated with the exponential growth phase (from

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**Table 2.** Activity of 0.275% (v/v) amine oxide on biofilm of different maturation (starting inoculum 200 μL of $10^5$ cfu/mL)

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Log₁₀ cfu/cm² (±SD)</th>
<th>Log₁₀ reduction of amine oxide (0.275% v/v) at contact time of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 min</td>
</tr>
<tr>
<td>16</td>
<td>5.57 (0.32)</td>
<td>1.09</td>
</tr>
<tr>
<td>40</td>
<td>7.94 (0.42)</td>
<td>0.00*</td>
</tr>
<tr>
<td>64</td>
<td>7.74 (0.43)</td>
<td>0.00*</td>
</tr>
</tbody>
</table>

*No reduction in cell number compared with the control.

**Table 3.** Activity of 1.1% (v/v) amine oxide for 2 min against biofilms and resuspended biofilm cells of different maturation (starting inoculum 200 μL of $10^5$ cfu/mL)

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Control log₁₀ cfu/cm² (±SD)</th>
<th>Log₁₀ reduction after 2 min contact time (±SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>biofilms</td>
</tr>
<tr>
<td>16</td>
<td>4.40 (0.23)</td>
<td>4.40 (0)</td>
</tr>
<tr>
<td>40</td>
<td>8.22 (0.27)</td>
<td>0.75 (0.11)</td>
</tr>
<tr>
<td>64</td>
<td>8.34 (0.17)</td>
<td>0.38 (0.46)</td>
</tr>
</tbody>
</table>

*Experiments were performed in triplicate.
Table 5. Effect of pH on amine oxide (0.018% v/v) activity against S. mutans using a quantitative suspension test

<table>
<thead>
<tr>
<th>pH</th>
<th>Log10 reduction (±SD)</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3−5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
</tr>
<tr>
<td>6</td>
<td>3.12</td>
<td>3.34</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.15)</td>
<td>(0.42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>2.43</td>
<td>3.55</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td>&gt;5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.16)</td>
<td>(0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.45</td>
<td>0.90</td>
<td>1.07</td>
<td>1.56</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.02)</td>
<td>(0.01)</td>
<td>(0.11)</td>
<td>(0.09)</td>
<td></td>
</tr>
</tbody>
</table>

*aUnadjusted pH.

Effect of pH on the antibacterial activity of amine oxide

When pH alone was investigated, only very low values (i.e. pH 3) were shown to have an effect on the survival of S. mutans. Under these conditions, a 2.33 log10 reduction after 30 min and a 3.28 log10 reduction after 60 min were observed. Other pHs, from 4 to 8 were found to have no effect with a maximum of 0.46 log10 reduction in number observed (data not shown). However, pH was shown to have an effect on the bactericidal activity of amine oxide (0.018% v/v; Table 5). Below pH 5, a potentiation of amine oxide activity was noticed, whereas at an alkaline pH (i.e. >8), amine oxide was significantly less bactericidal (P < 0.001; Student’s t-test).

Discussion

Amine oxides are amphoteric surfactants, which have been shown to possess a broad spectrum of activity against a variety of microorganisms.14,17–20 In this study, amine oxide (C10-C16-alkyldimethyl N-oxides) had a substantial antibacterial activity against planktonic cells of S. mutans. A concentration as low as 0.006% (v/v) was bacteriostatic. In addition, an amine oxide concentration of 0.018% (v/v) was shown to be bactericidal within 30 min, whereas a concentration of 0.275% (v/v) produced a >5 log10 reduction in cell concentration within 5 min. The variation in length of the hydrocarbon tail is thought to influence the antimicrobial activity of amine oxide. Its miscelle-forming capability is also correlated with its antimicrobial activity.21 Here, the testing for activity of amine oxide mixtures (i.e. C10-C16) was thought to provide an appropriate spectrum of antibacterial activity.

The protocols used to grow S. mutans biofilm on HA discs and to resuspend biofilm cells for enumeration proved to be highly reproducible. The role of saliva and particularly the role of a salivary pellicle have been deemed to be critical for the adherence of bacteria and thus plaque formation.5 Furthermore, we have based our model on the use of hydroxyapatite discs, in order to reflect the enamel of the tooth surface, since it is known that the nature of the substratum might affect antimicrobial activity.22

The use of bacterial biofilm model systems is particularly relevant to the study of microorganisms associated with caries and periodontal diseases.1,11,22 Since sessile bacteria have been shown to be much less susceptible to antimicrobials than planktonic cells.12,13 Our results confirmed that S. mutans grown in a planktonic state were more susceptible to amine oxide than when grown as a biofilm. Furthermore, resuspended biofilm cells were shown to have an intermediate susceptibility phenotype (i.e. between planktonic and sessile bacteria) when exposed to the biocide. In addition, this investigation showed that amine oxide was active against bacterial biofilm, although the age of the biofilm affects the bactericidal efficiency of the biocide. Bacterial phenotypic adaptation following attachment to a surface and within a biofilm plays an important role in the emergence of resistance to antimicrobials.23,24 Other mechanisms such as the impairment of biocide penetration into the biofilm matrix, the quenching of activity by the exopolysaccharides, and enzyme inactivation have also been described.12,13 The former mechanism (i.e. phenotypic adaptation) may have played a role in the adaptation of S. mutans to amine oxide exposure as demonstrated by the difference in susceptibility of mature biofilm and cells resuspended from these biofilms, although the latter mechanisms cannot be eliminated.

Several biocides, particularly cationic and phenolic agents, are part of the formulation of many oral care products because of their antibacterial activity and sometimes their substantivity.1,5,25 However, these biocides are usually used at a higher concentration than that of amine oxide used in this study. Here, low concentrations of amine oxide were deliberately used to enable the determination of the cells’ susceptibility and response patterns to amine oxide and to pin-point the limitations to its usage. In this study, no residual activity was observed when HA discs were pre-exposed to amine oxide 1.1% (v/v) for 5 min. Several biocides used in oral-care products are selected on the basis of their overall antimicrobial activity but also because of a significant residual effect. The use of such a low concentration of biocides is currently at the centre of a debate on the possible emergence of bacterial resistance to both biocides and antibiotics.26–29 A recent in vivo study which investigated the effect of chlorhexidine-containing mouthwash showed the biguanide induced a change in the oral flora by inhibiting the most susceptible cells while resistant microorganisms increased.30 Walsh and colleagues31 were unable to produce Gram-positive or -negative resistant mutants to amine oxide, even through the use of several different protocols, ranging from direct exposure to the biocides to stepwise training by which bacteria were exposed to gradually increasing concentrations of the biocides.

Acidification of the oral environment is an important factor in the formation of dental caries as it initiates dissolution of the tooth enamel.32 Here amine oxide was shown not to alter acidification of the medium, although low concentrations were investigated. It is certainly correct to assume that at a high concentration (e.g. 1.1% v/v), the significant antibacterial effect against planktonic and sessile S. mutans cells would also stop the production of acids from these microorganisms and thus acidification. In addition, it was clearly observed that amine oxide activity was potentiated by a low pH and so the production of acidic conditions within the depth of the biofilm will promote increased biocidal activity.

In conclusion, this in vitro oral biofilm system proved to be a useful model for the pre-clinical testing of amine oxide as a prospective anti-plaque agent at clinically relevant concentrations. Our results indicate that amine oxide is a good biocidal candidate for the early control of oral biofilms and could be beneficial in oral care formulations.
Amine oxide activity against biofilms

Acknowledgements

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Transparency declarations

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