XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of Staphylococcus aureus including biofilms

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Objectives: Slow-growing and non-dividing bacteria exhibit tolerance to many antibiotics. However, membrane-active agents may act against bacteria in all growth phases. We sought to examine whether the novel porphyrin antibacterial agents XF-70 and XF-73, which have rapid membrane-perturbing activity against Staphylococcus aureus, retained antistaphylococcal activity against growth-attenuated cells.

Methods: The killing kinetics of XF-70, XF-73 and various comparator agents against exponential phase cultures of S. aureus SH1000 were compared with effects on cells held at 4°C, non-growing cultures expressing the stringent response induced by mupirocin and bacteria in the stationary phase. Biofilms of S. aureus SH1000 were generated with the Calgary device to examine the activities of XF-70 and XF-73 under a further system exhibiting diminished bacterial growth.

Results: Cold culture, stringent response and stationary phase cultures remained susceptible to XF-70 and XF-73, which caused ≥5 log reductions in viability over 2 h. During this period the most active comparator agents (chlorhexidine and cetyltrimethylammonium bromide) only promoted a 3 log drop in viability. XF-70 and XF-73 were also highly active against biofilms, with both agents exhibiting low biofilm MICs (1 mg/L) and minimum biofilm eradication concentrations (2 mg/L).

Conclusions: XF-70 and XF-73 remained highly active against various forms of slow-growing or non-dividing S. aureus. The results support the hypothesis that membrane-active agents may be particularly effective in eradicating slow- or non-growing bacteria and suggest that XF-70 and XF-73 could be utilized to treat staphylococcal infections where the organisms are only dividing slowly, such as biofilm-associated infections of prosthetic devices.

Keywords: drug action, porphyrins, bactericidal activity

Introduction

During infection, bacteria often encounter unfavourable conditions that lead to periods of limited growth in which the organisms enter a quiescent state and persist within the host.1,2 Dormant bacteria frequently exhibit tolerance to antibiotics that are active against rapidly dividing cells, and this contributes to the prolonged treatment periods required for persistent infections.3,4 Consequently, antibacterial drugs that retain bactericidal activity under growth-arrested conditions may have clinical advantages over those that do not display such activities.3,5

The nature and properties of drugs with the ability to kill slow-growing bacteria have not been defined.3 Nevertheless, it is apparent that agents with membrane-perturbing activity might possess the required properties because they avoid the requirement for interaction with targets in active biosynthetic pathways, a process that is characteristic of many antibiotics that kill growing cells.3,5

Bacterial biofilms are increasingly recognized as a factor in the inability of licensed antibiotics to successfully eradicate various infections. The formation of biofilms has been implicated in the development of dental caries, cystic fibrosis pneumonia and infections associated with indwelling medical devices.6 Biofilm cultures are typically highly refractory to eradication by chemotherapy, without developing genotypic resistance. The number of therapeutic options is limited and the development of novel antimicrobial agents with antibiofilm activity is increasingly important. It is believed that the presence of metabolically
**Materials and methods**

**Bacteria, antibiotics and reagents**

S. aureus strain SH1000 was used throughout this study. It forms biofilms and is the same strain that has been used in our extensive studies on the biochemical mode of action of the XF agents. Antibiotics and chemicals were from Sigma-Aldrich (Poole, UK) with the exception of XF-73 and XF-70 (Destiny Pharma, Brighton, UK), daptomycin (Cubist Pharmaceuticals, Lexington, MA, USA), chlorhexidine (MP Biomedical, Illkirch, France), fosfomycin (Biochemie, Vienna, Austria), ciprofloxacin (Bayer, Leverkusen, Germany), fluocoxacinilin (CP Pharmaceuticals, Wrexham, UK), fusidic acid (Leo Pharma, Ballerup, Denmark), mupirocin (Pliva Pharma, Zagreb, Croatia) and meropenem (AstraZeneca Pharmaceuticals, Wilmington, DE, USA).

**Growth media and agar**

Bacteria were propagated in Mueller–Hinton broth (MHB) (Oxoid Ltd, Cambridge, UK) and grown on Mueller–Hinton agar (MHA) (Oxoid Ltd). In studies with daptomycin, MHB was supplemented with calcium chloride (50 mg/L).

**Antibacterial activity**

MIC values were determined using the broth microdilution method as specified by BSAC guidelines. Biofilm MICs (bMICs) and minimum biofilm eradication concentrations (MBECs) were determined using a modified version of the method developed by Ceri et al. Briefly, S. aureus SH1000 biofilms were grown at 37°C for 24 h using a Calgary biofilm device lid fitted to a microtitre plate (Nunc Inc., Roskilde, Denmark). Each polystyrene peg supports a biofilm containing ~10^7 cfu. Biofilms were washed in saline before being exposed to antimicrobial compounds diluted in MHB for 24 h. bMIC was measured as the lowest concentration of antimicrobial agent that inhibited planktonic growth. Subsequently, biofilms were washed twice in saline and transferred to fresh MHB. After a 24 h incubation period, the MBEC was measured as the lowest concentration of drug that effectively sterilized the biofilm, allowing no further planktonic growth.

**Killing kinetics**

The antimicrobial activities of XF-73, XF-70 and a range of comparator agents were studied using standard time–kill methodology. S. aureus SH1000 cultures were grown to early exponential phase (optical density at 600 nm (OD600 of 0.2) in MHB, before being exposed to antibiotic agents at 4× the MIC. An untreated culture served as the negative control. Cultures were maintained at 37°C, and samples were taken at 30 min intervals for 300 min, and were then serially diluted in PBS. Diluted culture was spread on MHA and was incubated at 37°C for 18–24 h before the number of cfu was counted. This method was also used to study the activity of antimicrobial agents against slow-growing cultures (see below for details). The lowest limit of reliable detection of organisms was considered to be 10 cfu/mL.

**Cultures expressing the stringent response**

The antibiotic mupirocin is a strong inducer of the stringent response in S. aureus and causes starvation of charged isoleucyl-tRNA by potentiating the stringent response. Strangency was induced in SH1000 cultures by adding mupirocin (4 mg/L) to cells in the early exponential growth phase (OD600 of 0.2). Cultures were incubated with mupirocin for 30 min before sampling began.

**Cold cultures**

Cold cultures were prepared by growing SH1000 cells to early exponential phase (OD600 of 0.2) at 37°C. Cultures were then centrifuged and the cell pellet was resuspended in MHB pre-chilled to 4°C. The killing kinetics of antimicrobial agents were studied as described in the section above.
with the exception that cultures were maintained at 4°C over the 5 h sampling period.

**Stationary phase cultures**

A growth curve was constructed to identify when SH1000 cultures enter and leave stationary phase. MHB (50 mL) was inoculated with 500 μL of SH1000 overnight culture and was maintained at 37°C with agitation for 8 days. The culture turbidity at OD<sub>600</sub> was measured at regular intervals using a Jenway 6300 spectrophotometer with a 1 cm light path (Jenway, Essex, UK).

SH1000 cultures were grown to early, mid and late stationary phase at 37°C by incubation for 24, 48 and 72 h, respectively. Cultures were centrifuged, the supernatant was removed and then a portion of the cell pellet was resuspended in the counterpart supernatant to an OD<sub>600</sub> of 0.2 (10<sup>8</sup> bacteria/mL). A time–kill assay was then performed on these stationary phase suspensions to study the effects of antimicrobial agents on bacterial cell viability.

**Results**

**Antibiofilm activity of XF drugs**

XF-70 and XF-73 exhibited MICs of 1 mg/L for planktonic cultures of *S. aureus* SH1000 (Table 1). Both XF drugs also demonstrated excellent activity against *S. aureus* SH1000 biofilms compared with the other antimicrobial agents examined (Table 1). This was reflected in low bMIC values, which extended to potent biofilm eradication activity (MBEC), a property not exhibited by the other antimicrobial agents used as controls (Table 1).

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Planktonic MIC (mg/L)</th>
<th>bMIC (mg/L)</th>
<th>MBEC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XF-70</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>XF-73</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.5</td>
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</tr>
<tr>
<td>Chlorhexidine</td>
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<td>1</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>2</td>
<td>4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>CTAB</td>
<td>2</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1</td>
<td>2</td>
<td>&gt;256</td>
</tr>
<tr>
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<td>Fusofomycin</td>
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<td>8</td>
<td>&gt;256</td>
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<td>Fusidic acid</td>
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<td>0.5</td>
<td>&gt;256</td>
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<tr>
<td>Gentamicin</td>
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</tr>
<tr>
<td>Meropenem</td>
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<td>0.5</td>
<td>&gt;256</td>
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<tr>
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<tr>
<td>Tetracycline</td>
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<td>&gt;256</td>
</tr>
<tr>
<td>Vancorycin</td>
<td>1</td>
<td>2</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

CTAB, cetyltrimethylammonium bromide. MICs were determined by microdilution in MHB. bMICs and MBECs were determined in MHB using the Calgary biofilm device.<sup>14,15</sup>

**Effects of XF drugs on *S. aureus* expressing the stringent response**

The activity of the XF drugs against *S. aureus* SH1000 that had been prevented from growing by the addition of mupirocin was examined. Previous studies have demonstrated that the stringent response completely abolishes the bactericidal activity of β-lactam antibiotics and fosfomycin against *S. aureus*.<sup>16</sup> Fosfomycin was included as a control in the present studies. At 4x the MIC, fosfomycin exhibited modest bactericidal activity against exponential phase bacteria during a period of 5 h (Figure 2), which was completely attenuated under conditions of stringency (Figure 3). Similar effects were observed for rifampicin (Figures 2 and 3). In contrast, XF-70 and XF-73 retained potent bactericidal activity against cultures of SH1000 prevented from growth by induction of the stringent response (Figures 2 and 3). Other agents with known membrane-disrupting properties (nisin, daptomycin, cetyltrimethylammonium bromide and chlorhexidine)<sup>5,17,18</sup> retained some bactericidal activity under stringent conditions (compare Figures 2 and 3), but this was not as predominant as that displayed by XF-70 and XF-73.

**Effects of XF drugs on cold cultures**

The effect of XF drugs on the viability of cold culture cells was determined over a period of 5 h (Figure 4). Low temperature had no effect on the activity of XF-70 and XF-73, which retained potent bactericidal activity against *S. aureus* SH1000 whose growth had been arrested by the temperature shift (Figure 4). Under these conditions both daptomycin and nisin retained some bactericidal activity, but the ability of other agents to kill the organisms was abolished (Figure 4).

**Effects of XF drugs on stationary phase cultures**

The beginning and end of stationary phase were established for *S. aureus* SH1000 cultured in MHB at 37°C by examining growth curves for the organism over extended periods (data not shown). Cells were defined as entering stationary phase after 24 h of growth and exiting at 96 h, after which culture turbidity declined, indicating bacterial lysis and death. Therefore, early stationary phase was considered to begin 24 h after inoculation, mid stationary phase at 48 h and late stationary phase at 72 h. In order to avoid inoculum effects for susceptibility testing associated with the high cell densities achieved in stationary phase cultures, organisms were recovered at the 24, 48 and 72 h timepoints and diluted to 10<sup>8</sup>bacteria/mL in the spent growth medium from these cultures prior to determination of the bactericidal activities of inhibitors.

XF-70 and XF-73 retained potent bactericidal activity against cells recovered from all timepoints in the stationary phase. Representative data for cells in mid stationary phase are illustrated in Figure 5. Although the initial killing rates exhibited by XF-70 and XF-73 against stationary phase cells appeared to be slightly less than those against exponential phase cultures (compare Figures 2 and 5), the activity of comparator agents against stationary phase cultures was poor (Figure 5). However, nisin and chlorhexidine exhibited some bactericidal activity against stationary phase cells, which appeared to be...
Infections that involve slow-growing and dormant bacteria represent a significant clinical problem as the associated organisms are difficult to eradicate with conventional antibiotics. Furthermore, there is no clear research strategy to suggest how new antibiotic classes might be discovered, or designed, to eliminate slow-growing organisms. Nevertheless, a consensus is beginning to emerge that antibacterial agents with bacterial cell membrane-perturbing activities might possess the required features to eliminate slow-growing bacteria because they do not depend upon inhibition of targets in active biosynthetic pathways.

When nutrients become limiting for growth, bacteria adjust their metabolism from one that supports growth to one that permits survival in the absence of nutrients. In many bacteria a key facilitator of this physiological switch, known as the stringent response, is accumulation of guanosine pyrophosphate and pentaphosphate. XF-70 and XF-73 had almost identical potent antibacterial activity against S. aureus expressing the stringent response (Figure 3) to that against exponentially growing cultures (Figure 2), demonstrating that metabolic activity is not required for their bactericidal activity.
Cold shock is an alternative method for arresting the growth of *S. aureus* to permit studies with antibiotics. Under these conditions bacteria enter a slow-growing state due to activation of stress responses that decrease membrane fluidity and reduce enzymatic activity within the cell. In addition to physiological changes within the cell, low temperatures will undoubtedly influence the susceptibility of bacteria to antimicrobial agents that target intracellular metabolic targets by decreasing their rates of diffusion and molecular interaction with the organisms. Again, XF-70 and XF-73 demonstrated potent antibacterial activity against cold culture cells (Figure 4). In contrast, the comparator agents utilized in both the stringent response (Figure 3) and cold culture experiments (Figure 4) demonstrated decreased antibacterial activities under these conditions.

The bactericidal activity of antibiotics against stationary phase cultures that have resulted from nutrient depletion is often used to assess the efficacy of the agents against non-dividing cells. However, the physiology, and hence susceptibility, of such non-dividing bacteria to agents will differ depending upon whether they have just entered the stationary phase, or have been subject to more prolonged nutrient deprivation. We therefore chose to examine the susceptibility of early, mid
and late stationary phase cultures to XF-70, XF-73 and comparator agents. XF-70 and XF-73 demonstrated potent antibacterial activity irrespective of the stage within the stationary phase that was tested. This suggests that any changes in physiology that occur whilst the bacteria are in stationary phase do not have an effect on the antibacterial activity of XF-70 and XF-73. In contrast, the activity of nisin and particularly chlorhexidine, two membrane-targeting agents, appeared to increase as the stationary phase progressed, suggesting that changes in cell membrane physiology might occur in late stationary phase.

It is well established that S. aureus and other bacteria grow more slowly in biofilms and that the diminished growth rates are an important factor in the increased resistance of bacteria within biofilms to many antibiotics. The bMIC and MBEC results for XF-70 and XF-73 are within one order of magnitude of the planktonic MIC (Table 1), demonstrating that the drugs are equally active against S. aureus within biofilms, a property not shared with all of the other comparators utilized in this study. Thus, XF-70 and XF-73 killed bacteria within the biofilm (low MBEC value), as well as preventing the shedding of bacteria from the biofilm (low bMIC value). These results for XF-70 and XF-73 are encouraging because the presence of staphylococcal biofilms on catheters and other indwelling medical devices presents significant medical problems. Consequently, there is a particular need to identify antimicrobial agents with the ability to eradicate biofilms once they are formed. The results presented here for XF-70 and XF-73 support the growing consensus that drugs that interfere with bacterial membrane integrity retain activity against organisms where normal growth is arrested. We have described the activities of XF-70 and XF-73 against a single well-defined strain of S. aureus that has also been used in our earlier studies on the mode of action of these agents. Confirmation of the results presented here with a wider range of S. aureus strains, including recent clinical isolates, would be desirable. Nevertheless, XF-70 and XF-73 show promise for the treatment of staphylococcal infections involving slow- or non-growing organisms such as prosthetic device infections, endocarditis and osteomyelitis.

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Transparency declarations

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References


