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XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity

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***Objectives*: XF-73 is a novel porphyrin antibacterial agent previously reported to inhibit a range of Gram-positive bacterial species, including *Staphylococcus aureus*. Its mode of action is unknown. Using *S. aureus* as a model organism we sought to examine the basis of its antibacterial activity.**

***Methods*: The effects of XF-73 on the growth and survival of *S. aureus* SH1000 were investigated by viable count and culture absorbance techniques. Inhibition of macromolecular synthesis and disruption of membrane integrity after exposure to XF-73 were examined by radiolabelling experiments, the BacLight fluorescent dye assay and measurement of K^+ and ATP leakage from the cell. The effect of XF-73 on a staphylococcal coupled transcription–translation system was also investigated.**

***Results*: XF-73 was rapidly bactericidal against *S. aureus* SH1000 and demonstrated more rapid killing kinetics than all other comparator agents when tested at an equivalent multiple (4×) of the MIC. Exposure of *S. aureus* to XF-73 for 10 min completely inhibited DNA, RNA and protein synthesis. XF-73 had no effect on transcription and translation *in vitro*. Cells exposed to XF-73 gave a positive response in the BacLight assay, which detects membrane damage. The drug also caused substantial loss of K^+ and ATP from the cell, but did not promote bacterial lysis.**

***Conclusions*: XF-73 exhibited rapid membrane-perturbing activity, which is likely to be responsible for inhibition of macromolecular synthesis and the death of staphylococci exposed to the drug.**

Keywords: drug action, membrane permeability, membrane integrity, *Staphylococcus aureus*

Introduction

There has been a marked decline in antibiotic research and development in recent years and it is now essential to discover new agents effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and other antibiotic-resistant pathogens.[1,2]

Structurally novel inhibitors that inhibit new molecular targets are particularly desirable since such agents are unlikely to be susceptible to existing mechanisms of bacterial resistance.[2] In this regard the XF antimicrobial drug series, exemplified by XF-73, is particularly interesting in view of its unique dicationic porphyrin structure (Figure 1), which is distinct from all other antimicrobial classes.

XF-73 is rapidly active against a broad range of Gram-positive species, including *S. aureus*.[3] Investigations into the likelihood of mutational resistance to XF-73 in *S. aureus* have demonstrated that, unlike some comparator antibiotics, there was no evidence for reduced susceptibility to the agent even after 55 repeat passages at subinhibitory concentrations.[4,5] The lack of resistance emergence may relate to a unique mode of action for XF-73. However, its mechanism of action is currently unknown. Using *S. aureus* as a model organism we sought to examine the basis of its antibacterial activity. In this article we define the biochemical events that are responsible for the antistaphylococcal activity of XF-73.

Materials and methods

Bacterial strains antibiotics and chemicals

S. aureus SH1000[6] was used for most of the studies reported here. Antibiotics and chemicals were from Sigma-Aldrich (Poole, UK) with the exception of XF-73 (Destiny Pharma, Brighton, UK),

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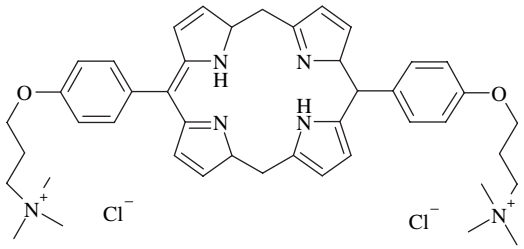

Figure 1. Structure of XF-73.

daptomycin (Cubist Pharmaceuticals, Lexington, MA, USA), the Live/Dead BacLight kit and the ATP determination kit (both from Invitrogen Life Technologies, Paisley, UK) and ethanol (Fisher Scientific Ltd, Loughborough, UK). The following radiolabelled chemicals were from GE Healthcare (Little Chalfont, Buckinghamshire, UK): [*methyl*-^{3}H]thymidine (70–95 Ci/mmol), [5,6-^{3}H]uridine (31–56 Ci/mmol) and L-[G-^{3}H]glutamine (20–50 Ci/mmol).

Growth media

Bacteria were grown in Mueller–Hinton broth (MHB; Oxoid Ltd, Cambridge, UK). This was supplemented with calcium chloride (50 mg/L) for studies with daptomycin.

MIC determinations

MICs were determined by 2-fold serial dilutions of antibacterial agents in MHB (supplemented with calcium for daptomycin) according to the broth microdilution guidelines of the BSAC.[7]

Effects of XF-73 on culture turbidity

Pre-warmed MHB (100 mL) was inoculated with 1 mL of an overnight culture of strain SH1000 and the fresh cultures were grown aerobically with shaking at 37°C until the turbidity reached ~0.2 absorbance units at 600 nm (~10^8 cfu/mL) measured in a 1 cm light path Jenway 6300 spectrophotometer (Jenway, Essex, UK). Cultures were divided into aliquots, some of which received XF-73 at 4× MIC, whilst others served as drug-free controls. Incubation of the cultures continued as before and samples were removed at defined intervals for determination of the culture absorbance at 600 nm.

Effects of antimicrobial agents on bacterial viability

Bactericidal tests were performed on exponential-phase cultures of *S. aureus* SH1000 in MHB (~10^8 cfu/mL), or on bacteria grown to early exponential phase in MHB and then, following centrifugation, resuspended at 10^8 cfu/mL in HEPES buffer (5 mM, pH 7.2) containing glucose (5 mM). Media were supplemented with calcium chloride for studies with daptomycin. Samples from MHB cultures, or buffer suspensions, were serially diluted in PBS and plated onto Mueller–Hinton agar (MHA) at various defined intervals following exposure to XF-73 and control agents at 4× MIC. Colonies were counted after incubation at 37°C for 18–24 h.

Macromolecular synthesis assays

DNA, RNA and protein synthesis were monitored in mid-exponential-phase cultures of *S. aureus* SH1000 in MHB (culture absorbance 0.2 units at 600 nm; ~10^8 cfu/mL) by the incorporation of the radiolabelled precursors [*methyl*-^{3}H]thymidine, [5,6-^{3}H]uridine and L-[G-^{3}H]glutamine into the macromolecular (trichloroacetic acid-precipitable) fraction as previously described.[8] Final concentrations were 1 μCi/mL for each of the precursors, which were added to cultures 10 min before the addition of antibiotics (4× MIC). After a further 10 min incubation at 37°C in the presence of antibiotics, samples were taken for determination of radioactive incorporation into DNA, RNA or protein and the data expressed as a percentage of incorporation into a drug-free control as previously described.[9]

In vitro coupled transcription–translation

Extracts from the nuclease-deficient strain *S. aureus* MW2[10] for coupled transcription–translation were prepared and assayed as previously described.[11]

Fixed timepoint measurements of membrane damage

Measurements of membrane integrity using the BacLight assay were made following a 10 min exposure to XF-73 and comparator antibiotics at 4× MIC as previously described in detail.[9]

Leakage of intracellular components over a time-course

Leakage of K^+ and ATP from cells resuspended in 5 mM HEPES buffer (pH 7.2) supplemented with 5 mM glucose was determined over a 2 h time-course as previously described.[12]

Results

Effect of XF-73 on the growth and survival of S. aureus and comparison with other agents

To perform subsequent microbiological and biochemical studies with XF-73, and to compare this agent with others possessing established modes of action, comparative MICs were determined for the test strain *S. aureus* SH1000 (Table 1). The MIC of XF-73 was 1 mg/L, comparable to that of daptomycin, tetracycline and vancomycin.

Experiments of a biochemical nature with antimicrobial agents are routinely performed at 4× MIC using early exponential-phase cultures (~10^8 bacteria/mL).[9,12] Consequently the microbiological effects of XF-73 at 4× MIC on cultures of *S. aureus* SH1000 were examined in order subsequently to relate these responses to biochemical events within the cell. The use of XF-73 at 4× MIC would also generate data for comparison with published studies on other agents.

The effects of XF-73 and comparator agents at 4× MIC on the survival of *S. aureus* SH1000 were determined over a 60 min time course for liquid cultures grown in MHB (Figure 2). XF-73 exhibited rapid bactericidal activity with substantial loss of viability after only 5 min of drug exposure and virtual sterilization of cultures after 60 min. The bactericidal activity of XF-73 was greater than that observed for all other agents tested (Figure 2). Loss of viability was not accompanied by lysis of bacteria, since culture turbidity did not decline even after 18 h of incubation in the presence of 4× MIC of XF-73 (data not shown).

Table 1. MICs of XF-73 and other agents for *S. aureus* SH1000 and their effects on membrane integrity

Antimicrobial compound	MIC (mg/L)	Percentage membrane integrity (±SD)[a]
No drug	NA	100±0
XF-73	1	0±0
Nisin	2	4.6±2.6
Daptomycin	1	94.7±14.1
CTAB	2	0±0
Valinomycin	2	27.4±8.5
Tetracycline	1	90.9±22.2
Rifampicin	0.008	ND
Ciprofloxacin	2	ND
Vancomycin	1	100±0
Moxifloxacin	2	86.8±4.5

CTAB, cetyltrimethylammonium bromide; NA, not applicable; ND, not determined.
MICs were determined by microdilution in MHB.
Membrane integrity was determined using the BacLight assay following exposure of bacteria to antimicrobial agents at 4× MIC for 10 min.
[a]Values shown are the means of at least three replicate determinations from three independent experiments.

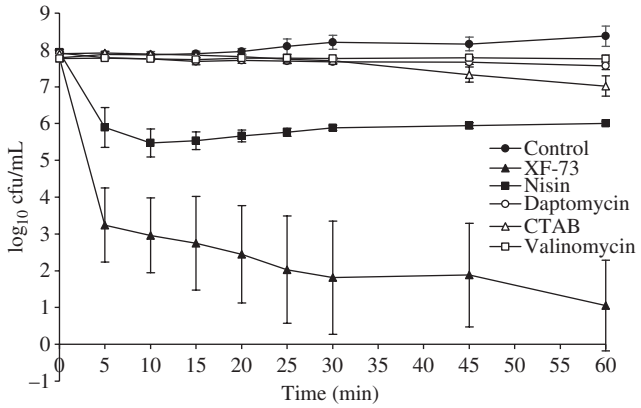

Figure 2. Effects of XF-73 and comparator agents on the survival of *S. aureus* SH1000 in MHB during a 60 min period. At time zero agents were added (4× MIC) to samples of early-logarithmic-phase cultures in MHB. Aliquots were then taken at the times indicated for determination of viable bacteria. The remainder of the cultures served as drug-free controls. Values shown are the means of at least three replicate determinations from three independent experiments.

Effect of XF-73 on macromolecular synthesis in S. aureus SH1000

XF-73 was tested at 4× MIC to evaluate its effect on the incorporation of radioactive precursors into DNA, RNA and protein during a period of short exposure (10 min) to the agent. The effects observed with XF-73 were compared with those exhibited by known, specific, inhibitors of DNA, RNA and protein synthesis (ciprofloxacin, rifampicin and tetracycline, respectively) each at 4× MIC. XF-73 caused substantial inhibition of incorporation of precursors into all three macromolecular synthesis pathways and there was no evidence for preferential inhibition of a single biosynthetic process (Figure 3).

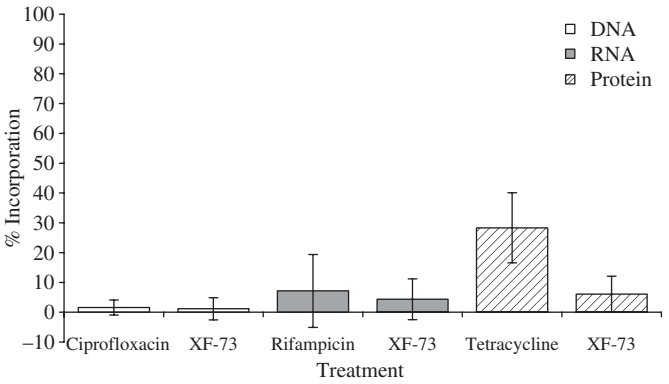

Figure 3. Effect of XF-73 and control agents at 4× MIC on DNA, RNA and protein synthesis in *S. aureus* SH1000 measured by incorporation of radiolabelled precursors. Incorporation is shown as a percentage of that in drug-free controls. Values shown are the means of at least three replicate determinations from three independent experiments.

Effects of XF-73 on an S. aureus in vitro coupled transcription–translation system

XF-73 (0.125–10 mg/L) had no effect on coupled transcription–translation in extracts from *S. aureus* MW2 (data not shown). SH1000 cannot be used for these studies since it contains nuclease activity, which destroys the plasmid template in the assay. However, the MIC of XF-73 for strain MW2 was 1 mg/L, identical to that for strain SH1000.

Evaluation of the bacterial membrane-damaging effects of XF-73 in a fixed timepoint (10 min) BacLight assay

The rapid bactericidal activity of XF-73 accompanied by inhibition of macromolecular synthesis described above may be due to lethal interaction of the drug with the staphylococcal cytoplasmic membrane. We therefore used the fixed time-point BacLight membrane damage assay[12] to examine XF-73 and comparator agents (Table 1).

Tetracycline, which inhibits protein synthesis, and vancomycin, a peptidoglycan synthesis inhibitor, gave negative results in the BacLight assay. Nisin, which is a pore-forming antibiotic causing physical disruption of the membrane bilayer,[12] and the membrane-active quaternary ammonium compound cetyltrimethylammonium bromide (CTAB)[13] substantially reduced membrane integrity. The K^+ ionophore valinomycin,[13] which does not cause physical disruption of the membrane bilayer,[12] gave a strong positive result in the BacLight assay. Daptomycin caused no detectable membrane damage in the BacLight assay and appears to promote later (post-10 min) membrane-damaging effects.[12]

XF-73 compromised membrane integrity within 10 min as measured by the BacLight assay. On this basis it may cause physical damage to the membrane. Leakage of intracellular components (an indicator of physical damage to the membrane) was examined in more detail (see next section).

Effect of XF-73 on the leakage of intracellular components

The effects of XF-73 on the kinetics of membrane disruption were examined in more detail with cells resuspended in HEPES

buffer, supplemented with glucose. This permitted concurrent measurement of the leakage of K^+ and ATP from the cell. The bactericidal activity of XF-73 under these conditions was also determined. Membrane integrity and bactericidal data were also generated for a number of control agents known to disrupt membrane structure or function. Buffer-based experiments are necessary to avoid the high background levels of K^+ and ATP that would otherwise interfere with leakage assays.[12]

The rapid bactericidal activity of XF-73 observed for cultures in MHB was also exhibited for bacteria resuspended in HEPES buffer, although the extent of killing was less for bacteria in the buffered system (see Figures 2 and 4). Nisin and the quaternary ammonium agent CTAB were also rapidly bactericidal under these conditions. However, valinomycin did not cause death and even though the buffer contained calcium for studies with daptomycin, killing by this antibiotic was slower than in MHB (Figure 4).

Cell death mediated by XF-73 was accompanied by extensive loss of intracellular K^+ (Figure 5) and ATP (Figure 6) from the cell. Nisin and CTAB both had rapid and extensive effects on the retention of K^+ and ATP by the staphylococcal cell (Figures 5 and 6). Daptomycin (in the presence of calcium) promoted a broadly similar profile although the effects were delayed compared with those occurring in the presence of nisin, CTAB or XF-73 (Figures 5 and 6). Valinomycin did not cause ATP leakage (Figure 6) and the K^+ leakage assay described here is not appropriate for measuring valinomycin-mediated transfer of the ion across the cytoplasmic membrane since valinomycin promotes influx, rather than efflux, of K^+.[13]

The behaviour of the known membrane-damaging agents in these assays was consistent with published data.[12,13]

Discussion

XF-73 is a promising new candidate for the treatment of staphylococcal infections since it demonstrates excellent antistaphylococcal activity, encompassing methicillin-susceptible *S. aureus*, MRSA and glycopeptide-intermediate *S. aureus*[3] and no resistant mutants are selected after 55 repeat passages at sub-lethal concentrations.[4,5] The potent bactericidal action of XF-73 may also be advantageous compared with the bacteriostatic activity of agents such as linezolid and tigecycline, recently approved for the treatment of certain staphylococcal infections.

The ability of XF-73 to kill *S. aureus* (Figures 2 and 4), and to cause release of K^+ and ATP from the cell with similar kinetics (Figures 5 and 6) suggests that these events are correlated i.e. that the rapid bactericidal effect of XF-73 results from its interaction with the staphylococcal cytoplasmic membrane. Since an intact cytoplasmic membrane is required for many essential processes in bacteria,[13] perturbation of its structure by XF-73 is consistent with a lethal action of the drug. Membrane disruption mediated by XF-73 is also indicated by the results of the BacLight assay (Table 1) and is consistent with the profile of macromolecular synthesis inhibition in whole cells (Figure 3).[9,14] Furthermore, the failure of XF-73 to inhibit transcription and translation *in vitro*, at concentrations up to 10× the whole-cell MIC, suggests that inhibition of macromolecular

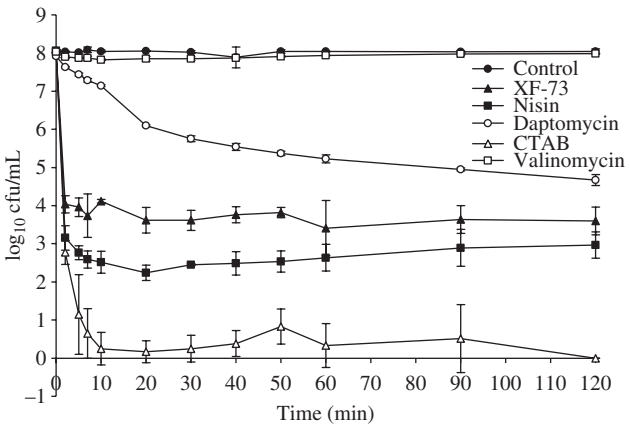

Figure 4. Effect of XF-73 and comparator agents at 4× MIC on survival of *S. aureus* SH1000 resuspended in HEPES buffer supplemented with glucose and in the case of daptomycin also with calcium chloride (50 mg/L). Values shown are the means of at least three replicate determinations from three independent experiments.

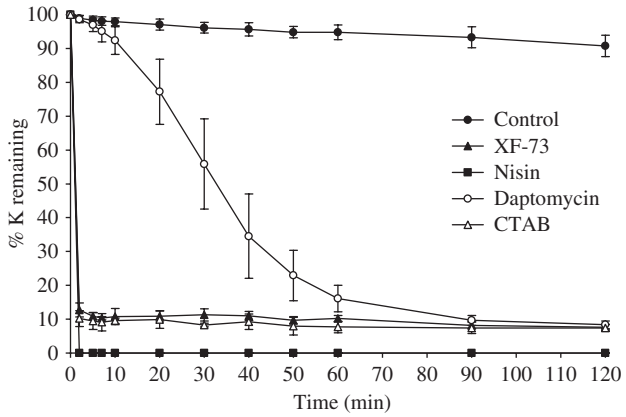

Figure 5. Effect of XF-73 and comparator agents at 4× MIC on retention of K^+ by *S. aureus* SH1000 resuspended in HEPES buffer supplemented with glucose and in the case of daptomycin also with calcium chloride (50 mg/L). Values shown are the means of at least three replicate determinations from three independent experiments.

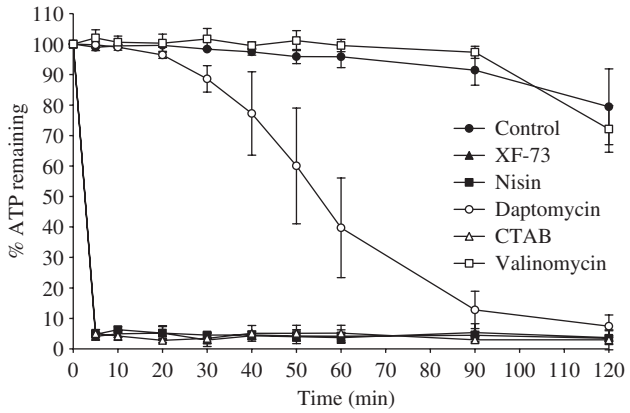

Figure 6. Effect of XF-73 and comparator agents at 4× MIC on retention of ATP by *S. aureus* SH1000 resuspended in HEPES buffer supplemented with glucose and in the case of daptomycin also with calcium chloride (50 mg/L). Values shown are the means of at least three replicate determinations from three independent experiments.

synthesis in whole cells is mediated by effects at the cell membrane and probably does not require entry of XF-73 directly into the cytoplasm.

The precise mechanism of the interaction of XF-73 with the *S. aureus* cytoplasmic membrane bilayer cannot be definitively established from the results reported here. However, the fact that XF-73 promotes leakage of chemically unrelated components (K^+, ATP) suggests that the drug binds to the membrane and causes physical disruption of the bilayer. It is not known why XF-73 achieves greater killing of MHB cultures (Figure 2) compared with cells resuspended in HEPES buffer with glucose (Figure 4), but it is probably related to differences in the physiology of cells in the two environments.

Although XF-73, daptomycin and nisin all appear to target the staphylococcal cytoplasmic membrane they are unlikely to have the same modes of action. None of the inhibitors are structurally related and biological data favour distinct modes of action. Unlike XF-73, daptomycin exhibits a calcium-dependent mode of action.[12] The biochemical profiles obtained with daptomycin in the BacLight and membrane leakage assays also differ from those obtained with XF-73. The biochemical profiles obtained with XF-73 and nisin are similar, namely positive responses in the BacLight assay and rapid leakage of potassium and ATP from the cell. However, a mutant of *S. aureus* SH1000 has recently been selected in our laboratories displaying a 64-fold decrease in susceptibility to nisin, but without altered susceptibility to XF-73 (A. J. O'Neill, K. Marriner and I. Chopra, unpublished data). Although the mechanism of resistance to nisin in this mutant is unknown, the results imply separate targets for nisin and XF-73.

The data discussed above suggest a lethal interaction of XF-73 with the cytoplasmic membrane. However, death is not accompanied by a decline in culture absorbance. Consequently, killing of *S. aureus* by XF-73 does not result from lysis of the cells. Although the precise molecular basis of interaction of XF-73 with the staphylococcal membrane is currently unknown, membrane targeting may explain why it has not been possible to generate XF-73-resistant mutants of *S. aureus* during prolonged laboratory subculture in its presence.[4,5] If the lethal effect of XF-73 results from insertion into the membrane bilayer, then only major changes in membrane composition and hence structure might confer resistance. Such changes could be difficult to achieve because multiple mutations would be required. Furthermore, even if such mutations arose, the alterations in membrane structure might well be incompatible with cellular survival.

Exploiting the bacterial cytoplasmic membrane as a drug target has been difficult due to functional and structural similarities between prokaryotic and mammalian cell membranes. Nevertheless, the systemically administered antibiotic daptomycin appears to exhibit selective targeting of the cytoplasmic membrane in Gram-positive bacteria. Currently XF-73 is being evaluated as a topical antibacterial agent to prevent and treat infections caused by Gram-positive bacteria, including MRSA.

In conclusion the results reported here show that XF-73 exhibits potent, non-lytic, bactericidal activity against *S. aureus*. A similar mode of action probably explains its antimicrobial activity against a wide range of other Gram-positive species.[3] Consequently, XF-73 is a promising candidate that is already in clinical development as a novel drug.

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Transparency declarations

I. C. is a member of the scientific advisory board of Destiny Pharma Ltd. W. R.-W. and W. L. are employees of Destiny Pharma Ltd. All other authors: none to declare.

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