Investigation of the Potential for Mutational Resistance to XF-73, Retapamulin, Mupirocin, Fusidic Acid, Daptomycin, and Vancomycin in Methicillin-Resistant *Staphylococcus aureus* Isolates during a 55-Passage Study[▽]

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XF-73 is a dicationic porphyrin drug with rapid Gram-positive antibacterial activity currently undergoing clinical trials for the nasal decolonization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus* (MRSA). In multistep (55-passage) resistance selection studies in the presence of subinhibitory concentrations of XF-73, retapamulin, mupirocin, fusidic acid, and vancomycin against four Network on Antimicrobial Resistance in *Staphylococcus aureus* MRSA strains, there was no >4-fold increase in the MIC for XF-73 after 55 passages. In contrast, there was an increase in the MICs for retapamulin (from 0.25 μg/ml to 4 to 8 μg/ml), for mupirocin (from 0.12 μg/ml to 16 to 512 μg/ml), for fusidic acid (from 0.12 μg/ml to 256 μg/ml), and for vancomycin (from 1 μg/ml to 8 μg/ml in two of the four strains tested). Further investigations using *S. aureus* NRS384 (USA300) and daptomycin demonstrated a 64-fold increase in the MIC after 55 passages (from 0.5 μg/ml to 32 μg/ml) with a >4-fold increase in the MIC obtained after only five passages. Sequencing analysis of selected isolates confirmed previously reported point mutations associated with daptomycin resistance. No cross-resistance to XF-73 was observed with the daptomycin-resistant strains, suggesting that whereas the two drugs act on the bacterial cell membrane, their specific site of action differs. XF-73 thus represents the first in a new class of antibacterial drugs, which (unlike the comparator antibiotics) after 55 passages exhibited a ≤4-fold increase in MIC against the strains tested. Antibacterial drugs with a low propensity for inducing bacterial resistance are much needed for the prevention and treatment of multidrug-resistant bacteria both within and outside the hospital setting.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is increasingly prevalent throughout the world and is endemic in some hospital and community settings (5, 15, 25). Community-associated MRSA (CA-MRSA) is also becoming more widespread; in the United States, 59% of skin and soft tissue infections presenting to emergency departments are caused by CA-MRSA (25). The most prevalent CA-MRSA clone in the United States, USA300, which carries the staphylococcal cassette chromosome (SCC) *mec* type IVa and Panton-Valentine leukocidin genes, now accounts for 61 to 87% of MRSA isolates from skin and soft tissue infections in North American emergency departments (1, 19) and is also emerging as a serious cause of health care-associated infections (14, 22, 29). There is therefore a critical need for new antibacterials, particularly those with no cross-resistance to currently available antibacterial drugs and with little or no likelihood of inducing resistance.

XF-73 is a dicationic porphyrin drug (Fig. 1), and previous studies have shown that XF-73 has potent Gram-positive *in vitro* bactericidal activity against a range of clinically important

S. aureus isolates, including methicillin-sensitive *S. aureus* (MSSA), health care-associated MRSA (HA-MRSA), and CA-MRSA (minimal bactericidal concentration at which 90% of strains tested are killed [MBC$_{90}$], 0.5 μg/ml) (10). XF-73 has rapid activity, with a >4-log reduction in CFU obtained after 5 min incubation in growth medium (27).

Investigations into the mechanism of action of XF-73 against *S. aureus* demonstrated that the drug exhibits a rapid bacterial cell membrane-perturbing activity, which is likely to be responsible for the inhibition of macromolecular synthesis and the death of staphylococci exposed to the drug (26, 27). The presence of known resistance mechanisms was found not to have any effect on the antibacterial activity of XF-73 (10), suggesting that the mechanism of action of XF-73 is novel. XF-73 has also been shown to be equally active against nongrowing *S. aureus* and against *S. aureus* within biofilms (28). XF-73 is being developed as a topical drug to prevent and treat *S. aureus*, and MRSA infections and clinical trials are under way to assess the potential for the nasal decolonization of *S. aureus*.

The purpose of this study was to investigate the propensity of XF-73 and selected comparators to select for endogenous mutational resistance during prolonged passages at subinhibitory concentrations. The topical antibiotics mupirocin, fusidic acid, and retapamulin were selected as direct comparators to XF-73, and vancomycin, which has been previously demonstrated to be refractive to the emergence of mutational resistance in multipassage studies (3, 6, 7, 20, 21), was also in-

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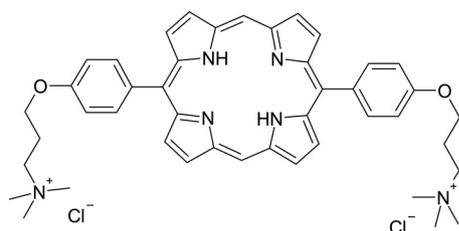
FIG. 1. Chemical structure of XF-73.

cluded. As the mechanism of action (MOA) of daptomycin (16, 30) is also proposed to act on the bacterial membrane, it was also tested against USA300, and molecular genetic studies to characterize the mechanism(s) of daptomycin resistance in isolated clones were also undertaken. Phenotypic stability of any resistant isolates obtained during the studies involving USA300 and cross-resistance with the other test compounds were also investigated.

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MATERIALS AND METHODS

Bacteria. Four Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) strains of MRSA, comprising prevalent and endemic HA- and CA-MRSA clones, were selected for serial passage (Table 1).

Antimicrobial agents and MIC testing. XF-73 and retapamulin extract were provided by Destiny Pharma Ltd., Brighton, United Kingdom. Vancomycin and fusidic acid were obtained from Sigma, mupirocin was obtained from Mast, France, and daptomycin was obtained from Cubist Pharmaceuticals Inc., Cambridge, MA 01239.

MICs were determined by the broth microdilution method according to approved M7-A7E standards of the CLSI (8), except that Iso-Sensitest broth was used as the test medium. For daptomycin susceptibility testing, the medium was supplemented with calcium to a final concentration of 50 μg/ml.

Multipassage resistance selection studies. Multipassage resistance selection studies using XF-73, mupirocin, fusidic acid, retapamulin, and vancomycin were performed with all four NARSA isolates listed in Table 1, while daptomycin was tested against *S. aureus* NRS384 (USA300) only. A dilution series of 10 concentrations was prepared for each test compound based on the previous MIC at 2× the required final concentration. Initial inocula (1×10^8 to 2×10^8 CFU/ml) were prepared in Iso-Sensitest broth from overnight agar cultures and diluted 100× in fresh broth, and 0.5 ml was added to 0.5 ml of each antimicrobial dilution series and the control bijou bottle (no antimicrobial). The bijou bottles were incubated at 35°C for 24 h, and the cultures were passaged daily for 55 days by using 0.5-ml inocula from the bijou bottle corresponding to 0.5× the MIC determined from the previous passage to inoculate a fresh series of drug dilutions and control bijou bottles. All drugs were passaged for the entire 55 passages, irrespective of the MIC. Isolates were saved at intervals throughout the

passage experiment, and resistant mutants emerging during the course of the study were also saved and stored frozen at −70°C.

After 55 passages, the stability of acquired resistance in the *S. aureus* NRS384 (USA300) passage experiment was investigated by MIC determinations after 10 successive passages on drug-free agar for cultures isolated from four different passage numbers. The cross-resistance of the isolates derived from USA300 was evaluated by determining the MIC against the other test compounds and comparing them to the baseline (passage 0).

In this study, resistance was defined as a >4-fold increase in the initial MIC (4, 7, 21).

Molecular characterization of daptomycin-resistant clones. Three isolates with reduced susceptibility for daptomycin, identified during the daptomycin serial passage, were selected for genetic characterization to determine the mechanism of resistance. DNA from the three isolates, wild-type USA300, and the control strain MW2 was extracted and amplified by PCR. Nine target DNA sequences, previously identified as sites where mutations resulted in elevated MICs for daptomycin, were investigated (11).

Genomic DNA purification. DNA was extracted from the isolates using the DNA template preparation kit (Roche) according to the manufacturer's instructions. An additional cell lysis solution (250 U lysozyme/ml, 25 U lysostaphin, 10 mM EDTA, 10 mM Tris [pH 8.0]) was used to facilitate cell lysis.

PCR amplification and DNA sequencing. PCR and sequencing primers were as used in a previous investigation into the genetic changes correlating with reduced susceptibility by daptomycin by *S. aureus* (11) with the exception of two additional primer sequences for the intergenic region upstream from *rspU* (1654504C-T AltF [GTTTTAACGGCTTCACAA], located at gene position 1654708–1654725, and 1654504C-T AltR [GAGGGAGTCAACAGAGGTA], located at gene position 1654291–1654309). PCR amplification and sequencing were undertaken by Quotient Bioresearch Ltd. as a service for a fee.

RESULTS

For XF-73, compared with the initial MIC values, there was a 2-fold increase for three strains, and a 4-fold increase for the fourth, in the MIC after 55 passages (Table 2). During the course of the passage experiment, MIC values for XF-73 showed no more than a 4-fold increase from the starting MIC value, indicating that no resistance (defined as a >4-fold increase in MIC [4, 7, 21]) developed during the 55 passages (Table 2).

For mupirocin, fusidic acid, and daptomycin, a >4-fold increase in the original MIC was observed for all four strains tested within seven repeat passages (Table 2). For vancomycin, a >4-fold increase was observed for two of the four strains tested (Table 2).

For retapamulin, the initial MIC of the extracted sample corresponded well with previously reported MIC values (0.25 μg/ml compared to an MIC_{90} of 0.12 μg/ml [17, 32]), confirming the validity of the sample. A >4-fold increase over the initial MIC was observed after 3 to 16 repeat passages (Table 2) for all four strains tested.

Daptomycin showed a steady increase in MIC during the course of the passage experiment in *S. aureus* NRS384 (USA300), with a final MIC of 32 μg/ml obtained (Table 2).

TABLE 1. MRSA isolates used for resistance testing

MRSA strain	Alternative name	Description	Source
NRS123	USA400	Multiply susceptible CA-MRSA carrying Panton-Valentine leukocidin genes and additional virulence genes distinct from those of HA-MRSA strains	NARSA
NRS382	USA100	Predominant US HA-MRSA strain, also known as the New York/Japan clone	NARSA
NRS384	USA300	CA-MRSA carrying Panton-Valentine leukocidin genes—now one of the most common isolates responsible for MRSA infections both in the community and, increasingly, in health care settings	NARSA
NRS387	USA800	Also known as the "pediatric clone," commonly isolated from children	NARSA

TABLE 2. Multipassage resistance selection results

Strain	Antibacterial	Initial MIC (μg/ml)	Passage at which resistance[a] emerges	Final MIC (μg/ml) at passage 55
S. aureus NRS123 (USA400; MRSA)	XF-73	0.5	No resistance	1
	Vancomycin	1	No resistance	4
	Retapamulin	0.25	3	8
	Fusidic acid	0.12	5	256
	Mupirocin	0.12	7	32
S. aureus NRS382 (USA100; MRSA)	XF-73	0.25	No resistance	1
	Vancomycin	1	32 (35[b])	8
	Retapamulin	0.25	9 (13[b])	8
	Fusidic acid	0.12	2	256
	Mupirocin	0.12	2	16
S. aureus NRS384 (USA300; MRSA)	XF-73	0.25	No resistance	0.5
	Vancomycin	1	20 (46[b])	8
	Daptomycin	0.5	5	32
	Retapamulin	0.25	16 (19[b])	4
	Fusidic acid	0.12	2	256
	Mupirocin	0.12	2	512
S. aureus NRS387 (USA800; MRSA)	XF-73	0.25	No resistance	0.5
	Vancomycin	1	No resistance	4
	Retapamulin	0.25	15	8
	Fusidic acid	0.12	1	256
	Mupirocin	0.12	2	256

[a] Resistance is defined as >4-fold increase in original MIC (4, 7, 21).
[b] Passage number at which three successive elevated MICs were first obtained.

Representative resistance development profiles for XF-73 and comparators versus *S. aureus* NRS384 (USA300) are presented in Fig. 2.

Stability of acquired resistance. The elevated MICs for mupirocin, fusidic acid, and daptomycin after 5, 24 (25 for fusidic acid), and 55 passages were found to be stable (defined as having an MIC after 10 drug-free passages within one dilution of the MIC before the drug-free passages [7, 21]).

Cross-resistance of acquired mutations. The cross-resistance profiles of XF-73-, mupirocin-, fusidic acid-, or daptomycin-incubated *S. aureus* NRS384 (USA300) isolates from passages 0, 5, 24
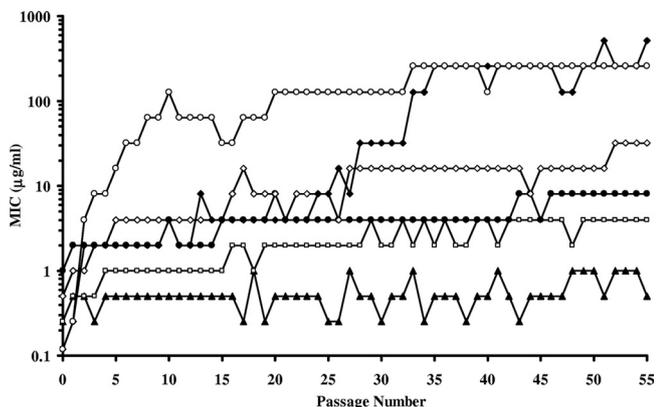

FIG. 2. Plot of MICs (μg/ml) of XF-73, mupirocin, fusidic acid, retapamulin, daptomycin, and vancomycin for *S. aureus* NRS384 (USA300) during 55 serial passages at 0.5 MIC (determined by the previous passage): ▲, XF-73; □, retapamulin; ●, vancomycin; ◇, daptomycin; ◆, mupirocin; ○, fusidic acid.

(25 for fusidic acid), and 55 were determined against a range of antimicrobial comparators: XF-73, mupirocin, fusidic acid, daptomycin, and two glycopeptide antibiotics, vancomycin and teicoplanin, after 10 passages in nonselective conditions on drug-free agar.

There was no evidence of cross-resistance with XF-73 in any of the resistant isolates tested. With one exception, none of the resistant isolates showed any cross-resistance with the other antibiotic comparators tested. Daptomycin-resistant isolates from passage 55 showed a >16-fold increase in teicoplanin MIC (passage 0 MIC, 0.25 μg/ml; passage 55 MIC, 4 μg/ml).

Genetic characterization of isolates of *S. aureus* NRS384 (USA300) with elevated daptomycin MICs. DNA sequences obtained from selected isolates of *S. aureus* NRS384 (USA300) obtained during the daptomycin 55-passage study with increasing daptomycin MIC values were compared to those of the parent strain [*S. aureus* NRS384 (USA300), no serial passages] for each of the targets selected (11). DNA sequences obtained from MW2 were compared to the published complete genome of MW2 (NCBI accession number BA000033) as a sequencing control.

S. aureus NRS384 (USA300) at passage 5, with a raised daptomycin MIC of 2 μg/ml (8-fold increase in initial MIC), was found to have an amino acid change at location A302V in the *mprF* gene. By passage 24, the daptomycin MIC had increased to 8 μg/ml (16-fold increase in initial MIC) and a further amino acid change in the *mprF* gene at location N352K and in the *yycG* gene at location L331S was detected. At passage number 55, with a daptomycin MIC of 32 μg/ml (64-fold increase in original MIC), no further amino acid changes were observed in the targets sequenced in this investigation.

DISCUSSION

No increase of the XF-73 MIC >4-fold was observed for any of the four MRSA strains tested in this study after 55 successive passages at subinhibitory concentrations. In contrast, there was a significant increase in the MIC for three of the comparators against all the strains tested and two of four strains for vancomycin using the same conditions. XF-73 has a very rapid bactericidal activity (26, 27) and acts on both dividing and nongrowing bacterial cells (28), and investigations suggest that it has a novel MOA (26, 27). The results reported in this study confirm that such an antibacterial profile is likely to reduce the potential for the emergence of bacterial mutational resistance.

The results obtained with mupirocin and fusidic acid in this study are in line with results reported in previous passage studies (3, 20). Mupirocin is widely used for the elimination of *S. aureus* nasal carriage and the prophylactic intranasal eradication of MRSA (23, 33, 35). However, this has led to increased resistance rates and calls to limit its use to outbreak situations only, rather than for routine decolonization of *S. aureus* (18, 24, 34). Recent estimates suggest that mupirocin resistance rates in HA-MRSA are in the order of 15 to 18% (9, 31, 36). Of particular concern is the recent finding that high-level mupirocin-resistant MRSA strains are more likely to be associated with CA-MRSA, tend to occur more commonly in MRSA-colonized patients, and are more likely to have coresistance to fusidic acid (31). A recent demonstration (2) that nasal decolonization of MSSA using mupirocin of elective surgical hospital patients can have a significant positive impact on MSSA infection rates is encouraging. However, the use of mupirocin as a prophylactic for decolonization of the much larger numbers of patients carrying nasal MSSA (30 to 40%) might be considered a strategy which may drive mupirocin resistance rates even higher and to the point of impotence.

Retapamulin is a new topical antibiotic approved in 2007, but increases in retapamulin MICs in *in vitro* passage studies were reported in 2006 (20): three strains were chosen for prolonged passage to the maximum 50 passages, at which point the MICs for retapamulin were found to be 4 to 16 µg/ml, a 67- to 533-fold increase over the initial MIC. Subsequent studies have suggested that there may be a fitness cost associated with mutations that result in a reduction of activity of retapamulin (12), but clinical isolates with MICs of ≥2 µg/ml for retapamulin have already been reported (13).

Vancomycin has been used as a comparator in a significant number of multipassage studies (3, 4, 6, 7, 20, 21), and the emergence of resistance has been reported in only one of these (3). In our study, the initial MIC for vancomycin increased >4-fold in two of the four strains tested. In comparison, using the same strains, no such increase in the MIC of XF-73 was observed.

As the proposed antibacterial MOA for daptomycin (16, 30) is similar to that proposed for XF-73 (26, 27), both of which appear to target the bacterial membrane, resulting in membrane depolarization and loss of intracellular components, it was also included in this study. *In vitro* reduced susceptibility to daptomycin in *S. aureus* NRS384 (USA300) emerged after only five repeat passages and remained stable after 10 serial passages in drug-free conditions. This result is in accordance with results reported in previous passage studies (3, 4, 21).

The genetic characterization of the mutations that had occurred which resulted in a reduced susceptibility to daptomycin confirmed that the initial mutations were located in previously identified sites in the genome (11). The mutations which were responsible for the elevation of the daptomycin MIC from 8 µg/ml to 32 µg/ml were not located in any of the nine locations investigated in this study, suggesting that mutations can occur at other sites. The final MIC obtained in our study (32 µg/ml) was higher than that reported in the source reference (11), and thus it is plausible that the mutations responsible were not identified in the previous study.

No effect on the antibacterial activity of XF-73 was observed with any of the daptomycin isolates tested from different passage numbers, suggesting that while XF-73 and daptomycin may both act on the bacterial cell membrane, XF-73 does not appear to share any common target sites with daptomycin.

The results of this multistep passage resistance study clearly demonstrate that XF-73 has potential significant advantages over existing topical antibacterial compounds with regards to the emergence of mutational resistance at subinhibitory concentrations. No >4-fold increase in MICs for XF-73 was observed after 55 successive passages at subinhibitory concentrations for four NARSA strains. XF-73 may potentially provide a significant addition to the antibacterial armamentarium for topical bacterial infection prophylaxis and treatment. XF-73 is currently undergoing clinical trials for the nasal decolonization of *S. aureus*, including MRSA.

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