

1 Assessing the potential for *Staphylococcus aureus* to evolve
2 resistance to XF-73

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Antibiotic resistance in pathogenic bacteria has emerged as a fundamental threat to human health by increasing the mortality rates and economic costs associated with bacterial infections. The commensal pathogen *Staphylococcus aureus* provides a clear illustration of the AMR crisis. The clinical use of antibiotics has driven the global spread of ‘waves’ of resistant strains, methicillin-resistant *S.aureus* (MRSA) are now a global problem in healthcare settings, and a growing problem in the community[1, 2]. Approximately 1/3 of people show asymptomatic nasal carriage of *S.aureus*, and nasal carriage is associated with an elevated risk of developing invasive *S.aureus* infections. One strategy to combat *S.aureus* is to decolonize individuals who are carriers, thereby reducing the risk of future infection. For example, the topical antibiotic mupirocin has been widely used for nasal decolonization of *S.aureus*. However, the use of mupirocin has driven the spread of mupirocin resistance, highlighting the need to develop alternative decolonization agents[1]. XF-73 (exeporfinium chloride) is a novel, di-cationic porphyrin drug with high *in vitro* potency[3] that is being developed as a potential product for use in *S.aureus* decolonization.

Antibiotic use generates natural selection for resistant mutants that are able to grow in the presence of antibiotic, and the spread of resistance in bacterial pathogens is a simple and elegant example of Darwinian evolution. The rate at which pathogens evolve resistance to antibiotics is therefore an important factor contributing to the long-term utility of antibiotics. Given this, there is growing recognition that the risk of evolution to new antibacterial agents such as XF-73, should be assessed as part of pre-clinical drug development[4, 5].

Assessing the risk for S.aureus to evolve mutational resistance to XF-73

The simplest possible mechanism for bacteria to evolve resistance to antibiotics is by acquiring chromosomal mutations that confer resistance, usually by modifying antibiotic targets. Serial passage experiments evaluate the potential for bacteria to evolve mutational resistance by exposing bacterial strains to a range of antibiotic concentrations and passaging the culture of the strain that shows growth at the highest antibiotic concentration into fresh media containing the same range of antibiotic concentrations. If resistant mutants are present in the culture of cells that are passaged, the strain will be able to grow at higher concentrations of antibiotic. By serially passaging strains over multiple cycles (typically 20-50), these experiments provide a powerful tool to study the step-wise evolution of resistance (Figure 1). Farrell *et al*[6] passaged cultures of 4 MRSA strains in the presence of XF-73 and set of comparator antibiotics for 55 serial passages (Table 1). Resistance to the comparator antibiotics evolved rapidly and repeatedly apart for vancomycin where MRSA resistance emergence appeared later. These results are in line with previous studies [7, 8], and highlight the incredible ability of *S.aureus* to evolve mutational resistance to antibiotics. In contrast, no significant increases in resistance to XF-73 were observed in any of the MRSA strains.

Does this striking result mean that XF-73 is an ‘evolution-proof’ antibacterial drug? One way to explore this question further is to calculate the number of resistance mutations that were effectively screened in this passage experiment. Farrell *et al* passaged 5×10^5 cells into 6 or more lethal concentrations of XF-73 for 55 passages in 4 strains, and the total number of cells that were subject to XF-73 resistance selection was therefore: 5×10^5 cells/passage/strain*55 passages*6 tests/passage*4 strains = 6.6×10^8 . If we assume that mutations occur at random, the Poisson distribution implies that the number of cells screened for resistance must be 7 times higher than the mutant frequency to ensure a 99.9% probability of a least one mutant occurring during an experiment. According to this logic, any mutations that are present at a frequency greater than $(1 \text{ mutant}/6.6 \times 10^8 \text{ cells})/7 = 1 \times 10^{-8}$ should have theoretically been tested for resistance to XF-73 during the passage experiment. Quantitative culturing studies have estimated that the average number of *S.aureus* cells in the nares of MRSA carriers varies between 100[9] and 20,000[10] cells. Given these titres, we can roughly estimate that XF-73 resistant mutants should be found in between $1/10^6$ and $2/10^4$ carriers. Note, however, that these calculations assume that the mutation rate is the same *in vitro* and *in vivo*.

Assessing the potential for resistance to evolve by horizontal gene transfer (HGT)

Many of the most clinically important resistance genes in pathogenic bacteria, including *S.aureus*, have been acquired by horizontal gene transfer (HGT). The conventional approach to estimate the potential for pathogenic bacteria to acquire resistance genes by HGT is to transform the target bacterium with a library of expression plasmids containing DNA that has been cloned from microbial communities in bulk, and this has been applied very successfully to *E.coli*[4, 5]. However, this method relies on the ability of the target pathogen to be transformed with very high efficiency, and functional metagenomics has yet to be applied to Gram positive bacteria.

Recent bioinformatic studies have identified putative donor taxa for many of the acquired resistance genes that are found in *S.aureus*[11]. One potential approach to assess the possibility of *S.aureus* to acquire resistance to XF-73 by horizontal gene transfer is to screen these putative resistance gene donors for phenotypic resistance to XF-73. This approach does not assume that currently characterized resistance genes will confer cross-resistance to XF-73; rather, it assumes species with a history of transferring resistance genes to *S.aureus* may be important sources of mobile XF-73 resistance genes. Importantly, some of the most likely sources of XF-73 resistance genes have been shown to be sensitive to XF-73, including *S.epidermidis*, *S.haemolyticus*, and *S.saprophyticus*, but it would clearly be desirable to extend XF-73 susceptibility testing to other coagulase-negative staphylococci[11]. Whilst this provides a reasonable approach to test for resistance that may potentially be transferred among closely related bacteria, it is clearly not practical to apply this approach to more distantly related groups that are likely to have transferred resistance genes to *S.aureus* in the past, such as *Bacilliales* and *Clostridiales*[11].

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94 *Conclusions and outlook*

95 In summary, the available evidence suggests that *S.aureus* has low potential to evolve
96 resistance to XF-73 relative to antibiotics. However, it is important to bear in mind that there
97 are multiple routes to resistance evolution (HGT) that have yet to be investigated, and further
98 experimental work would allow for a more refined analysis of the potential for *S.aureus*
99 resistance to XF-73 to evolve. For example, passage experiments using hyper-mutator strains
100 would provide more power to detect very low frequency ($<10^{-8}$) XF-73 resistance mutations.
101 It is also important to emphasize that that therapeutic benefit of a drug comes from its ability
102 to function successfully *in vivo*; it is noteworthy that XF-73 has already demonstrated clinical
103 anti-Staphylococcal efficacy by the US National Institutes for Allergy and Infectious Diseases
104 [12].

105 *Acknowledgements*

106 The author is supported by Wellcome Trust Grant 106918/Z/15/Z and this project was
107 supported by funding from Destiny Pharma plc.

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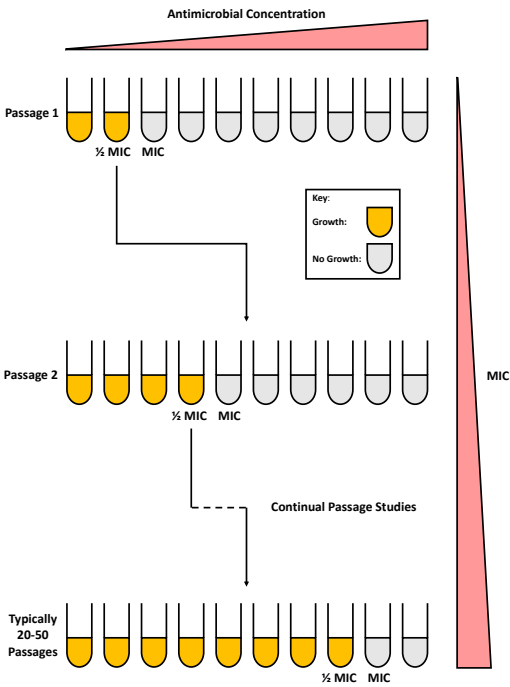


Figure 1: Serial Passage Experiments. This figure shows a schematic of the design of serial passage experiments, as described in the text.

Table 1: Summary of XF-73 and comparator serial passage experiments:

Drug	Number of strains that evolved resistance during passaging	Median passage of resistance emergence	Median fold increase in resistance (MIC) during passaging
XF-73	0/4	None	None
Vancomycin	2/4	>43.5	6
Retapamulin	4/4	12	32
Fusidic acid	4/4	2	2048
Mupirocin	4/4	2	1200

This table summarizes serial passage data from reference [6] showing that mutational resistance to XF-73 does not evolve in serial passage experiments.