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Caspofungin (CAS), Colistin (CST), and Polymyxin B (PMB) Enhance Moxifloxacin (MXF) Activity against *Staphylococcus aureus* Biofilms by Disturbing Matrix exopolysaccharides

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ABSTRACT

Objective: Biofilms are poorly responsive to anti-staphylococcal antibiotics. Our aim was to examine whether combining MXF with amphiphilic drugs without antistaphylococcal activity such as CST, PMB or CAS, could improve its activity on biofilms from reference strains and clinical strains from epidemic clones (acute infections).

Methods: Biofilms were grown for 24 h in 96-wells plates in Trypticase Soy Broth (TSB) supplemented with 2% NaCl and 1% glucose. 24h-old (mature) biofilms were exposed for 48 h to a broad range of MXF conc. (to obtain full conc.-response curves), combined with CAS, CST, or PMB at 2 X MIC. Viability in biofilms was quantified using the redox indicator resazurin (1). MXF penetration in biofilms was assessed by confocal microscopy (λ_{exc} 400 nm / λ_{em} 520 nm). Exopolysaccharides (EPS) were extracted and assayed as described by Kolodkin-Gal *et al* (2) and Albalasmeh *et al.* (3) EPS macromolecular size was measured by dynamic light scattering.

Results: CAS, CST, and PMB had MICs of 40, 50, and 50 mg/L, respectively and did not show any activity by themselves against biofilms at 2x MIC. MXF alone was poorly active (50 % reduction in viability reached only for ATCC33591 and 1 clinical isolate at conc. ≤ 20mg/L). Combination with CAS, CST or PMB allowed reaching 50 % reduction for 6/7, 3/7 and 4/7 clinical strains respectively. CAS, CST or PMB increased up to 30-fold MXF penetration, reduced approx. 3-fold EPS content, and decreased at least 300-fold EPS size for biofilms formed by strain 2005/179 (high responder) but not by 2003/651 (poor responder).

Conclusion: Amphiphilic molecules like CAS, PMB or CST reduce EPS content and size in biofilms, which may contribute to enhance MXF penetration and activity. Combination with amphiphiles appears thus as a promising strategy to act upon *S. aureus* biofilms, warranting the search of more potent molecules.

INTRODUCTION

- The ability of *S. aureus to* produce biofilms is considered as a main reason for persistence or recurrence of infections. Biofilm formation is a multi-step process, which involves bacterial attachment to a support, formation of complex aggregates of adhering microorganisms, and production of a matrix rich in exopolysaccharides (EPS).
- Within biofilms, bacteria are poorly responsive to antibiotics. We showed that biofilms formed by clinical isolates of *S. aureus* are particularly reluctant to antibiotic action [1]. Now therapeutic strategies are therefore needed to try improving antibiotic activity against bthese specific forms of infection.
- Antiseptic agents are in general much more active against biofilms, possibly due to their amphipathic character. Yet they cannot be used for deep tissues infections because of their intrinsic toxicity.

OBJECTIVE

- The aim of our study was to examine whether combining an antistaphylococcal antibiotic with amphiphilic drugs showing no intrinsic activity on staphylococci, like the anti-gram-negative polymyxin B (PMB) and colistin (CST) or the antifungal caspofungin (CAS), may improve activity on preformed biofilms.
- To this effect, we used moxifloxacin as an exemplative antibiotic and biofilms formed by 7 clinical isolates (epidemic clones isolated from various body sites) + a reference strain [1].
- Data were correlated with changes in moxifloxacin penetration within biofilms and in biofilm exopolysaccharides (EPS) content and size.

RESULTS

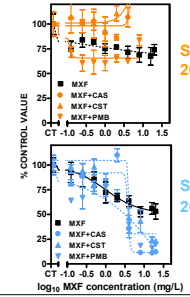
MICs in broth and relative potency of moxifloxacin alone or in combination with CAS, PMB, CST against mature biofilms

strains[a]	MXF MIC (mg/L)	moxifloxacin concentrations (mg/L) needed to reach 50 % reduction of viability in biofilms[b]			
		MXF	MXF + CAS[c]	MXF + PMB[c]	MXF + CST[c]
ATCC33591 (MRSA)	0.032	4	0.1	0.125	0.125
Surv2003/1083 (MSSA)	0.125	>20[d]	18	>20	18.6
2011S027 (MSSA)	0.125	1	0.68	0.8	0.6
2009S025 (MRSA)	0.125	>20	2	3.6	4
Surv 2003/651 (MRSA)	4	>20	>20	>20	>20
Surv2005/104 (MRSA)	4	>20	18	>20	>20
Surv2005/179 (MRSA)	2	>20	3.7	3.7	4
2009S028 (MRSA)	2	>20	4	>20	>20

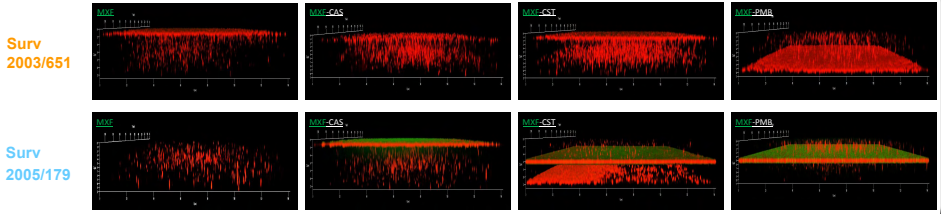
[a] all clinical isolates belong to the epidemic CC5 or CC8 clonal complexes
[b] calculated using the Hill equation of conc.-response curves (see examples in the figures)
[c] used at 2x their respective MIC
[d] effect not reached at 20 mg/L

CAS, CST, PMB were synergistic with moxifloxacin for most of the strains; no synergy was observed for 2003/651 strain.

Concentration-response curves: viability (% control values) in biofilms exposed to moxifloxacin alone or in combination for 2 exemplative clinical isolates

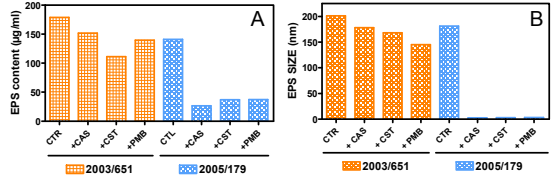


Influence of CAS, CST, PMB on moxifloxacin penetration within biofilms of strain 2005/179 or 2003/651.



CAS, CST, PMB increase moxifloxacin penetration in biofilms from 2005/179 but not from 2003/651

Influence of CAS, CST, PMB on EPS content (A) and EPS size (B) in biofilms of strains 2005/179 or 2003/651



CAS, CST, PMB reduce exopolysaccharides (EPS) content and size in 2005/179 biofilm but not in 2003/651 strain

METHODS

- Biofilms were cultivated in polystyrene 96-well plates in TSB; 2% NaCl; 1% glucose at 37°C for 24h with an initial inoculum adjusted to an OD_{620nm} of 0.005. Biofilms were exposed to increasing concentrations (0.125-20mg/L) of moxifloxacin alone or with combination with CAS (80 mg/L), CST (100mg/L), PMB (100mg/L) during 48h. Biofilm mass was evaluated by measuring the OD of crystal violet and viability of bacteria, using the redox indicator resazurin (reduced to fluorescent resorufin by viable bacteria) [1].
- Moxifloxacin penetration within biofilms was evaluated taking advantage of its intrinsic fluorescence ($\lambda_{exc\ 400nm}$ / $\lambda_{em\ 520nm}$). 24 h biofilms-overgrown cover slips were incubated for 1h with 20 mg/L moxifloxacin and thereafter stained with 0.5 mM CTC Kit [1] ($\lambda_{exc\ 488nm}$ / $\lambda_{em\ 620nm}$) for 30 min in the dark. After washing, biofilms were imaged using confocal microscopy.
- Expolysaccharides were purified as described [2] and quantified by a method described to measure carbohydrates [4], using glucan as a standard. The diameter of exopolysaccharide supramolecular particles was measured by dynamic light scattering.

CONCLUSION

- **Caspofungin, colistin and polymyxin B, can reduce EPS content and size in *S. aureus* biofilms, which enhances moxifloxacin diffusibility and activity in these biofilms.**
- **The reasons why some clinical isolates remain poorly susceptibible to these effects need to be further investigated.**

References
[1] Siala *et al.* 2014. Antimicrob. Ag. Chemother. Epub ahead of print; PMID: 25114142
[2] Kolodkin-Gal *et al.* 2012. Cell. 149:684-92
[3] Albalasmeh *et al.* 2013. Carbohydrate Polymers 97, 253.

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