Revealing moxifloxacin activity against biofilms of *S. aureus* isolated from persistent infections by means of polycationic and amphiphilic substances.

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Abstract

Objective: Moxifloxacin has low activity against *S. aureus* biofilms [1]. Positively-charged hydrophilic molecules and cationic detergents prevent *S. aureus* biofilm formation [2; 3]. We have examined whether these 2 types of molecules could also modulate moxifloxacin activity on preformed biofilms, in relation with moxifloxacin diffusibility through the biofilm. Norspermidine was selected as hydrophilic polycation, and caspofungin (antifungal echinocandin) as amphiphilic polycation because, contrary to detergents, it has no intrinsic activity on *S. aureus.*

Methods: Biofilms were grown for 24 h in 96-wells plates, using 7 clinical isolates from recurrent infections, and exposed for 48 h to increasing concentrations of moxifloxacin, combined with 200µM norspermidine or 73µM caspofungin. Biofilm mass was quantified by crystal violet absorbance; viability in the biofilm, using the redox indicator resazurin. Antibiotic diffusion through the biofilm was determined as described by anderl et *al.*, [4].

Results: Caspofungin and norspermidine alone were ineffective. Moxifloxacin caused 50 % reduction in viability at concentrations < 20mg/L for 2/7 strains. This effect was reached for 6/7 strains when combined with caspofungin, with no change in biomass. Norspermidine did not show any synergistic effect. Moxifloxacin diffusibility was > 70% through 6/7 biofilms, but only 20% through the biofilm remaining resistant to moxifloxacin-caspofungin combinations.

Conclusion: A cationic amphiphile like caspofungin improves moxifloxacin potency against *S.aureus* biofilms, provided the antibiotic can diffuse through the structure. Molecular mechanisms responsible for synergy are being studied.

Introduction

Staphylococcus aureus is an important human pathogen causing chronic infections that are difficult to treat. Biofilm contributes to the persistence of infections, by protecting bacteria from immune system and antimicrobial agents.

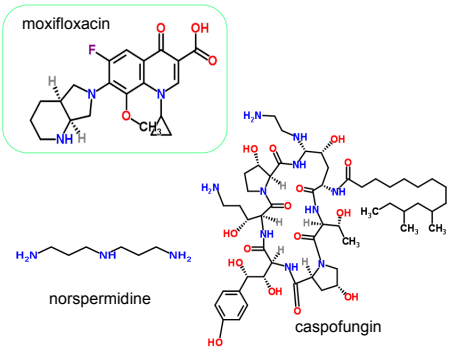
Specifically, we recently showed that many antibiotics are poorly active on biofilms, including the fluoroquinolone moxifloxacin [1], especially when using clinical isolates from persistent infections. Exploring new therapeutic strategies capable of acting on these biofilms is therefore needed.

It has been suggested that cationic amphiphilic compounds like detergents can prevent biofilm formation [2] while polycationic compounds like spermidine or norspermidine can impair biofilm growth by triggering its disassembly [3].

Aim of the study

Our aim was to examine whether cationic amphipathic molecules or polycations can improve the activity of moxifloxacin against biofilms form *S.aureus* clinical isolates.
We selected

- the antifungal agent caspofungin as exemplative cationic amphiphile because, in contrast to detergents, it has no intrinsic activity on *S. aureus;*
- norspermidine as polycationic compound.



Methods

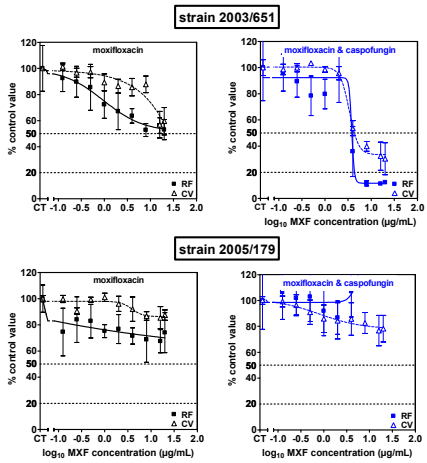
Eight *S.aureus* strains were used in this study: one reference strain [ATCC33591 (MRSA)] and seven clinical isolates selected in the collection of the Belgian reference center of *S.aureus*. These strains were selected from patients suffering from recurrent or persistent infections.

Biofilms were cultivated in polystyrene 96-well plates in TGN medium (Trypticase Soy Broth (TSB); 2% NaCl; 1% glucose) at 37°C for 24h with an initial inoculum adjusted to an OD_{620nm} of 0.005. Biofilms are exposed to increasing concentration (0.125 to 20µg/mL) of moxifloxacin alone or with combination with caspofungin (73µM) or norspermidine (200µM) 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).

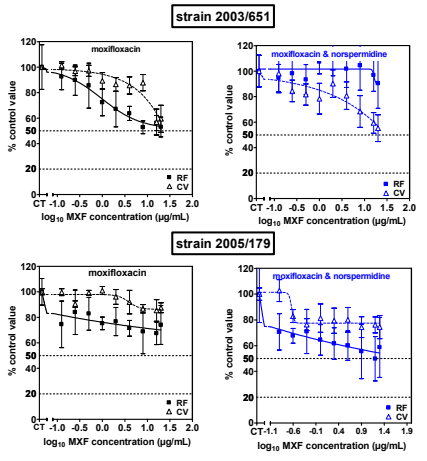
Results

Concentration-response activity of moxifloxacin combinations against biofilms from 2 exemplative clinical strains.
Preformed biofilms were exposed to increasing concentrations combined with a fixed concentration of either caspofungin or norspermidine for 48 h.
RF: viability evaluated by resorufin fluorescence; CV: biofilm mass evaluated by crystal violet absorbance, both expressed in % control [untreated biofilm].

1 - moxifloxacin & caspofungin



2 - moxifloxacin & norspermidine



Caspofungin increased moxifloxacin activity [both efficacy and potency] towards viability and matrix for strain 2005/179 but not for strain 2003/651; norspermidine was not synergistic.

Summary for all strains:

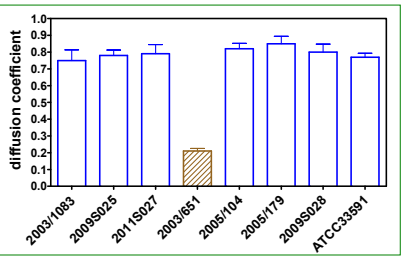
strains	moxifloxacin concentrations (mg/L) needed to reach the specified effect on viability as estimated based on the equations of the concentration-response curves					
	moxifloxacin alone		moxifloxacin & caspofungin		moxifloxacin & norspermidine	
	50 % reduction	80% reduction	50 % reduction	80 % reduction	50 % reduction	80% reduction
2003/1083	> 20	> 20	18	> 20	> 20	> 20
2009S025	> 20	> 20	2	8	> 20	> 20
2011S027	1	20	0.68	1.2	10	> 20
2003/651	> 20	> 20	> 20	> 20	> 20	> 20
2005/104	> 20	> 20	18	> 20	> 20	> 20
2005/179	> 20	> 20	3.7	5.8	> 20	> 20
2009S028	> 20	> 20	4	> 20	> 20	> 20
ATCC33591	1	> 20	0.1	2	0.7	8

Caspofungin was synergistic with moxifloxacin for all strains (blue background) except 2003/651 (brown background). Norspermidine increased moxifloxacin activity essentially against the ATCC33591 reference strain (as shown in blue).

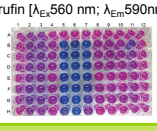
Diffusion of moxifloxacin through biofilms.

The graph shows the diffusion coefficient of moxifloxacin for each biofilm, calculated as the ratio between
(1) the drug concentration having diffused from the agar medium [E] to the disk [B] when a biofilm [A] had been grown between the two membranes [C] and [D] and
(2) in the absence of biofilm (maximal value).

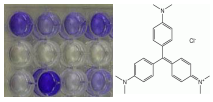
Moxifloxacin was capable of diffusing through most biofilms (as shown in blue) except the 2003/651 biofilm (as shown in brown), which is the one refractory to the synergistic effect of caspofungin.



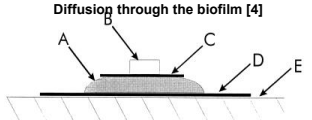
Redox indicator rezasurin assay:
blue-colored wells: dead bacteria ; pink-colored wells: viable bacteria (blue rezasurin reduced in the pink, fluorescent compound resorufin [λ_{Ex}560 nm; λ_{Em}590nm])



Quantification of matrix
by non-specific staining of biofilm constituents with crystal violet



Diffusion through the biofilm [4]



A:Biofilm; **B**: a concentration disk; **C**:Polycarbonate membrane filters (13mm diameter) ; **D**:Polycarbonate membrane filters (25mm diameter) **E**: agar culture medium containing 20mg/L moxifloxacin

Conclusions

- **The polycationic compound norspermidine did not markedly modify moxifloxacin activity, showing synergy only against the reference strain.**
- **The antifungal caspofungin improves moxifloxacin activity against most *S. aureus* biofilms from clinical strains.**
- **Moxifloxacin diffuses through those biofilms that are more susceptible to the combination moxifloxacin & caspofungin, suggesting that caspofungin does not act by increasing antibiotic diffusibility but requires optimal antibiotic availability to exert its synergistic effect.**
- **This result demonstrates the potential interest of specific combinations in biofilm eradication, even though the molecular mechanism of the synergy remains to be established.**

References
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