Corso di Dottorato di Ricerca in Scienze della Vita e dell'Ambiente, Ciclo XXXIX

Efflux Pump Inhibitors against antibiotic resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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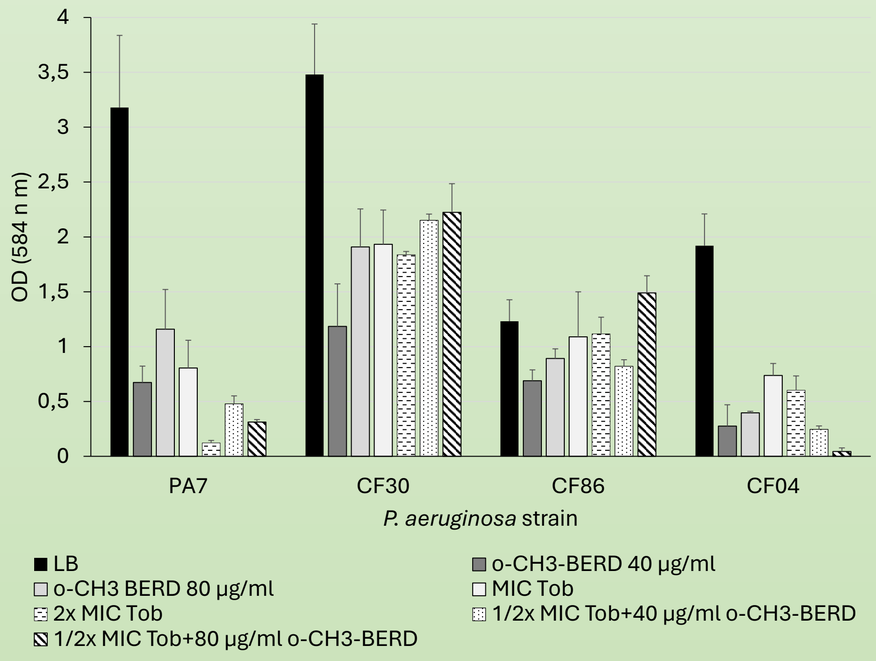
Background

Antibiotic resistance is one of the most pressing issues of global public health. Nevertheless, strategies can be used to minimize the emergence and impact of antibiotic resistance [1]. The aim of the project is to explore alternative strategies to counteract antibiotic resistance in *P. aeruginosa* and *S. aureus* strains by testing compounds able to act in association with antibiotics to prevent biofilm formation and enhance antibiotic susceptibility of the strain. Efflux Pump Inhibitors (EPIs) have been tested against *P. aeruginosa* and *S. aureus* strains targeting MexXY-OprM and NorA Efflux Pumps (EP) respectively, furthermore given the involvement of efflux pumps in biofilm formation [2], EPIs were tested to evaluate their effect on biofilm production.

Pseudomonas aeruginosa

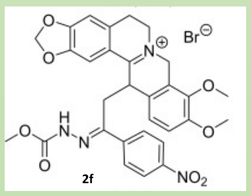
Berberine derivatives as EPIs of the MexXY-OprM efflux pump in *P. aeruginosa* clinical strains

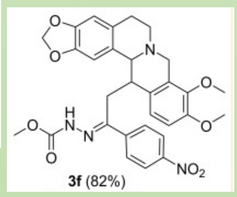
Based on the synergy results [3] between berberine derivatives and tobramycin, the activity of the o-CH_3-BERD alone or in combination with antibiotic in the inhibition of biofilm production was investigated in 4 aminoglycoside resistant *P. aeruginosa* strains.



o-CH3-BERD used alone at 40 µg/ml (rather than at 80 µg/ml), caused a greater reduction of biofilm production respect to tobramycin (1XMIC).
Only in PA7 and CF04 the o-CH3-BERD at 40 and 80 µg/ml in association with tobramycin (1/2 MIC) caused a reduction greater than those determined by tobramycin or EPI alone [3].
Hence, the antibiofilm activity of o-CH3-BERD in association with tobramycin seems to be strain-dependent.

Assessment of the activity of new berberine derivatives as tobramycin adjuvants to mitigate resistance and inhibit biofilm production in *P. aeruginosa* strains isolated from cystic fibrosis patient

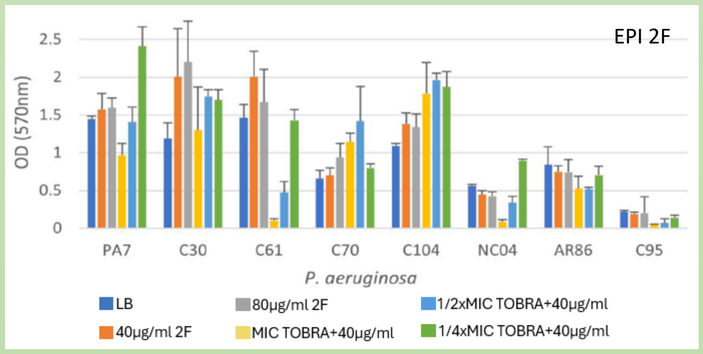




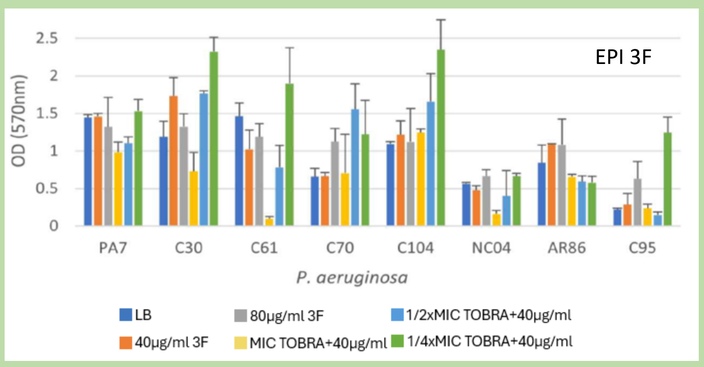
Synergism

Strain	Tobramycin MIC (µg/ml)	Tobramycin MIC (µg/ml) in presence of EPIs (20µg/ml)	
		2F	3F
PA7	128	32	64
C15	64	16	16
C25	16	8	8
C30	32	16	16
C61	128	32	32
C70	64	32	16
C95	32	16	16
C105	128	32	32
NC04	16	8	8
NC06	32	16	8
AR48	32	8	8
AR86	1024	256	256

Biofilm inhibition assays by EPI-Tobramycin



Biofilm production in LB broth and in LB supplemented with 2F alone or in association with tobramycin.



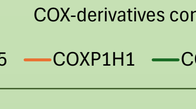
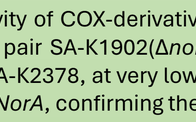
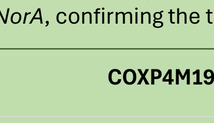
Biofilm production in LB broth and in LB supplemented with 3F alone or in association with tobramycin.

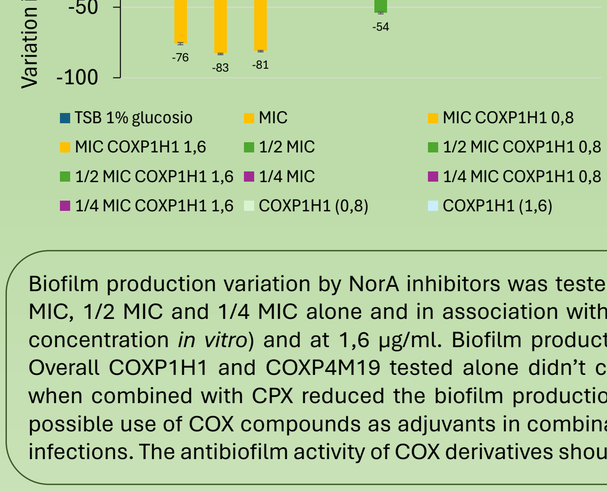
The association Berberine derivatives/tobramycin caused a reduction in resistance to tobramycin in all *P. aeruginosa* clinical strains, although it did not restore the susceptible phenotype. It can be explained by the presence in most strains of other resistance mechanisms besides the expression of EP. Biofilm production results showed a marked variability in response to different experimental conditions in the different strains. Overall, an increase of biofilm production was observed in most strains using 2F and 3F alone and in combination with antibiotic. Efflux pump inhibition by EPIs could limit the extrusion of QS signals causing an intracellular accumulation of autoinducers (AI) followed by an increased biofilm production.

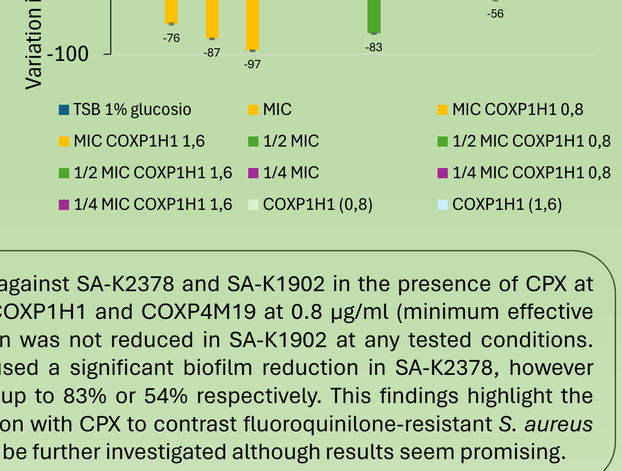
Staphylococcus aureus

Celecoxib (COX) derivatives targeting NorA efflux pump in Ciprofloxacin (CPX) resistant *S. aureus*

The growth of the *norA*-overexpressing mutant SA-1199B in presence of different COX-derivatives concentrations resulted in a MIC reduction from 4- to 16-fold of ciprofloxacin (CPX). These results were interpreted as synergism between EPIs and CPX. Indeed, in the wild type strain SA1199 *norA,* with a low resistance level to CPX, no synergy was observed as demonstrated by no or 2-fold MIC reduction of CPX in presence of EPIs.

		***S. aureus* 1199B**					***S. aureus* 1199**				
			Synergism					Synergism			
CODE	STRUCTURE	MIC (µg/ml)	MIC/fold	Conc. of inhib. (µg/ml)	CPX MIC (µg/ml)	N° fold CPX MIC reduction	MIC (µg/ml)	MIC/fold	Conc. of inhib. (µg/ml)	CPX MIC (µg/ml)	N° fold CPX MIC reduction
COXP1H5		12,5	1	12,5	0,6	16	12,5	1	12,5	No growth	/
					/	/				No growth	/
			1/4	3,125	2,5	4		1/4	3,125	/	/
COXP1H1		25	1/2	12,5	1,2	8	25	1/2	12,5	0,16	2
					1,2	8				0,16	2
			1/4	6,25	2,5	4		1/4	6,25	0,16	2
COXP4M19		>50	<1/4	12.5	2,5	4	>50	<¼	12.5	0,16	2
					1,2	8				0,16	2
COXP4M13		>50	<1/4	12.5	2,5	4	>50	<1/4	12,5	0,16	2
					2,5	4				0,16	2
					2,5	4				/	/
S. aureus 1199 MIC CPX=0,32µg/ml											
S. aureus 1199B MIC CPX=10µg/ml											





The activity of COX-derivatives against NorA was further assessed by checkerboard assays using the *S. aureus* isogenic pair SA-K1902(Δ*norA*)/SA-K2378(*norA*+). All the four derivatives caused a 4-fold reduction of the CPX MIC in SA-K2378, at very low concentrations (0,2-0,8 µg/ml). No reduction was observed for SA-K1902 carrying a deleted *NorA*, confirming the target specificity of the compounds.

Biofilm production variation by NorA inhibitors was tested against SA-K2378 and SA-K1902 in the presence of CPX at MIC, 1/2 MIC and 1/4 MIC alone and in association with COXP1H1 and COXP4M19 at 0.8 µg/ml (minimum effective concentration *in vitro*) and at 1,6 µg/ml. Biofilm production was not reduced in SA-K1902 at any tested conditions. Overall COXP1H1 and COXP4M19 tested alone didn't caused a significant biofilm reduction in SA-K2378, however when combined with CPX reduced the biofilm production up to 83% or 54% respectively. This findings highlight the possible use of COX compounds as adjuvants in combination with CPX to contrast fluoroquinilone-resistant *S. aureus* infections. The antibiofilm activity of COX derivatives should be further investigated although results seem promising.

Material and methods

P. aeruginosa strains, from fibrosis cystic patients, were provided by the Ancona Regional hospital whereas *S. aureus* strains belonged to our collection. They were cultured in pseudomonas agar and mannitol salt agar medium respectively. Berberine derivatives were provided from the chemistry group of Prof.ssa Galeazzi, COX-compounds were kindly provided from Prof. Sabatini from Università di Perugia. MIC assays were performed using broth microdiluition method according to CLSI guidelines. Checkerboard and biofilm inhibition assays were performed as previously described [3,4], testing *P. aeruginosa* and *S. aureus* strains with antibiotics alone or in association with EPIs.

References

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[2] Alav, J. M. Sutton, K. M. Rahman, *J. Antimicrob. Chemother.* 2018, *73*, 2003–2020.
[3] Giorgini et al. 2012 doi.org/10.1002/cmdc.202300568
[4] H.D. Isenberg, Clinical Microbiology Procedures Handbook ASM, Washington DC, in:1992.
[5] Stepanovic S, 2000 Apr;40(2):175-9. doi: 10.1016/s0167-7012(00)00122-6. PMID: 10699673.