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Reversal of drug resistant phenotype of *Staphylococcus* clinical strains by synergistic action of P128 and antibiotics

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Abstract

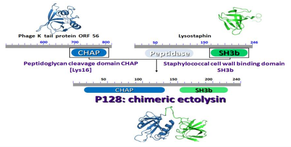
Infections caused by drug resistant strains of *S. aureus* and coagulase-negative staphylococci (CoNS) are leading causes of morbidity and mortality all over the world. To overcome the challenge of drug resistance, various approaches are being followed to either discover new therapeutics with a novel mechanism of action or that potentiate the efficacy of existing drugs. P128 is a chimeric recombinant bacteriophage derived ectolysin (phage lysin involved in cleaving the peptidoglycan from outside the bacterium during DNA injection) which shows potent bactericidal activity on planktonic and biofilm embedded cells of *S. aureus* and CoNS species. Moreover, combinations of P128 and a number of antibiotics have been found to be synergistic on planktonic cells and biofilms of *S. aureus* including MRSA. To determine whether this synergistic effect would extend to drug-resistant strains, P128 was tested in combination with oxacillin, vancomycin or linezolid by checkerboard assays on strains individually resistant to one of these drugs. On four MRSA strains with oxacillin MIC of >16 µg/ml and P128 MIC of 0.5 - 1.0 µg/ml, a combination of sub-MIC P128 (0.025-0.20 µg/ml) and 0.5 µg/ml of oxacillin resulted in inhibition of bacterial growth, indicating a strong synergy. Similar results were seen with other drugs wherein combinations of sub-MIC of P128 and clinically sensitive breakpoint concentrations of vancomycin, linezolid, daptomycin or ciprofloxacin could effectively inhibit the growth of clinical *S. aureus* strains which were individually resistant to these drugs. In a mouse model of bacteremia using the USA300 MRSA strain, a combination of 2.5 mg/kg of P128 (single dose) and 100 mg/kg of oxacillin (4 doses 6 hours apart) led to survival of 81% of infected animals, whereas oxacillin or P128 individually protected 31% and 50% of mice respectively, demonstrating superior efficacy of the drugs in combination. Taken together, these results suggest that a combination of P128 and antibiotics has the potential to be developed to treat infections caused by drug resistant strains of staphylococci.

Introduction

P128: an engineered Ectolysin with unique properties

- P128 is a chimeric recombinant ectolysin* that is being developed to treat systemic and topical *Staphylococcus* infections.
- P128 shows rapid bactericidal activity on antibiotic sensitive and resistant strains of *S. aureus* and coagulase negative staphylococci (CoNS).
- P128 has demonstrated potent anti biofilm activity on MRSA and CoNS strains in various *in vitro* models including those involving clinically relevant mixed bacterial species.
- P128 is highly synergistic with standard-of-care (SoC) drugs in inhibiting *S. aureus* and CoNS cells especially those embedded in biofilms.
- P128 specifically kills *Staphylococcus* spp. and shows no inhibition of other bacterial species or eukaryotic cells even at concentrations as high as 1 mg/ml.
- These properties make it an attractive candidate for antibacterial development.

*Ectolysin - phage lysin involved in cleaving the peptidoglycan from outside the cell during DNA injection.

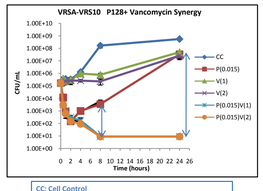


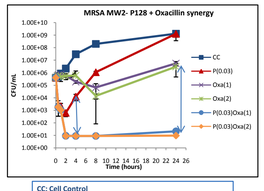
- Staphylococci are commensal or otherwise non-pathogenic natural flora in some settings and yet sometimes threatens our life as a tenacious pathogen.
- In addition to its ability to outwit our immune system, its multi-drug resistance phenotype makes it one of the most intractable pathogenic bacteria in the history of antibiotic chemotherapy. It has conquered most of the antibiotics that have been developed since 1940s.
- The overall burden of staphylococcal disease, particularly that caused by MRSA is increasing in many countries in both healthcare and community settings.
- Due to the emergence of resistance to conventional antibiotics, efforts to develop non-small-molecule antibacterial therapeutics have been increasing.
- Rapid killing, low rates of resistance and profound anti-biofilm activity are a few key properties of phage lysins, which can be exploited to develop clinically useful therapeutics.
- This study investigates the *in vitro* and *in vivo* efficacy of P128 in combination with SoC antibiotics on respective drug resistant strains of *Staphylococcus*.

Methods and Results

A) Synergistic effect of combinations of P128 and antibiotics on planktonic cells of drug resistant Staphylococcal isolates – Time Kill Kinetics (TKK)

- Time kill kinetics assay with P128 and antibiotics was performed as per modified CLSI guidelines.
- A $\geq$ 2 $\log_{10}$ difference in viability between the combination and most active single drug was considered as the cut off value to score for synergy.



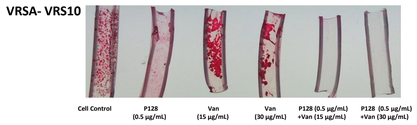


Combination of P128 + oxacillin and P128 + vancomycin at their sub MICs, exhibited significant synergy, suggested by 5 & 6 log reduction in CFU respectively on MRSA and VRSA at the end of 24 h, indicating reversal of drug resistant phenotype.
The synergistic effect of the combinations is also aided by the rapid killing property of P128 .

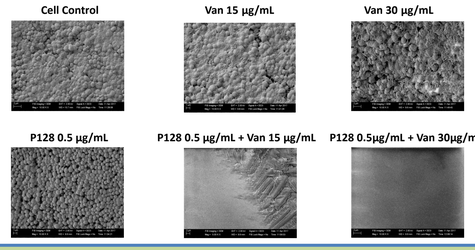
D) Synergistic effect of sub-inhibitory concentrations of P128 and antibiotics in combination on biofilms on catheters

- Biofilms were grown on catheters (Nair *et. al.*, 2016)
- Challenged with P128 and antibiotics individually and in combinations.
- The treated catheters were analysed using SEM for the presence of biofilm.

Catheters stained with safranin



SEM images of biofilm on catheters



P128 and Oxacillin / Vancomycin combinations at their sub MBICs, cleared the biomass indicating synergistic eradication of biofilm.

B) Synergistic effect of combinations of P128 and antibiotics on planktonic cells of drug resistant *Staphylococcus* – Checkerboard assay

- Checkerboard assay with P128 and antibiotics was performed as per modified CLSI guidelines.
- The combination was considered synergistic when the ΣFIC is ≤ 0.5, additive when the ΣFIC is > 0.5 to 1.0, indifferent when > 1.0 and antagonistic when the ΣFIC is ≥ 2.0

Strains resistant to	Strain	MIC µg/ml			FICI
		P128	Oxa	P128 + Oxa	
Oxacillin	MRSA, USA 300	0.45	> 16	0.025 + 0.50	0.05
	MRSA, MW2	0.48	> 16	0.03 + 0.50	0.07
	S. epidermidis NRS867	8.0	> 8.0	2.0 + 0.50	0.28
			Van	P128 + Van	
Vancomycin	VRSA, VRS 3b	0.97	32	0.24 + 0.25	0.20
	VRSA, VRS 10	3.90	16	0.24 + 1.0	0.12
			Dap	P128 + Dap	
Daptomycin	*S. epidermidis* NRS867	32	2.0	1.0 + 0.50	0.28
	S. epidermidis NRS853	16	2.0	2.0 + 0.50	0.28
			Lin	P128 + Lin	
Linezolid	LRSA- NR45924	2.0	> 32	0.25 + 2.0	0.15
	LRSA- NR45930	1.0	8.0	0.25 + 2.0	0.50
			Cip	P128 + Cip	
Ciprofloxacin	MRSA, BK#13228	4.0	32	0.25 + 8.0	0.37
	MRSA, B9241	8.0	8.0	2.0 + 0.25	0.28

P128 and antibiotic combinations exhibited a high degree of synergy, suggested by the low FIC indices. The combination with P128 lowered the MIC of antibiotics to clinically sensitive breakpoints on strains individually resistant to one of these drugs, indicating reversal of drug resistant phenotype.

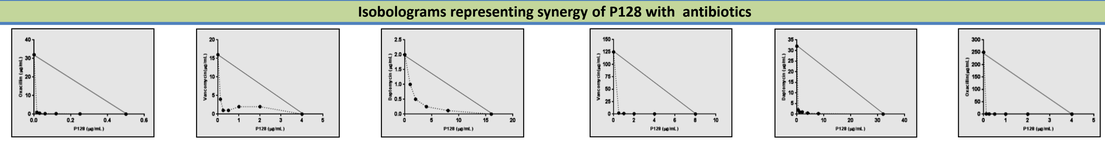
C) Synergistic effect of combinations of P128 and antibiotics on biofilms of drug resistant Staphylococcal isolates – Checkerboard assay

- Biofilms were grown in 96 well microtitre plate (Nair *et. al.*, 2016)
- A checker board assay was performed (as per modified CLSI guidelines) with combination of P128 and antibiotics.
- The combination effect was quantified by MTT assay and corresponding FICI values were calculated.
- The combination was considered synergistic when the ΣFIC is ≤ 0.5, additive when the ΣFIC is > 0.5 to 1.0, indifferent when > 1.0 and antagonistic when the ΣFIC is ≥ 2.0

Strains resistant to	Strain	MIC µg/ml			FICI
		P128	Oxa	P128 + Oxa	
Oxacillin	MRSA, MW2	4.0	> 250	0.50 + 0.24	0.12
			Van	P128 + Van	
Vancomycin	VRSA, VRS10	8.0	125	2.0 + 0.49	0.25
			Dap	P128 + Dap	
Daptomycin	*S. epidermidis* NRS867	32	15.6	2.0 + 3.9	0.18
			Lin	P128 + Lin	
Linezolid	LRSA, NR45924	16	> 250	2.0 + 3.9	0.14
			Cip	P128 + Cip	
Ciprofloxacin	MRSA, BK#13228	25	> 250	12.5 + 0.48	0.50

P128 and antibiotic combinations exhibited significant synergy on 72 h- preformed staphylococcal biofilms, suggested by the low FIC indices. This indicated reversal of drug resistant phenotype in antibiotic resistant staphylococci.

Isobolograms representing synergy of P128 with antibiotics



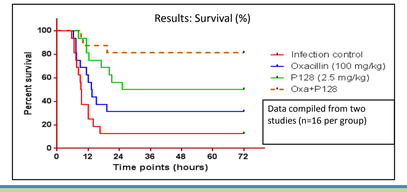
Synergy of P128 with vancomycin, oxacillin and daptomycin on planktonic cells of *S. aureus* VRSA, MRSA and *S. epidermidis* DRSE

Synergy of P128 with vancomycin, oxacillin and daptomycin on biofilms of *S. aureus* VRSA, MRSA and *S. epidermidis* DRSE

The individual MIC or MBIC values of P128 and the drugs have been joined by a solid line, while the MIC or MBIC values obtained in P128 and drug combinations have been joined by a dotted line, showing the synergistic effect of the combinations.

E) Synergistic effect of treatment with sub-therapeutic doses of oxacillin and P128 in a mouse model of bacteremia.

- 8 - 9 weeks old female BALB/c mice were challenged (IP) with *S. aureus* USA300 (10^9 CFU per animal in 5% mucin).
- Two hours post-challenge the mice were treated with P128 (2.5 mg/kg, IP) or oxacillin (100 mg/kg, IM, 4 doses at 6 h intervals) or both.
- Observed for survival for 72 hours.



A combination of 2.5 mg/kg of P128 (single dose) and 100 mg/kg of oxacillin (4 doses 6 hours apart) led to survival of 81% of infected animals, whereas oxacillin or P128 individually given at the same sub therapeutic dose protected 31% and 50% of mice respectively, demonstrating superior efficacy of the drugs in combination and reversal of oxacillin resistant phenotype.

Conclusions

- **P128, an ectolysin, shows potent bactericidal activity when tested in combination with oxacillin, vancomycin, daptomycin, ciprofloxacin and linezolid by checkerboard assays on strains individually resistant to one of these drugs.**
- **P128 at sub-MIC concentration can lower the MIC of antibiotics to clinically sensitive breakpoints on strains individually resistant to one of these drugs, indicating reversal of drug resistant phenotype.**
- **The extent and rapidity of kill exhibited in combination TKK experiment, demonstrates remarkable synergy of P128 with antibiotics on *Staphylococcus*.**
- **Checkerboard studies on biofilms of MRSA, VRSA, LRSA, and daptomycin resistant *S. epidermidis* showed that combinations of P128 and individual antibiotics were effective in inhibiting the growth of bacteria in preformed biofilms. These combinations were also effective in eradicating biofilms formed on catheters.**
- **In a mouse model of bacteremia using the USA300 MRSA strain, a combination of P128 and oxacillin led to survival of 81% of infected animals, whereas oxacillin or P128 at pre-selected sub-therapeutic doses individually protected 31% and 50% of mice respectively, demonstrating superior efficacy and reversal of drug resistant phenotype by the drugs in combination compared to single drugs.**
- **The property of synergistic killing by P128 with SoC antibiotics (irrespective of antibiotic class) resulting in reversal of drug resistant phenotype, should allow development of effective combination therapy for treating chronic *Staphylococcus* infections.**

References

- **Kelley PG, Gao W, Ward PB, Howden BP.** 2011. J. Antimicrob. Chemother. 66:1057–1060. doi: 10.1093/jac/dkr066.
- **Stewart PS. 2015. Microbiol. Spectr. 3:3.** doi: 10.1128/microbiolspec.MB-0010-2014.
- **Nair S, Desai S, Poonacha N, Vipra A, Sharma U.** 2016. Antimicrob. Agents Chemother. **60**:7280-7289. doi:10.1128/AAC.01118-16.
- **Chung PY, Toh YS.** 2014. Pathog. Dis. 70:231–239. doi: 10.1111/2049-632X.12141.
- **Paul VD, Rajagopalan SS, Sundarrajan S, George SE, Asrani JY, Pillai R, Chikkamadaiah R, Durgaiah M, Sriram B, Padmanabhan S.** 2011. BMC Microbiol 11:226. doi: 10.1186/1471-2180-11-226.
- **Pastagia M, Schuch R, Fischetti VA, Huang DB.** 2013. J. Med. Microbiol. 62:1506–1516. doi: 10.1099/jmm.0.061028-0.
- **Hiramatsu K, Katayama Y, Matsuo M, Sasaki T, Morimoto Y, Sekiguchi A, Baba T.** 2014. J. Infect. Chemother. 2014 Oct;20(10):593-601. doi: 10.1016/j.jiac.2014.08.001.

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