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Combination of Polymyxin B and Rifampicin is Synergistic Against Intracellular *P. aeruginosa*

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Abstract

Background:
P. aeruginosa (Pa), an opportunistic pathogen, is a leading agent of hospital-acquired infections and chronic infections in patients with cystic fibrosis, causing morbidity and mortality [1, 2]. Those infections are hard to eradicate as Pa is intrinsically resistant to many common antibiotics, largely due to low permeability of outer membrane [3], which hampers the entrance of antibiotics. In addition, Pa evades immune system and benefits from diminished local concentrations of antibiotics by forming biofilms [4], and hiding inside phagocytic [5] and epithelial cells [6]. Polymyxins are synergistic with many drugs by improving their penetration inside Gram-negative bacteria. Our aim was to examine the effect of polymyxin B in combination with rifampicin, a disused antibiotic for which Pa resistance is low, against intracellular forms of infection.

Methods:
ATCC27853 and PAO1 were used. Extracellular activity of the combination was measured by cfu counting in cation-adjusted MHB. Intracellular activity was evaluated using an established model of infected THP-1 cells [5]. Synergy was assessed assuming dose additivity (Loewe's model) and using Hill-Langmuir equation for concentration-response curves.

Results:
Unexpectedly, rifampicin alone had higher efficacy on intracellular bacteria compared to those growing in broth. A strong synergy was observed when combined with polymyxin B against both strains intracellularly while zero interaction was observed in broth. In an attempt to explain these results, extracellular activity was also measured in broth acidified to pH 5.5, plausibly mimicking the pH that Pa encounters in vacuoles. In these conditions, the strong synergistic interaction seen during intracellular infection was reproduced.

Conclusion:
The combination of rifampicin and polymyxin B is highly synergistic against intracellular Pa, possibly thanks to the acidic pH of the vacuoles hosting bacteria in infected cells.

References

[1] Lyczak *et al.* 2000 Microbes Infect 2:1051-60.
[2] Rowe *et al.* 2005 N Engl J Med 352:1992-2001.
[3] Hancock 1998 Clin Infect Dis 27:S93-9.
[4] Høiby *et al.* 2010 Int J Antimicrob Agents 35:322-32.
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[6] Sana *et al.* 2015 MBio 6:e00712

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Background and Aims

P. aeruginosa (Pa) causes recalcitrant infections [1, 2] that are hard-to-treat due to pathogen's low permeability to antibiotics [3] and its hiding in biofilms [4] and inside host cells [5-6]. Polymyxins work by disrupting bacterial membranes of Gram-negative bacteria and, in doing so, are the antidote of permeability issues encountered by many anti-Gram negative antibiotics.

Our laboratory has developed pharmacodynamic models to assess the extra- and intracellular efficacy and potency of antibiotics in a quantitative manner [5].

Our aim was to examine the potential of polymyxin B (PMB) in combination with rifampicin (RIF), a disused antibiotic for which Pa resistance is low, against intracellular forms of infection.

Methods

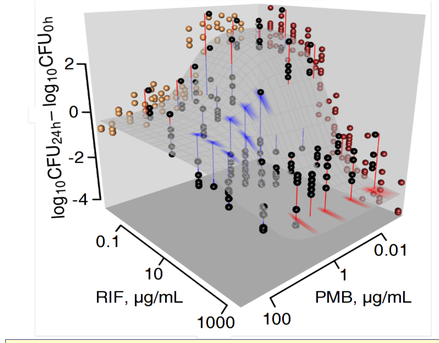
1. Strains: *P. aeruginosa* ATCC 257853 and PAO1.
2. MIC determinations: microdilution (CLSI recommendations) with susceptibility assessed according to the EUCAST interpretive criteria (http://www.eucast.org).
3. Antibiotic activity against intracellular bacteria: (i) phagocytosis of bacteria by human THP-1 monocytes; (ii) elimination of non-internalized bacteria by exposure to gentamicin; (iii) incubation with a wide range of extracellular concentrations (0.003-100 x MIC) of RIF and PMB for 24 h to obtain full spectrum of concentration-response outcomes. Intracellular activity evaluated at 24h as the change in CFU (log_{10} units) from the initial inoculum. Concentration-response results were fitted with four-parameter logistic regression (Hill-Langmuir equation) fixing the slope at 1.
4. Antibiotic activity against bacteria in broth: overnight culture of bacteria was diluted to 0.5 x 10^6 CFU/mL in fresh cation adjusted Mueller-Hinton medium and subjected to wide range of antibiotic concentrations (0.003-100 x MIC) of RIF and PMB for 24 h at 37 °C, 130 rpm in test tubes at a final volume of 2 mL. The activity of antibiotics was evaluated as described above for intracellular bacteria.
5. The zero interaction effect of combinatorial drug treatment (mere additivity of the drugs) was predicted from concentration-response curves assuming dose additivity (Loewe's model).

Key Conclusions and Outlook

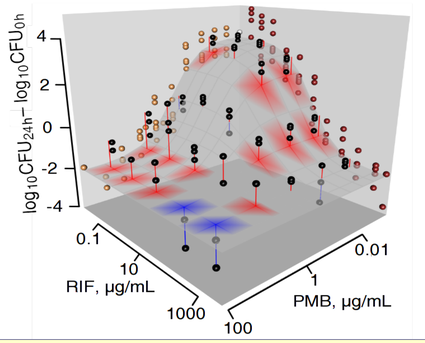
- Combination of polymyxin B and rifampicin is markedly synergistic, it eradicates two orders of magnitude more intracellular bacteria than expected from the combined effects of monotherapies.
- Some of the synergy is possibly potentiated by the acidic environment *Pseudomonas aeruginosa* encounters in vacuoles where it resides in monocytes during intracellular infection.
- We are currently working to explore the generality of this observation on other Gram-negative ESKAPE pathogens and to elucidate the molecular foundations of synergy.

Results

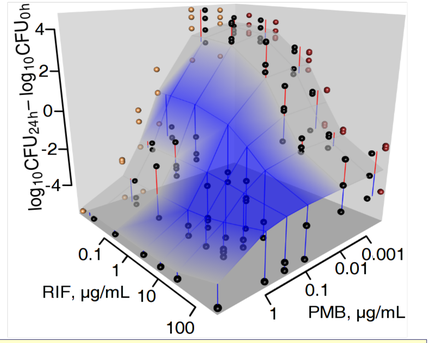
1. Polymyxin B and rifampicin against intracellular *P. aeruginosa* ATCC27853



2. Polymxin B and rifampicin against *P. aeruginosa* ATCC27853 in broth at neutral pH

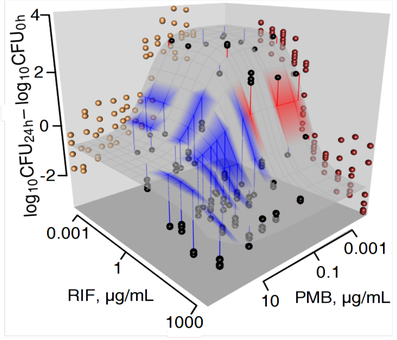


3. Polymxin B and rifampicin against *P. aeruginosa* ATCC27853 in broth at pH 5.5



Certain combinations of polymyxin B and rifampicin are synergistic in acidic conditions. Circles on figures 1-4 stand for change in CFUs after 24h of antibiotic treatment from an initial inoculum of 10^6 CFU/mL. Red circles stand for the results of rifampicin and yellow for polymyxin monotherapy. From the results of monotherapies, the expected outcome was calculated for the combinations of the two drugs assuming there is no drug-drug interaction (grey surface). The measured experimental outcome of combinations is recorded as black figures. For better visualization: (1) the combination results (black circles) are connected with lines to the zero interaction surface, (2) lines and regions of surface are colored blue for synergies and red for antagonisms.

4. Intracellular synergy holds for other *P. aeruginosa* strains (shown here for PAO1)



5. Combination of polymyxin B and rifampin results in two orders of magnitude better killing than expected

Growth conditions	Polymyxin B (mg/L)	Rifampicin (mg/mL)	Measured true effect [a]	Zero interaction [b]	Δ [c]	p-value [d]
Intracellular [e]	0.3	4.8	-1.3	0.6	**-1.9**	***
	1	4.8	-2.5	0.3	**-2.8**	***
	3	4.8	-2.1	-0.1	**-2**	***
Broth pH 5.5 [f]	0.075	0.024	-2	0.2	**-2.2**	*
	0.075	0.24	-2.4	0.1	**-2.5**	*
	0.075	8	-4.1	-1.2	**-2.9**	*
Broth pH 7.4 [g]	0.3	4.8	0.3	0.2	0.1	NS
	3	4.8	-0.8	-1.6	0.8	NS

[a] log_{10} change in CFUs after 24h incubation with antibiotics from starting value (10^6 CFU/mL)
[b] additive effec of Loewe calculated from the effects of monotherapies
[c] difference between predicted zero interaction (no synergy/antagonism) and true measured effect
[d] p-value for 0-hypothesis that measured effect is not different from zero interaction; *** < 0.001, * < 0.05, NS not significant
[e] THP-1 monocytes infected with an average of 1 bacteria/cell after phagocytosis and washing
[f] cation-adjusted Mueller-Hinton broth with 100 mM MES at pH 5.5
[g] cation-adjusted Mueller Hinton broth

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