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DnpA, a putative de-*N*-acetylase involved in *Pseudomonas aeruginosa* persistence, reduces intracellular ciprofloxacin activity

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Abstract (revised)

Objectives: Persisters are now recognized as a possible cause of antibiotic failure. The putative de-*N*-acetylase DnpA has been shown to increase persister levels in *Pseudomonas aeruginosa*, conferring tolerance specifically to fluoroquinolones in broth[1]. *P. aeruginosa* is capable of invading epithelial and phagocytic cells. Intracellularly, the bacteria are less responsive to antibiotics, with a maximal efficacy only between 1 (β-lactams) and 2.5 (fluoroquinolones) $\log_{10}$ CFU decrease from the post-phagocytosis inoculum[2], leaving an important intracellular bacterial load. Our objective was to assess a possible role of DnpA in this intracellular persistence of *P.aeruginosa* using ciprofloxacin as exemplative fluoroquinolone and meropenem as a representative antibiotic from another antipseudomonal class.

Methods: Strains: PAO1; a *dnpA* deletion mutant (ΔdnpA) and a *dnpA* overexpressing strain (generated in the PAO1 background). Susceptibility testing: MICs determined according to CLSI recommendations in cation-adjusted MHB. Intracellular infection and activity of antibiotics: these experiments were performed exactly as described[2], with activity expressed as change from the initial inoculum after 24 h of exposure to antibiotics. Data were used for fitting a concentration-response curve (Hill equation) to calculate the E_{max} and C_{static} pharmacodynamic parameters (see definitions in the Table).

Results: The Table shows the relevant pharmacodynamic parameters and MIC values. All strains showed similar MICs for each antibiotic. For ciprofloxacin, E_{max} was significantly larger (more negative value) for the ΔdnpA strain, while E_{max} was slightly lower (less negative value) and C_{static} was slightly higher for the PAO1(dnpA) overexpressing strain. For meropenem, no differences were observed among strains.

	Intracellular pharmacodynamic parameters and MIC values							
	Ciprofloxacin (CIP)				Meropenem (MEM)			
Strain	E_{max} [a,b]	MIC[c]	C_{static} [d]	R^2	E_{max} [a,b]	MIC[c]	C_{static} [d]	R^2
PAO1	-2.34 ± 0.17 (A)	0.25	0.34	0.99	-0.75 ± 0.27 (A)	2	5.03	0.95
ΔdnpA	-3.94 ± 0.27 (B)	0.25	0.27	0.99	-1.24 ± 0.24 (A)	2	3.01	0.98
PAO1(dnpA)	-2.49 ± 0.33 (A)	0.5	0.88	0.94	-0.70 ± 0.29 (A)	4	6.80	0.94

[a] maximum decrease in log CFU compared to the post-phagocytosis inoculum for an infinitely high concentration in antibiotic
[b] statistical analyses per column (ANOVA; Tukey post-hoc test): values with different letters in upper case are significantly different from one another
[c] minimal inhibitory concentrations (in mg/L)
[d] Concentration (in mg/L) resulting in no apparent bacterial growth (number of CFU identical to the post-phagocytosis inoculum)

Conclusion: The expression of *dnpA* impairs the intracellular activity of ciprofloxacin but not of meropenem, suggesting it triggers persistence and fluoroquinolone tolerance intracellularly, extending this model to the observations previously made in broth. Targeting DnpA appears to be an appealing strategy to improve fluoroquinolone efficacy against intracellular *P. aeruginosa*.

Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* is capable of invading epithelial and phagocytic cells. Intracellularly, the bacteria are less responsive to antibiotics[1]. The putative de-*N*-acetylase DnpA has been shown to increase persister levels in *P.aeruginosa*, conferring tolerance specifically to fluoroquinolones in broth[2].

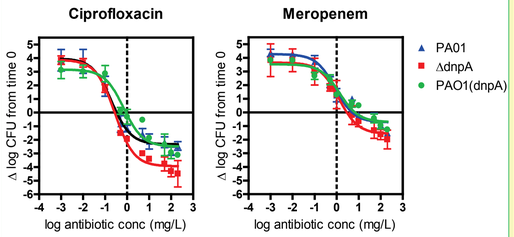
A possible role of persisters in recalcitrance of intracellular *P. aeruginosa* to antimicrobial agents has not yet been documented.

Objectives

To assess a possible role of DnpA in the poor responsiveness of intracellular *P. aeruginosa* to antibiotics, using:

- **ciprofloxacin** as a representative fluoroquinolone
- **meropenem** as a representative antibiotic from another anti-pseudomonal class.

Results

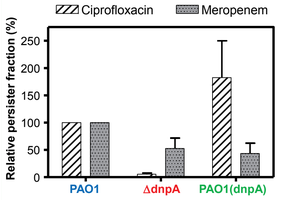


Concentration-response curves of antibiotics against intracellular *P. aeruginosa* strains. The graphs show the changes in the number of CFU per milligram of cell protein in THP-1 cells after 24 h of incubation at increasing extracellular antibiotic concentrations. Data are means ± standard deviations (n=3).

Intracellular pharmacodynamic parameters and MIC values

Strain	Ciprofloxacin				Meropenem			
	E_{max}*	MIC	C_{static}	R^2	E_{max}*	MIC	C_{static}	R^2
PAO1	-2.34 ± 0.17 (A)	0.25	0.34	0.99	-0.75 ± 0.27 (A)	2	5.03	0.95
ΔdnpA	-3.94 ± 0.27 (B)	0.25	0.27	0.99	-1.24 ± 0.24 (A)	2	3.01	0.98
PAO1(dnpA)	-2.49 ± 0.33 (A)	0.5	0.88	0.94	-0.70 ± 0.29 (A)	4	6.80	0.94

E_{max} : maximal decrease in inoculum (in $\log_{10}$ units) compared to the post-phagocytosis inoculum as extrapolated for an infinitely large antibiotic concentration
MIC: minimal inhibitory concentrations (in mg/L)
C_{static} : static concentration i.e., the extracellular concentration (in mg/L) resulting in no apparent bacterial growth (number of CFU identical to the post-phagocytosis inoculum)
(*) statistical analyses per column (ANOVA; Tukey post-hoc test): values with different letters in upper case (A,B) are significantly different from one another



Relative persister fractions of extracellular *P.aeruginosa* strains.

CFU obtained for persisters of dnpA and PAO1(dnpA) are expressed in relation to that of PAO1, which was set to 100% Antibiotics were used at a concentration of 1x MIC.

- All strains showed similar MIC values for the respective antibiotics.
- For **ciprofloxacin:**
  - significantly larger E_{max} (more negative value) for the ΔdnpA strain.
  - slightly lower E_{max} (less negative value) and higher C_s for the *dnpA* overexpressing strain.
- For **meropenem:**
  - no significant differences observed among strains.

Materials & Methods

Intracellular infection assay

Bacterial growth (Overnight at 37°C) → **Opsonization** (1 h, 37°C) 9 mL RPMI-1640+ 1 mL human serum → **Phagocytosis** (2 h, 37°C, 5% CO_2) 10 bacteria/THP-1 cell → **Killing extracellular bacteria** (1 h, 37°C) 100 mg/L gentamicin → **Incubation** (24 h, 37°C, 5% CO_2) Increasing concentration of antibiotic → **Cell harvesting, lysis, CFU counting & cell protein content determination**

Bacterial strains

- *P. aeruginosa* PAO1: wildtype
- ΔdnpA: Deletion of *dnpA*
- PAO1(dnpA): Overexpression of *dnpA*

Persistence assay

Bacterial growth (Overnight at 37°C) → **Repeat with 1:200 dilution** (Overnight at 37°C) → **1:200 dilution + antibiotic 1:200 dilution + H_2O** (5 h at 37°C) → **CFU counting** → **% Persister fraction** = $\frac{\text{CFU after antibiotic treatment}}{\text{CFU after } H_2O \text{ treatment}}$ x 100

Conclusions

- ✓ Deletion of *dnpA* decreases persister formation upon fluoroquinolone exposure in broth.
- ✓ Deletion of *dnpA* improves the intracellular activity of ciprofloxacin but not that of meropenem.
- ✓ The results extend the previously described observations of extracellular model.

Discussion

- Expression of *dnpA* might trigger intracellular persistence and fluoroquinolone tolerance.
- Only fluoroquinolone-related persister formation is affected, suggesting an interference with the activity of these antibiotics that needs to be further explored.
- Targeting DnpA appears to be an appealing strategy to improve fluoroquinolone efficacy against intracellular *P. aeruginosa*.

References

1. Liebens *et al.*, Pathog Dis 2014, 71:39–54
2. Buyck *et al*, AAC 2013, 57:2310-8

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