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Subcellular localisation of *S. aureus* small colony variant (SCV) *vs.* its normal phenotype (NP) counterpart in a model of airway epithelial cells: Impact on the intracellular activity of oritavancin, gentamicin, oxacillin and moxifloxacin

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ABSTRACT

Background: Recurrence of *S. aureus* infections in cystic fibrosis (CF) is partly related to the presence of SCVs, which are able to survive within eucaryotic cells. Treatments should therefore include antibiotics that accumulate and act in the infected compartment. Using a model of airway epithelial cells we have examined (i) the subcellular localization of an SCV and its normal phenotype (NP) counterpart isolated from the same CF patient; (ii) the intracellular activity of antibiotics with different routes of cellular penetration and subcellular distribution (GEN and ORI: endocytosis and accumulation in lysosomes ; OXA and MXF: diffusion, and distributed throughout the cell).

Methods: MICs were determined by microdilution in cation-adjusted MH broth. Intracellular activity was measured at 24 h using a wide range of AB concentrations to obtain full pharmacological dose-effect responses. For confocal microscopy, bacteria were stained with fluorescein-isothiocyanate, and lysosomes, with LysoTracker Red.

Results: Confocal microscopy at 3h post-infection showed that SCV remained confined with lysosomes whereas NP appeared also to be free in the cytoplasm. The Table shows the MICs and intracellular activities.

antibiotic	SCV			NP		
	MIC[a]	EC_{50}[b]	Emax[c]	MIC[a]	EC_{50}[b]	Emax[c]
GEN	0.125	1.88*	-0.78*	0.5	0.54	-0.44
ORI [d]	0.015	62.47	-1.68*	0.03	81.47	-0.72
		601.9*	-2.87*		1141	-1.63
OXA	0.125	2.02*	-1.03	0.5	0.20	-0.84
MXF	0.125	0.87*	-0.63*	0.25	0.46	-1.14

[a]: in µg/mL; determined in the presence of 0.002% tween 80 for ORI
[b]: concentr. causing 50% of the maximal effect as deduced from the sigmoidal regression of dose-effect relationships; expressed as multiples of the MIC
[c]: maximal effect, as deduced from the sigmoidal regression of dose-effect relationships; expressed as ∆log CFU from Time 0
[d]: 2 successive sigmoidal regressions with variable slope to fit the data
*: p<0.05 for SCV vs NP

Conclusion: SCV and NP do not localise within the same subcellular compartment of airway epithelial cells, which can explain why GEN and ORI show greater extent of kill towards SCV than NP, and why OXA and MXF are active at lower concentr. towards NP than SCV. ORI, however, has the highest max. effect against both phenotypes.

INTRODUCTION

Small Colony Variants (SCVs) are now recognized as a major cause for the persistent and recurrent character of infections due to *S. aureus* such as cystic fibrosis (CF) (1). Moreover, SCVs are capable to survive within host cells, where they are protected from host defenses and antibiotics (2).

PK/PD suggest that, for acting upon intracellular SCVs, antibiotics should (a) exert a bactericidal effect even against a slowly growing organism, and (b) accumulate in sufficient amount in the subcellular compartment where bacteria sojourn (3).

Using a model of human macrophages, we previously showed that SCVs remained confined within acidic vacuoles and were poorly susceptible to most antibiotics, with the exception of oritavancin, rifampin, moxifloxacin, and quinupristin/dalfopristin (4).

Airway epithelial cells may however constitute a more relevant model for CF. In particular, recent data suggest that *S. aureus* can escape from endosomes and gain access to the cytosol of epithelial cells, this process being still more efficient in CF cells (5).

REFERENCES

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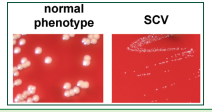
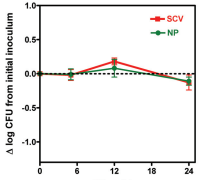
RESULTS

Strains used in this study,

(24 h of incubation on Columbia blood agar)



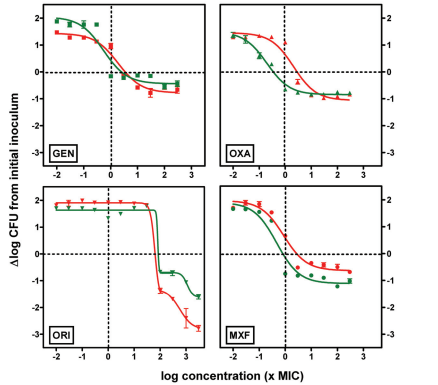
Intracellular growth of SCV and NP within airway epithelial cells



10 mg/L lysostaphin was present during the whole incubation period to avoid extracellular contamination

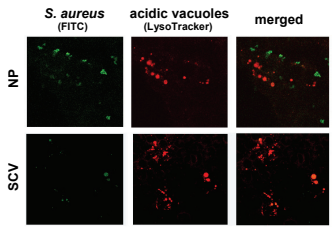
Intracellular activity of antibiotics

SCV NP



Dose-response curves of antibiotics against intracellular SCV and NP. The graphs show the change in the number of CFU (∆ log CFU from initial inoculum) per mg of cell protein in airway epithelial cells after 24 h incubation at the extracellular concentrations shown in the abscissa (expressed in multiples of MIC of each drug measured in MH broth).

Confocal microscopy of airway epithelial cells observed 3 h after phagocytosis of *S. aureus*



Merged images suggest that NP does not localize within acidic vacuoles, in contrast with SCV.

Pertinent regression parameters of dose-response curves for intracellular activity against SCV and NP

ATB	SCV					NP				
	MIC [a]	E_{max} (CI) [b]	EC_{50} (CI) [c]	C_{static} [c]	R^2	MIC [a]	E_{max} (CI) [b]	EC_{50} (CI) [c]	C_{static} [c]	R^2
GEN	0.125	-0.78	1.88 *	1.73	0.959	0.5	-0.44	0.54	1.50	0.905
		(-0.91 to -0.64)	(1.27 to 2.77)				(-0.64 to -0.23)	(0.29 to 1.00)		
ORI [e]	0.015	-1.68 *	62.47	6.12	0.994	0.03	-0.72	81.47	6.91	0.976
		(-1.81 to -0.55)	(53.81 to 72.51)				(-0.90 to -0.53)	(74.39 to 88.55)		
		-2.87 *	601.9 *	NA	0.829		-1.63	1141	NA	0.938
		(-3.17 to -2.45)	(239.3 to 1395)				(-1.76 to -1.45)	(758.8 to 1598		
OXA	0.125	-1.03	2.02 *	1.59	0.948	0.5	-0.84	0.20 *	0.64	0.970
		(-1.24 to -0.87)	(1.33 to 3.23)				(-1.93 to -0.76)	(0.14 to 0.27)		
MXF	0.125	-0.63 *	0.87 *	1.58	0.957	0.25	-1.14	0.46 *	0.93	0.945
		(-0.78 to –0.48)	(0.58 to 1.31)				(-1.29 to -0.93)	(0.29 to 0.74)		

[a] mg/L
[b] CFU decrease (in $\log_{10}$ units) at time = 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration = ∞
[c] concentrations (in multiples of the MIC) causing a reduction of the inoculum half-way between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation.
[d] concentration (in multiples of the MIC) resulting no apparent bacterial growth (number of CFU identical to the initial inoculum), as determined by graphical intrapolation.
[e] 2 successive sigmoidal regressions were use to fit to actual intracellular data
* p < 0.05 for SCV versus NP

AIM OF THE STUDY

To study the activity

- of 4 antibiotics selected for their contrasting cellular pharmacokinetics (3):
  - Gentamicin (GEN): low cellular accumulation (2-4 x); localized in lysosomes
  - Oritavancin (ORI): very high cellular accumulation (> 100 x); localized in lysosomes
  - Oxacillin (OXA): low cellular accumulation (~ 1 x); mainly localized in the cytosol
  - Moxifloxacin (MXF): high accumulation (~ 15 x); mainly localized in the cytosol but probably able to easily redistribute between cell compartments
- against isogenic strains of *S. aureus* isolated from a same CF patient but showing SCV or normal phenotype.
- in a model of normal airway epithelial cells.

METHODS

- **Bacteria:** we used a stable thymidine-auxotrophic SCV and its isogenic normal phenotype isolated from the same CF patient (6).
- **Susceptibility testing:** MICs were determined by microdilution method in MH broth with reading made after 24 h and 48 h of incubation, respectively for SCV and NP. Polysorbate 80 (0.002 %) was added to oritavancin solution to prevent adherence to plastic surfaces (7).
- **Cell infection and determination of intracellular activity:** Phagocytosis of bacteria by airway epithelial cells (kind gift of Prof. Vaerman, Unité de Médecine Expérimentale, UCL) was allowed during 2 h, with an inoculum of 25 bacteria/cell. Cells were then incubated with 50 mg/L gentamicin for 1 h (to eliminate non-internalized bacteria) and reincubated in fresh medium containing the tested antibiotic or lysostaphin at 10 mg/L (control) to prevent extracellular growth of bacteria. The post-phagocytosis inoculum typically ranged from 0.8 to $1.0x10^6$ CFU/mg cell protein and the extracellular bacteria contamination was minimal (< 0.5%). Intracellular activity was measured after 24 h of exposure to antibiotic with a wide range of concentrations to obtain full pharmacological dose-response curves. CFU were counted after 24 h (NP) or 48 h (SCV) incubation of serial dilutions of cell lysates plated on BHI agar.
- **Confocal microscopy:** Infection was done as described above with bacteria labeled with fluorescein-isothiocyanate (FITC). To stain the acidic compartments, 1 µM LysoTracker Red DND-99 was added to the medium 1 h prior harvesting the cells. At 3 h post-infection, cells were washed and fixed with 3.7% paraformaldehyde. After washing, specimens were dried and mounted in 2.5% DAPCO in Mowiol. Observations were made on an confocal microscope and images were digitally recorded with a Focus Graphics image recorder and treated with the Confocal Assistant software.

CONCLUSIONS

- Lysosomotropic antibiotics (GEN, ORI), show improved efficacy (higher E_{max}) against SCV as compared to NP.
- Cytosolic antibiotics (OXA, MXF), show higher potency (lower EC_{50}) against NP than against SCV.
- These differences may be rationalized by the apparent different subcellular localization of both strains, antibiotics proving more effective or potent in the compartment where they accumulate.
- Oritavancin is the most active drug in any case.
- Our results suggest the interest of in vitro models for understanding the parameters that can determine intracellular activity of antibiotics.

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