Characterization of the Intracellular Growth of Menadione and Hemin-dependent Small-Colony Variants (SCV) of *Staphylococcus aureus* in a Model of THP-1 Macrophages: Consequences for Antibiotic Intracellular Activity

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ABSTRACT

BACKGROUND: Small-colony variants (SCVs) of *Staphylococcus aureus* are a major cause of persistent infections. This is related to their capacity of surviving within eukaryotic cells, where they are protected from immune defenses. We have developed a model of *S. aureus*-infected THP-1 macrophages and used it here to compare the intracellular growth of a MRSA strain and its two stable menadione- and hemin-dependent SCVs and to assess their intracellular susceptibility to antibiotics.

METHODS: Strains: MRSA COL (K7), *menD* disruptant (K8), *hemB* disruptant (K9), and *hemB* complemented strain (K10). Extracellular growth: measured by change in OD620nm in MH broth (complemented or not with menadione or hemin for K8 and K9, respectively). Growth in infected cells: assessed from the change in CFU from post-phagocytosis value after 24 h of incubation. Antibiotic activity: measured as the change in CFU from post-phagocytosis value after 24 h of incubation with drug concentrations varying from 0.0.1 to 100 x the MIC, with calculation of pertinent pharmacodynamic parameters (static concentration [no apparent change of intracellular inoculum] and Emax [decrease in log CFU compared to post-phagocytosis inculum for an infinitely large antibiotic concentration], based on regression parameters of the Hill equation [sigmoid dose-response).

RESULTS: Extracellular growth: K8 and K9 displayed only minimal growth over 8h. Addition of menadione (2 µg/mL) allowed for partial reversion K8 growth, whereas addition of hemin (2 µg/mL) allowed full reversal for K9 (similar growth rate as K7 or K10). Growth in THP-1 cells: K9 multiplied at the same rate as its parental strain K7 or the K10 complemented strain (3-3.5 log CFU/mg prot over 24 h), whereas K8 intracellular growth was much slower (1 log CFU/mg prot over 24h). Antibiotic intracellular maximal efficacies (Emax) for K8, K7 and K9 were for vancomycin: -0.74, -0.31, and -0.69; for doripenem, -1.10, -0.84, and -0.83; for rifampin: -1.42, -1.75, and -1.24; for moxifloxacin: -1.55, -1.58, and -0.86; for daptomycin, -1.58, -0.84, and -0.27; for gentamicin: -1.86, -0.99, and -0.66; and for oritavancin: -2.68, -1.08, and -1.39). Static concentrations for intracellular bacteria were close to their MIC in broth for all tested antibiotics.

CONCLUSION: The marked difference in cell-associated growth between K8 and K9 strains suggests that the intracellular milieu may contain nutrients essential for the growth of a hemin-dependent strain and, to a lesser extent for a menadione-dependent strain. Yet, this slower growth does not affect the intracellular efficacy of antibiotics, which appears similar or even higher than against strains showing more rapid intracellular growth. The study, therefore, shows that antibiotic intracellular activity is not systematically linked to the growing capabilities of the intracellular bacteria.

INTRODUCTION

S. aureus Small-Colony Variants (SCVs) play a critical role in the pathogenesis associated with staphylococcal diseases, especially in antibiotic-refractory, recurrent and persistent infections such as chronic airway infections in cystic fibrosis patients (1).

S. aureus SCVs are auxotrophic for distinct growth factors such as thymidine, menadione and/or hemin. They showed phenotypic characteristics including slow growth, decreased pigmentation, intracellular persistence, low coagulase activity, and reduced hemolytic activity. The SCV phenotype is nearly restored to normal by growth with appropriate medium supplements (2-4).

SCVs have a propensity to survive within eucaryotic cells (5-6), which can contribute to the persistence of infection and the difficulty to eradicate them with antibiotics. We previously showed that a thymidine-dependent SCV was poorly susceptible to most antibiotics in a model of intracellular infection, with only oritavancin reaching a bactericidal effect after 24 hours of incubation (7). Yet, there is now data so far regarding the activity of antibiotics against hemin or menadione-dependent intracellular SCVs. Hemin and menadione are two compounds involved in the synthesis of the electron carriers cytochrome and menaquinone. Hemin or menadione-dependent phenotypes are unstable, but stable in vitro mutants can be obtained by inactivation of the *hemB* gene (one of the genes encoding enzymes of the porphyrin biosynthetic pathway) (6) or of the *menD* gene (one of the genes required for menadione biosynthesis) (8).

AIM OF THE STUDY

The aim of this work was to compare the extracellular and intracellular growth of the MRSA strain *S. aureus* COL, in comparison with its stable *menD* and *hemB* mutants with SCV phenotype and with a *hemB* complemented strain and to examine in parallel the intracellular activity of seven antistaphylococcal drugs against these strains.

METHODS

Bacterial strains: *S. aureus* COL (K7; MRSA), its respective *menD* (K8) and *hemB* (K9) mutants and *hemB* complemented strain (K10) were used in this study. The *hemB and menD* mutants were constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene and an *ermC* cassette-inactivated *menD* gene, respectively (6; 8).

Extracellular growth and supplementation: It was measured by change in OD 620 nm in Mueller Hinton Broth supplemented or not with menadione or hemin.

Susceptibility testings: MICs were measured by microdilution in Mueller Hinton Broth.

Cells: Experiments were performed with human THP-1 cells (ATCC TIB-202, Manassas,VA), a myelomonocytic cell line displaying macrophage-like activity.

Cell infection and determination of the intracellular activities of antibiotics: Phagocytosis was initiated at a bacteria per macrophage ratio of 10 (1h at 37°C), followed by elimination of non-phagocytosed bacteria by exposing the cells to 50 mg/L gentamicin (45min). Cells were then transferred to fresh medium supplemented with increasing concentrations of antibiotics (0.001- to 150 mg/L) for 24 hours. Results, expressed as the change in the intracellular inoculum at 24 h compared to time 0, were used to fit a Hill equation to allow determination of the values of two key pharmacological descriptors of antibiotic activity (static concentration and minimal and maximal relative efficacy).

RESULTS (1)

Figure 1. Extracellular growth and Supplementation

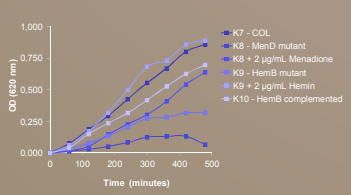
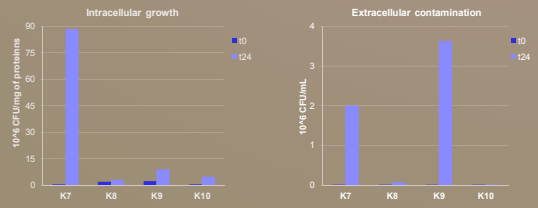


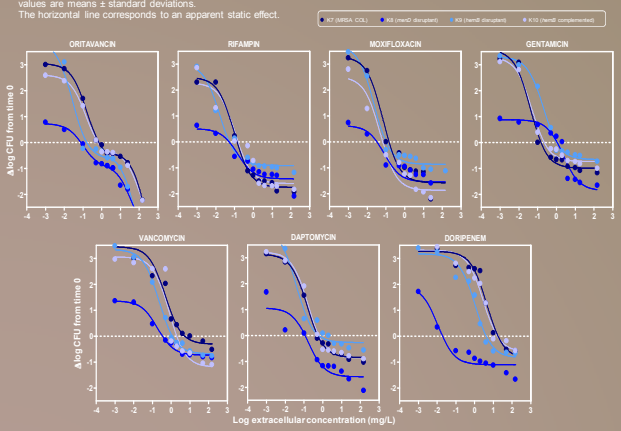
Figure 2. Intracellular growth



RESULTS (2)

Figure 2. Intracellular activity of antibiotics: Dose-Response Curves

Dose-response curves of antibiotics towards the different isogenic strains of *S. aureus* phagocytized by THP-1 cells.
Cells were incubated with the antibiotic for 24 h at the concentrations (total drug) indicated on the abscissa.
The ordinate shows the change in the number of CFU per mg of cell protein as compared to the post-phagocytosis inoculum. All values are means ± standard deviations.
The horizontal line corresponds to an apparent static effect.



➪ ORI shows bimodal effects (2 zones of concentration dependency separated by a plateau)

➪ ORI, RIF and MXF display an intense intracellular activity against all strains

➪ GEN, VAN and DAP show lower but still high significant intracellular activity against all strains, especially against K8

➪ DOR regained activity against intracellular SCVs of MRSA

Table 1. Intracellular activity of antibiotics: Pharmacological Descriptors

AB	K7			K8			K9			K10		
	E_{min}	E_{max}	E_{min}-E_{max}	E_{min}	E_{max}	E_{min}-E_{max}	E_{min}	E_{max}	E_{min}-E_{max}	E_{min}	E_{max}	E_{min}-E_{max}
ORI	2.95	> - 2	> 4.95	0.19	> - 2	> 2.19	3.68	> - 2	> 5.68	2.50	> - 2	> 4.50
MXF	3.29	-1.58	4.87	0.63	-1.55	2.18	3.74	-0.86	4.60	2.52	-1.87	4.38
RIF	2.50	-1.75	4.25	0.51	-1.42	1.94	3.02	-0.91	3.93	2.28	-1.61	3.88
GEN	3.57	-0.99	4.56	0.88	-1.86	2.74	3.29	-0.66	3.95	3.29	-0.73	4.02
VAN	3.45	-0.31	3.76	1.36	-0.74	2.10	3.34	-0.69	4.03	3.06	-1.19	4.25
DAP	3.15	-0.84	3.99	1.07	-1.58	2.65	4.08	-0.27	4.35	3.23	-0.85	4.08
DOR	3.27	-0.84	4.11	1.93	-1.10	3.03	3.17	-0.83	4.00	3.31	-0.59	3.91

E_{min}: Max. increase in log CFU compared to initial inoculum for an infinitely low AB conc. (expressed in Δlog CFU from time 0);
E_{max}: Max. decrease in log CFU compared to initial inoculum for an infinitely high AB conc. (expressed in Δlog CFU from time 0);
$E_{min} - E_{max}$: Amplitude of the antibacterial response (expressed in Δlog CFU from time 0).

AB	K7		K8		K9		K10	
	MIC	C_{stat}	MIC	C_{stat}	MIC	C_{stat}	MIC	C_{stat}
ORI	0,125 – 0,25	0.71	0.03	0.27	0.03	0.14	0,125 - 0.25	0.65
MXF	0.03	0.14	0.125	0.03	0.125	0.10	0.03	0.07
RIF	0.03	0.13	0.03	0.05	0.03	0.05	0.03	0.15
GEN	0.125	0.15	2	1.05	1	1.05	0.25	0.23
VAN	2	5.21	1	0.34	2	1.14	2	2.00
DAP	0.5	0.56	0.5	0.11	0.25	0.60	1	0.60
DOR	16	17.30	32	0.02	16	4.10	16	15.97

C_{stat}: Extracellular concentration of antibiotic yielding no apparent change in CFU after 24 hours compared to the post-phagocytosis inoculum (expressed in mg/L, in log).

All parameters were calculated from sigmoidal dose-response with Hill coefficient of 1 as shown in Figure 2.
For oritavancin, 2 successive sigmoidal curves were fitted to the data and maximal effect was not yet reached at the highest concentration tested.

CONCLUSIONS

Intracellular growth
- The menadione dependent stain show a much slower intracellular growth than the menadione-dependent strain or the normal phenotype parent, which is associated with a lower ability to contaminate the extracellular medium. This suggest that the intracellular medium may contain in appropriate concentrations the nutriments needed to the growth of the hemin-dependent strain, but not of the menadione-dependent strain.

SCV versus other strains
- Extracellularly, all drugs show similar intrinsic activity (MIC) against SCVs and parental strain, except gentamicin which is less active against SCV, as usually observed for SCVs (9).
- Intracellularly, the menadione-dependent SCV (K8) shows a slower growth (lower Emin). Yet, this does not impair the efficacy of antibiotics (similar or higher Emax than for other strains).

Antibiotic intracellular activity
- Oritavancin, moxifloxacin, and rifampin are the only drugs capable of reducing the intracellular counts of more than 1 log at 24 hours for all strains. These drugs also proved the more efficient against a thymidine-dependent SCV in the same intracellular model (7).
- Oritavancin shows bimodal effects against all strains (2 successive zone of concentration-dependent activity, as already described against intracellular thymidine-dependent SCV [4]). This may denote the multiple modes of action of this drug (10).
- As previously described for other β-lactams and MRSA strains (9), doripenem regains activity intracellularly against the COL MRSA and its SCV variants as a consequence of the effect of the acidic pH of the phagolysosomes on PBP2a conformation (11).

Clinical implication
- Selection of antibiotics showing intracellular activity against SCVs may be important when dealing with infections for SCVs play an important role, as is the in cystic fibrosis or persistent osteomyelitis, for example (1).

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