Effect of Menadione Supplementation on the Intracellular Growth of a Menadione Auxotrophic Small-Colony Variant (SCV) Mutant of *Staphylococcus aureus* in a Model of THP-1 Macrophages: Consequences for Antibiotic Intracellular Activity

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Abstract

BACKGROUND: We showed that a menD SCV mutant of *Staphylococcus aureus* grew much more slowly intracellularly than a hemin-dependent SCV, suggesting that the intracellular milieu does not contain required nutrients. We have now examined how menadione supplementation affects intracellular growth and antibiotic activity in infected THP-1 cells.

METHODS: Strains: MRSA COL (K7) and its Δ*menD* mutant displaying the SCV phenotype (K8). Infected THP-1 incubated with AB in medium supplemented with 5 mg/L menadione-sodium bisulfite (MSB); activity measured as the change in CFU from post-phagocytosis value after 24 h incubation with AB (conc: 0.01-100 x MIC). PD parameters calculated using Hill equation.

RESULTS: Iintracellular growth (Emin) was reduced by 2 log for K8 but restored by MSB. AB efficacy (Emax) was similar or lower (0.5-1 log) for K8 vs. K7 but higher (0.5-1 log) for RIF, DOR, MXF, and DAP in MSB-Suppl medium. ORI was equally efficient against K7, K8, and suppl. K8 and the most effective of all AB tested (Emax > - 2 log).

CONCLUSION: MSB suppl. restores intracellular growth and efficacy of antibiotics, suggesting that poor intracellular activity of most antibiotics was due to slow growth. The fact that ORI was as effective in all conditions indicates a mode of action independent of growth, which may be useful for eradicating persistent infections.

Introduction

Small-Colony Variants of *Staphylococcus aureus* play a critical role in the pathogenesis associated with staphylococcal diseases, especially in persistent, recurrent and antibiotic-refractory infections (1-3) such as chronic airway infections in cystic fibrosis patients (4).

SCVs are auxotrophic for distinct growth factors such as thymidine, menadione and/or hemin. They show 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; 5-6).

SCVs have a propensity to survive within eucaryotic cells (7-8), which can contribute to the persistence of infection and the difficulty to eradicate them with antibiotics. We previously demonstrated that a menadione-dependent strain showed much slower intracellular growth than an hemin-dependent strain or its normal phenotype parent (ICAAC 2010; abs. A1-678). This suggests that the intracellular medium may contain appropriate concentrations of the nutriments required for growth of the hemin-dependent strain, but not of the menadione-dependent strain.

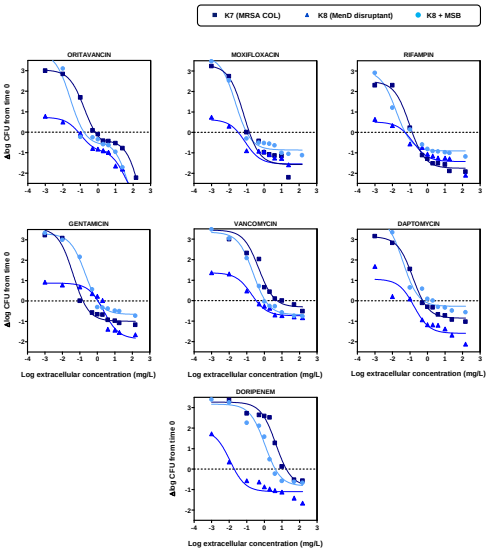
Menadione is required by the bacteria for the synthesis of menaquinone. In vivo, menadione-dependent phenotypes are unstable, but stable in vitro mutants can be obtained by inactivation of the *menD* gene [one of the genes required for menadione biosynthesis (9)].

The aim of this work was to study the effect of menadione supplementation on the intracellular growth of the *menD* mutant and on the activity of seven antistaphylococcal drugs in infected THP-1 cells.

Results (1)

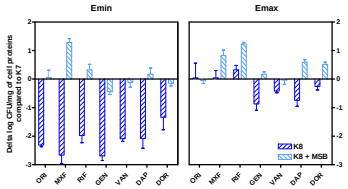
Figure 1. Intracellular activity of antibiotics

Dose-response curves of antibiotics against 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 (n=3). The horizontal line corresponds to an apparent static effect.



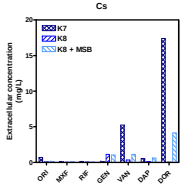
Results (2)

Figure 2. Relative intracellular activity of antibiotics:
Minimal (Emin), maximal (Emax) change in bacterial counts in dose-effect experiments



Antibiotics: Oritavancin (ORI), Moxifloxacin (MXF), Rifampin (RIF), Gentamicin (GEN), Vancomycin (VAN), Daptomycin (DAP), Doripenem (DOR)
Emin: Minimal efficacy (maximal intracellular growth observed for an infinitely low antibiotic concentration [data expressed as difference in log CFU versus K7]) - *A negative value means a lower intracellular growth compared to K7*
Emax: Maximal efficacy (decrease in log CFU compared to initial inoculum for an infinitely high antibiotic concentration [expressed as difference in log CFU versus. K7]) - *A positive value means a reduced efficacy compared to K7*

Figure 3. Relative potency of antibiotics at Static concentration (Cs; intracellular)



Cs: Extracellular concentration of antibiotic yielding no apparent change in CFU after 24 hours compared to the post-phagocytosis inoculum - *The lower the Cs, the higher the potency*
All parameters were calculated from sigmoidal dose-response with Hill coefficient of 1 as shown in Figure 1.

Methods

Bacterial strains: *S. aureus* COL (K7; MRSA) and its respective *menD* mutant (K8) were used in this study. The *menD* mutant was constructed by allelic replacement with an *ermC* cassette-inactivated *menD* gene (8).

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 (10): 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 CFU at 24 h compared to the intracellular inoculum at 0 h, 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).

Suppplementation: Medium was supplemented with or without Menadione Sodium Bisulfite (5 µg/mL).

Conclusions

Intracellular growth

- MSB supplementation completely restores the intracellular growth of the menadione-dependent strain.

Antibiotic intracellular activity

- MSB restores efficacy of antibiotics, suggesting that poor intracellular activity of most antibiotics was due to slow growth.
- Oritavancin, moxifloxacin, and rifampin are the most efficient, being the only drugs capable of reducing the intracellular counts by more than 1 log at 24 hours for all strains. They are also the most potent, with low static concentrations, and no influence of MSB on potency.
- As previously described for other β-lactams and MRSA strains (12), doripenem regains activity intracellularly against the COL MRSA parental strain and its menadione-dependent variant as a consequence of the effect of the acidic pH of the phagolysosomes on PBP2a conformation (13).
- Oritavancin shows a bimodal effect against all strains [2 successive zone of concentration-dependent activity, as already described against intracellular thymidine-dependent SCV (11)]. It is also the only drug that showed the same efficacy against the parental strain, its menadione-dependent mutant and the complemented strain. This may denote the fact that oritavancin has multiple modes of action independent of growth, which may be useful for eradicating persistent infections.

References

(1) Von Eiff. 2008. *Int J Antimicrob Agents*. 31:507-10; **(2)** Proctor et al. 1995. *Clin Infect Dis*. 20:95-102; **(3)** Proctor et al. 1994. *Infect Agents Dis*. 3:302-312; **(4)** Tuchscherr et al. 2010. *J Infect Dis*. 202:1031-40; **(5)** Von Eiff et al. 2001. *Clin Infect Dis*. 32:1643-1647; **(6)** Kahl et al. 1998. *J Infect Dis*. 177:1023-1029; **(7)** Becker et al. 2004. *J Clin Microbiol*. 42:4988-4995; **(8)** Von Eiff et al. 1997. *J Bacteriol*. 179:4706-4712; **(9)** Bates et al. 2003. *J Infect Dis*. 187:1654-1661; **(10)** Barcia-Macay et al. 2006. *Antimicrob Agents Chemother*. 50:841-851; **(11)** Van Bambeke et al. 2008. *Trends Pharmacol Sci*. 29:124-34; **(12)** Lemaire et al. 2007. *Antimicrob Agents Chemother*. 51:1627-1632; **(13)** Lemaire et al. 2008. *J Biol Chem*. 283:12769-76.

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