PHARMACODYNAMIC EVALUATION OF THE INTRACELLULAR ACTIVITY OF ANTIBIOTICS AGAINST HEMIN AND MENADIONE-DEPENDANT SMALL-COLONY VARIANT (SCV) MUTANTS OF *STAPHYLOCOCCUS AUREUS* IN A MODEL OF ACTIVATED OR NOT THP-1 CELLS

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ABSTRACT

BACKGROUND: *Staphylococcus aureus* has been associated with recurrent and persistent infections in Cystic Fibrosis patients. This may be ascribed to the presence of intracellular (IC) Small-Colony Variants (SCV), which are protected from immune defenses and antibiotics (AB). We have developed a model of *S. aureus*-infected THP-1 cells and used it to assess the IC activity of AB against a MRSA strain and its two stable menadione- and hemin-dependent SCVs. As the menadione-dependent strain shows a slower IC growth (see abstract 1130322), we also examined the effect of Menadione Sodium Bisulfite (MSB) supplementation on AB activity against this strain.

METHODS: Activation: Incubation of THP-1 monocytes with PMA (200 nM, 48 h). AB accumulation: Incubation of cells with AB for 2 h; cellular concentrations measured by fluorimetry for MXF and microbiological assays for RIF and GEN. Strains: MRSA COL (K7), *menD* disruptant (K8), *hemB* disruptant (K9) and *hemB* complemented strain (K10). IC activities: Change in CFU from the post-phagocytosis inoculum after 24 h incubation with AB (extracellular concentration: 0.01-100 x MIC); PD parameters calculated using Hill equation of dose-response (AAC, 2006, 50:841-851). MSB suppl.: Infected THP-1 incubated with AB in medium suppl. with 5 mg/L MSB.

RESULTS: In non-activated THP-1, IC growth was slower (lower Emin) as well as the amplitude of the dose-response (Emin-Emax) for K8 than for the other strains, but both parameters were restored upon MSB supplementation. RIF and MXF reached a Emax > 1 log reduction against the three strains, and GEN and DOR against K8 only (note that DOR was active against IC SCV even if MRSA, as described for non-SCV strains [AAC 2007, 51:1627-32]). Static concentrations (Cs: extracellular concentration for which no change in IC inoculum is observed) were all close to the MICs (not shown). Dose-response curves for K10 matched those of K7. Activation of THP-1 cells is accompanied by an increase in AB accumulation (1.7, 1.8 and 3.9 for RIF, MXF and GEN, respectively) and a 2- to 15-fold increase in their AB potency (decrease in Cs [not shown]).

CONCLUSION: In non activated THP-1, the global amplitude of the response was dependent on the growth rate of the strain. RIF, MXF - two bactericidal AB- are the most effective, as observed also for a thymidine-dependent SCV in the same model (AAC 2009, 53:1434–42). Moreover, activation of monocytes in macrophages enhances the potency of AB, probably in relation to their increased accumulation.

INTRODUCTION

Staphylococcus aureus can produce subpopulations which are phenotypically very different from the parent strain. These naturally occurring subpopulations grow slowly, leading to colonies that are named “Small Colony Variants” (SCVs). The biochemical basis of this phenotypic abnormality is an auxotrophism for distinct growth factors such as menadione, hemin and/or thymidine (1).

Related to their slow metabolism as well as to their propensity to persist and survive inside eucaryotic cells, 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 (2-4).

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.

Due to metabolic and morphological similarities, the human monocytic cell line THP-1 can be differentiated to macrophages by the protein kinase C activators Phorbol 12-Myristate 13-Acetate (5). These adherent THP-1 cells showed remarkable phenotypic changes demonstrating their activated state, as an increased phagocytic activity (6) and an increased in the intracellular activity of antibiotics against some bacteria (7).

The aim of this study were, firstly, to study the effect of menadione supplementation on the intracellular growth of the *menD* mutant. and secondly, to compare unstimulated and phorbol-ester-differentiated THP-1 cells for the uptake of different antibiotics and for their activity against phagocytosed *S. aureus* and its SCVs mutants.

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 (7, 8).

Cells: Experiments were performed with human THP-1 cells (ATCC TIB-202, Manassas, VA), a myelomonocytic cell line displaying macrophage-like activity. Activation of THP-1 was obtained by the addition of 200 ng/mL of PMA during 48 hours.

Accumulation: Cells were incubated with an extracellular concentration equal to the Cmax measured in the serum of patients, for 2 hours, at 37°C. MXF was measured by fluorimetry; RIF and GEN by microbiological assay.

Cell infection and determination of the intracellular activities of antibiotics (9): 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).

Suppplementation experiments: Medium was supplemented with Menadione Sodium Bisulfite (5 µg/mL).

CONCLUSION

Accumulation
- Differentiation of monocytes into macrophages leads to an increased accumulation of antibiotics (1.7, 1.8 and 3.9-fold for RIF, MXF, and GEN, respectively);

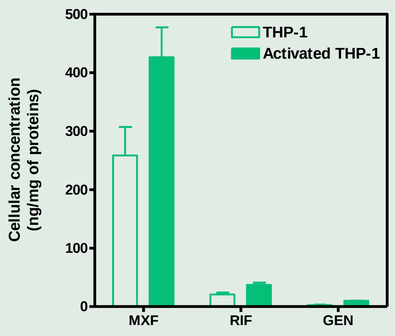
Intracellular growth
- MSB supplementation completely restores the intracellular growth of the menadione-dependent strain.

Intracellular activity of antibiotics
- MSB restores efficacy of antibiotics, suggesting that poor intracellular activity of most antibiotics was due to slow growth;
- Activation of monocytes in macrophages enhances the potency of antibiotics against *S. aureus* and its SCV forms (Cs decreased of 2 to 15 times). This could result from their higher cellular accumulation. In contrast, maximal efficacy is not affected.

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 (2).

RESULTS

FIGURE 1. Accumulation of antibiotics



Antibiotic	Accumulation ratio*
MXF	1.65
RIF	1.79
GEN	3.88

* Data expressed as the ratio of accumulation in activated vs. non-activated cells

FIGURE 2. Cell infection protocole

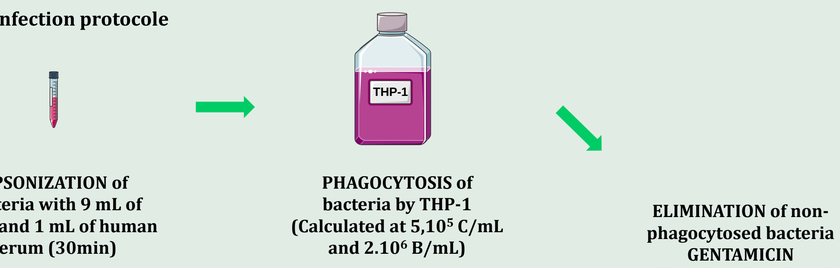
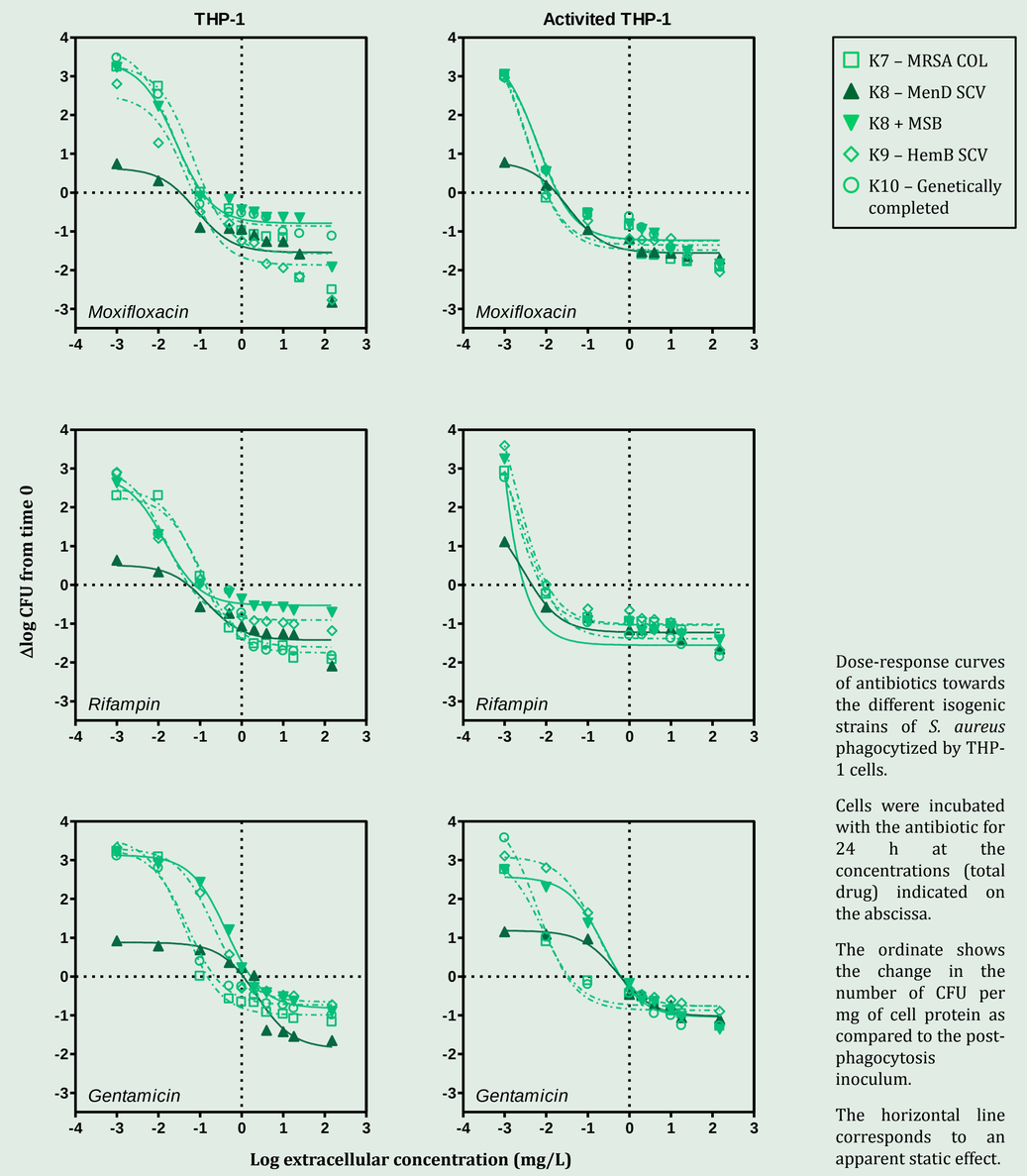


FIGURE 3. Intracellular potencies: Static concentration (Cs)

Antibiotic	Intracellular potency ratio (Cs)			
	K7	K8	K9	K10
RIF	0.06	0.08	0.20	0.28
MXF	0.07	0.51	0.19	0.15
GEN	0.23	0.55	0.62	0.13

Cs: Extracellular concentration of AB yielding no apparent change in CFU after 24 hours compared to the post-phagocytosis inoculum (intrapolated from dose-response curve); data expressed as the ratio of this value in activated vs. non-activated cells

FIGURE 4. Intracellular activities: 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.

The horizontal line corresponds to an apparent static effect.

REFERENCES

(1) Proctor et al. 2006. *Nat Rev Microbiol.* 4:295–305; **(2)** Von Eiff. 2008. *Int J Antimicrob Agents*. 31:507-10; **(3)** Proctor et al. 1995. *Clin Infect Dis.* 20:95-102; **(4)** Proctor et al. 1994. *Infect Agents Dis.* 3:302-312; **(5)** Tsuchiya et al. 1992. *Cancer Res.* 42:530–6; **(6)** Tominga et al. 1998. *J Exp Med.* 186:99–11; **(7)** Bates et al. 2003. *J Infect Dis.* 187(10):1654-1661; **(8)** Von Eiff et al. 1997. *J Bacteriol.* 179:4706-4712; **(9)** Barcia-Macay et al. 2006. *Antimicrob Agents Chemother*. 50:841-851.

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