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Influence of the Protein Kinase C Activator Phorbol Myristate Acetate on the Intracellular Activity of Antibiotics against Hemin- and Menadione-Auxotrophic Small-Colony Variant Mutants of *Staphylococcus aureus* and Their Wild-Type Parental Strain in Human THP-1 Cells

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In a previous study (L. G. Garcia et al., Antimicrob. Agents Chemother. 56:3700–3711, 2012), we evaluated the intracellular fate of *menD* and *hemB* mutants (corresponding to menadione- and hemin-dependent small-colony variants, respectively) of the parental COL methicillin-resistant *Staphylococcus aureus* strain and the pharmacodynamic profile of the intracellular activity of a series of antibiotics in human THP-1 monocytes. We have now examined the phagocytosis and intracellular persistence of the same strains in THP-1 cells activated by phorbol 12-myristate 13-acetate (PMA) and measured the intracellular activity of gentamicin, moxifloxacin, and oritavancin in these cells. Postphagocytosis intracellular counts and intracellular survival were lower in PMA-activated cells, probably due to their higher killing capacities. Gentamicin and moxifloxacin showed a 5- to 7-fold higher potency (lower static concentrations) against the parental strain, its *hemB* mutant, and the genetically complemented strain in PMA-activated cells and against the *menD* strain in both activated and nonactivated cells. This effect was inhibited when cells were incubated with *N*-acetylcysteine (a scavenger of oxidant species). In parallel, we observed that the MICs of these drugs were markedly reduced if bacteria had been preexposed to H_2O_2. In contrast, the intracellular potency of oritavancin was not different in activated and nonactivated cells and was not decreased by the addition of *N*-acetylcysteine, regardless of the phenotype of the strains. The oritavancin MIC was also unaffected by preincubation of the bacteria with H_2O_2. Thus, activation of THP-1 cells by PMA may increase the intracellular potency of certain antibiotics (probably due to synergy with reactive oxygen species), but this effect cannot be generalized to all antibiotics.

Small-colony variants (SCVs) of *Staphylococcus aureus* have a propensity to survive within eukaryotic cells, and this propensity has been associated with the persistent and/or recurrent character of the infections that they cause (26, 40–42, 51), most conspicuously in cystic fibrosis patients (49, 54). Moreover, their slow growth and metabolic defects impair the activity of many antibiotics (8, 25).

In a previous paper (20), we compared the intracellular growth of isogenic menadione- and hemin-dependent SCVs with that of their parental strain in human THP-1 cells and examined the activity of antibiotics against the intracellular forms of these bacteria. These cells are indeed widely used for studying the intracellular activity of antibiotics against phagocytized bacteria (6, 27, 30, 33–35, 38, 46). We observed that the menadione-dependent mutant grew much more slowly in THP-1 cells than the hemin-dependent strain but remained as susceptible to antibiotics in terms of maximal reduction of the inoculum compared to the postphagocytosis values.

THP-1 cells were originally obtained from a patient suffering from acute monocytic leukemia. Although displaying many of the characteristics of macrophages (47), these cells maintain their monomyelocytic phenotype in culture and have notoriously poor antibacterial defense mechanisms. THP-1 cells, however, can be differentiated into macrophage-like cells by exposure to the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) (43). The most remarkable changes accompanying this differentiation are a capacity to adhere to surfaces, increased phagocytic activity toward latex beads, and stimulation of superoxide production (19, 43). The consequences of these changes concerning the uptake and intracellular survival of bacteria and how they modulate the intracellular activity of antibiotics remain, however, poorly understood. In the present study, we examined how differentiation of THP-1 cells by PMA affects the intracellular fate of an *S. aureus* strain with a normal phenotype and its menadione- and hemin-auxotrophic SCV mutants when exposed to antibiotics. Antibiotics were selected from among those having proved the most and least effective in nonactivated cells, namely, moxifloxacin and oritavancin (20), with the aim to examine whether cell activation could contribute to further improvement of their activity. Gentamicin was studied in parallel as a control drug showing less activity against intracellular bacteria but being highly efficient against extracellular bacteria.

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MATERIALS AND METHODS

Antibiotics and main reagents. Moxifloxacin and oritavancin were obtained as microbiological standards from Bayer HealthCare (Leverkusen, Germany) and from The Medicines Company (Parsippany, NJ), respectively. Gentamicin was obtained as the branded product commercialized in Belgium for human use (Geomycin [distributed by GlaxoSmithKline S.A.-N.V., Genval, Belgium]). Pooled human serum from healthy volunteers was purchased from Lonza Ltd. (Basel, Switzerland) and stored at −80°C until use. Cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA), and other reagents were from Sigma-Aldrich (St. Louis, MO) or Merck KGaA (Darmstadt, Germany).

Bacterial strains and MIC determinations. Four isogenic strains were used through this study, namely, strain COL (wild-type, hospital-acquired methicillin-resistant *Staphylococcus aureus*), its *menD* and *hemB* SCV mutants (constructed by allelic replacement with an *ermB* cassette-inactivated *hemB* gene and an *ermC* cassette-inactivated *menD* gene, respectively [7, 52]), and a *hemB* genetically complemented (*hemB*gc) strain. SCVs were grown either in Mueller-Hinton broth (MHB) or in the same medium supplemented with 2 μg/ml menadione sodium bisulfite (MSB) or hemin (20). Unless stated otherwise, MICs were determined in MHB adjusted to pH 7.4 or pH 5.5 after 24 h and following the general recommendations of the Clinical and Laboratory Standards Institute (CLSI) (12, 13).

Cells and differentiation method. All experiments were conducted with human THP-1 monocytic cells (ATCC TIB-202 [American Type Culture Collection, Manassas, VA] [47]) differentiated to adherent macrophages by incubation with PMA (200 μg/liter; Sigma-Aldrich) for 48 h at 37°C (45).

Cell infection and intracellular activity of antibiotics. Cell infection and assessment of antibiotic activities were performed exactly as described previously (20).

Influence of H_2O_2 on antibiotic activity. Bacteria were preincubated for 30 min in the dark with 10 mM H_2O_2 in MHB. This concentration was selected as minimally affecting the growth of normal-phenotype strains in the absence of added antibiotic (37). Bacteria were then pelleted and resuspended in fresh broth for determination of the MICs as described above, but with readings made after both 24 and 48 h. Both values were recorded because significant differences were seen when dealing with bacteria preexposed to H_2O_2.

Curve-fitting and statistical analyses. Curve-fitting analyses and determination of the pertinent regression parameters of the concentration-response experiments were made with GraphPad Prism software (version 4.03; GraphPad Software, San Diego, CA). Data were used to fit monophasic or biphasic sigmoidal functions as previously described (20). Statistical analyses were performed with GraphPad Instat software (version 3.06; GraphPad Software).

RESULTS

Phagocytosis and intracellular growth. In a first series of experiments (Fig. 1, top), we examined the influence of cell activation by PMA on phagocytosis and intracellular growth of the wild-type strain, its *menD* and *hemB* SCVs, and the *hemB* genetically complemented strain. In nondifferentiated cells and as previously described, SCVs, detected by determination of the numbers of CFU (viable bacteria), were less avidly internalized than the parental or the genetically complemented *hemB*gc strains (20). In differentiated cells, the internalization of all strains was further drastically decreased (15- to 30-fold). After 24 h of incubation, all strains except for the *menD* mutant showed an approximately 1.5-log-CFU increase per mg of cell protein in nonactivated cells but only an approximately 0.5-log-CFU increase per mg cell protein in PMA-activated cells; the *menD* mutant did not grow in either cell type. The actual counts of viable bacteria associated with the cells were therefore 150- to 400-fold lower in PMA-activated cells than nonactivated cells at 24 h for all strains except the *menD* mutant, for which there was only a 50-fold difference between PMA-activated and nonactivated cells. Interestingly, because of their lower initial value, the supplemented *menD* mutant and the *hemB* mutant reached 2- to 10-fold lower CFU counts than the parental strain in each cell type. However, by normalizing all values to those of the postphagocytosis inoculum of each strain (Fig. 1, bottom), all strains (except the *menD* mutant) were found to have a similar growth rate when considering a given cell type, but this rate was about 2-fold lower in PMA-activated cells than nonactivated cells.

Intracellular activity of antibiotics. The activity of the three selected antibiotics in PMA-activated THP-1 cells at concentrations ranging from 0.001 to 150 mg/liter was then examined after 24 h of incubation. This time point was selected because such a length of time allows the reproducible growth of bacteria (except for the *menD* mutant, which grows more slowly) and comparison with data previously obtained with nonactivated cells (20). Results are presented in Fig. 2 for both activated and nonactivated cells. Sigmoidal functions could be fitted to all data, with the best fits obtained using a monophasic function for gentamicin and a biphasic function for moxifloxacin and oritavancin. There was no significant difference between the functions describing the individual behaviors of the parental strain, the *hemB* mutant, the genetically complemented strain (*hemB*gc), and the *menD* mutant grown in MSB-supplemented medium. All data from these strains were therefore used together to build a single function, as illustrated in Fig. 2. In contrast, the *menD* mutant showed a considerably lower minimal efficacy (E_{min}) value because of its slower intracellular growth. Table 1 compares the pharmacodynamic parameters of these curves with those observed in nonactivated cells. The more salient observation concerns the differences in antibiotic potencies (apparent static concentrations [C_ss]). Thus, the potency of gentamicin against the parental strain, the *hemB* mutant, and the genetically complemented *hemB* mutant was about 7-fold higher (7-fold lower C_s) in PMA-activated cells than nonactivated cells, whereas no significant difference was seen for the *menD* mutant, whether it was tested in the presence of MSB or not. The potency of moxifloxacin was about 5-fold higher (5-fold lower C_s) against all strains (except the *menD* mutant) when measured in PMA-activated cells than nonactivated cells. In sharp contrast, the apparent static concentration of oritavancin toward all strains was similar in both cell types. No systematic differences in maximal efficacy (E_{max}) could be observed within the limits of our experimental conditions (but true E_{max} values could not be determined with confidence for several antibiotic and strain combinations due to the biphasic character of several of the concentration-effect responses and the limitations to the highest antibiotic concentrations that could be used without toxic effects to the THP-1 cells), yet global analysis of the curves showed a significant effect of cell activation on the activity of gentamicin and moxifloxacin against all strains except the *menD* mutant; these differences clearly rely on improved potency (lower apparent static concentrations) in activated cells.

Influence of *N*-acetylcysteine on antibiotic intracellular activity. Differentiation of THP-1 cells by PMA is known to activate cell defense mechanisms, including the production of reactive oxygen species (ROS) (43). We therefore examined whether the general antioxidant *N*-acetylcysteine, a scavenger of oxidative species widely used to counteract the pro-oxidant effects of phorbol esters

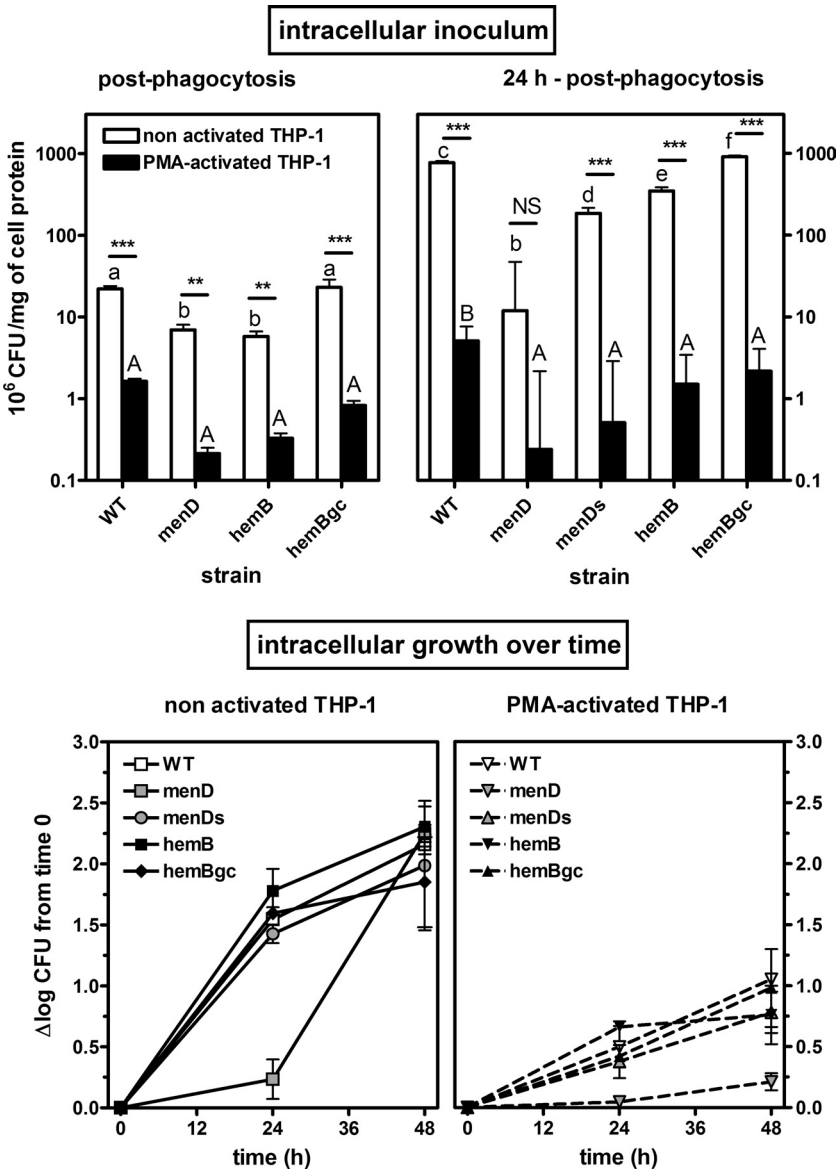

FIG 1 Comparative phagocytosis and intracellular survival of *S. aureus* of the normal phenotype (wild type [WT]), SCVs (*menD* [supplemented {menDs} or not {menD} with 2 μg/ml MSB for intracellular survival] and *hemB*), and the *hemB* genetically complemented strain (*hemB*gc) in nonactivated THP-1 cells (open bars) or in THP-1 cells activated by 48 h of incubation with 200 μg/liter PMA (solid bars). (Top) Enumeration of cell-associated CFU after 1 h of phagocytosis (left) and after 24 h of incubation postphagocytosis (right). (Bottom) Comparative growth of the strains over 48 h of incubation, with data expressed as changes from the initial inoculum. Data are means of 3 to 6 independent experiments performed in triplicate. Statistical analysis was by analysis of variance with the Tukey *post hoc* test) for differences between activated and nonactivated cells (***, $P < 0.001$; **, $P < 0.01$; NS, not significant) and differences between strains for a given type of cell (lowercase letters, comparison between strains in nonactivated cells; uppercase letters, comparison between strains in activated cells; different letters show significant differences with P values of <0.05).

(1, 11, 50), modulated the potency of antibiotics. For this purpose, infected cells were incubated for 24 h with each of the investigated antibiotics at its C_s determined from the results of the concentration-effect experiments [Table 1]) alone or in the presence of 25 mM *N*-acetylcysteine. Figure 3 shows that *N*-acetylcysteine markedly decreased the activity of gentamicin and moxifloxacin against all strains in both nonactivated and PMA-activated cells. Thus, rather than an apparent static effect, we observed substantial bacterial growth (about 1 to 3 log CFU) compared to what was observed for these antibiotics in the absence of *N*-acetylcysteine. The effect was more important in PMA-activated cells for gentamicin toward all strains except the parental strain and for moxifloxacin toward the parental and the *hemB*gc strains. The effect of *N*-acetylcysteine on oritavancin activity was less marked, with a gain of only 0.5 to 1.5 $\log_{10}$ CFU for the parental, *menD*, *hemB*, and *hemB*gc strains and a slight decrease in the numbers of CFU for the supplemented *menD* mutant (*menD*s).

Influence of H_2O_2 on antibiotic extracellular activity. To further investigate the potential cooperation between antibiotics and the oxidant species produced by phagocytes suggested by the previous experiment, we evaluated the influence of H_2O_2 on the activity of antibiotics. To determine this effect, MICs were measured

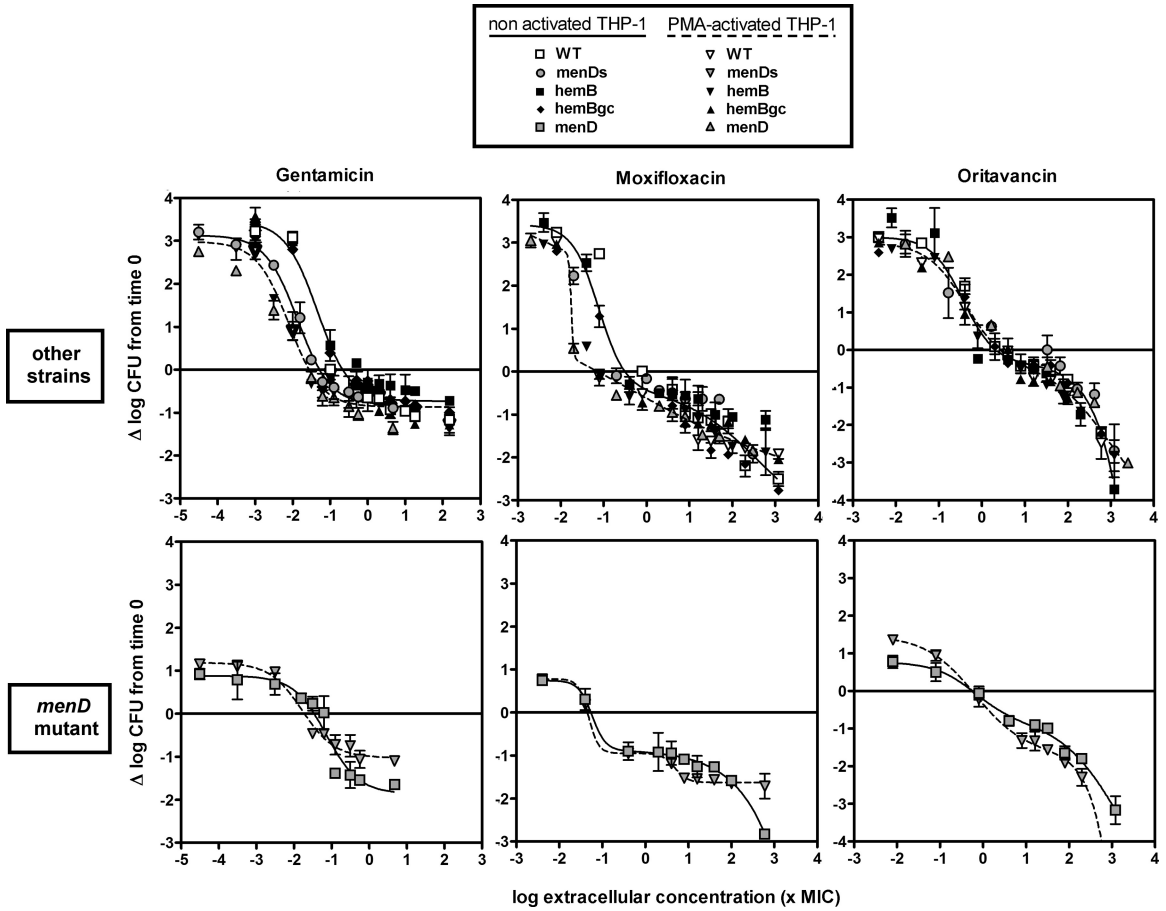

FIG 2 Concentration-response curves of gentamicin, moxifloxacin, and oritavancin in nonactivated THP-1 cells (solid line) or in cells activated by 48 h of incubation with 200 μg/liter PMA (dotted line) against the *S. aureus* parental strain with the wild-type phenotype, its *menD* mutant in medium supplemented by 2 μg/ml MSB (menDs), its *hemB* mutant (hemB) and the *hemB* genetically complemented mutant (hemBgc) (top), or its *menD* mutant under control conditions (menD) (bottom). Infected cells were incubated in the presence of increasing concentrations of antibiotics (total drug) for 24 h. The ordinate shows the change in the number of CFU (log scale) per mg of cell protein compared to the postphagocytosis inoculum. The solid horizontal line corresponds to an apparent static effect. The abscissa shows on the log scale the drug concentration in the culture medium expressed in multiples of the MIC measured at pH 5.5 (for all strains except the *hemB* strain) or pH 5.5 in the presence of hemin (for the *hemB* strain, based on data having demonstrated the availability of hemin-like compounds in the cellular medium [20]). All values are means ± standard deviations (SDs) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments were reproduced 3 times with similar results.

in broth at neutral as well as at acidic pH for bacteria preexposed to 10 mM H_2O_2 for 30 min and compared with those for unexposed bacteria. Table 2 shows that preexposing bacteria to H_2O_2 caused a drastic decrease of the MIC of gentamicin and moxifloxacin toward all strains when using readings made at 24 h. Values, however, were higher when readings were made after 48 h. Interestingly, preincubation with H_2O_2 restored the susceptibility of the *menD* mutant (supplemented or not with MSB) to gentamicin at acidic pH, and this effect partially persisted at 48 h. In contrast to the other two antibiotics, the MIC of oritavancin was essentially unaffected by preincubation of the bacteria with H_2O_2.

DISCUSSION

Although differentiation of monocytes in macrophages by PMA has been quite extensively studied, the influence of PMA on the phagocytosis of bacteria and their intracellular survival, as well as on the intracellular activity of antibiotics, has rarely been described. The present study is, therefore, one of the first to have examined these parameters in detail. We used THP-1 cells and *S. aureus* because this pair has been extensively studied by us and others using nondifferentiated cells (6, 21, 33, 35, 36, 39). To make the study as informative as possible with respect to the role played by the persistence of intracellular bacteria in the relapsing and recurrent character of staphylococcal infection, we examined wild-type *S. aureus* cells and also their SCV counterparts using a series of isogenic strains, the behavior of which in nonactivated cells has recently been characterized (20).

Considering the phagocytosis and intracellular survival of the bacteria first, we show that cell activation by PMA causes an apparent decrease in the number of viable bacteria associated with the cells after phagocytosis, as well as reduces intracellular growth upon further incubation. The reduction in the number of phagocytosed bacteria is *a priori* surprising since PMA is known to favor phagocytosis by THP-1 cells. However, this effect of PMA was studied only with latex beads (14, 43, 55), the uptake of which is nonspecific. In contrast, phagocytosis of *S. aureus* requires its attachment at the cell surface mainly via fibronectin-binding proteins FnBP-A and FnBP-B that connect to cellular integrins via

TABLE 1 Pertinent regression parameters[a] and statistical analysis of the dose-response curves illustrated in Fig. 2

	Nonactivated THP-1 cells							PMA-activated THP-1 cells							
				C_s							C_s				
Antibiotic	Strain	E_{min}[b]	E_{max}[c]	Multiple of MIC[d]	μg/ml[e]	Fract[f]	R^2	Strain	E_{min}	E_{max}	Multiple of MIC	μg/ml	Fract	R^2	ANOVA[g]
Gentamicin	WT, *hemB*, *hemB*gc	3.46 (3.15 to 3.77)a,A[h]	−0.74 (−0.87 to −0.60)a,A	0.22	0.11–0.22	NA[i]	0.97	WT, *menD*s, *hemB*, *hemB*gc	2.99 (2.67 to 3.30)a,A	−0.87 (−1.01 to −0.73)a,A	0.03	0.02–0.03	NA	0.95	<0.001
	menD	0.88 (0.50 to 1.26)b,A	−1.86 (−2.38 to −1.33)b,A	0.03	0.96	NA	0.95	*menD*	1.19 (0.95 to 1.42)b,A	−1.03 (−1.24 to −0.82)a,B	0.02	0.64	NA	0.98	>0.05
	*menD*s	3.13 (2.89 to 3.37)a,A	-0.82 (−1.02 to −0.62 a,A	0.05	1.06	NA	0.99								>0.05
Moxifloxacin	WT, *menD*s, *hemB*, *hemB*gc	3.43 (2.80 to 4.04)a,A	<−2 c	0.32	0.04–0.08	0.52	0.94	WT, *menD*s, *hemB*, *hemB*gc	4.44 (−4.39 to 13.27)c,A	<−2 b	0.07	0.01–0.02	0.33	0.99	<0.05
	menD	0.85 (0.52 to 1.18)b,A	<−2 c,A	0.07	0.02	0.72	0.99	*menD*	0.78 (−2.55 to 4.10)b,A	−1.63 (−1.86 to −1.39)c,B	0.05	0.01	0.72	1.00	>0.05
Oritavancin	WT *menD*s, *hemB*, *hemB*gc	3.10 (2.72 to 3.49)a,A	<−2 c	3.0	0.38–0.75	0.50	0.95	WT, *menD*s, *hemB*, *hemB*gc	2.85 (2.46 to 3.24)a,A	<−2 b	2.63	0.33–0.66	0.44	0.98	>0.05
	menD	0.77 (0.41 to 1.06)b,A	<−2 c	0.63	0.08	0.36	0.99	*menD*	1.48 (0.63 to 3.32)b,A	<−2 b	0.56	0.07	1.14	1.00	>0.05

[a] Calculated on the basis of the sigmoidal regressions with a Hill coefficient of 1 for extracellular data and for intracellular data with gentamicin and on biphasic sigmoidal regressions with Hill coefficients of 1 for intracellular data with moxifloxacin and oritavancin.

[b] E_{min}, increase in the number of CFU (in $\log_{10}$ units) from the corresponding original inoculum extrapolated for an infinitely low concentration of antibiotics (mean with the 95% confidence interval in parentheses).

[c] E_{max}, decrease in the number of CFU (in $\log_{10}$ units) from the corresponding original inoculum extrapolated for an infinitely large concentration of antibiotics (mean with the 95% confidence interval in parentheses). For moxifloxacin and oritavancin, the absence of clear plateaus in the change in the number of intracellular CFU at the highest antibiotic concentrations tested prevented us from calculating accurate E_{max} values. For some curves, the plateau was not reached at the maximal concentration tested; the maximal effect was therefore estimated as being greater than a 2-log-unit decrease in the inoculum (<−2 in the table).

[d] Concentration (multiple of the MIC) resulting in no apparent bacterial growth determined by graphical interpolation. MICs (readings made at 24 h) were used as follows: values at pH 7.4 were used for extracellular activity, values at pH 5.5 were used for intracellular activity for all strains by the *hemB* mutant, and values at pH 5.5 were used in the presence of hemin for the *hemB* mutant on the basis of previous data suggesting the availability of hemin-like compounds in the cellular medium (20).

[e] Concentration calculated on the basis of the MICs given in Table 2; a range is given when a single fit was applied to strains having different MICs.

[f] Fract, fraction (proportion) of the total response that could be ascribed to the first wave of decrease in the number of CFU in the biphasic curve.

[g] Statistical analysis comparing all data points from each condition between nonactivated and PMA-activated cells using the fitted Hill functions (one-way analysis of variance with the Tukey test for multiple comparisons).

[h] Statistical analysis comparing the E_{min} and E_{max} values of each data set: (i) analysis per column (one-way analysis of variance with the Tukey test for multiple comparisons between each parameter for all drugs; data with different lowercase letters are significantly different from each other [$P < 0.05$]); (ii) analysis per row (unpaired, two-tailed *t* test between nonactivated and PMA-activated cells; data with different uppercase letters are significantly different from each other [$P < 0.05$]).

[i] NA, not applicable.

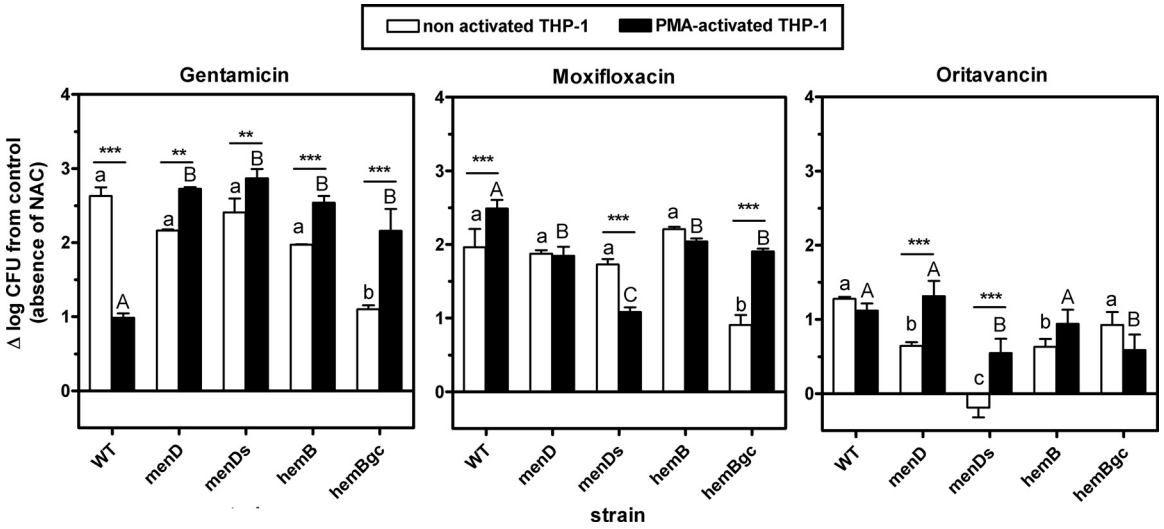

FIG 3 Influence of *N*-acetylcysteine on the intracellular activity of antibiotics in nonactivated THP-1 cells (open bars) or in cells that have been activated by incubation with 200 μg/liter PMA for 48 h (solid bars). Antibiotics were used at their C_s, i.e., the concentration at which no apparent change from the initial inoculum was observed, as interpolated from concentration-effects studies (Table 1), and the number of viable bacteria (CFU) per mg of cell protein was determined after 24 h of incubation in the presence of 25 mM *N*-acetylcysteine. Values are expressed as the difference in CFU from the values recorded in the absence of *N*-acetylcysteine (NAC; close to 0 in all cases). All values are means ± standard deviations (SD) of three independent determinations (when not visible, the SD bars are smaller than the size of the symbols). Experiments have been reproduced 3 times with similar results. Statistical analysis was by analysis of variance with the Tukey *post hoc* test, and data with different letters indicate significant differences ($P < 0.05$) between strains (lowercase letters, in nonactivated cells; uppercase letters, in activated cells) and cell types (***, $P < 0.001$; **, $P < 0.01$).

soluble fibronectin and trigger internalization (23, 24, 44). The COL strain used in the present study, however, is described to be deficient in fibronectin-binding proteins (48), which may explain why internalization may not be favored in PMA-activated cells, despite their increased capacity to bind fibronectin (19). PMA also induces a marked stimulation of the production of oxidant species (19, 43). Oxidative burst is a major mechanism by which phagocytes defend themselves against bacterial invasion, including invasion by *S. aureus* (17, 22). Thus, we may reasonably hypothesize that phagocytized bacteria are more quickly killed in PMA-activated THP-1 cells than unstimulated cells. This would explain (i) the differences in recovery and the apparent slower growth of phagocytized wild-type bacteria since the endpoint (number of CFU) is the net result of the two opposite processes of bacterial growth and killing (as already observed by us in *Listeria monocytogenes*-infected THP-1 cells upon stimulation of the production of reactive oxygen species by exposure to gamma interferon [38]) and (ii) the still lower recovery of SCVs compared to that of the parent strain, because SCVs are hypersusceptible to oxidant agents *in vitro* (53).

TABLE 2 Influence of preincubation with H_2O_2 on MICs of antibiotics against the COL strain, its SCVs, and the genetically complemented *hemB* strain, with readings made at 24 h and 48 h[a]

Antibiotic	pH	H_2O_2	MIC (mg/liter)					
				menD mutant		*hemB* mutant		
			Wild type	Without MSB	With MSB	Without hemin	With hemin	*hemB*gc
Gentamicin	7.4	−	0.25/0.25	1/1	1/1	0.5/0.5	0.125/1	0.25/0.5
		+	0.03/1	0.125/1	0.125/1	0.125/1	0.125/0.5	0.03/1
	5.5	−	1/1	32/64	32/64	32/32	0.5/0.5	1/1
		+	0.125/2	0.125/2	0.125/4	0.125/2	0.06/2	0.125/2
Moxifloxacin	7.4	−	0.03/0.06	0.125/0.125	0.125/0.125	0.125/0.25	0.06/0.125	0.03/0.06
		+	0.008/0.125	0.008/0.125	0.008/0.125	0.002/0.06	0.008/0.06	0.008/0.125
	5.5	−	0.125/0.25	0.25/0.5	0.5/0.5	0.25/0.5	0.125/0.125	0.125/0.5
		+	0.002/0.125	0.002/0.125	0.002/0.125	0.001/0.125	0.002/0.125	0.002/0.125
Oritavancin	7.4	−	0.25/1	0.03/0.06	0.03/0.06	0.125/0.125	2/4	0.25/0.25
		+	0.25/1	0.03/0.06	0.03/0.06	0.125/0.125	2/4	0.25/0.25
	5.5	−	0.25/1	0.125/0.125	0.06/0.125	0.125/0.25	0.25/0.5	0.25/1
		+	0.25/1	0.125/0.125	0.06/0.125	0.125/0.25	0.25/0.5	0.25/1

[a] Preincubation was in medium supplemented or not in menadione sodium bisulfite (MSB; 2 μg/ml) or in hemin (2 μg/ml). Data are for readings made at 24 h/48 h. The slow growth of SCVs sometimes made readings difficult to obtain at 24 h. *hemB*gc, genetically complemented *hemB* strain.

Moving now to the analysis of the data concerning antibiotic activity, a first striking observation is that moxifloxacin and gentamicin share a similar response to cell activation by PMA, characterized by an increased potency (lower apparent static concentration) compared to that against the parental strain, the genetically complemented strain, and the *hemB* mutant which becomes indistinguishable from that measured against the *menD* strain in nonactivated cells. Two complementary pieces of evidence suggest that this effect results from cooperation between these antibiotics and the oxidant cell defense mechanisms that have been stimulated by PMA. First, their activity is impaired in cells coincubated with the general antioxidant *N*-acetylcysteine. Second, the MICs of both drugs markedly decreased when bacteria were preincubated with an oxidant species like H_2O_2. This is consistent with the fact that the bactericidal mode of action of these two drugs involves the formation of radical species within the bacteria (15, 28, 29). The fact that MICs were higher at 48 h is probably due to the short duration of the effect of H_2O_2 over time. Of note, however, the activity of the two drugs appeared to be unaffected by activation of cells when tested with the *menD* strain. A plausible explanation is that the deficiency in electron transport in this strain already makes it sensitive to the basal level of oxidant species produced by nonactivated cells, in relation to the fact that SCVs have been described to be hypersensitive to oxidants (53). The *hemB* mutant does not show this hypersusceptibility, but this strain behaves like the complemented strain when exposed to the intracellular medium (20), probably because this milieu contains heme-rich compounds capable of restoring its normal metabolic activity. Interestingly, an oxidant environment is also able to restore the activity of gentamicin against both types of SCVs, suggesting that it can compensate for the defect in transmembrane electrical potential ($\Delta\Psi$) responsible for the intrinsic resistance of SCVs to aminoglycosides (8, 40).

A second and contrasting observation is that the concentration-response profile of oritavancin is not altered by cell activation by PMA, regardless of the strain examined. This suggests that, in contrast to aminoglycosides or fluoroquinolones, the bactericidal activity of oritavancin is less dependent on the formation of radical species. We show indeed that the oritavancin MIC is not affected upon preincubation of bacteria with H_2O_2 and its intracellular activity is less affected by *N*-acetylcysteine. While aminoglycosides or fluoroquinolones act upon intrabacterial targets, oritavancin bactericidal activity is assumed to result from inhibition of cell wall synthesis and anchoring to the bacterial membrane, causing depolarization and alteration of membrane permeability (9). Indeed, both *in vitro* and animal studies document that oritavancin and the two other classes of drugs interact very differently with cell host defenses. First, at clinically relevant concentrations oritavancin does not modify the production of reactive oxygen species by THP-1 cells (32) and neither increases nor decreases the capacity of phagocytic cells to kill pathogens that are out of its spectrum of activity, like *Acinetobacter baumannii* or *Candida albicans* (5). In contrast, moxifloxacin induces the release of reactive oxygen species by THP-1 cells (21). Second, oritavancin is highly effective in neutropenic animal models of infection (2), i.e., in the absence of oxidative defenses, whereas higher doses of fluoroquinolones are needed to reach a bacteriostatic effect when used in neutropenic versus immunocompetent animals (3). We also know that aminoglycosides show shorter postantibiotic effects in neutropenic animals (18).

Another and mutually nonexclusive explanation for the difference between oritavancin and the other drugs with respect to the influence of PMA on their potency could result from the delayed growth of bacteria in PMA-activated cells. Moxifloxacin suffers from an inoculum effect (31), which may explain why it is less active against intracellular bacteria when the amount of viable organisms is large. Gentamicin is more active against highly dividing bacteria (16), and we have seen that THP-1 differentiation slows the growth of intracellular *S. aureus*. In contrast, oritavancin remains bactericidal against both small and large inocula and remains bactericidal against slow-growing bacteria (4, 10). Yet, because of the pleiotropic effects exerted by PMA on THP-1 cells (43), we cannot exclude other concomitant mechanisms. We must also acknowledge that intracellular antibiotic activity was examined at a single time point; cooperation with cell defense mechanisms could have been different if shorter or longer incubation times had been used.

In conclusion, the data presented here show that activation of THP-1 cells by PMA affects the intracellular potency (apparent static concentration) of antibiotics to an extent that may reflect the dependence of their action on reactive oxygen species, without affecting their maximal efficacy. This may point to a useful process of cooperation between antibiotics and host defenses for certain agents, such as gentamicin and moxifloxacin. Conversely, agents for which cell activation has a minimal effect (like oritavancin) may remain as effective when host defense mechanisms are weakened.

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REFERENCES

1. **Allegra L, et al.** 2002. Human neutrophil oxidative bursts and their in vitro modulation by different N-acetylcysteine concentrations. Arzneimittelforschung **52**:669–676.
2. **Ambrose PG, Drusano GL, Craig WA.** 2012. In vivo activity of oritavancin in animal infection models and rationale for a new dosing regimen in humans. Clin. Infect. Dis. **54**(Suppl 3):S220–S228.
3. **Andes D, Craig WA.** 2002. Pharmacodynamics of the new fluoroquinolone gatifloxacin in murine thigh and lung infection models. Antimicrob. Agents Chemother. **46**:1665–1670.
4. **Arhin FF, Sarmiento I, Parr TR, Jr, Moeck G.** 2012. Activity of oritavancin and comparators in vitro against standard and high inocula of Staphylococcus aureus. Int. J. Antimicrob. Agents **39**:159–162.
5. **Baquir B, et al.** 2012. Macrophage killing of bacterial and fungal pathogens is not inhibited by intense intracellular accumulation of the lipoglycopeptide antibiotic oritavancin. Clin. Infect. Dis. **54**:S230–S232.
6. **Barcia-Macay M, Seral C, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F.** 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against Staphylococcus aureus in a model of THP-1 macrophages. Antimicrob. Agents Chemother. **50**:841–851.
7. **Bates DM, et al.** 2003. Staphylococcus aureus menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. J. Infect. Dis. **187**:1654–1661.
8. **Baumert N, et al.** 2002. Physiology and antibiotic susceptibility of Staphylococcus aureus small colony variants. Microb. Drug Resist. **8**:253–260.

9. **Belley A, et al.** 2010. Oritavancin disrupts membrane integrity of Staphylococcus aureus and vancomycin-resistant enterococci to effect rapid bacterial killing. Antimicrob. Agents Chemother. **54**:5369–5371.
10. **Belley A, et al.** 2009. Oritavancin kills stationary-phase and biofilm Staphylococcus aureus cells in vitro. Antimicrob. Agents Chemother. **53**: 918–925.
11. **Bergstrand H, et al.** 1986. Stimuli-induced superoxide radical generation in vitro by human alveolar macrophages from smokers: modulation by N-acetylcysteine treatment in vivo. J. Free Radic. Biol. Med. **2**:119–127.
12. **Clinical and Laboratory Standards Institute.** 2011. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. M7-A9, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
13. **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing. Twenty-second informational supplement. MS100-S22. Clinical and Laboratory Standards Institute, Wayne, PA.
14. **Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH.** 2010. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. PLoS One **5**:e8668. doi:10.1371/journal.pone.0008668.
15. **Dwyer DJ, Kohanski MA, Collins JJ.** 2009. Role of reactive oxygen species in antibiotic action and resistance. Curr. Opin. Microbiol. **12**:482–489.
16. **Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE.** 1991. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob. Agents Chemother. **35**:1824–1828.
17. **Fang FC.** 2011. Antimicrobial actions of reactive oxygen species. mBio **2**(5):e00141–11. doi:10.1128/mBio.00141-11.
18. **Fantin B, Ebert S, Leggett J, Vogelman B, Craig WA.** 1991. Factors affecting duration of in-vivo postantibiotic effect for aminoglycosides against gram-negative bacilli. J. Antimicrob. Chemother. **27**:829–836.
19. **Faull RJ, Kovach NL, Harlan JM, Ginsberg MH.** 1994. Stimulation of integrin-mediated adhesion of T lymphocytes and monocytes: two mechanisms with divergent biological consequences. J. Exp. Med. **179**:1307–1316.
20. **Garcia LG, et al.** 2012. Pharmacodynamic evaluation of the activity of antibiotics against hemin- and menadione-dependent small-colony variants of Staphylococcus aureus in models of extracellular (broth) and intracellular (THP-1 monocytes) infections. Antimicrob. Agents Chemother. **56**:3700–3711.
21. **Hall IH, Schwab UE, Ward ES, Ives TJ.** 2003. Effects of moxifloxacin in zymogen A or S. aureus stimulated human THP-1 monocytes on the inflammatory process and the spread of infection. Life Sci. **73**:2675–2685.
22. **Hampton MB, Kettle AJ, Winterbourn CC.** 1996. Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of Staphylococcus aureus by neutrophils. Infect. Immun. **64**:3512–3517.
23. **Hauck CR, Ohlsen K.** 2006. Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by Staphylococcus aureus. Curr. Opin. Microbiol. **9**:5–11.
24. **Hoffmann C, Ohlsen K, Hauck CR.** 2011. Integrin-mediated uptake of fibronectin-binding bacteria. Eur. J. Cell Biol. **90**:891–896.
25. **Idelevich EA, et al.** 2011. Comparative in vitro activity of finafloxacin against staphylococci displaying normal and small colony variant phenotypes. J. Antimicrob. Chemother. **66**:2809–2813.
26. **Kahl B, et al.** 1998. Persistent infection with small colony variant strains of Staphylococcus aureus in patients with cystic fibrosis. J. Infect. Dis. **177**:1023–1029.
27. **Khan A, Sarkar S, Sarkar D.** 2008. Bactericidal activity of 2-nitroimidazole against the active replicating stage of Mycobacterium bovis BCG and Mycobacterium tuberculosis with intracellular efficacy in THP-1 macrophages. Int. J. Antimicrob. Agents **32**:40–45.
28. **Kohanski MA, Dwyer DJ, Collins JJ.** 2010. How antibiotics kill bacteria: from targets to networks. Nat. Rev. Microbiol. **8**:423–435.
29. **Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ.** 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell **130**:797–810.
30. **Lecaroz C, Blanco-Prieto MJ, Burrell MA, Gamazo C.** 2006. Intracellular killing of Brucella melitensis in human macrophages with microsphere-encapsulated gentamicin. J. Antimicrob. Chemother. **58**: 549–556.
31. **Lee SY, Fan HW, Sutherland C, DeRyke AC, Nicolau DP.** 2007. Antibacterial effects of moxifloxacin and levofloxacin simulating epithelial lining fluid concentrations against community-acquired methicillin-resistant Staphylococcus aureus. Drugs R. D. **8**:69–77.
32. **Lemaire S, et al.** 2010. Influence of oritavancin on phagocytosis, generation of reactive oxygen species and killing of intracellular S. aureus in murine and human macrophages and comparison with azithromycin and vancomycin, poster A1-1360. Abstr. 50th Intersci. Conf. Antimicrob. Agents Chemother., Boston, MA. American Society for Microbiology, Washington, DC.
33. **Lemaire S, et al.** 2011. Activity of moxifloxacin against intracellular community-acquired methicillin-resistant Staphylococcus aureus: comparison with clindamycin, linezolid and co-trimoxazole and attempt at defining an intracellular susceptibility breakpoint. J. Antimicrob. Chemother. **66**:596–607.
34. **Lemaire S, et al.** 2010. Cellular pharmacodynamics of the novel biaryloxazolidinone radezolid: studies with infected phagocytic and nonphagocytic cells, using Staphylococcus aureus, Staphylococcus epidermidis, Listeria monocytogenes, and Legionella pneumophila. Antimicrob. Agents Chemother. **54**:2549–2559.
35. **Nguyen HA, et al.** 2009. Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a Staphylococcus aureus small-colony variant strain isolated from a cystic fibrosis patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. Antimicrob. Agents Chemother. **53**: 1434–1442.
36. **Nguyen HA, Grellet J, Dubois V, Saux MC, Quentin C.** 2007. Factors compromising the activity of moxifloxacin against intracellular Staphylococcus aureus. J. Antimicrob. Chemother. **59**:755–758.
37. **Olivier AC, Lemaire S, Van Bambeke F, Tulkens PM, Oldfield E.** 2009. Role of rsbU and staphyloxanthin in phagocytosis and intracellular growth of Staphylococcus aureus in human macrophages and endothelial cells. J. Infect. Dis. **200**:1367–1370.
38. **Ouadrhiri Y, Scorneaux B, Sibille Y, Tulkens PM.** 1999. Mechanism of the intracellular killing and modulation of antibiotic susceptibility of Listeria monocytogenes in THP-1 macrophages activated by gamma interferon. Antimicrob. Agents Chemother. **43**:1242–1251.
39. **Paillard D, Grellet J, Dubois V, Saux MC, Quentin C.** 2002. Discrepancy between uptake and intracellular activity of moxifloxacin in a Staphylococcus aureus-human THP-1 monocytic cell model. Antimicrob. Agents Chemother. **46**:288–293.
40. **Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD.** 1995. Persistent and relapsing infections associated with small-colony variants of Staphylococcus aureus. Clin. Infect. Dis. **20**:95–102.
41. **Proctor RA, et al.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat. Rev. Microbiol. **4**:295–305.
42. **Sachse F, Becker K, von Eiff C, Metze D, Rudack C.** 2010. Staphylococcus aureus invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. Allergy **65**:1430–1437.
43. **Schwende H, Fitzke E, Ambs P, Dieter P.** 1996. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. J. Leukoc. Biol. **59**:555–561.
44. **Sinha B, et al.** 1999. Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. Cell. Microbiol. **1**:101–117.
45. **Stokes RW, Doxsee D.** 1999. The receptor-mediated uptake, survival, replication, and drug sensitivity of Mycobacterium tuberculosis within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. Cell. Immunol. **197**:1–9.
46. **Takemura H, et al.** 2000. Evaluation of a human monocytic cell line THP-1 model for assay of the intracellular activities of antimicrobial agents against Legionella pneumophila. J. Antimicrob. Chemother. **46**: 589–594.
47. **Tsuchiya S, et al.** 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer **26**: 171–176.
48. **Vaudaux P, et al.** 2002. Increased expression of clumping factor and fibronectin-binding proteins by hemB mutants of Staphylococcus aureus expressing small colony variant phenotypes. Infect. Immun. **70**:5428–5437.
49. **Vergison A, et al.** 2007. National survey of molecular epidemiology of Staphylococcus aureus colonization in Belgian cystic fibrosis patients. J. Antimicrob. Chemother. **59**:893–899.
50. **Villagrasa V, et al.** 1997. Inhibitory effects of N-acetylcysteine on super-

oxide anion generation in human polymorphonuclear leukocytes. J. Pharm. Pharmacol. **49**:525–529.
51. **von Eiff C, et al.** 2001. Intracellular persistence of Staphylococcus aureus small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. Clin. Infect. Dis. **32**:1643–1647.
52. **von Eiff C, et al.** 1997. A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly. J. Bacteriol. **179**:4706–4712.
53. **von Eiff C, et al.** 2006. Phenotype microarray profiling of Staphylococcus aureus menD and hemB mutants with the small-colony-variant phenotype. J. Bacteriol. **188**:687–693.
54. **Yagci S, Hascelik G, Dogru D, Ozcelik U, Sener B.** 28 November 2011. Prevalence and genetic diversity of Staphylococcus aureus small-colony variants in cystic fibrosis patients. Clin. Microbiol. Infect. [Epub ahead of print.] doi:10.1111/j.1469-0691.2011.03742.x.
55. **Zhou L, et al.** 2010. Retinoid X receptor agonists inhibit phorbol-12-myristate-13-acetate (PMA)-induced differentiation of monocytic THP-1 cells into macrophages. Mol. Cell. Biochem. **335**:283–289.