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Influence of Oxidant Stress on the Activity of Antibiotics against the Extracellular and Intracellular (THP-1 cells) Forms of *S. aureus* and its Small Colony Variants (SCV) Mutants

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Abstract

BACKGROUND. Intracellular survival of *S. aureus* (SA) is associated with persistent infections, especially by SCVs. As reactive oxidant species (ROS) may be critical in antibiotic-dependent bacterial killing, we have examined how activation of monocytes modulate antibiotic (AB) activity against wild-type phenotype MRSA COL and its *menD* and *hemB* SCV mutants.

METHODS. Strains and antibiotics are shown in the Table. MICs: microdilution in MHB at pH 5.5 (mimicking phagolysosomes, with preincubation with H_2O_2 when indicated). Cells: THP-1 monocytes activated with Phorbol Myristate Acetate (PMA). Intracellular activity: Change in CFU from post-phagocytosis inoculum after 24 h incubation with AB; N-acetylcysteine (NAC) used as a scavenger of ROS; relative potency and efficacy calculated from the Hill equation of conc.-response curve.

RESULTS.

Antibiotic	Strain	Extracellular		Intracellular		
		MIC (µg/mL)[a]	Reduction in MIC upon preincubation with H_2O_2 (fold dilutions)[b]	Cs (X MIC) in THP-1 cells [c]	Gain in potency upon cell activation by PMA (fold reduction in Cs) [d]	Loss of efficacy upon scavenging of oxidant species by NAC (Δlog CFU) [e]
Gentamicin (GEN)	WT	1	3	0.15	4.4	2.6
	menD	32	8	0.03	1.7	2.2
	hemB	0.5	2	0.35	17.4	2
Moxifloxacin (MXF)	WT	0.125	6	0.50	6.6	2
	menD	0.25	7	0.05	1.2	1.9
	hemB	0.125	7	0.24	4.7	2.2
Oritavancin (ORI)	WT	0.25	0	2.45	0.8	1.3
	menD	0.125	0	0.57	1.0	0.6
	hemB	0.25	1	0.38	0.2	0.7

[a] value measured at pH 5.5 to mimic the phagolysosomal environment (infected compartment of the cells)
[b] difference (fold dilution) between the MIC measured at pH 5.5 in control conditions and for bacteria that had been pre-exposed for 30 min to 10 mM H_2O_2 before antibiotic addition
[c] static concentration (i.e. extracellular conc. resulting in no apparent intracell. bacterial growth after 24 h of incubation of infected cells with the antibiotic); determined by graphical interpolation using sigmoidal regressions of data from conc-effects studies ; value expressed in X MIC at acidic pH
[d] ratio between the Cs calculated as described above in undifferentiated THP-1 cells and in THP-1 cells that had been pre-incubated for 48 h with 200 µg/L of Phorbol Myristate Acetate (PMA)
[e] change in intracellular inoculum (-log CFU) in THP-1 cells incubated for 24 h with antibiotics at their static concentrations in the presence of 25 mM N-acetylcysteine (NAC, general scavenger of oxidant species) as compared to control conditions (no NAC).

In broth, preincubation with H_2O_2 markedly decreased the MIC of GEN and MXF but not that of ORI. Cell activation by PMA increased the intracellular potency of GEN and MXF (wild-type strain and *hemB* mutant), while NAC reduced their activity by 2 log in non-activated cells. In contrast, cell activation had no effect on intracellular ORI potency and NAC reduced its efficacy by 1 log only.

CONCLUSIONS. Activation of THP-1 cells by PMA may increase the intracellular relative potency of AB according to the dependence of their activity on ROS (cooperation with host defenses). Conversely, agents for which cell activation has minimal effect (like ORI), may remain as effective when host defense mechanisms are weakened.

Introduction

Small-Colony Variants (SCVs) of *Staphylococcus aureus* have a propensity to survive within eukaryotic cells, which has been associated with the persistent and/or recurrent character of the infections they cause.

Reactive oxidant species (ROS) are critical for antibiotics such as fluoro-quinolones or aminoglycosides to express their bactericidal activity in broth [1].

We therefore wondered whether ROS produced by monocytes upon activation by PMA [2] could also enhance the activity of bactericidal antibiotics towards intra-phagocytic SCVs.

Aims and set-up

Our aim was to assess the influence of ROS on the extracellular and intracellular activity of 3 bactericidal antibiotics with distinct modes of action (gentamicin, moxifloxacin and oritavancin) against the extracellular and intracellular forms of the MRSA COL strain and its isogenic menadione- and hemin-dependent SCVs.

We used a model of infected THP-1 human monocytes in which a menadione-dependent mutant grows much more slowly than an hemin-dependent strain but remains as susceptible to antibiotics in terms of maximal reduction of the inoculum compared to the post-phagocytosis values [3].

For extracellular activity, ROS were generated by preexposure to H_2O_2. For intracellular activity, THP-1 cells were activated with phorbol myristyl acetate (PMA) and scavenged by addition of N-acetyl-cysteine (NAC) [4].

Results

Table 1. Influence of pre-incubation with H_2O_2 on MICs of antibiotics against the COL strain and its SCVs (supplemented or not in MSB or in hemin)

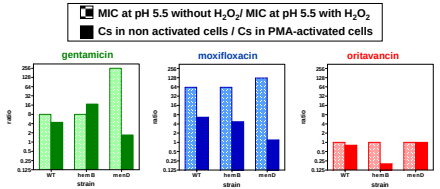
Antibiotic	pH	H_2O_2	MIC (mg/L)				
			Wild-type	*menD* mutant		*hemB* mutant	
				- MSB	+ MSB	- hemin	+ hemin
Gentamicin	7.4	-	0.25	1	1	0.5	0.125
		+	**0.03**	**0.125**	**0.125**	**0.125**	0.125
	5.5	-	1	32	32	32	0.5
		+	**0.125**	**0.125**	**0.125**	**0.125**	**0.06**
Moxifloxacin	7.4	-	0.03	0.125	0.125	0.125	0.06
		+	**0.008**	**0.008**	**0.008**	**0.002**	**0.008**
	5.5	-	0.125	0.25	0.5	0.25	0.125
		+	**0.002**	**0.002**	**0.002**	**0.001**	**0.002**
Oritavancin	7.4	-	0.25	0.03	0.03	0.125	2
		+	0.25	0.03	0.03	0.125	2
	5.5	-	0.25	0.125	0.06	0.125	0.25
		+	0.25	0.125	0.06	0.125	0.25

MICs were measured in broth at pH 7.4 or 5.5 for bacteria pre-exposed or not to10 mM H_2O_2 for 30 min

- MICs of gentamicin and moxifloxacin were reduced if bacteria were preincubated with H_2O_2.
- Those of oritavancin remained unchanged.

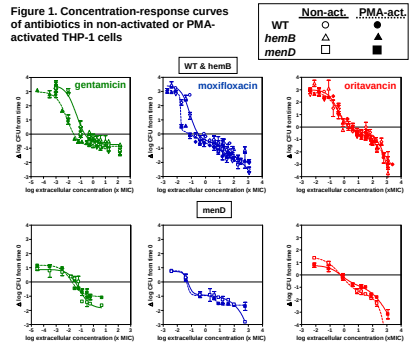
Figure 2. Comparison of the change in extracellular and intracellular potency induced by exposure to H_2O_2 (extracellular) or by PMA activation (intracellular)

MICs were measured for bacteria preexposed or not to 10 mM H_2O_2 for 30 min.
Static conc. (Cs) were determined by graphical interpolation using the conc-effect curves.



- For gentamicin and moxifloxacin, there is a marked gain in potency
- extracellularly, in the presence of H_2O_2 towards all strains
- intracellularly, upon PMA activation, towards the WT strain and the *hemB* mutant
- For oritavancin, neither H_2O_2 nor PMA activation were able to modify extracellular or intracellular potency, respectively

Figure 1. Concentration-response curves of antibiotics in non-activated or PMA-activated THP-1 cells

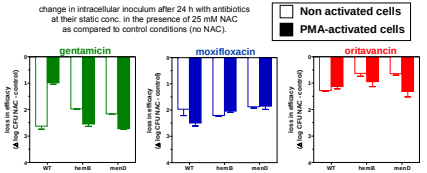


Infected cells were exposed during 24 h to antibiotics at increasing concentrations.
Data are expressed as changes from the post-phagocytosis inoculum, with concentrations expressed in X MIC at pH 5.5 (+ hemin for *hemB* strain that grows normally within the cells [1]).

- Gentamicin and moxifloxacin showed higher potency (lower Cs) against the WT and *hemB* mutant in PMA-activated cells than in non-activated cells.
- Oritavancin has a similar potency in both cell types irrespective of the strain.

Figure 3. Loss of antibiotic intracellular efficacy (at their static conc.) induced by the addition of NAC in non-activated or PMA-activated cells

change in intracellular inoculum after 24 h with antibiotics at their static conc. in the presence of 25 mM NAC as compared to control conditions (no NAC).



- Loss in intracellular efficacy in the presence of NAC was greater for gentamicin and moxifloxacin than for oritavancin in both cell types for all strains.

Methods

- **Bacterial strains**: strain COL (wild-type, HA-MRSA); its *menD* and *hemB* SCV mutants [5,6], supplemented or not with 2 µg/mL menadione sodium bisulfite (MSB) or hemin.
- **Extracellular activity:** MIC determination according to CLSI recommendations [7] in CA-MHB adjusted at pH 7.4 or 5.5, for bacteria preincubated or not for 30 min with 10 mM H_2O_2 in the dark.
- **Cell activation:** Incubation with 200 µg/L PMA for 48 h at 37°C.
- **Intracellular activity:** infection of cells by preopsonized bacteria, antibiotic exposure and assessment of activity determined after 24h incubation following published procedure [3]. Curve fitting of concentration-effects performed uisng sigmoid or biphasic sigmoidal regressions. When applicable, coincubation with 25 mM NAC and antibiotics at their static concentration during 24 h.

Conclusions

- **For gentamicin or moxifloxacin,** activation of cells increases their intracellular potency against normal phenotype and hemin-dependent strains (but not against the menadione-dependent strain because it is intrinsically more susceptible). These drugs may, therefore, depend on host defense mechanisms to fully exert their activity.
- Conversely, cell activation did not modify the intracellular activity of **oritavancin**, suggesting that this drug is not dependent on oxidant species for activity and that it may remain effective even when oxygen-dependent host defense mechanisms are weakened.

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