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Activity of quinupristin/dalfopristin against extracellular and intracellular *Staphylococcus aureus* with various resistance phenotypes

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Objectives: Treatment of chronic or recurrent *Staphylococcus aureus* infections may require using antibiotics with activity against intracellular multiresistant organisms. Quinupristin/dalfopristin (3:7) has been examined in this context.

Methods: Quinupristin and dalfopristin were used separately or mixed. Strains used were: (i) methicillin-susceptible and -resistant *S. aureus* (MSSA and MRSA); (ii) one *vat*(B) MSSA and *msr*(A/B) MRSA; (iii) *erm*(A)$^+$ [MSSA, MRSA, vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA)]; and (iv) one *erm*(A/B)$^+$ *cfr*$^+$ MRSA resistant to quinupristin, dalfopristin and their combination. Assessment of activity was determined by: (i) MICs (CLSI method); and (ii) concentration–response curves in broth and after phagocytosis by THP-1 macrophages, with descriptors of the model (E_{min}) and the pharmacodynamic response [maximal relative efficacy (E_{max}), relative potency (EC_{50}) and apparent static concentration (C_{static})].

Results: *erm*(A)-positive strains were all susceptible to quinupristin/dalfopristin (except strain CM05), with MICs not adversely influenced by acid pH or by the MRSA, VISA or VRSA character of the strain. In concentration–response experiments, quinupristin/dalfopristin showed similar patterns for all strains (except strain CM05), with a >3 $\log_{10}$ cfu decrease in broth and a 1.3 [*erm*(A) strain] to 2.6 [fully susceptible, *vat*(B) and *msr*(A/B) strains] $\log_{10}$ cfu decrease for intracellular bacteria at the maximal extracellular concentration tested (25 mg/L). Maximal extracellular and intracellular activity was obtained for a quinupristin/dalfopristin ratio of 3:7. For strain CM05, quinupristin/dalfopristin was static in all conditions.

Conclusions: Based on historical comparisons with rifampicin, fluoroquinolones, lipoglycopeptides and other antistaphylococcal drugs with a large accumulation in eukaryotic cells, quinupristin/dalfopristin appears to be one of the most active antibiotics against intracellular *S. aureus* studied in this model so far, largely irrespective of its resistance phenotype.

Keywords: THP-1 macrophages, maximal relative efficacy, relative potency, static concentration, pharmacodynamics, *erm*(A/B), *cfr*

Introduction

The capacity of *Staphylococcus aureus* to survive and replicate within eukaryotic cells is now recognized as a determining factor in the persistent or recurrent character of many infections.[1] The treatment of such infections requires that antibiotics reach the infected compartment(s) within cells and express their activity therein.[2] Beyond the necessity to select antibiotics based on specific pharmacokinetic and pharmacodynamic properties, treatment options are further complicated by the emergence of multidrug-resistant strains for which safe and effective alternatives are scarce. Among methicillin-resistant *S. aureus* (MRSA), strains harbouring the MLS_B phenotype (resistance to macrolides, lincosamides and streptogramins B, most often constitutively expressed[3,4]) are now widespread.[5] More worryingly, vancomycin-intermediate *S. aureus* (VISA) strains with decreased susceptibility to glycopeptides are increasingly reported.[6] While vancomycin-resistant *S. aureus* (VRSA) strains and linezolid-resistant strains are still very rare today,[7,8] they constitute true superbugs against which the medical community must be prepared.

First described for its potent activity against Gram-positive cocci in the early 1990s,[9] Synercid® (hereafter referred to as quinupristin/dalfopristin) is the trade name of a preparation associating two constituents, quinupristin (a streptogramin B) and dalfopristin (a streptogramin A), in a fixed proportion (3:7). Together with pristinamycin, quinupristin/dalfopristin belongs to the class of streptogramins (ATC code J01FG; http://www.whocc.no/atcddd/). Like macrolides and lincosamides, streptogramins interact with the 50S ribosomal subunit.[10,11] While being bacteriostatic when tested alone, quinupristin and dalfopristin become highly bactericidal when used in combination,[12] thanks to an interaction of dalfopristin with rRNA that enhances the affinity of quinupristin for the ribosome.[13] Although genetically related, resistance to each of the quinupristin/dalfopristin components has been described [*erm* encoding a methylase (MLS_B phenotype),[14,15] *msr*(A) causing active efflux (MS_B and M phenotypes), *vgb* encoding an inactivating lyase (S_B phenotype)[15–18] for streptogramin B, and *vat* encoding acetyltransferases and *vga* causing active efflux (S_A phenotype)[18–20] for streptogramin A], quinupristin/dalfopristin often retains useful antibacterial activity and remains clinically active against MLS_B-resistant strains.[12,21,22] As this phenotype is often co-associated with those resistant to methicillin (MRSA) and with reduced susceptibility to vancomycin (VISA), quinupristin/dalfopristin now appears to be one of those last resource drugs for the clinician facing staphylococcal infections with such multiresistant isolates.[23]

Both components of quinupristin/dalfopristin have been shown to accumulate to high levels inside phagocytic cells,[24] and quinupristin/dalfopristin has proven active in models of intracellular infections caused by *S. aureus*,[24,25] including VISA strains[26] and small colony variants.[27] In the present study, we have compared the extracellular and intracellular activities of quinupristin/dalfopristin against a panel of *S. aureus* (reference strains and clinical isolates) displaying specific mechanisms of resistance to streptogramins A or B. We also used a linezolid-resistant clinical isolate that was resistant to quinupristin/dalfopristin. The panel of tested strains also included staphylococcal isolates with other resistance phenotypes (MRSA, VISA and VRSA) such as can be found in patients now or in the future. We used a well-established model of infected human macrophages[28] that allows pharmacological comparisons to be drawn and has already been applied successfully to many resistant strains.[26,29,30]

Materials and methods

Materials

Quinupristin and dalfopristin were provided as microbiological standards by Nordic Pharma Ltd, Paris, France. The two compounds were separately dissolved in DMSO at a concentration of 50 g/L and diluted in water to a final concentration of 1 g/L. The two stock solutions were mixed extemporaneously in a 3:7 w/w ratio to obtain a quinupristin/dalfopristin solution with the same proportion as that of quinupristin/dalfopristin. Cell culture media and fetal calf serum were purchased from Invitrogen (Paisley, Scotland, UK) and Difco (Sparks, MD, USA). Human serum for opsonization of *S. aureus* was obtained from healthy volunteers and stored at −80°C as pooled samples until use. Biochemical and other reagents were purchased from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich-Fluka (St Louis, MO, USA).

Bacterial strains and determination of extracellular activity of antibiotics

S. aureus ATCC 25923 and *S. aureus* ATCC 33591 strains were obtained from the ATCC (Manassas, VA, USA), and NRS18, NRS126 and VRS2 were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* programme (NARSA; Herndon, VA, USA) supported under NIAID/NIH contract # HHSN272200700055C. The other strains were clinical isolates. The following clinical isolates were provided by Y. Glupczynski. MG1 [MSSA erythromycin resistant/clindamycin resistant/*erm*(A), ID no. N6113072]; MG2 [MSSA erythromycin resistant/clindamycin susceptible/*msr*(A/B), ID no. N6112967]; and MG3 (MRSA erythromycin susceptible/clindamycin susceptible, ID no. N6111729). O. Denis provided clinical isolate S103 [erythromycin susceptible/clindamycin susceptible/dalfopristin resistant/*vat*(B)] J. Quinn (Rush University Medical Center, IL, USA) provided clinical isolate CM05 (erythromycin resistant/clindamycin resistant/quinupristin resistant/dalfopristin resistant); in this strain, an rRNA methyltransferase encoded by *cfr* (conferring resistance to oxazolidinones, phenicols, pleuromutilins, lincosamides and streptogramins A)[31] and *erm*(A/B) (conferring resistance to macrolides, lincosamides and streptogramins B) are constitutively co-expressed within the same *mlr* operon.[32–34] To avoid phenotype drifts during the time taken to carry out this work, we prepared a large number of aliquots for each strain that were kept frozen until use and discarded after performing each series of experiments.

MICs were determined in Mueller–Hinton broth according to CLSI recommendations with the following modifications: (i) for the combination of quinupristin/dalfopristin, arithmetic dilutions were used to determine the MIC with greater accuracy when <1 mg/L; and (ii) for measurements at acid pH, the broth was adjusted by addition of 2 N HCl to a value of 5.4, and this value was rechecked before and after incubation. Killing curve experiments were performed by incubating bacteria for 24 h under shaking in Mueller-Hinton broth in the presence of antibiotic, as described previously.[35] Cfu values were determined after spreading on tryptic soy agar and overnight incubation using an automated scanning system (Bio-Rad Gel Doc, Bio-Rad, Hercules, CA, USA). In preliminary experiments, we checked for the absence of a carry-over effect by comparing bacterial counts from plates where the antibiotic dilutions were spread on the agar before the bacteria and from control plates. As no effect was seen as long as the concentration of the antibiotic solution remained <30× the MIC, samples were systematically diluted to lower their concentrations accordingly. This also sets the limit of detection of bacteria to a value corresponding to a decrease of 4 $\log_{10}$ units from the initial inoculum (~10^6 cfu/mL for experiments in broth; for experiments with macrophages, the dilution of the sample was always such that no carry-over effect could take place).

Cell viability upon exposure to quinupristin/dalfopristin

The viability of infected THP-1 cells exposed to quinupristin/dalfopristin for 24 h was determined over a 1–25 mg/L concentration range using a Trypan Blue staining assay to mimic what had been observed in the serum of humans during Phase I trials.[36]

Cell infection and determination of intracellular activity

All experiments were conducted with THP-1 macrophages, exactly as described previously.[28,35] Briefly, opsonized *S. aureus* were mixed with cells for 1 h, extracellular bacteria were eliminated by exposure to gentamicin (100× the MIC; 45 min) and cells were incubated with the antibiotic for 24 h at 37°C. After cell harvesting and plating, cfu values were determined and their number expressed per mg of cell protein.

Analysis of the dose–response curves and statistical analysis

Data from the dose–response experiments were used to derive a pharmacological model based on the Hill equation (response versus

$\log_{10}$ of drug concentration)[17] allowing calculation of four key descriptors pertinent to the model and the expression of activity of the antibiotic under study,[28] namely: (i) the relative minimal efficacy (E_{min}) corresponding to the bacterial growth for an infinitely low antibiotic concentration; (ii) the maximal relative efficacy (E_{max}) corresponding to the reduction of cfu for an infinitely large antibiotic concentration (a higher maximal relative activity corresponds to a lower, more negative E_{max} value); (iii) the relative potency (EC_{50}), defined as the drug concentration causing a response half-way between the minimal (E_{min}) and the maximal (E_{max}) responses; and (iv) the apparent static concentration (C_{static}) corresponding to the concentration of drug causing no apparent change in cfu compared with the initial inoculum. The values of the first three descriptors are obtained by fitting a sigmoid function to the data (non-linear regression) and (i) using the corresponding bottom (E_{max}), top (E_{min}) and EC_{50} function parameters (together with their standard error and 95% confidence interval values) and (ii) by graphical intrapolation for C_{static}.

MLS$_B$ resistance genotyping

The ribosomal methylases encoded by *erm*(A) and *erm*(C), the macrolide efflux pumps encoded by *msr*(A) and *msr*(B), and the acetyltransferase-inactivating streptogramin A encoded by *vat*(B) were characterized by PCR exactly as previously described.[3]

Curve fitting and statistical analyses

All curve fittings and statistical analyses were performed using GraphPad Prism® version 4.02 for Windows and Instat® version 3.06 (GraphPad Prism Software, San Diego, CA, USA).

Results

Susceptibility testing

Table 1 shows: (i) the origin of the strains used in the present study; (ii) their resistance phenotypes with respect to methicillin, vancomycin and linezolid; (iii) their MLS genotype; and (iv) their MICs of erythromycin, clindamycin, quinupristin and dalfopristin (tested separately and with the value of their MIC ratio) and quinupristin/dalfopristin. Quinupristin, dalfopristin and quinupristin/dalfopristin were also tested at pH 5.4 (to mimic the pH of phagolysosomes where phagocytized *S. aureus* reside in THP-1 macrophages).[37,38] As anticipated from the corresponding genotypes,[15,22] the S103 *vat*(B) strain was 16-fold less susceptible to dalfopristin than to quinupristin, while the MG2 *msr*(A/B) strain showed an elevated MIC of erythromycin (32 mg/L) but to a lesser extent of quinupristin (2 mg/L), consistent with an efflux mechanism of resistance. All *erm*(A) strains tested showed elevated MICs of erythromycin, clindamycin and quinupristin, but (i) remained susceptible to dalfopristin and (ii) displayed low MICs of quinupristin/dalfopristin, demonstrating the advantage of this combination. In contrast, the CM05 linezolid-resistant strain with a *cfr* and *erm*(A/B) genotype was resistant to all molecules tested, including dalfopristin, and, as a result, also showed an elevated MIC of quinupristin/dalfopristin. Acid pH did not affect the MICs of quinupristin, dalfopristin or quinupristin/dalfopristin, which remained similar or only one dilution lower than those measured at neutral pH [see Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Table 1. Resistance phenotypes and genotypes, and antibiotic susceptibility of the strains used in this study

Strain	Origin	Resistance		MIC (mg/L)[a]					
		phenotype	MLS genotype	erythromycin	clindamycin	quinupristin	dalfopristin	quinupristin/dalfopristin MIC ratio	quinupristin/dalfopristin (3:7)
ATCC 25923	reference strain	MSSA	—	0.5	0.25–0.5	0.5–1	2	0.25–0.5	0.45
MG3 (N6111729)	throat swab	MRSA	—	0.5	0.25–0.5	0.5–1	4	0.125–0.25	0.40
S103	clinical	MSSA	*vat*(B)	0.5	0.5	0.5	8	0.0625	0.40
MG2 (N6112967)	wound swab	MSSA	*msr*(A/B)	32	0.25	2	2	1	0.50
MG1 (N6113072)	wound swab	MSSA	*erm*(A)	256	256	32	1	32	0.40
ATCC 33591	reference strain	MRSA	*erm*(A)	256	256	32–64	2	16–32	0.40
NRS18	wound swab	MRSA/VISA	*erm*(A)	256	256	64	2	32	0.35
NRS126	bloodstream	MRSA/VISA	*erm*(A)	256	256	32	1	32	0.40
VRS2	wound swab	VRSA	*erm*(A)	>128	>128	64	2	32	0.40
CM05	nasal swab	MRSA/LZD^R	*erm*(A/B)	256	256	64	>128	<0.5	5

LZD^R, linezolid resistant.
[a]As determined at pH 7.4. No marked difference ($\leq$1 $\log_2$ dilution) was seen for quinupristin, dalfopristin or quinupristin/dalfopristin when tested at pH 5.4 [see Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. All MICs were determined on freshly thawed aliquots from the corresponding strains and are representative of those used in the other experiments described in this paper.

Concentration–effect relationships

In preliminary experiments, the cytotoxicity of quinupristin/dalfopristin towards infected THP-1 cells was examined by assessing the cell permeability to the vital dye Trypan Blue. A 2-fold increase in the proportion of stained cells over control values (from ∼15% to ∼30%) was seen after exposure of infected cells for 24 h to quinupristin/dalfopristin concentrations of 25 mg/L. Further increasing this concentration caused a more marked increase in stained cells that reached ∼90% at 100 mg/L. For this reason, all subsequent experiments used a quinupristin/dalfopristin concentration limited to a maximum of 25 mg/L.

In a first series of experiments, the activity of quinupristin/dalfopristin was examined over a 0.01–25 mg/L concentration range against bacteria in broth (extracellular activity) and bacteria phagocytized by THP-1 macrophages (intracellular activity). Figure 1 illustrates the data obtained for the fully susceptible strain (ATCC 25923) and for four strains selected for harbouring distinct MLS genotypes. Table 2 shows the pertinent descriptors of the concentration–response curves [with goodness of fit values (R^2) and statistical analyses] for all strains (thus also including the values obtained for the VISA strains NRS18 and NRS126 and for the VRSA strain VRS2). In all cases, a Hill equation (using a slope factor of 1) could be fitted to the data. All strains grew to similar levels extracellularly, as denoted by the lack of a significant difference in their respective E_{min} parameters (∼3 $\log_{10}$ cfu increase), indicating no loss of fitness related to the presence of resistance mechanisms in broth. In THP-1 macrophages, most strains grew to similar levels as extracellularly, except S103, VRS2 and CM05, which achieved a somewhat lower growth (∼2.1 $\log_{10}$ cfu increase).

The most salient observation from these studies is that all strains, except CM05 (which will be described below), showed an essentially similar dose–response pattern, with maximal relative efficacies (E_{max}) spanning from a −2.12 to −3.83 $\log_{10}$ cfu decrease for extracellular bacteria and from a −1.19 to −2.65 $\log_{10}$ cfu decrease for intracellular bacteria. Of interest, the ratio of intracellular to extracellular E_{max} values spanned from 0.46 to 1.14, indicating that the overall efficacy of quinupristin/dalfopristin for killing bacteria was only moderately or even not impaired intracellularly compared with what could be observed in broth. Considering the intracellular relative potencies (EC_{50}) and static concentrations (C_{static}), these were quite close to the corresponding MIC measured in broth at pH 7.4 (with ratios to MICs spanning from 0.43 to 2.70 for EC_{50} and 0.55 to 2.30 for C_{static}), indicating that the intracellular milieu did not adversely affect the potency of quinupristin/dalfopristin.

In contrast to what was seen with all other strains, quinupristin/dalfopristin showed a considerably lower relative potency (higher EC_{50}) and higher static concentration (C_{static}) towards the CM05 strain, both extracellularly and intracellularly. Whereas its extracellular C_{static} matched the MIC, the intracellular C_{static} (as determined by graphical intrapolation) was much higher than anticipated, with a ratio to the MIC of ∼4.3, indicating a substantial loss of potency of quinupristin/dalfopristin towards the intracellular forms of CM05 compared with the other strains. The maximal relative efficacies (E_{max}) of quinupristin/dalfopristin towards CM05 were also markedly reduced, with values of only a 1.7 $\log_{10}$ cfu decrease extracellularly and close to 0 (static effect) intracellularly, denoting the inability to overcome this resistance mechanism by simply increasing the antibiotic concentration in either condition.

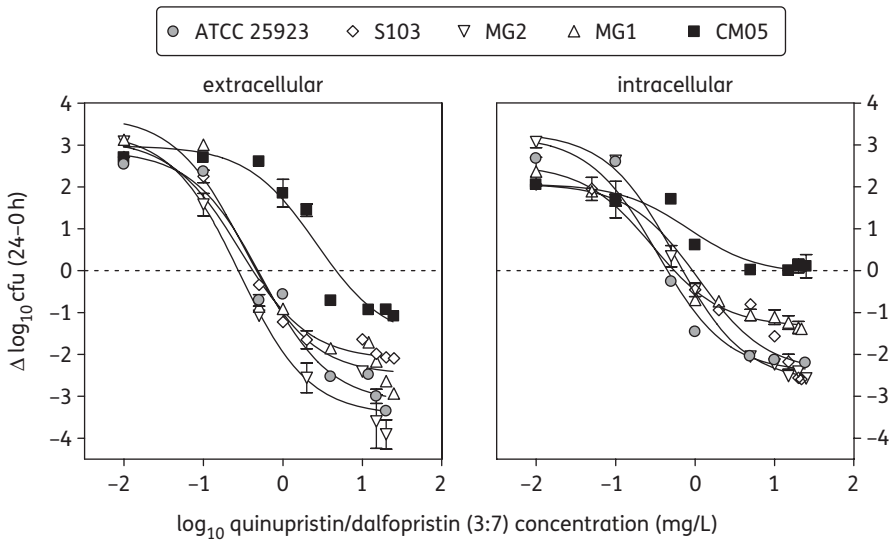

Figure 1. Concentration–response curves for the extracellular (left) and intracellular (right) activity of quinupristin/dalfopristin (3:7) against *S. aureus* strains ATCC 25923 (fully susceptible), S103 [*vat*(B)], MG2 [*msr*(A/B)], MG1 [*erm*(A)] and CM05 [*erm*(A/B), *cfr*] in broth (left) or after phagocytosis by THP-1 macrophages (right). The ordinate shows the change in cfu (in log scale) per mL (extracellular) or per mg of cell protein (intracellular) after 24 h of incubation in the presence of increasing concentrations of quinupristin/dalfopristin as compared with the initial inoculum. The horizontal broken line shows a static effect. The limit of detection corresponds to −4 $\log_{10}$ cfu. Data are means ± SD of three independent experiments (most of the SD bars are smaller than the symbols). Data obtained for each strain were used to fit sigmoidal equations. See Table 2 for regression and pertinent parameters with statistical analysis of their differences.

Table 2. Pertinent regression parameters [with confidence intervals (CIs)] and statistical analysis of the concentration–response curves for extracellular and intracellular activity of quinupristin/dalfopristin against all tested strains illustrated in Figure 1

	Extracellular activity					Intracellular activity				
Strain	E_{min}[a] (CI)	E_{max}[b] (CI)	EC_{50}[c] (CI)	C_{static}[d]	R^2	E_{min}[a] (CI)	E_{max}[b] (CI)	EC_{50}[c] (CI)	C_{static}[d]	R^2
ATCC 25923	2.87 a;A (1.69–4.04)	−3.16 ad;A (−3.95 to −2.36)	0.53 a;A (0.22–1.30)	0.48	0.967	3.21 ad;A (1.93–4.50)	−2.42 a;B (−3.27 to −1.58)	0.34 a;A (0.12–0.94)	0.46	0.970
S103	3.14 a;A (2.38–3.89)	−2.12 bcd;A (−2.50 to −1.75)	0.28 a;A (0.16–0.51)	0.41	0.981	2.07 bc;B (1.39–2.75)	−2.41 a;A (−3.08 to −1.74)	1.08 bcd;B (0.43–2.74)	0.92	0.957
MG2	3.31 a;A (1.75–4.86)	−3.45 a;A (−4.32 to −2.58)	0.29 a;A (0.10–0.81)	0.28	0.974	3.29 a;A (2.99–3.61)	−2.65 a;B (−2.83 to −2.46)	0.56 ac;B (0.45–0.71)	0.69	0.997
MG1	3.70 a;A (2.20–5.19)	−2.48 ab;A (−3.22 to −1.75)	0.31 a;A (0.11–0.86)	0.47	0.948	2.53 ab;B (2.21–2.85)	−1.34 be;B (−1.50 to −1.18)	0.29 a;A (0.18–0.45)	0.55	0.994
NRS18	3.65 a;A (2.75–4.55)	−2.82 abd;A (−3.60 to −2.04)	0.33 a;A (0.16–0.66)	0.43	0.969	2.47 ab;A (1.78–3.15)	−1.89 ae;B (−2.35 to −1.44)	0.51 ac;A (0.25–1.03)	0.67	0.971
NRS126	3.28 a;A (2.86–3.70)	−2.26 abd;A (−2.60 to −1.91)	0.29 a;A (0.20–0.42)	0.42	0.992	2.66 ab;A (1.77–3.55)	−1.19 b;B (−1.59 to −0.80)	0.23 a;A (0.10–0.56)	0.53	0.961
VRS2	3.14 a;A (2.30–3.93)	−3.83 a;A (−4.95 to 2.72)	0.88 b;A (0.44–1.75)	0.71	0.969	2.19 bd;B (1.28–3.09)	−1.77 ce;B (−2.30 to −1.24)	0.17 a;B (0.06–0.51)	0.22	0.943
CM05	2.98 a;A (2.16–3.80)	−1.67 cd;A (−2.79 to −0.56)	2.62 c;A (0.89–7.69)	4.68	0.944	2.07 c;B (1.48–2.67)	−0.08 d;B (−0.52 to 0.36)	0.83 d;B (0.22–3.18)	21.93[e]	0.933

Statistical analysis: (i) analysis per column (one-way analysis of variance with Tukey post-test for multiple comparisons between each parameter of all drugs), figures with different lower case letters are significantly different from each other ($P<0.05$); and (ii) analysis per row (unpaired, two-tailed *t*-test between corresponding parameters of extracellular and intracellular activities), figures with different upper case letters are significantly different from each other ($P<0.05$).

[a]Cfu increase (in $\log_{10}$ units) at 24 h from the corresponding post-phagocytosis inoculum, as extrapolated for an infinitely low antibiotic concentration (minimal relative efficacy).

[b]Cfu decrease (in $\log_{10}$ units) at 24 h from the corresponding post-phagocytosis inoculum, as extrapolated for an infinitely large antibiotic concentration (maximal relative efficacy). All cfu counts were above the minimal detection level.

[c]Relative potency expressed as the concentration (mg/L) causing a reduction of the inoculum half-way between the E_{min} and E_{max} values, as obtained from the Hill equation (using a slope factor of 1).

[d]Static concentration (mg/L) for which no apparent bacterial growth was detected (no change from the initial inoculum) as determined by graphical intrapolation.

[e]This value (calculated by graphical intrapolation of *x*-axis crossing of the Hill equation) remains largely undetermined as E_{max} is barely below 0 for this strain compared with all other strains.

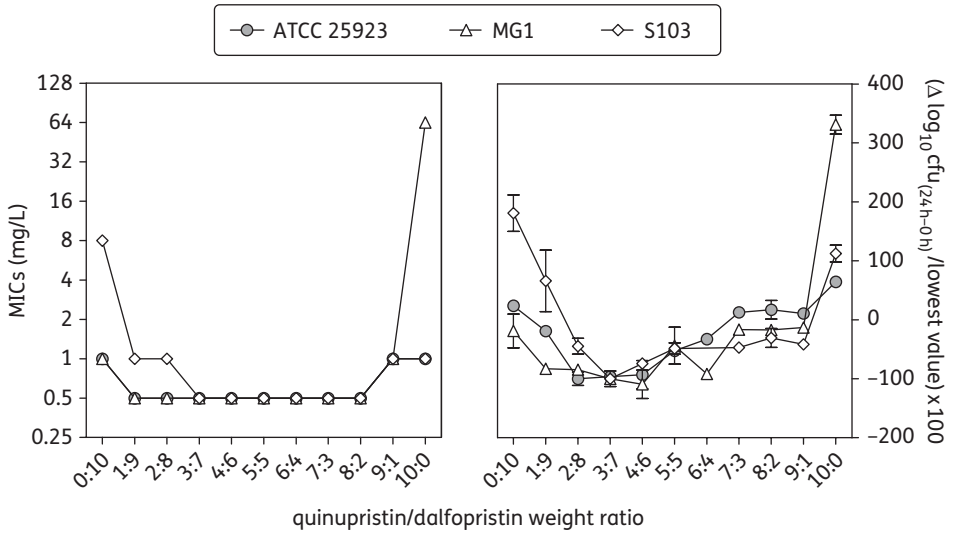

Figure 2. Activities of quinupristin/dalfopristin mixed in variable proportions and tested for MIC determination in broth at pH 7.4 (left) and activity against intracellular *S. aureus* (right) at a fixed total extracellular concentration of 10 mg/L (24 h of incubation; the ordinate shows the decreases in $\log_{10}$ cfu compared with the initial inoculum and expressed as the percentage of the maximal value observed for each strain [ATCC 25293, -1.87 ± 0.211 (2:8 ratio); MG1, -0.985 ± 0.217 (4:6 ratio); S103, -0.791 ± 0.104 (3:7 ratio)]. Data are means ± SD of three independent experiments; when not visible the SD bars are smaller than the symbols.

Activity of quinupristin/dalfopristin at variable concentration ratios

While the original description of quinupristin/dalfopristin showed that the synergy between dalfopristin and quinupristin could be observed in broth over a wide range of concentration ratios,[12] little is known about the expression of this synergy towards intracellular bacteria. We therefore examined how modifying the composition of the combination from 1:0 to 0:1 in 0.1 increments could modulate the intracellular activity weight ratio, while keeping the total extracellular concentration to a constant value of 10 mg/L. Strains ATCC 25923, S103 and MG1 were used in this study as they presented three typical and different resistance phenotypes. Their MICs in broth at pH 7.4 were also measured in parallel for reference purposes. Figure 2 shows that activity was maximal (lowest MIC and largest decrease of $\log_{10}$ cfu) for a quinupristin/dalfopristin weight ratio of ~3:7–8:2 in broth and 3:7–4:6 in THP-1 macrophages.

Discussion

To our knowledge, this study is the first to examine in depth the *in vitro* extracellular and intracellular activity of quinupristin/dalfopristin combination on a panel of *S. aureus* strains with clinically relevant mechanisms of resistance to antibiotics acting on the 50S subunit of the ribosome or the assembly of the ribosomal subunits, as well as to other major antistaphylococcal drugs such as β-lactams and glycopeptides. The main message is that this combination of streptogramins is quite active against the intracellular forms of *S. aureus*, making it one of the most active agents in this context,[28] including against MRSA, VISA or VRSA isolates.

Considering extracellular bacteria and conventional susceptibility testing first, we confirm that resistance to one of its constituents does not adversely affect the activity of quinupristin/dalfopristin at least in *S. aureus*,[12] which, beyond the fact that the drug is only used sparingly in the clinical arena, may explain why overall susceptibilities of this organism have remained stable in all surveyed areas since its authorization for human use in the late 1990s.[39–47] Reporting the quinupristin/dalfopristin MIC ratio, as done here, may be a useful indicator of the presence of a resistance determinant to one of the quinupristin/dalfopristin components, which will not appear from the simple determination of the MIC of the commercial mixture. Extending this type of analysis to larger collections would be of interest to further validate it as potential diagnostic tool. In contrast, the simultaneous presence of *erm*(A) (determinant of the MLS_B phenotype) and of *cfr* (determinant of resistance to oxazolidinones, phenicols, pleuromutilins, lincosamides and streptogramins A[31,33]) (as seen in CM05) results in resistance to the two components of quinupristin/dalfopristin, a marked increase in the MIC and loss of bactericidal activity. Originally described in animal staphylococci,[31,48] the multiresistance *cfr* has now been observed in human *S. aureus* isolates.[33,49,50] Its dissemination could result in cross-resistance of staphylococci to many antibiotics, in both human and veterinary medicine, and should, therefore, be carefully monitored. This is all the more important for quinupristin/dalfopristin, as (i) *cfr* and *erm*(A) are both localized on highly mobile genetic elements and (ii) streptogramins have been used and lincosamides are still used in animals, creating a risk of simultaneous transfer of both genes to human isolates.

Concerning the concentration–effect relationships for intracellular bacteria, our data also showed unanticipated properties for quinupristin/dalfopristin and susceptible strains, namely: (i) that the intracellular static concentrations and EC_{50} values are close to the MICs in broth; and (ii) that the maximal intracellular relative efficacy (E_{max}) values are quite high and globally

only half of those observed against extracellular bacteria. The intracellular static concentrations and EC_{50} values, and their relationship to MICs in broth, are globally similar to those recently reported for clarithromycin and the ketolides CEM-101 and telithromycin.[51] It could be argued that this effect is related to a permeabilization of the cells during the incubation time, giving quinupristin/dalfopristin free access to intracellular bacteria. There was indeed a modest but detectable increase in the proportion of cells stained with Trypan Blue at the maximum concentration tested. However, such a permeabilization would tend to make E_{max} values for intracellular bacteria similar to those of extracellular bacteria (as observed in the same model with the membrane-permeabilizing antibiotic CSA-13),[52] which was not the case here. It is more tempting to speculate that this may result from the cellular accumulation of quinupristin and dalfopristin. Previous studies using radiolabelled compounds found accumulation levels of 34 and 50 for quinupristin and dalfopristin, respectively,[24] a level globally similar to that of macrolides and ketolides in this set-up.[28,51] Their subcellular localization has not been established, but their weak basic character, and the impairment of accumulation seen in cells exposed to acidic pH,[51] strongly suggest a proton-trapping mechanism in acidic membrane-bounded vacuoles, as demonstrated for macrolides[53] and many other weak organic bases.[54] In this context, it is important to emphasize that acidic pH does not impair the activity of quinupristin/dalfopristin or of its constituents (in contrast to what is seen with macrolides and ketolides,[51] or as was shown specifically for ATCC 25923 with gentamicin in the same models).[55] This could contribute to the maintenance of its potency in the intracellular milieu. The results of our experiments in which we varied the quinupristin/dalfopristin weight ratio and for which results pointed to an optimal value of ~3:7 as for extracellular bacteria also suggest that both streptogramins are handled in a similar way by THP-1 cells, making synergy as effective intracellularly as in broth.

With respect to its intracellular maximal relative maximal efficacies (E_{max}), quinupristin/dalfopristin more effectively reduces the intracellular inoculum, compared with vancomycin or linezolid (both essentially bacteriostatic),[29,56] β-lactams, including ceftobiprole (for which E_{max} values almost never exceed a 1–1.5 $\log_{10}$ cfu decrease),[28,30,38] or daptomycin (~1.6 $\log_{10}$ cfu decrease).[57] Globally speaking, the intracellular maximal relative efficacy of quinupristin/dalfopristin compares favourably with that of highly bactericidal agents such as telavancin or oritavancin (when tested against MSSA and MRSA at high extracellular concentrations)[26,28,29] or moxifloxacin (towards strains with low MICs).[28] The activity of quinupristin/dalfopristin is, however, clearly impaired by the acquisition of mechanisms of resistance against both of its constituents, as evidenced by the behaviour of the CM05 strain. This limit affects not only the relative potency but also, quite intriguingly, its maximal relative efficacy, which should largely prevent any useful use of the drug even at increased dosages. Of note, the intracellular medium thus seems to exacerbate differences between CM05 and the susceptible strains, to the extent that the drug becomes essentially static (making the value of C_{static} only indicative). This could arise from changes in gene expression (including potential up-regulation of those involved in resistance),[58] an overall decrease in bacterial metabolism or envelope structural changes when *S. aureus* is intracellular and, thereby, exposed to acidic pH.[59]

In a more general context, this work opens up renewed perspectives for the treatment of multidrug-resistant *S. aureus* infections. While a large panel of new anti-MRSA drugs has been under development over recent years, only daptomycin, tigecycline and, more recently, telavancin (but in the USA only so far) have been authorized for human use on a wide scale. Each of these drugs has limits in terms of tolerance at the registered dosages.[60–62] Moreover, since the current susceptibility breakpoints of all these drugs, based on their currently registered dosages, is very close to the upper limit of the MIC distribution of the wild-type populations (see http://www.eucast.org), a dosage increase will be needed in the case of emergence of even low-level resistance, which will further increase the risk of toxicity.

The model presented here is only partial and suffers from several limitations[28–30,51,56] that make simple extrapolations to human therapy quite uncertain. Yet, a recent study has shown fairly good agreement between the findings with this *in vitro* model and those obtained from an animal model as far as intracellular *S. aureus* infection is concerned.[63] The present cell model also allows for direct comparison between drugs, giving insight into their intrinsic potential against intracellular infection. Thus, while the safety and tolerability issues associated with the administration of quinupristin/dalfopristin cannot be ignored,[64] and the existence of fully resistant strains may limit its use, our findings may help in reappraising the use of this drug in difficult to treat staphylococcal infections where eradication of intracellular bacteria could be of importance for successful therapy.

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Transparency declarations

Nothing to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary material

Table SP1: MICs (mg/L) of quinupristin, dalfopristin and quinupristin/dalfopristin (3:7) against strain ATCC 25923 at neutral and acidic pH [a]

Quinupristin		Dalfopristin		Q-D	
pH 7.4	**pH 5.4**	**pH 7.4**	**pH 5.4**	**pH 7.4**	**pH 5.4**
0.5-1	0.5-1	2	2	0.45	0.40
0.5-1	0.5-1	4	4	0.40	0.40
0.5	0.5	8	2-4	0.40	0.40
2	2	2	2	0.50	0.40
32	32	1	1	0.40	0.30
32-64	32-64	2	1	0.40	0.40
64	32-64	2	2	0.35	0.40
32	64	1	1	0.40	0.30
64	64	2	1	0.40	0.30
64	32	> 128	16	5	4

[a] See Baudoux et al.[1] for comparisons of the effect of pH on the MICs of oxacillin and gentamicin towards this strain

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