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Activities of antistaphylococcal antibiotics towards the extracellular and intraphagocytic forms of *Staphylococcus aureus* isolates from a patient with persistent bacteraemia and endocarditis

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ABSTRACT

Decreased susceptibility of *Staphylococcus aureus* to antistaphylococcal agents may be associated with inability to eradicate intracellular forms, which could explain therapeutic failures. This hypothesis was tested using clinical isolates obtained from a patient with persistent staphylococcal bacteraemia under therapy. Four isogenic isolates (three from tissue, one from blood) with increased MICs for vancomycin (1–4 mg/L) and for daptomycin (1–4 mg/L) were collected after an initial 16-day treatment with vancomycin–rifampicin–gentamicin, followed by 13–20 days of treatment with daptomycin–rifampicin–gentamicin. Isolates were tested for MICs and for: (i) vancomycin (BODIPY-FL-vancomycin) and daptomycin binding; (ii) cell wall turnover (loss of *N*-acetyl-D-[1-^{14}C]glucosamine in 30 min after 1 h of labelling); and (iii) Triton X-100-induced autolysis. Extracellular (broth) and intracellular (THP-1 macrophages) activities of rifampicin, linezolid and fusidic acid at C_{max}, and of vancomycin, daptomycin, quinupristin–dalfopristin and oritavancin over a wide range of extracellular concentrations (with pharmacological modelling to determine E_{max}), were measured at 24 h. Increases in vancomycin MICs correlated with increased drug binding, and decreased cell wall turnover and detergent-induced autolysis. Increases in daptomycin MICs correlated with decreased daptomycin binding. Intracellular activity was weak (E_{max} <1 $\log_{10}$ CFU decrease) for vancomycin against all isolates, and for daptomycin against isolates with MICs >1 mg/L. Among all antibiotics tested, only quinupristin–dalfopristin and oritavancin provided close to bactericidal intracellular activities (1.6–2.5 $\log_{10}$ CFU decreases at C_{max}). Determination of the intracellular susceptibility of *S. aureus*, combined with improved methods of diagnosis, could be useful when dealing with persistent staphylococcal infections and could improve therapy.

Keywords Daptomycin, fusidic acid, linezolid, macrophages, oritavancin, quinupristin–dalfopristin, resistance, rifampicin, *S. aureus*, vancomycin

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INTRODUCTION

Staphylococcus aureus is an aggressive pathogen that poses a significant public health threat. Treatment of staphylococcal diseases involves two main challenges. First, this pathogen has become increasingly resistant to currently available antibiotics, narrowing the choice of useful agents [1–3]. In this context, vancomycin-intermediate *S. aureus* (VISA) isolates are being increasingly encountered, although detection problems with VISA and lack of agreement on definitions of the hetero-VISA phenotype have complicated the situation [4]. These strains appear to develop from pre-existing methicillin-resistant *S. aureus* in patients undergoing vancomycin therapy [5]. They are somewhat unique, owing to their

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increased cell wall thickness, the precise reason for which is unknown but which is associated with: (i) accumulation of free D-alanyl-D-alanine termini in the peptidoglycan (acting as false target sites for vancomycin [6,7]); and (ii) resistance to detergent-induced autolysis [8]. Second, there is growing evidence that *S. aureus* invades and persists within eukaryotic cells [9,10]. This is reflected clinically by its propensity to cause a variety of recurrent and relapsing infections, such as osteomyelitis, skin and skin structure infections, or endocarditis [11–13], offering an explanation for the frequency and recurrence of such invasive infections [9,14].

Although the metabolic and phenotypic characteristics of laboratory-generated VISA have been described by several groups [8,15,16], a detailed analysis of isogenic strains has not been frequently attempted [17]. Hence, this study aimed to investigate the global properties of a series of isogenic methicillin-resistant *S. aureus* (MRSA) isolates recovered from a patient with a prosthetic aortic valve who, as a complication of bacteraemic pacemaker infection, developed an endocardial abscess [18]. In addition, given that the intracellular persistence of *S. aureus* may reflect an important nidus for severe endocarditis, the extracellular and intracellular activities of several antibiotics commonly recommended or under investigation for the treatment of staphylococcal infections were also compared. Concentrating on macrophages, and using previously established models [19,20], this study provides a potential rationale for the poor clinical and microbiological response in patients infected with these variants, while suggesting potential benefit in using newly developed antistaphylococcal agents.

MATERIALS AND METHODS

Case report

The patient (a 65-year-old, with aortic valve replacement in 1991, coronary artery disease, sick-sinus syndrome, and diabetes mellitus) was hospitalized for fevers that developed 2 weeks after pacemaker placement, with serosanguinous discharge from the pacemaker site, and MRSA bacteraemia (vancomycin MIC 2 mg/L, daptomycin MIC 1 mg/L). Details on pertinent clinical events and therapeutic regimens in relation to sample collection are presented in a synoptic fashion in Table 1. Bacteraemia was documented over a 33-day span, and resolved only after debridement of an abscess underneath the aorta sewing ring and aortic valve replacement, strongly suggesting that the maintenance of tissue foci of infection was the main cause of the persistent character of the infection. One year after discharge, the patient was clinically well and showed no sign of relapse.

Table 1. Time course of the infection, successive antistaphylococcal therapies and vancomycin and daptomycin MICs for *Staphylococcus aureus* isolated from the blood of the patient at the initiation of and during therapy

			MIC (mg/L)[a]	
Day	**Event**	**Antistaphylococcal treatment**	**Vancomycin**	**Daptomycin**
0	First hospitalization and blood sampling Start of initial antibiotic treatment	Start vancomycin/gentamicin/rifampicin	2	1
6	Removal of pacemaker			
13	Transfer to Hershey Medical Center Isolation of VISA		4	1
15	Isolation of daptomycin-resistant *S. aureus*		2–4[b]	1–2[b]
16	Confirmation of vegetation on prosthetic aortic valve Modification of initial treatment	Start daptomycin Stop vancomycin and rifampicin		
29	Isolation of VISA and daptomycin-resistant *S. aureus*	Continue gentamicin	4–8[c]	4[c]
31	Change in antibiotic treatment	Start linezolid Stop gentamicin and daptomycin		
35	Debridment of endocardial abscess; replacement of aortic valve; sample collection from infected endocardial abscess	Transiently add vancomycin and gentamicin	2–8[d]	1–8[d]
37–38	Change in antibiotic treatment	Stop vancomycin and gentamicin Continue linezolid Add quinupristin–dalfopristin	[e]	
49	Myalgia (related to quinupristin–dalfopristin)	Stop quinupristin–dalfopristin Continue linezolid Add trimethoprim–sulphamethoxazole		
65	Hyperkalaemia, renal failure	Stop trimethoprim–sulphamethoxazole Continue linezolid		
77	End of antistaphylococcal treatment	Stop linezolid		

[a]Determined at the Clinical Laboratory of the Hershey Medical Center (macrobroth dilution method and confirmed by microbroth and Etest methods); the values shown are the extremes observed (see notes a–d).
[b]Between day 11 and day 27, 14 isolates were obtained: one with vancomycin/daptomycin MICs of 2/1 mg/L, 12 with vancomycin/daptomycin MICs of 4/1 mg/L, and one with vancomycin/daptomycin MICs of 4/2 mg/L.
[c]Between day 29 and day 32, four isolates were obtained: one with vancomycin/daptomycin MICs of 4/1 mg/L, and three with vancomycin/daptomycin MICs 8/4 mg/L.
[d]On day 35, nine isolates were obtained: eight with vancomycin/daptomycin MICs of 2/1 mg/L, and one with vancomycin/daptomycin MICs of 8/8 mg/L.
[e]All blood cultures were negative for *S. aureus* (methicillin-resistant *S. aureus* or vancomycin-intermediate *S. aureus* (VISA)) as from day 36.

Table 2. Clinical isolates, source of isolation and susceptibilities to selected antibiotics (all isolates are resistant to oxacillin, ciprofloxacin, moxifloxacin, and rifampicin (see text)) in comparison with a fully sensitive reference strain

		MIC (mg/L)[b]						
Isolate/strain[a]	Source of isolation	VAN	TEC	DAP	LZD	Q/D	FUS	ORI
HMC 546 (#3)	Wound; aortic valve[c]	1 (1.25)	2	1–2	1	0.5	0.5	0.125
HMC 547 (#6)	Subvalvular tissues[c]	2 (2)	4	3	1	0.5	0.5	0.125
HMC 548 (#10)	Annulus vegetations[c]	4[e] (3.5)	8	4	1	0.5	0.5	0.5
HMC 549 (#17)	Blood[d]	4[e] (4.0)	8	4	1	0.5	0.5	0.5
ATCC 25923	Reference strain	1 (0.9)	1–2	0.125–0.25	1	0.5	0.5	0.06

VAN, vancomycin; TEC, teicoplanin; DAP, daptomycin; LZD, linezolid; Q/D, quinupristin–dalfopristin; FUS, fusidic acid; ORI, oritavancin.
[a]Numbers in parentheses are those used in the original description of the isolates [18].
[b]As determined by geometric (log_2 progression) dilution for all antibiotics by microdilution [20] (for vancomycin, numbers in parentheses refer to the MICs determined by arithmetic dilutions using 0.25 mg/L increments).
[c]Isolated at day 35 (see Table 1).
[d]Isolated at day 15 (see Table 1).
[e]Defined as vancomycin-intermediate *S. aureus* on the basis of the CLSI definition (MIC ≥2 mg/L [52]); those isolates are, however, still considered susceptible according to the EUCAST clinical breakpoints (≤4 mg/L; http://www.srga.org/eucastwt/MICTAB/MICglycopeptides.html) but fall 1 log_2 dilution above the EUCAST epidemiological cut-off (2 mg/L; http://217.70.33.99/Eucast2/SearchController/regShow.jsp?Id = 13901).

Bacterial isolates and reference strains, and determination of MICs and of *agr* polymorphism

The clinical isolates used in the present study (Table 2) have been described previously [18]. In parallel, a fully sensitive *S. aureus* strain (ATCC 25923) was used. A vancomycin-intermediate *S. aureus* strain (NRS 126, obtained from NARSA, Herndon, VA, USA (vancomycin MIC 4 mg/L)) was also used for control purposes. MICs were determined independently at both institutions and yielded very similar results. The methods used at the Hershey Medical Center have been described in detail [18]. At the Université catholique de Louvain, a microdilution method was used, following the recommendations of the CLSI, with both geometric and arithmetic dilutions. For oritavancin, all media contained 0.002% polysorbate (Tween-80 or 2-(2-(3,4-bis(2-hydroxyethoxy)oxolan-2-yl)-2-(2-hydroxythoxy)ethoxy)ethyl(*E*)-octadec-9-noate) as advised by the manufacturer to prevent drug binding to plastic. The accessory gene regulator (*agr*) polymorphism was determined by PCR [21,22].

Binding of vancomycin and daptomycin to whole bacteria

Drug binding was measured using boron dipyrromethene difluoride-labelled vancomycin (BODIPY®FL vancomycin; Invitrogen Corp., Carlsbad, CA, USA) or unmodified daptomycin. Bacteria were exposed to either drug (BODIPY-FL-vancomycin, 1 mg/L; daptomycin, 50 and 100 mg/L) for 30 min at 37°C in Mueller–Hinton Broth containing 2% NaCl and adjusted to pH 7.4, harvested by centrifugation, washed with ice-cold phosphate-buffered saline, and lysed as described previously [23]. Both drugs were then assayed by fluorimetry (Fluorocount TM (multiplate reader), Packard Instruments, PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA), with excitation and emission wavelengths set at 485 and 530 nm for vancomycin (assay linearity: 0.6–4 mg/L; R^2 >0.99), and 380 and 425 nm for daptomycin [24] (linearity: 1–250 mg/L; R^2 >0.99 (due to instrument limitations, the optimal emission wavelength (460 nm) could not be used, but this reduced the signal value by only *c.* 50%)). Protein content was determined in parallel by using the Folin–Ciocalteu/biuret method [25].

Autolysis assay

Triton X-100-stimulated autolysis in 50 mM glycine buffer (pH 8) was assessed after 30 min following a previously described protocol [17].

Cell wall turnover

Cells were labelled for 1 h at 37°C with 5 μg of *N*-acetyl-D-[1-^{14}C]glucosamine (55 mCi/mmol; Amersham plc, Little Chalfont, Buckinghamshire, UK). After labelling, bacteria were harvested by centrifugation and pellets were resuspended in isotope-free medium containing 5 mM non-radioactive *N*-acetyl-D-glucosamine [16], and collected either immediately (time 0 h) or after 30 min of incubation at 37°C. After being washed in ice-cold phosphate-buffered saline, bacteria were resuspended in 10% ice-cold trichloroacetic acid/100 mM *N*-acetyl-D-glucosamine [26], and radioactivity was measured by liquid scintillation counting. Cell wall turnover was expressed as the percentage of radioactivity released during the 30-min post-labelling incubation period.

Determination of extracellular and intracellular activities

Extracellular activities were measured in broth as described previously [27]. Intracellular activities were measured on bacteria phagocytozed by THP-1 macrophages (ATCC TIB-202), a human myelomonocytic cell line displaying macrophage-like activity (American Tissue Collection; supplied through LGC Promochem Ltd, Teddington, UK) as also described previously ([20,23]; the procedure used: (i) implies a 2000-fold dilution of the cell samples on a volume basis, minimizing interference of the carried-over antibiotic on CFU counting [28]; and (ii) limits the incubation time for CFU counting to 24 h, so that the occurrence of so-called 'small-colony variants' is not taken into account [20]). Typical starting inocula were *c.* 1-2 × 10^6 CFU/mL (broth) or per mg of cell protein (macrophages).

Antibiotics and main reagents

The following antibiotics were obtained as microbiological standards from their corresponding manufacturers: linezolid

from Pfizer Inc., New York, NY; quinupristin–dalfopristin (30 : 70 mass ratio) from Nordic Pharma, Paris, France; oritavancin from Targanta Therapeutics Corp., Cambridge, MA, USA; daptomycin from Novartis AG, Basel, Switzerland; teicoplanin from Sanofi-Aventis, Paris, France, rifampicin from Merrell Dow Pharmaceuticals, Strasbourg, France; and ciprofloxacin and moxifloxacin from BayerHealthCare, Leverkusen, Germany. Gentamicin and vancomycin were obtained as the corresponding branded products (Geomycin®; Vancocin®) as distributed in Belgium (and complying with the provisions of the European Pharmacopoeia for human use) by Glaxo-SmithKline s.a., Genval, Belgium (pilot studies showed that clinical formulations and microbiological standards of these products had similar activities). Oxacillin and fusidic acid were purchased from Sigma-Aldrich, St-Louis, MO, USA. Cell culture media and sera were from Invitrogen and other reagents from Sigma-Aldrich or Merck KGaA, Darmstadt, Germany.

Statistical analyses

Curve-fitting analyses were made using GRAPHPAD PRISM version 4.02 for Windows (GraphPad Prism Software, San Diego, CA, USA).

RESULTS

Antibiotic susceptibilities (MICs)

Table 2 shows the clinical isolates used for the present study with their anatomical source and the MICs of selected antibiotics (all of these isolates were resistant to oxacillin (MIC ≥16 mg/L; all strains carried the *mecA* and the *SCCmec* group IVa genes), to fluoroquinolones (ciprofloxacin MIC ≥32 mg/L; moxifloxacin MIC ≥2 mg/L) and to rifampicin (MIC ≥4 mg/L)). As compared to the fully susceptible strain ATCC 25923 and with the exception of strain HMC 546, all clinical isolates also showed reduced susceptibility to vancomycin and teicoplanin. Susceptibility to daptomycin was also impaired, even for the two vancomycin-susceptible *S. aureus* (VSSA) strains (HMC 546 and HMC 547) and still more for the two VISA strains. In contrast, the MICs of fusidic acid, quinupristin–dalfopristin and linezolid for all clinical isolates were low and similar to those observed for ATCC 25923, and in the same range as observed in a recent survey of MRSA and VISA isolates [29]. For quinupristin–dalfopristin, the MLSB phenotype was not established, but the fact that VISA strains remain susceptible to this antibiotic combination is not surprising, as this has been observed in other isolates, not only in our laboratory (Baudoux P, Lemaine S, Van Bambebe F, Tulkens PU, unpublished data) but also elsewhere [30,31]. For oritavancin, MICs were two-fold to eight-fold higher for the clinical isolates than for ATCC 25923 but still 2–3 $\log_2$ dilutions lower than those of vancomycin.

Characterization of reduced susceptibility to vancomycin

VISA strains have been reported to contain more cell wall subunits able to bind vancomycin [7], to have reduced cell wall turnover [16] and to be more resistant to Triton X-100 autolysis [16] in comparison with susceptible strains. These characteristics were therefore examined for four selected clinical isolates with different susceptibilities to vancomycin (HMC 546 and HMC 547 (VSSA) and HMC 548 and HMC 549 (VISA)). As shown in Fig. 1, vancomycin binding increased, and cell wall turnover and Triton X-100-induced autolysis decreased roughly in parallel with the increase in MIC, consistent with the concept that decreased susceptibility to vancomycin is related to thickening of the bacterial cell wall.

Earlier studies demonstrated that vancomycin treatment failure is often associated with group II polymorphism of the *agr* locus [32]. All clinical isolates belonged to *agr* group II on the basis of PCR analysis.

Characterization of daptomycin resistance

Because all clinical isolates (including the two VSSA isolates HMC 546 and HMC 547) showed MICs above the clinical susceptibility breakpoint (1 mg/L) of EUCAST and of the US labelling, the binding of the drug to whole bacteria preparations from these isolates (HMC 546 and HMC 549) was measured in comparison with the fully susceptible reference strain ATCC 25923 and a reference VISA strain (NRS 126). As shown in Fig. 2, binding of daptomycin (100 mg/L) for both clinical isolates was considerably reduced compared to what was observed for the fully sensitive strain and there was also a clear correlation between reduced binding and increase in MIC when all data points were considered (experiments made at a daptomycin concentration of 50 mg/L yielded values almost half of those shown in Fig. 1, demonstrating that binding was linear in the concentration span used).

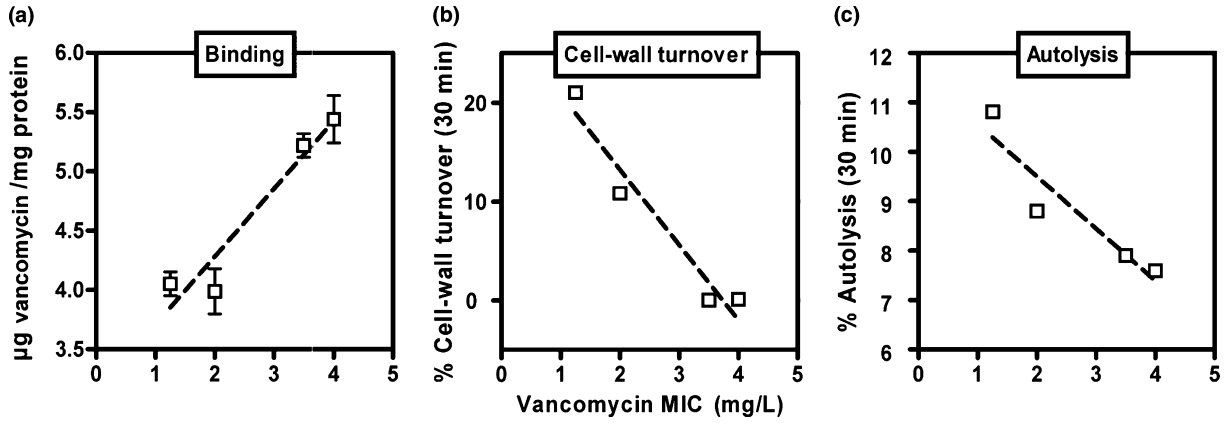

Fig. 1. Correlation between vancomycin susceptibility (MIC, as determined by arithmetic dilution) and vancomycin binding (a), cell wall turnover (b) and Triton X-100-induced autolysis (c) of clinical isolates (from left to right in each graph: HMC 546 (MIC 1.25 mg/L); HMC 547 (MIC 2 mg/L); HMC 548 (MIC 3.5 mg/L), and HMC 549 (MIC 4 mg/L)). All values are means ± SD (n = 3; when not visible, SD bars are smaller than the symbols). The dotted lines show the result of a linear regression analysis of each set of data. Values for the fully sensitive ATCC 25923 strain (MIC 1 mg/L) were: vancomycin binding, 4.3 ± 0.2 µg/mg protein; cell wall turnover, 23.3 ± 0.3%; autolysis, 12.3 ± 0.2%.

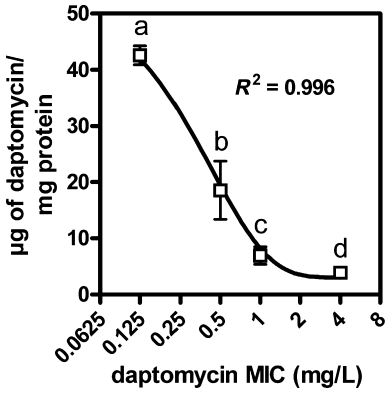

Fig. 2. Correlation between binding of daptomycin (100 mg/L) to whole bacterial preparations (±SD (n = 3)) and MIC (as determined by geometric dilution (in duplicate; no difference between individual values)) for (a) the fully sensitive reference strain ATCC 25923 (MIC 0.125 mg/L), (b) the vancomycin-intermediate *Staphylococcus aureus* reference strain NRS 126 (MIC 0.5 mg/L) and the two clinical isolates (c) HMC 546 (MIC 1 mg/L) and (d) HMC 549 (MIC 4 mg/L). The graph shows the regression function obtained by fitting a sigmoid equation to the data. Statistical analysis (ANOVA): data points with different letters are significantly different from each other ($p < 0.01$).

Assessment of the intracellular activities of antibiotics

In earlier studies, the activity of antibiotics was, generally speaking, markedly impaired intracellularly as compared to what can be observed for bacteria maintained in broth or in culture medium [20,27,33]. As this phenomenon could explain therapeutic failures in spite of acceptable susceptibility as determined by conventional MIC measurement, the extracellular and intracellular activities of antistaphylococcal antibiotics towards HMC 546 were systematically compared to the fully susceptible ATCC 25923, using a model of infected THP-1 macrophages.

In the first approach, all drugs were compared at a reference concentration, which was arbitrarily set at their observed or estimated C_{max} (total drug) when administered to humans at conventional doses (rifampicin, 4 mg/L; vancomycin, 50 mg/L; daptomycin, 77 mg/L; linezolid, 20 mg/L; fusidic acid, 50 mg/L; quinupristin–dalfopristin, 10 mg/L; oritavancin, 40 mg/L). The results are presented in Fig. 3, and show that the loss of activity of all antibiotics when tested against an intracellular form of *S. aureus* was as important for the clinical isolates as for the reference strain (the loss was marginal for fusidic acid, but this antibiotic is essentially bacteriostatic against both forms). For vancomycin, activity was not much different between the reference strain and HMC 546 and HMC 549, indicating that the concentration used (50 mg/L) was giving maximal activity, disregarding the differences in MIC. In contrast, the decreased susceptibility of HMC 546 and HMC 549 to daptomycin in comparison with the reference strain (MIC 1–2 vs. 0.12–0.25 mg/L) resulted in this drug almost completely losing its ability to kill intracellular bacteria, even though it was used at a concentration

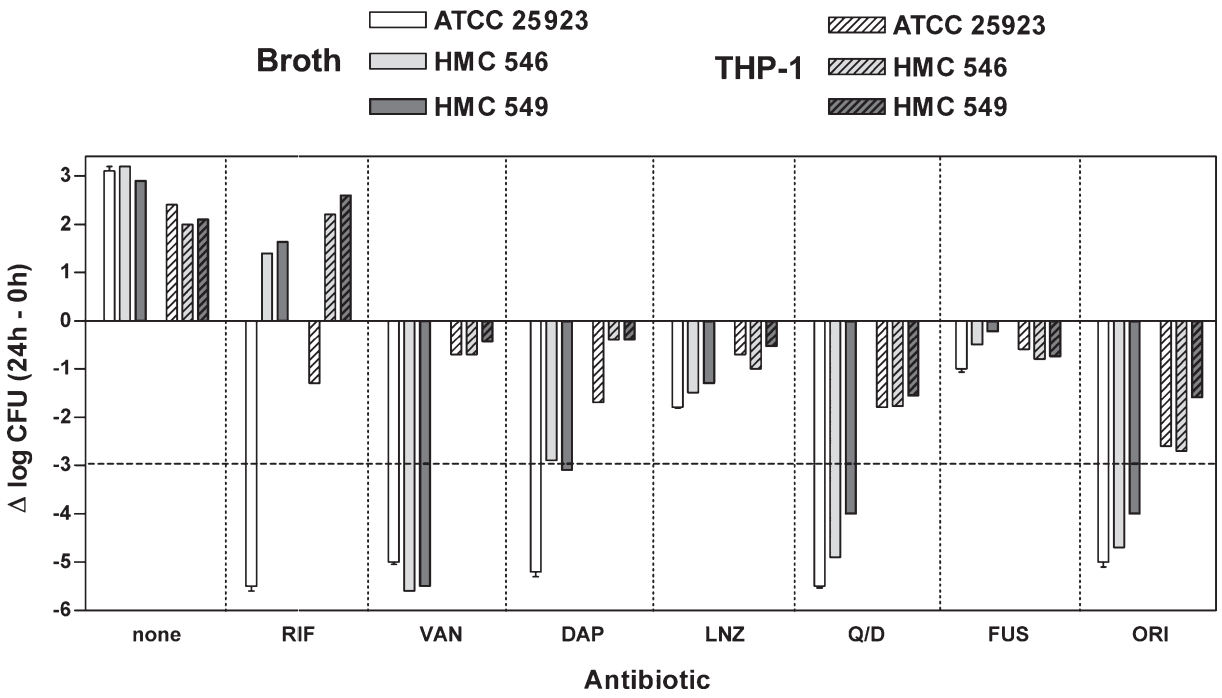

Fig. 3. Comparison between extracellular (broth; unhatched bars) and intracellular (THP-1 macrophages; hatched bars) activities of antistaphylococcal antibiotics against the fully susceptible ATCC 25923 strain (white bars), the HMC 546 isolate (vancomycin-susceptible *Staphylococcus aureus*; light grey bars), and the HMC 549 isolate (dark grey bars). The ordinate shows the change in the number of CFU (Δ log CFU) per mL of culture medium (broth) or per mg of cell protein (THP-1). Each antibiotic was added for 24 h at a concentration corresponding to its known or estimated human serum C_{max} (total drug: rifampicin (RIF) 4 mg/L; vancomycin (VAN) 50 mg/L; daptomycin (DAP) 77 mg/L; linezolid (LNZ) 20 mg/L; fusidic acid (FUS) 50 mg/L; quinupristin–dalfopristin (Q/D) 10 mg/L; oritavancin (ORI) 40 mg/L). The dotted horizontal line shows the decrease in CFU (3 $\log_{10}$ units, commonly taken as an indication of a bactericidal effect [53].

much above its MIC (77 mg/L). Quinupristin–dalfopristin and oritavancin showed intermediate activity. For oritavancin, this decrease was parallel with its increase in MIC. Linezolid and fusidic acid activities were essentially bacteriostatic in macrophages, and their activities were not markedly different between the clinical and the reference strains. Finally, the resistance of HMC 546 and HMC 549 to rifampicin in broth (MIC 4 mg/L) translated into a complete loss of activity against its intracellular form when used at that concentration. None of the antibiotics tested had bactericidal activity against the intracellular forms of either the reference or the clinical strains (defined by a 3 $\log_{10}$ decrease of the cell-associated CFUs [34]), even though oritavancin approached it for ATCC 25923 and HMC 546, but not for HMC 549. Fig. 4 shows the data of Fig. 3 expressed as a function of the concentration/MIC ratio for drugs with bactericidal effect in broth (rifampicin, vancomycin, daptomycin, quinupristin–dalfopristin, and oritavacin). It is clear that the decrease in maximal intracellular activity is *c.* 1.5–2 $\log_{10}$ CFU, and is reached once the concentration/MIC ratio exceeds 50, independently of the antibiotic–strain combination considered. In this context, the lower response to daptomycin and the lack of response to rifampicin of HMC 546 and HMC 549 are related to their lower concentration/MIC ratios.

To gain more insight into the concentration–effect relationships of the antistaphylococcal antibiotics and the clinical isolates HMC 546 (VSSA) and HMC 549 (VISA), in comparison with the fully sensitive reference strain ATCC 25923 [20,27], full dose–response experiments were run with vancomycin, daptomycin, quinupristin–dalfopristin and oritavancin, using a wide range of extracellular concentrations, spanning from sub-MIC values to C_{max} or above (vancomycin, 0.005–50 mg/L; daptomycin, 0.01–250 mg/L; quinupristin–dalfopristin, 0.01–25 mg/L; oritavancin, 0.01–40 mg/L). The data were then used to determine the corresponding Hill function parameters. This approach allows an accurate distinction between changes that affect drug

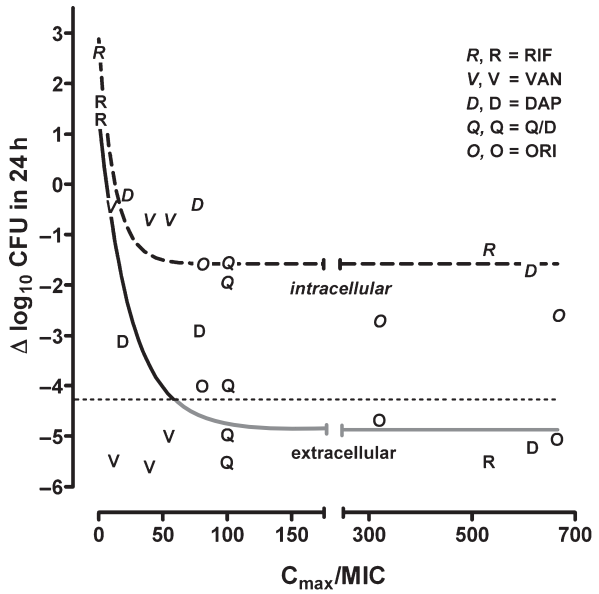

Fig. 4. Extracellular and intracellular activities of rifampicin, vancomycin, daptomycin, quinupristin–dalfopristin and oritavancin against ATCC 25923, HMC 546 and HMC 549 when plotted against the corresponding C_{max}/MIC ratio (see Fig. 3 for activity and C_{max} values and Table 2 for MICs). Data were used to fit 'one-phase exponential decay' functions (dotted line, intracellular bacteria; solid line, extracellular bacteria) to help identify the corresponding apparent maximal killing values (-1.6 $\log_{10}$ CFU for intracellular and -4.9 $\log_{10}$ CFU for extracellular bacteria, as compared with the original inoculum) and the C_{max}/MIC ratio at which these are neared. The horizontal dotted line indicates the limit of reliability of bacterial counts by the technique used (see [20] for details). The real maximal killing activity against extracellular bacteria could, therefore, be more extensive than estimated here.

efficacy (by calculating the E_{max} parameter (relative efficacies)) and those that affect drug potency (by calculating the corresponding EC_{50} parameter (relative potency)) [20]. Data are presented graphically in Fig. 5, and the corresponding Hill equation descriptors are presented in Table 3, together with the regression parameters, an estimation of the bacteriostatic concentration of each antibiotic (C_s) and the C_{max}/C_s ratios. In all cases, activity was related to concentration, conforming to the pharmacological model previously described for the reference strain [20]. This revealed significant differences in relative efficacies (E_{max}), the absolute values of which were significantly: (i) larger for quinupristin–dalfopristin and oritavancin than for vancomycin or daptomycin for all strains; and (ii) lower for daptomycin when comparing the two clinical isolates to the ATCC reference strain. Interestingly, however, no statistically significant difference in E_{max} was noted between HMC 546 (VSSA) and HMC 549 (VISA) for each of the antibiotics tested, indicating that the reduced susceptibility of the VISA isolate to vancomycin and daptomycin, as detected by the measurement of MICs, was only relative (and could be compensated for by an increase in concentration). Differences in relative potencies (EC_{50}) were also noted between vancomycin and daptomycin on the one hand, and quinupristin–dalfopristin and oritavancin on the other (variations in the responses to oritavancin, however, were not not statistically significant). Finally, whereas the C_{max}-to-bacteriostatic concentration ratio ranking was clearly in favour of daptomycin for the reference strain ATCC 25923, this ranking became quinupristin–dalfopristin > vancomycin ≈ oritavancin ≫ daptomycin for HMC 546 (VSSA), and quinupristin/dalfopristin > oritavancin ≫ daptomycin ≈ vancomycin for HMC 549 (VISA).

DISCUSSION

Vancomycin has long been considered to be the drug of choice for the treatment of MRSA infections. However, isolates of *S. aureus* with reduced vancomycin susceptibility, first reported in Japan [35], now appear to be widespread [5] and call for both a better knowledge of the biochemical changes associated with this phenotype (thereby improving our diagnostic capabilities) and a critical assessment of the proposed therapies. The present article aims at extending our knowledge of these strains in three main directions.

First, it was possible to correlate three critical biochemical properties (binding of vancomycin, cell wall turnover, and Triton X-100-induced autolysis) with changes in vancomycin susceptibility in a series of isogenic MRSA isolates obtained from a single patient undergoing vancomycin and daptomycin treatments given in succession [18]. This should allow for a more confident interpretation of these altered properties, rather than examining variations occurring for unrelated clinical isolates. Thus, the decrease in susceptibility of the isolates collected during treatment with vancomycin is directly correlated with an increase in the drug binding to the bacteria, a reduction of the cell wall turnover, and a decreased susceptibility to Triton X-100 autolysis.

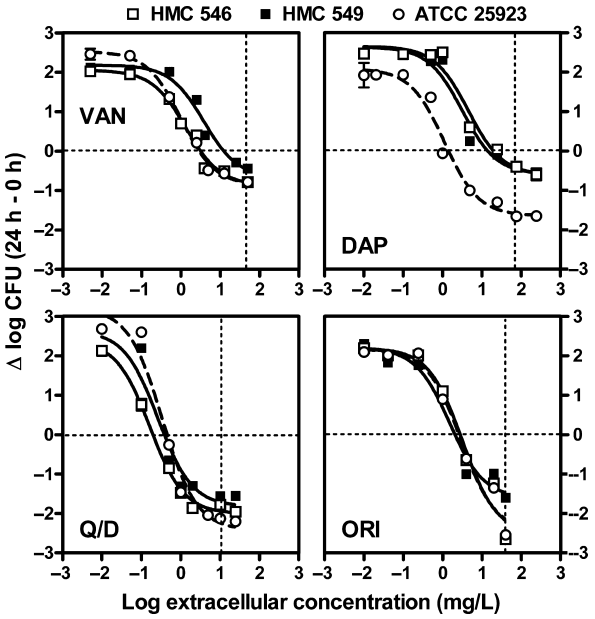

Fig. 5. Activities of four selected antibiotics (vancomycin (VAN); daptomycin (DAP); quinupristin–dalfopristin (Q/D); oritavancin (ORI)) against the intracellular forms of the reference, fully sensitive strain ATCC 25923 (open circles) and of the two clinical isolates, HMC 546 (vancomycin-susceptible *Staphylococcus aureus*; open squares) and HMC 549 (vancomycin-intermediate *S. aureus*; closed squares) after phagocytosis by THP-1 macrophages. Cells were incubated with the antibiotic for 24 h at the concentrations (total drug) indicated on the abscissa (VAN, 0.005–50 mg/L; DAP, 0.01–250 mg/L; Q/D, 0.01–25 mg/L; ORI, 0.01–40 mg/L). The graphs show the change in the number of CFUs (Δ log CFU) per mg of cell protein. All values are means ± SD (n = 3; when not visible, the SD bars are smaller than the size of the symbols). Data were used to fit sigmoid dose–response curves using a standard model with a slope factor (Hill coefficient) of 1 (see Table 3 for regression parameters). The horizontal dotted line corresponds to a bacteriostatic effect (no net change from the initial post-phagocytosis inoculum); the vertical dotted line indicates the extracellular concentration (total drug) corresponding to the observed or estimated serum C_{max} in patients receiving conventional dosages of the corresponding antibiotic and used for the comparison of activities shown in Fig. 3.

This supports the concept that the VISA phenotype developing *in vivo* is due to bacteria building up an 'antibiotic-trapping' mechanism through the thickening of the cell wall [6], which eventually prevents vancomycin from diffusing and reaching its lipid II target at the septal tip of dividing bacteria [36]. Combined with the detection of *agr* group II polymorphism (more frequent in VISA strains [37] and predictive of vancomycin treatment failures [32]), these biochemical characteristics could be useful in improving the early and reliable diagnosis of VISA isolates in clinical samples. Further studies using more clinical and laboratory strains, however, will be needed to confirm these correlations with the alterations in susceptibility, and to fully demonstrate the specificity and selectivity of these biochemical changes. Moreover, because these binding experiments or cell wall turnover measurements are difficult to implement in routine practice, more simple tests will need to be developed. This may actually become critical, given the present situation of poor recognition of the reduced susceptibility of *S. aureus* to vancomycin using available automated commercial devices, especially in cases of heterogeneously resistant phenotypes [38].

Second, the rapid emergence of resistance to daptomycin, already observed by others [39], is puzzling. The isolates studied here had both cell wall changes (reduction in muramic acid O-acetylation) and point mutations (in *mprF*, encoding lysylphosphatidylglycerol synthase) [18]. Previous studies have shown that whole cells and membranes of daptomycin-resistant *S. aureus* bind a reduced amount of daptomycin [40]. The present results confirm and expand this observation, in that increases in daptomycin MICs correlate with an almost commensurate reduction of drug binding to the bacteria (as assessed by fluorometric determination). A very recent study, of which we became aware during the revision process [41], has also identified a number of additional changes in daptomycin-resistant

Table 3. Pertinent regression parameters, statistical analysis and calculation of bacteriostatic concentrations from the dose–response curves illustrated in Fig. 2

Antibiotic	Strain	Regression parameter value (95% confidence intervals)[a]			Bacteriostatic concentration (mg/L)[b]	C_{max}/bacteriostatic concentration ratio
		E_{max} (mg/L)[c,d]	EC_{50} (mg/L)[d,e]	R^2		
Vancomycin	ATCC 25923	−0.87 (−1.14 to −0.60) a,A	0.99 (0.62–1.59) a,A	0.995	2.9	17
	HMC 546	−0.87 (−1.27 to −0.47) a,A	1.33 (0.70–2.51) a,A	0.979	3.1	16
	HMC 549	−0.68 (−1.32 to −0.04) a,A	3.79 (1.47–9.78) a,A	0.974	12	4.2
Daptomycin	ATCC 25923	−1.64 (−2.04 to −1.26) b,B,E	1.13 (0.59–2.16) a,A	0.980	1.4	55
	HMC 546	−0.61 (−1.16 to −0.06) a,A	3.20 (1.66–12.5) a,A	0.971	20	3.8
	HMC 549	−0.59 (−1.14 to −0.05) a,A	4.56 (1.11–9.16) a,A	0.967	14	5.4
Quinupristin–dalfopristin	ATCC 25923	−2.42 (−3.27 to −1.58) a,C,E	0.34 (0.12–0.94) a,C	0.973	0.46	21.7
	HMC 546	−2.01 (−2.25 to −1.78) a,C,E	0.16 (0.11–0.25) a,C	0.995	0.19	53
	HMC 549	−1.82 (−2.24 to −.50) a,C,E	0.26 (0.05–1.27) a,C,E	0.934	0.38	26
Oritavancin	ATCC 25923	−2.46 (−3.50 to −1.42) a,C	2.95 (1.02–8.49) a,A	0.976	2.6	15
	HMC 546	−2.54 (−3.79 to −1.57) a,C	3.24 (0.93–11.2) a,A	0.967	2.7	15
	HMC 549	−1.61 (−2.44 to −0.77) a,C,B	1.51 (0.46–4.81) a,A,D	0.968	2.0	20

[a]Statistical analysis (one-way ANOVA; Tukey's test for multiple comparisons): values with different letters are significantly different from each other within the pertinent comparison group ($p < 0.05$); lower-case letters, comparison between the three isolates for each condition (antibiotic); upper-case letters, comparison throughout all conditions.
[b]Concentration (mg/L) resulting in no apparent bacterial growth (the number of CFU was identical to that of the original inoculum), as determined by graphical interpolation.
[c]CFU decrease (in $\log_{10}$ units) at 24 h from the corresponding original inoculum, as extrapolated for antibiotic concentration at infinity.
[d]Total drug.
[e]Concentration (mg/L) causing a reduction of the inoculum halfway between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation (by using a slope factor of 1).

isolates (affecting membrane fluidity, translocation of the positively charged phospholipid lysylphosphotidylglycerol to the outer membrane leaflet, surface charge, susceptibility to daptomycin-induced depolarization, permeabilization, and autolysis), which will need to be explored in our strains. Decreased daptomycin susceptibility in VISA isolates is commonly ascribed to the thickening of the cell wall, which shields the daptomycin target (phophatidylglycerol) [7]. Although this could explain the decreased daptomycin binding seen with HMC 548 or HMC 549, it probably cannot account for our observations with HMC 546, as this isolate was vancomycin-susceptible and showed no increase in vancomycin binding or decreased susceptibility to detergent-induced autolysis. Therefore, a true reduction of target abundance or shielding through a mechanism other than cell wall thickening is dealt with here. This will need to be explored in more detail.

Third, although primarily known as an extracellular pathogen, *S. aureus* has been shown to invade and survive within the eukaryotic intracellular environment [12,42]. Persistence in tissue, most likely intracellularly, probably accounts for several aspects of relapsing staphylococcal diseases, long-term colonization, and persistence of bacteraemia. Previous studies using reference strains of *S. aureus* with various susceptibility phenotypes [20,34] showed that conventional antistaphylococcal agents are considerably less efficient against intracellular bacteria than against their extracellular counterparts. The present study extends this concept to clinical strains and shows that the pharmacological model derived from the analysis of the reference strain ATCC 25923 is also valid for these strains. In all cases, the lack of bactericidal effect of the antistaphylococcal agents against intracellular bacteria could be ascribed to a loss of relative efficacy (E_{max}), explaining why a large intracellular inoculum could persist regardless of the extracellular concentration of the antibiotic [20,34]. Moreover, (i) a minimal extracellular concentration/MIC ratio of 50 is probably necessary for near-maximal antibacterial effect; and (ii) even for highly bactericidal antibiotics such as oritavancin, quinupristin–dalfopristin, or rifampicin, little or no improvement is expected by further increasing the drug concentration/MIC ratio. The present study used macrophages, which may be only partly representative of the intracellular nidus seen in typical staphylococcal infections (which, beyond macrophages, involve keratinocytes [13], osteoblasts [11], or endothelial cells [12]). However, it may allow us to draw two important conclusions relative to potential treatment failures such as those seen with the patient presented in this study. First, a phenotype of resistance to rifampicin (MIC ≥4 on the basis of current CLSI breakpoints; EUCAST breakpoints are 0.06 (S) and 0.5 (R)) makes the drug completely inactive against the intracellular forms of *S. aureus*, even if used at a concentration that corresponds to the MICs for extracellular bacteria. This was surprising, as rifampicin accumulates in macrophages (*c.* 20-fold in the model used here [20]), and its activity is known to be enhanced by

acid pH (*c.* 30-fold decrease in MIC for ATCC 25923 [20]). Second, reduced susceptibility to vancomycin (MIC >2 mg/L [38]; see also the current CLSI breakpoint) or to daptomycin (MIC >1 [38]; see also the CLSI and the EUCAST breakpoints) makes both drugs poorly active against intracellular forms, even if used at extracellular concentrations far above their MIC. The accumulation of vancomycin and daptomycin in cells infected by HMC 546 or HMC 549 was not measured in comparison with those infected with ATCC 25923. The possibility that the decreases in intracellular activity observed for HMC 546 and HMC 549 are due to pharmacokinetic parameters (decreased accumulation) only cannot, therefore, be excluded. However, differences in pharmacodynamic parameters, related to target modifications, are more likely. Whatever the mechanism, however, it is tempting to speculate that the low intracellular efficacies of rifampicin, vancomycin and daptomycin against the clinical isolates studied here is the reason why infection could not be cured in the patient from whom the samples were obtained, and why the infected tissues eventually had to be surgically removed. It would be interesting, in this context, to examine the effects of drug combinations against the intracellular forms of the clinical strains of this patient, as combinations are known to be synergistic against susceptible strains [43]. This is probably most critical for vancomycin and daptomycin, because the pharmacological analysis (Fig. 5) shows that: (i) vancomycin has only limited efficacy against all strains; and (ii) daptomycin maximal efficacy is reduced against strains with an elevated MIC, in a manner than cannot be compensated for by an increase in its concentration.

This study also shows that fusidic acid, which in many countries is considered to be a useful resource for difficult-to-treat MRSA infections caused by organisms requiring MICs of <1 or 2 mg/L [44], is actually poorly effective against intracellular forms. Conversely, quinupristin–dalfopristin and oritavancin were quite active against the intracellular forms of all clinical isolates tested, regardless of their resistance phenotype. The approach used, however, did not allow exclusion of the occurrence of the so-called 'small-colony variants' [45], a point that will be examined in further studies. Given this limitation, the superiority of oritavancin, as compared with vancomycin, especially against VISA strains, is consistent with its dual mode of action, which involves not only its binding to D-Ala-D-Ala motifs, e.g. vancomycin, but also transglycosylase inhibition and membrane depolarization and permeabilization [46]. The mode of action of quinupristin–dalfopristin, for which synergism between the two components is due to induction of an increased ribosome affinity [47], is less suggestive, apart from the fact that this combination of streptogramins is bactericidal in broth, whereas single components are bacteriostatic only. Quinupristin–dalfopristin and oritavacin accumulate to large extents in cells [48,49], which could suggest that their superior activity against intracellular bacteria is mainly linked to this specific cellular pharmacokinetic parameter. Previous studies, however, show that there is no simple correlation between cellular accumulation and activity in the model used here when comparing a large number of antibiotics of different pharmacological classes [20]. A role of carried-over antibiotic (causing an artefactual decrease of CFU counts) can also be excluded, on the basis of the procedure used and considering that intracellular activity reaches a plateau even if the concentration/MIC ratio is increased to very large values (Fig. 4).

This model has many limitations, which have been discussed in detail in previous reports [20,34], e.g. the use of a constant exposure to the antibiotics, the impossibility of evaluating the influence of the inhibitory effect of protein binding on drug efficacy, the limited duration of drug exposure, and the many uncertainties concerning relevance to the *in vivo* situation. For some of the antibiotics, full dose–response curves were not established, limiting the conclusions concerning what has been observed at a concentration corresponding to the human C_{max} (total drug). All these aspects will need to be carefully studied in the future. For instance, the fact that aminoglycosides penetrate cells only very slowly [50] makes this model, although superior to other shorter-term models, insufficient for true assessment of the long-term effects of these drugs [51]. It may, nevertheless, prove useful for a comparative assessment of antistaphylococcal drugs and for characterization of better agents in the future. Combined with the improved diagnostic methods suggested above, an assay of intracellular activity could also contribute, already today, to improved choices of appropriate therapies among

available agents for difficult-to-treat staphylococcal infections when persistence of intraphagocytic *S. aureus* is suspected.

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