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RESEARCH ARTICLE

Tigecycline inhibition of a mature biofilm in clinical isolates of *Staphylococcus aureus*: comparison with other drugs

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Abstract

The purpose of our study was to evaluate the anti-staphylococcal biofilm activity of tigecycline, compared with a group of recently developed or commonly used antimicrobials such as linezolid, daptomycin, levofloxacin, tobramycin and rifampin, all possessing putative antibiofilm properties, on a sample of multi-drug-resistant methicillin-resistant *Staphylococcus aureus* grown as a planktonic and mature biofilm. We determined conventional minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for the planktonic forms, MICs of adherent cells and finally, minimum biofilm eradication concentrations (MBECs). No drug was able to inhibit adherent bacteria at the same concentration necessary for eradicating a mature biofilm; the latter concentrations varied from three to seven times higher than the ones inhibiting adhesion. The concentrations eradicating biofilm were reached by rifampin and daptomycin at lower concentrations with respect to the other antibiotics tested; tigecycline was able to inhibit mature biofilms at higher concentrations, while all the other antibiotics were only able to inhibit adhering cells.

Introduction

Biofilms are known for their complexity (Costerton *et al.*, 1999) and ability to resist just about everything in our current antimicrobial armory. Inside these well-organized and highly regulated structures, bacterial cells acquire different properties with respect to free-floating bacteria – among which metabolic and phenotypic specializations are acquired – diverse morphologies and a heterogeneous physiology. All these features may be responsible for the coexistence of aerobically growing, fermenting, dormant and dead cells.

It is well known that biofilms play a role in the pathogenesis of *Staphylococcus aureus* infections, causing many difficulties in the eradication of biofilm-associated infections (BAIs) and leading to a persistent and chronic state of many *S. aureus* diseases (Ceri *et al.*, 1999; Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004).

Mechanisms of biofilm resistance, still not totally clear, are usually multifactorial and vary from one organism to another. It has been suggested that these resistance mechanisms can be attributed to many factors: a delayed penetration of antimicrobials through the biofilm matrix; the presence of slow or nongrowing cells within the biofilm; the heterogeneity of the biofilm bacterial population in terms of the presence of subpopulations with different phenotypic levels of resistance; and the presence of persisters (Mah & O'Toole, 2001; Harrison *et al.*, 2005).In *Pseudomonas aeruginosa*, for example, the involvement of a cluster of genes, namely the *ndv*B locus, has been correlated with high-level biofilm-specific resistance (Mah *et al.*, 2003). Furthermore, antimicrobial biofilm resistance can be considered as a sort of 'population resistance' in which the sequestration of drugs can expose bacteria to antimicrobial concentrations below therapeutic doses, within infection sites, and also below the mutant prevention concentration (Dong *et al.*, 1999; Zhao & Drlica, 2001).

The activity of antimicrobials on biofilms depends on their molecular size, positive charges, permeability coefficient and bactericidal activity, and from this point of view, it is mandatory to test the antibiofilm activity of new drugs, potentially useful in BAIs.

Current standard assays and parameters such as minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs), performed normally on suspended cultures, are inadequate to evaluate the

antimicrobial activity of slow nongrowing sessile cells, and the use of several variables (MBEC, MBIC, MIB^{ADH}, MBC^{ADH}) and methods (Calgary device method, MBIC determination, quantification of viable cells by XTT assay) complicates the interpretation of *in vitro* results and the understanding of antimicrobial antibacterial activity (Ceri *et al.*, 1999; Labthavikul *et al.*, 2003; Sandoe *et al.*, 2006; Smith *et al.*, 2009).

The purpose of our study was to evaluate the anti-staphylococcal biofilm activity of tigecycline, compared with a group of recently developed or commonly used antimicrobials such as linezolid, daptomycin, levofloxacin, tobramycin and rifampin, all possessing putative antibiofilm properties, on a sample of multi-drug-resistant methicillin-resistant *S. aureus* (MRSA) grown as a planktonic and mature biofilm. We determined conventional MICs and MBCs for the planktonic forms, MICS of adherent cells and finally, minimum biofilm eradication concentrations (MBECs).

Materials and methods

Bacterial strains

Our sample included 30 previously identified biofilm-producing MRSA strains collected in our laboratory from central venous catheters or sputum of cystic fibrosis patients. Isolates were grown on mannitol–salt–agar (MSA) plates (Oxoid, Basingstoke, UK) identified by the coagulase test and the Api-Staph System (Bio-Merieux), and maintained at $-80\,^{\circ}C$ until use.

Biofilm production

The biofilm-forming ability of *S. aureus* was tested in a static system using a spectrophotometrical quantitative assay. Each strain was grown in tryptone soy broth (Oxoid), with the addition of 0.25% glucose (TSBG). These assays were performed in microtitre plates as described previously (Christensen *et al.*, 1985; Ziebuhr *et al.*, 1999; Cafiso *et al.*, 2007). Each reported value is the average of 12 measurements at 490 nm.

MICs and MBCs

Conventional MICs and MBCs of tigecycline, levofloxacin, linezolid, daptomycin, tobramycin and rifampin were determined in duplicate using Clinical and Laboratory Standard Institute (CLSI) (2009) guidelines.

Biofilm susceptibility tests

Each MIC^{adh} and MBEC experiment, conducted in parallel with MIC and MBC, was carried out in the presence of tigecycline, levofloxacin, linezolid, daptomycin, tobramycin and rifampin.

The MIC^{adh} was the lowest antimicrobial concentration at which there was no observable bacterial growth in the wells containing adherent microcolonies. Briefly, the MIC^{adh} test was performed and evaluated as published previously (Labthavikul *et al.*, 2003), with the following modifications: the bacterial suspensions used were at a density of $10^8\,CFU\,mL^{-1}$; this suspension was prepared in phosphate-buffered saline (PBS) and diluted 1 : 30 in TSBG.

The MBEC, read as the lowest antimicrobial concentration at which bacteria fail to regrow after exposure to the antibiotic, was performed using a modified version of the Calgary biofilm device method (Ceri *et al.*, 1999).

Isolates grown overnight in TSBG were adjusted to a turbidity of 1.0 McFarland standard, validated by viable counts on tryptone soy agar plates and diluted 1 : 30 in fresh TSBG. An 200-μl aliquot was then placed in each row of a 96-well flat-bottom microtitre plate (Nuclon Delta, Nunc, Denmark), covered with a 96-peg lid (Nunc-TSP; Nunc) and statically incubated overnight for biofilm formation on the pegs.

To remove planktonic cells, the peg lid was rinsed three times in $1\times$ sterile PBS, placed on a new plate filled with 200 μL of fresh broth containing serial dilutions of antibiotics as described by CLSI guidelines and incubated overnight at $37\,^{\circ}C$. The peg lid was then placed onto a plate containing a fresh medium and the sessile cells were removed by bath sonication for 5 min at 40 kHZ (Bransonic 2510, Branson Ultrasonics Corporation, Danbury). Finally, the peg lid was discarded and replaced with a new lid. The plates were incubated for 24 h at $37\,^{\circ}C$, and after this incubation, the MBEC was recorded. All assays were conducted in triplicate.

Results

The MICs and MBCs of the planktonic cells, and biofilm inhibition and eradication as determined by MIC^{adh} and MBEC measurements, respectively, are summarized in Table 1. The results obtained on planktonic forms showed that all antibiotics tested were variably bactericidal, with $MBCs_{90}$ ranging from one dilution higher than the MIC_{90} value to ≥ 3 dilutions higher for bacteriostatic agents.

The results on biofilm cells showed that MIC_{50}^{adh} of tigecycline, linezolid, daptomycin and rifampin were 0.5, 4 and $1\,mg\,L^{-1}$, respectively, similar to the inhibition values obtained in planktonic growth and remaining within the susceptibility breakpoints. Only the MIC_{50}^{adh} of levofloxacin ($8\,mg\,L^{-1}$) and tobramycin ($128\,mg\,L^{-1}$) were resistant values for the two antibiotics.

MIC_{90}^{adh} of tigecycline and rifampin had the same values as their MIC_{50}^{adh} (0.5 and $1\,mg\,L^{-1}$), whereas a value of one dilution higher than MIC_{50}^{adh} was registered in the MIC_{90}^{adh} both for daptomycin ($2\,mg\,L^{-1}$) and for levofloxacin ($16\,mg\,L^{-1}$). A MIC_{90}^{adh} value more than one dilution higher

Table 1. *In vitro* MICs and MBECs of tigecycline and comparative antibiotics against planktonic and adherent *Staphylococcus aureus* isolates

	Planktonic bacteria (mg L^{-1})						Adherent bacteria (mg L^{-1})					
Antimicrobial agent	MIC range	MIC_{50}	MIC_{90}	MBC range	MBC_{50}	MBC_{90}	MIC^{adh} range	MIC_{50}^{adh}	MIC_{90}^{adh}	MBEC range	$MBEC_{50}$	$MBEC_{90}$
TYC	0.06–0.25	0.12	0.25	0.5–2	2	2	0.06–1	0.5	0.5	1–64	16	64
LEV	1–32	8	16	8–64	16	32	1–64	8	16	>128	>128	>128
LNZ	2–8	2	4	8– >16	>16	>16	0.5–64	4	64	16– >128	>128	>128
DAP	0.25–1	0.5	0.5	0.5–2	0.5	1	1–4	1	2	8– >128	16	16
TOB	4–128	16	128	8–128	16	128	2– >128	128	>128	2– >128	>128	>128
RD	0.06–1	0.12	0.5	0.12–2	0.5	2	0.06–2	1	1	8–16	16	16

TYC, tigecycline (0.5 mg L^{-1}*); LEV, levofloxacin (1 mg L^{-1}*); LNZ, linezolid (4 mg L^{-1}*); DAP, daptomycin (1 mg L^{-1}*); TOB, tobramycin (4 mg L^{-1}*); RD, rifampin (1 mg L^{-1}*).

*CLSI range susceptibility; EUCAST range susceptibility.

than MIC_{50}^{adh} was found for linezolid and tobramycin, which showed 64 and > 128 mg L^{-1} MIC_{90}^{adh}, respectively.

The MIC_{90}^{adh} of tigecycline (0.5 mg L^{-1}) and rifampin (1 mg L^{-1}) remained within the susceptibility breakpoints.

Comparing MIC_{90}^{adh} with MIC_{90}, it is necessary to emphasize that tigecycline and rifampin MIC_{90}^{adh} presented only a one- to twofold increase with respect to their MIC_{90}, whereas for linezolid, daptomycin and tobramycin, a two- to fourfold increase was found. The levofloxacin MIC_{90} and MIC_{90}^{adh} were 16 mg L^{-1}.

$MBEC_{50}$ and $MBEC_{90}$ of rifampin and daptomycin were 16 mg L^{-1}, whereas for tigecycline, the $MBEC_{90}$ was 64 mg L^{-1} and the $MBEC_{50}$ was 16 mg L^{-1}.

Moreover, evaluating $MBEC_{90}$ and MBC_{90}, it was found that the $MBEC_{90}$ values were at least three dilutions higher than the MBC_{90} values for all tested antimicrobials, with values of 64 and 2 mg L^{-1} for tigecycline, 16 and 1 mg L^{-1} for daptomycin, 16 and 2 mg L^{-1} for rifampin, > 128 and > 16 mg L^{-1} for linezolid, > 128 and 32 mg L^{-1} for levofloxacin and > 128 and 128 mg L^{-1} for tobramycin.

Discussion

Biofilm formation, as described in numerous papers, represents a major obstacle for the clinical efficacy of antimicrobial agents. The results of conventional antimicrobial susceptibility testing are difficult to apply to bacteria in biofilms because traditional antimicrobial treatments fail to eradicate surface-attached bacteria (Patel, 2005). It seems logical that biofilm-eradicating concentrations of drugs are necessary for a positive therapeutic outcome in BAIs such as endocarditis, and foreign body and prosthetic infection (Ceri *et al.*, 1999; Zimmerli & Ochsner, 2003). Different research groups have investigated the activity of new anti-Gram-positive drugs such as tigecycline, alone or in combination with linezolid and daptomycin against biofilm-producing staphylococci, but not all studies are comparable in terms of methodologies and, ultimately, in terms of the concordance of the results obtained (Petersen *et al.*, 2002; Labthavikul *et al.*, 2003; Raad *et al.*, 2007; Hajdu *et al.*, 2009; Presterl *et al.*, 2009; Rose & Poppens, 2009; Smith *et al.*, 2009).

In the present study, all antibiotics tested acted diversely in inhibiting or eradicating structured and mature biofilms. No drug was able to inhibit adherent bacteria at the same concentration necessary for eradicating mature biofilm; the latter concentrations varied from three to seven times higher than those inhibiting adhesion. The concentrations eradicating biofilm were reached by rifampin and daptomycin at lower concentrations compared with the other antibiotics tested; tigecycline inhibited mature biofilms at higher concentrations, while all the other antibiotics were only able to inhibit adhered cells.

In conclusion, we used different *in vitro* susceptibility testing methods to examine how antimicrobial agents affect mature biofilms produced by clinical isolates of *S. aureus*. Our results suggest that tigecycline, daptomycin and rifampin are promising as useful agents for eradicating *S. aureus* biofilm infections, while the other drugs tested were only able to inhibit adherent cells. The concentrations required for biofilm eradication are three to five times higher than the concentrations required for inhibiting adherent cells. These effects may be an important factor in the selection of antimicrobial therapy for this virulent organism.

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