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Stability of the MICs of various antibiotics in different clonal populations of methicillin-resistant *Staphylococcus aureus*

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Sir,

We read with interest the recent report by Hiramatsu *et al.* of the first isolation of a strain of methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to vancomycin.[1] To date, clinical isolates of *S. aureus* have been uniformly susceptible only to glycopeptide antibiotics which have consequently become the drugs of choice for the treatment of patients with MRSA infections. However, as vancomycin-resistant isolates of *S. aureus* are likely to become more widespread, alternative antibiotics with activities against MRSA are needed urgently. Although arbekacin and ampicillin/sulbactam were used successfully to eradicate the Japanese isolate, thereby demonstrating the efficacy of existing non-glycopeptide drugs, little is known about the stability of the MICs of alternative compounds over time. In order to assess this property in clonally related MRSA strains isolated in the Düsseldorf area, we determined the MICs of a broad range of antimicrobial agents for 125 such isolates recovered over a period of 3 years.

The test isolates were selected from a collection of 489 MRSA strains from 183 different patients; the organisms had been referred to the Institute for Medical Microbiology and Virology, University Hospital Düsseldorf, between 1992 and 1995 by 11 regional hospitals. From these 489 strains, 183 were chosen for typing studies on the basis that they were the first isolates recovered from any site of a colonized or infected patient. All isolates were typed by pulsed-field gel electrophoresis (PFGE) which is regarded as the most discriminatory molecular technique for typing MRSA strains. This procedure identified 28 distinct MRSA types,[2] of which the three most common were types 1, 2 and 3 (comprising 75, 38 and 12 isolates respectively); these have been shown to be the predominant PFGE types found in western Germany. Carriage of the *mecA* and *coa* genes by the 125 isolates belonging to the three PFGE types was confirmed with a multiplex PCR.

Type 1 MRSA strains were isolated between July 1992 and June 1995 from 29 wards in five hospitals throughout the region. Those belonging to MRSA type 2 were isolated between November 1992 and April 1995 from 19 wards in three hospitals, while those belonging to MRSA type 3 were isolated between September 1994 and January 1995 from five wards in three hospitals.

The MICs of 20 antimicrobial agents for the 125 strains belonging to PFGE types 1, 2 and 3 were determined by a microbroth dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS);[3] the inoculum was approximately 10^8 cfu/L. All of the isolates were resistant to penicillin (MICs $>$ 32 mg/L), ampicillin (MICs $>$ 32 mg/L), amoxycillin–clavulanate (MICs $>$ 32 mg/L), piperacillin–tazobactam (MICs $>$ 128 mg/L), oxacillin (MICs $>$ 64 mg/L), imipenem (MICs $>$ 16 mg/L), ceftriaxone (MICs $>$ 32 mg/L), gentamicin (MICs $>$ 16 mg/L), tetracycline (MICs $>$ 8 mg/L), rifampicin (MICs $>$ 2 mg/L), erythromycin (MICs $>$ 16 mg/L) and clindamycin (MICs $>$ 8 mg/L). The MICs of the remaining agents are shown in the Table.

All of the isolates were susceptible to the glycopeptides, vancomycin and teicoplanin, the MICs of these agents remaining stable over the range 0.5–1 mg/L. Despite reports of the development of low-level vancomycin resistance in clinical isolates of coagulase-negative staphylococci and the first report of reduced susceptibility to vancomycin in *S. aureus*,[1] no increase in the MICs of glycopeptides for MRSA strains isolated in Düsseldorf was observed. These antibiotics therefore remain the drugs of choice for patients with MRSA infections, both in the Düsseldorf area and, indeed, throughout western Germany where the same PFGE types predominate.

The combination of quinupristin and dalfopristin (Synercid) was highly active against all of the MRSA isolates tested; moreover, the MICs were stable over the range 0.25–1 mg/L during the study period. As the combination is also active *in vitro* against strains exhibiting inducible MLS (macrolide, lincosamide and streptogramin-B) resistance, quinupristin and dalfopristin being poor inducers of the methylase (*erm*) genes, it shows promise as an alternative to the glycopeptides in patients with MRSA infections.[4]

Table. MICs of various antibiotics for 125 MRSA isolates belonging to three PFGE types

Antibiotic	MIC (mg/L) MRSA type 1 (n = 75)	MRSA type 2 (n = 38)	MRSA type 3 (n = 12)
Ciprofloxacin	16–32	0.125[a] 16–32[b]	16–32
Co-trimoxazole	⩽0.05	⩽0.05	⩽0.05
Minocycline	4	2–4	1–2
Quinupristin–dalfopristin	0.5–1	0.5–1	0.25–0.5
Sparfloxacin	2–4	0.06[a] 2–4[b]	4
Teicoplanin	0.5–1	0.5–1	0.5–1
Trovafloxacin	0.5–1	0.06[a] 0.5–1[b]	0.5
Vancomycin	0.5–1	0.5–1	1

[a]MICs for the first four isolates.
[b]MICs for the remaining 34 isolates.

Of the three fluoroquinolones tested, trovafloxacin was the most active; all but four strains were resistant to ciprofloxacin and the activity of sparfloxacin was modest. The MICs of these agents were stable only in types 1 and 3. Isolates belonging to PFGE type 2, on the other hand, could be subdivided into two groups according to their susceptibilities. The first four strains isolated (referred in November 1992), which comprised group 1, were susceptible to the three fluoroquinolones (MIC range = 0.06–0.125 mg/L), while the 34 strains isolated subsequently, which comprised group 2, were markedly less susceptible (range = 0.5–1 mg/L for trovafloxacin, 2–4 mg/L for sparfloxacin and 16–32 mg/L for ciprofloxacin); however, the MICs for the strains in the latter group remained stable over the 2.5 year period during which they were collected. Although there were no mutations in the *grlA*, *grlB*, *gyrA* and *gyrB* genes in the first four isolates, all 34 strains in the second group were characterized by mutations in *grlA* (Ser80 to Phe) and *gyrA* (Glu88 to Lys). Clearly, newer fluoroquinolones, such as trovafloxacin, exhibit excellent in-vitro activities. However, their efficacies as treatment of patients with MRSA infections have not been confirmed and, for the time being, they should be used in combination with other agents to which MRSA strains are susceptible when, for whatever reason, the administration of glycopeptides and streptogramins is precluded.

All of the MRSA isolates were susceptible to co-trimoxazole (MICs ⩽ 0.5 mg/L), which is active *in vitro* against up to 95% of MRSA strains and time–kill studies have shown it to be rapidly bactericidal at concentrations equivalent to 4 × MIC.[5]

Minocycline was active against all of the isolates tested (MICs = 1–4 mg/L). There have been reports of the successful use of minocycline as treatment of patients with staphylococcal infections, including endocarditis caused by MRSA, although the drug is rarely used in this clinical setting, presumably because of its exclusively bacteriostatic activity.[6]

In summary, the data presented here demonstrate the stability of the MICs of a range of antimicrobials for clonally related MRSA strains isolated in the Düsseldorf area. This stability is particularly noteworthy as the isolates were recovered over a period of up to 3 years from 29 different wards in five hospitals, each of which varied in terms of patient demographics and the extent and type of antibiotic usage. All of the MRSA isolates, most of which belonged to the three most common PFGE types isolated in the western regions of Germany, were stably susceptible to vancomycin, teicoplanin, quinupristin–dalfopristin, trovafloxacin, co-trimoxazole and minocycline. Clearly, while resistance amongst MRSA isolates to many antibiotics appears to be a stable phenomenon, stable susceptibility to antibiotics other than the glycopeptides suggests that there is a rational basis for prescribing alternative therapeutic agents in the management of patients with infections caused by these pathogens.

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Comparative in-vitro activities of GD-40 and other β-lactamase inhibitors against TEM-1 and SHV-2 β-lactamases

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Sir,

Group 2b (and 2be) Class A broad- and extended-spectrum β-lactamases have emerged as important causes of β-lactam treatment failure in patients infected with aerobic Gram-negative bacilli.[1] Clavulanic acid, a naturally occurring compound, and semisynthetic β-lactamase inhibitors, including sulbactam, tazobactam and 6β-bromo-penicillanic acid (brobactam), have been developed in order to overcome this resistance mechanism.[2–4] We previously described the synthesis of a new series of 6α-bromo-, 6α-chloro- and 6α-fluoro-2β-chloromethyl-2α-methylpenam-3α-carboxylic acid sulphones and the activities of these agents against the β-lactamase I of *Bacillus cereus*.[5] Of these novel compounds, 6α-chloro-2β-chloromethyl-2α-methylpenam-3α-carboxylic acid 1,1-dioxide (GD-40) had the greatest activity. In this study, we have compared the inhibitory activity of GD-40 with those of β-lactamase inhibitors currently available for clinical use and brobactam against TEM-1 and SHV-2β-lactamases.

Tazobactam was provided by Symphar Laboratories Inc., Edmonton, Canada, the lithium salt of clavulanic acid by SmithKline Beecham, Surrey, UK and brobactam by Leo Pharmaceuticals, Ballerup, Denmark; GD-40 and sulbactam were synthesized according to methods described previously.[5,6] TEM-1 and SHV-2 β-lactamases were obtained by sonicating overnight cultures of *Escherichia coli* J53(RGK) and *E. coli* J53.2 86-1 respectively, both of which were kindly provided by A. Medeiros, in Brain Heart Infusion broth (Difco, Detroit, MI, USA). The enzymes were partially purified by gel filtration with Sephadex G75 and stored at −20°C in 0.1 M phosphate buffer, pH 7.0, until ready for use. The purity of each enzyme was confirmed by demonstration of a single band at the expected isoelectric point (i.e. pI 5.4 for TEM-1 and pI 7.6 for SHV-2) following analytical isoelectro-focusing in broad-range (pH 3–10) gels; an ampicillin iodometric overlay was used for the detection of both enzymes and a ceftriaxone iodometric overlay for the detection of SHV-2.[7]

Hydrolysis of 100 μM nitrocefin (as substrate) in 100 mM phosphate buffer, pH 7.0, in the absence of inhibitor or in the presence of a broad range of concentrations of each inhibitor (final volume, 1 mL) was followed by measuring absorbance at 482 nm at room temperature (25°C) with a UV-Vis scanning spectrophotometer (Shimadzu) and a UVPC2101-coupled program; the range of concentrations was determined for each agent in preliminary experiments and each test was performed in duplicate. The reaction was initiated by adding enzyme (for TEM-1, 20 μL of a 1:5 dilution of a stock solution, containing 1.79 U/mL and 0.62 U/mg protein, in 100 mM phosphate buffer, pH 7.0, and, for SHV-2, 15 μL of a 1:5 dilution of a stock solution, containing 2.39 U/mL and 0.08 U/mg protein, in 100 mM phosphate buffer, pH 7.0). As reactions with suicide inhibitors are strongly dependent on the time course of the reaction, the hydrolysis rate for each enzyme–inhibitor combination (at each concentration) was determined from the slope of the tangent to the reaction curve after incubation for 5 min.

The effect of pre-incubation of enzyme and inhibitor was evaluated by incubating a 900 μL solution of each enzyme with each inhibitor at room temperature for 5 min. The reaction was initiated by adding 100 μL of a 1 M solution of nitrocefin, and was followed spectrophotometrically as described above; the mixing time was 15 s.

Relative inhibition of enzyme activity was calculated according to the following equation: $100 \times (1 - A_i/A_c)$, where A_i is the slope of the reaction obtained with the inhibitor and A_c is the slope obtained with the control (i.e. in the absence of inhibitor), in both cases at the end of the

Table. Comparative activities of GD-40 and other β-lactamase inhibitors

Inhibitor	Enzyme	Condition	IC_{50} (μM)
Brobactam	TEM-1	no pre-incubation	0.4884
		pre-incubation	0.0139
	SHV-2	no pre-incubation	0.0524
		pre-incubation	0.0003
Tazobactam	TEM-1	no pre-incubation	0.0456
		pre-incubation	0.0333
	SHV-2	no pre-incubation	0.0265
		pre-incubation	0.0075
Sulbactam	TEM-1	no pre-incubation	0.9826
		pre-incubation	1.0740
	SHV-2	no pre-incubation	0.1896
		pre-incubation	0.0676
Clavulanic acid	TEM-1	no pre-incubation	0.4119
		pre-incubation	0.2499
	SHV-2	no pre-incubation	0.3502
		pre-incubation	0.0072
GD-40	TEM-1	no pre-incubation	0.4910
		pre-incubation	0.6510
	SHV-2	no pre-incubation	4.0008
		pre-incubation	0.6500

period of incubation. Experimental IC_{50}s (the concentration of each agent that inhibited 50% of enzyme activity) were determined after plotting the percentage remnant activity (y) against the concentration of inhibitor (x) and analysing the raw data with Table-Curve v. 3.01 (Jandel Scientific, Chicago, IL).

The inhibitory activities of GD-40 and the other agents tested are summarized in the Table. The IC_{50}s of GD-40 were comparable to those of earlier β-lactamase inhibitors (clavulanic acid and sulbactam), but higher than those of more recently introduced compounds (tazobactam and brobactam). In common with other β-lactamase inhibitors, pre-incubation of enzyme and inhibitor significantly affected the IC_{50} with SHV-2, but had little effect on the value with TEM-1. Time-course analysis of enzyme inactivation (data not shown), together with the marked difference in the inhibition of SHV-2 with and without pre-incubation with the inhibitor (IC_{50}s of 0.65 μM and 4 μM respectively), suggest a branched reaction pathway, as previously observed with other β-lactamase inhibitors.[8]

The present study has demonstrated that GD-40 and other 6α-halo-2β-chloromethyl sulphones may be of value as inhibitors of both broad- and extended-spectrum β-lactamases.

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