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In-vitro activity and killing effect of quinupristin/dalfopristin (RP59500) on nosocomial *Staphylococcus aureus* and interactions with rifampicin and ciprofloxacin against methicillin-resistant isolates

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Quinupristin/dalfopristin (RP59500) is a novel streptogramin and a semisynthetic derivative of pristinamycins I_A and II_B. The following properties of RP59500 were investigated: (i) its in-vitro activity against 164 hospital isolates of *Staphylococcus aureus*, 101 of which were methicillin-resistant (MRSA); (ii) its killing effect against 24 MRSA and seven methicillin-susceptible (MSSA) isolates; (iii) its interactions with rifampicin and ciprofloxacin against 18 MRSA isolates, six susceptible to both rifampicin and ciprofloxacin and 12 resistant to both, at 1 x MIC, 2 x MIC and 4 x MIC. Rifampicin and ciprofloxacin were applied at a concentration equal to their mean serum levels in order to establish the clinical relevance of the results. The MIC_{50}, MIC_{90}, MBC_{50} and MBC_{90} of quinupristin/dalfopristin were, respectively, ≤0.015, 2, 0.12 and 2 mg/L for MRSA isolates and ≤0.015, 0.06, ≤0.015 and 0.25 mg/L for MSSA isolates. All isolates were inhibited by quinupristin/dalfopristin. Its killing effect varied with concentration and time, being optimal at 4 x MIC and after 24 h growth. Strains surviving 24 h exposure to this agent had much higher MICs than the parent strain, but only a limited number of them became resistant. Quinupristin/dalfopristin at 2 x MIC and 4 x MIC showed in-vitro synergy with rifampicin against highly resistant isolates mainly at 6 h and 24 h of growth involving 50–83% of MRSA isolates, and showed synergy with ciprofloxacin at 24 h involving 42–75% of isolates. The MIC increase in colonies surviving at 24 h was restricted by the presence of rifampicin or ciprofloxacin. In contrast, the above combinations acted synergically over the total number of MRSA strains susceptible to both rifampicin and ciprofloxacin. The above findings show that quinupristin/dalfopristin is a very potent antistaphylococcal agent, and that its activity against MRSA isolates is enhanced when it is combined with rifampicin or ciprofloxacin.

Introduction

In the hospital environment, *Staphylococcus aureus* resistant to a variety of classes of antimicrobials, such as β-lactams, quinolones and macrolides, are responsible for many life-threatening infections.[1] Treatment of these infections with newer streptogramins like quinupristin/dalfopristin (RP59500) is a therapeutic challenge. Quinupristin/dalfopristin is a injectable derivative dispensed as a 30:70 mixture of the water-soluble streptogramins quinupristin (RP57669) and dalfopristin (RP54476), which are semisynthetic derivatives of the insoluble istinamycins I_A and II_B respectively,[2] the former being a polyunsaturated macrolactone and the latter a cyclic hexadepsipeptide. Quinupristin/dalfopristin is active *in vitro* against *S. aureus* resistant to macrolides, lincosamides and streptogramin B (MLS_B phenotype), including both methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) isolates.[3]

Our aim was to evaluate the in-vitro activity of quinupristin/dalfopristin and its bactericidal effect over time for a large number of MRSA and MSSA hospital isolates, as well as to investigate its in-vitro interactions with rifampicin and ciprofloxacin against MRSA.

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Materials and methods

Determination of resistance to methicillin

Altogether 164 *S. aureus* isolates were tested. They were derived from different patients with a variety of nosocomial infections and were isolated from pus (152), blood (six), pleural fluid (three) and CSF (three). Resistance of the tested isolates to methicillin was determined using an oxacillin screen plate after plating single colonies of a fresh *S. aureus* isolate on to Mueller–Hinton agar (Becton Dickinson, Cockeysville, MD, USA) with 6 mg/L of incorporated oxacillin (Sigma, St Louis, MO, USA) and 4% NaCl. Growth of colonies after 18 h of incubation at 30°C was taken as indicative of resistance to methicillin.[4]

Susceptibility testing

MICs and MBCs of quinupristin/dalfopristin were determined for all tested isolates and were compared with those of ten other antimicrobial agents with activity against MRSA. Quinupristin/dalfopristin was provided by Rhône–Poulenc Rorer (Paris, France) as a pale yellowish powder, of which a 10 g/L stock solution was stored frozen at −70°C. This solution was diluted in Mueller–Hinton broth (Oxoid Ltd, London, UK) before use. The MICs of quinupristin/dalfopristin were determined by a microdilution technique with final volumes of 0.05 mL and of oxacillin, imipenem, gentamicin, ciprofloxacin, erythromycin, clindamycin, tetracycline, rifampicin, trimethoprim–sulphamethoxazole and vancomycin on ready-made microdilution plates (Sensititre Ltd, West Sussex, UK). A 5×10^5 cfu/mL log-phase inoculum was applied and the following ranges of antimicrobial concentrations were tested: quinupristin/dalfopristin, 0.015–32 mg/L; oxacillin, imipenem and gentamicin, 0.25–32 mg/L; ciprofloxacin, 0.5–4 mg/L; erythromycin and clindamycin, 0.12–16 mg/L; tetracycline, 2–16 mg/L; rifampicin, 0.5–4 mg/L; trimethoprim–sulphamethoxazole, 0.25/4.75–4/76 mg/L and vancomycin, 0.5–64 mg/L. MICs of oxacillin were determined in the presence of 2% NaCl.

The MIC was considered as the lowest concentration of antimicrobial that prevented visible bacterial growth after 18 h incubation. The following susceptibility breakpoints were used: quinupristin/dalfopristin, 2 mg/L;[5] oxacillin, 2 mg/L; imipenem and gentamicin, 4 mg/L; ciprofloxacin, 2 mg/L; erythromycin and clindamycin, 0.5 mg/L; tetracycline, 4 mg/L; rifampicin, 1 mg/L; trimethoprim–sulphamethoxazole, 2/38 mg/L; vancomycin, 4 mg/L.[6]

MBCs were determined by plating the content of all clear wells on to MacConkey agar (Becton Dickinson); the MBC was defined as the lowest concentration of antimicrobial that killed 99.9% of the applied inoculum.

Time–kill studies of quinupristin/dalfopristin

Twenty-four MRSA and seven MSSA from the above 164 isolates were exposed *in vitro* to quinupristin/dalfopristin at 1 × MIC, 2 × MIC and 4 × MIC for varying lengths of time. Four tubes were prepared for each tested strain; one growth control and three containing quinupristin/dalfopristin at the above concentrations. A 5×10^5 cfu/mL log-phase inoculum was added to each tube along with Mueller–Hinton broth, to give a final volume of 10 mL. All tubes were placed in a 37°C water-bath and, after 0, 2, 4, 6 and 24 h incubation, the bacterial growth in each tube was determined by performing four consecutive 1:10 (v/v) dilutions of a 0.1 mL aliquot of each tube in Mueller–Hinton broth and by plating a 0.1 mL volume of each dilution on to MacConkey agar. This procedure avoided any antimicrobial carryover effect and had a lower detection limit of 10 cfu/mL. A total of 124 killing curves were performed. A value of $\geqslant 3$ $\log_{10}$ decrease of viable cell counts at a specific time of growth compared with the growth control was considered as an adequate killing effect of quinupristin/dalfopristin.[7]

Colonies of each strain surviving 24 h exposure to quinupristin/dalfopristin were suspended in Mueller–Hinton broth and incubated until they reached a logarithmic phase of growth. The resulting inoculum served for MIC redetermination to quinupristin/dalfopristin as described above. MIC increases of $\geqslant$4-fold compared with the MIC of the parent strain were taken as a significant decrease in susceptibility to quinupristin/dalfopristin; development of resistance to quinupristin/dalfopristin occurred whenever this increase surpassed the MIC breakpoint to quinupristin/dalfopristin.

In-vitro interactions between quinupristin/dalfopristin and rifampicin or ciprofloxacin on MRSA

Twelve of the above 24 MRSA isolates highly resistant to both rifampicin (MIC > 4 mg/L) and ciprofloxacin (MIC > 4 mg/L) and six MRSA isolates derived from the initial pool of 101 strains (but not included in the time–kill assay described above) that were susceptible to both rifampicin (MIC $\leqslant$ 0.5 mg/L) and ciprofloxacin (MIC $\leqslant$ 1 mg/L) were exposed *in vitro* over time to quinupristin/dalfopristin (at 1 × MIC, 2 × MIC or 4 × MIC) combined with either 4 mg/L rifampicin or 2 mg/L ciprofloxacin. These concentrations of rifampicin and ciprofloxacin were selected because they represented their mean serum levels after administration of their conventional oral doses.[8,9] Twelve tubes were prepared for each tested isolate: a growth control; quinupristin/dalfopristin alone at 1 × MIC, 2 × MIC and 4 × MIC; 4 mg/L rifampicin alone or combined with quinupristin/dalfopristin at 1 × MIC, 2 × MIC and 4 × MIC; and 2 mg/L of ciprofloxacin alone or combined with quinupristin/dalfopristin at 1 × MIC, 2 × MIC and 4 × MIC. Rifampicin was provided by Marion Merrel

Dow (Richmond, Ontario, Canada) as an orange, amorphous powder, and was dissolved in methanol (Merck, Darmstadt, Germany) and made up in Mueller–Hinton broth so that the final methanol concentration was 0.001% (v/v). Ciprofloxacin was provided by Miles Pharmaceuticals (West Haven, CT, USA) as a white crystalline powder. In all 12 tubes a 5×10^5 cfu/mL log-phase inoculum was added along with Mueller–Hinton broth to give a final volume of 10 mL. A total of 216 killing-curves were performed and bacterial growth was determined as described above.

If a combination of quinupristin/dalfopristin with either rifampicin or ciprofloxacin caused a decrease in viable cell count of ≥ 2 $\log_{10}$ compared with the most active single agent, the effects of the combination were considered to be synergic. If the decrease in viable cell count was 1–2 $\log_{10}$ the effects of the combination were considered to be additive.[7] For colonies surviving 24 h exposure to the tested combinations, MICs of quinupristin/dalfopristin were redetermined as described above.

Results

Susceptibility testing

One hundred and one isolates were MRSA and 63 MSSA. The distribution of quinupristin/dalfopristin MICs and MBCs against these isolates is shown in Table I, and the susceptibilities of MRSA to 11 antimicrobial agents in Table II. The MIC_{50}, MIC_{90}, MBC_{50} and MBC_{90} of quinupristin/dalfopristin against MRSA were ≤ 0.015, 2, 0.12 and 2 mg/L respectively and those against MSSA were ≤ 0.015, 0.06, ≤ 0.015 and 0.25 mg/L respectively. Eighty-four (83.2%) of the MRSA isolates were resistant to both erythromycin and clindamycin, and all of these were inhibited by quinupristin/dalfopristin.

Table I. Distribution of MICs and MBCs of quinupristin/dalfopristin to 101 MRSA and 63 MSSA isolates

	MIC/MBC (mg/L)	No (%) of isolates										
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
MRSA	MIC	51 (50.5)	10 (9.9)	12 (11.9)	5 (4.9)	3 (2.9)	5 (4.9)	4 (3.9)	11 (10.9)	–	–	–	–
	MBC	24 (23.8)	12 (11.9)	11 (10.9)	8 (7.9)	12 (11.9)	10 (9.9)	8 (7.9)	10 (9.9)	3 (2.9)	3 (2.9)	–	–
MSSA	MIC	53 (84.1)	3 (4.8)	5 (7.9)	–	2 (3.2)	–	–	–	–	–	–	–
	MBC	33 (52.3)	9 (14.3)	10 (15.9)	3 (4.8)	4 (6.4)	3 (4.8)	1 (1.6)	–	–	–	–	–

Time–kill studies of quinupristin/dalfopristin

The in-vitro killing effect of quinupristin/dalfopristin on 24 MRSA and seven MSSA isolates is shown in Table III. As shown, 50% of the MRSA isolates were killed by $4 \times$ MIC of quinupristin/dalfopristin at 24 h as opposed to 100% of MSSA isolates at the same concentration and time interval. Three (12.5%) MRSA isolates became resistant to quinupristin/dalfopristin after 24 h incubation with $1 \times$ MIC, two (8.3%) of which became also resistant to it after a $2 \times$ MIC exposure, a phenomenon not observed after a $4 \times$ MIC exposure.

In-vitro interactions between quinupristin/dalfopristin and rifampicin or ciprofloxacin

The in-vitro synergy between quinupristin/dalfopristin and either rifampicin or ciprofloxacin against 18 MRSA isolates is shown in Table IV. Any isolate subject to synergy at 2, 4

Table II. Susceptibility patterns of 101 MRSA isolates to 11 antimicrobial agents

Antimicrobial	Susceptibility breakpoint (mg/L)	MIC (mg/L) range	MBC (mg/L)				% Inhibited
			MIC_{50}	MIC_{90}	MBC_{50}	MBC_{90}	
Quinupristin/dalfopristin	2	≤0.015–4	≤0.015	2	0.12	2	100.0
Oxacillin	2	8–≥32	≥32	≥32	≥32	≥32	0.0
Imipenem	4	≤0.25–≥32	≥32	≥32	≥32	≥32	14.8
Gentamicin	4	≤0.25–≥32	≥32	≥32	≥32	≥32	7.9
Ciprofloxacin	2	≤0.5–≥4	≥4	≥4	≥4	≥4	13.9
Erythromycin	0.5	≤0.12–≥16	≥16	≥16	≥16	≥16	9.9
Clindamycin	0.5	≤0.12–≥16	≥16	≥16	≥16	≥16	12.9
Tetracycline	4	≤2–≥16	≥16	≥16	≥16	≥16	8.9
Rifampicin	1	≤0.5–≥4	≥4	≥4	≥4	≥4	24.8
Co-trimoxazole	2/38	≤0.25/4.75–≥4/76	≥4/76	≥4/76	≥4/76	≥4/76	37.6
Vancomycin	4	≤0.5–4	1	1	1	2	100.0

Table III. Killing effect of quinupristin/dalfopristin on 24 MRSA and seven MSSA isolates

Time (h)	No (%) of MRSA strains killed by:			No (%) of MSSA strains killed by:		
	1 × MIC	2 × MIC	4 × MIC	1 × MIC	2 × MIC	4 × MIC
2	–	–	1 (4.2)	–	–	–
4	–	1 (4.2)	2 (8.3)	–	–	–
6	–	1 (4.2)	4 (16.7)	–	–	2 (28.6)
24	2 (8.3)	7 (29.2)	12 (50.0)	–	1 (14.2)	7 (100.0)
Susceptibility decrease among surviving colonies	11/24 (45.8)	9/24 (37.5)	10/24 (41.7)	3/7 (42.9)	2/7 (28.6)	1/5 (20.0)[a]
MIC_{90} (mg/L) before/after exposure to indicated concentration	4/0.12	4/0.12	2/0.12	0.5/≤0.015	0.5/≤0.015	0.5/≤0.015

[a]In two strains no colonies survived at 24 h.

Table IV. Synergic effect between quinupristin/dalfopristin (RP59500) and rifampicin or ciprofloxacin on 18 MRSA isolates

Susceptibility to rifampicin and ciprofloxacin	Synergy (no (%) of strains)					
	rifampicin + RP5900 at			ciprofloxacin + RP5900 at		
	1 × MIC	2 × MIC	4 × MIC	1 × MIC	2 × MIC	4 × MIC
Susceptible (n = 6)						
2 h	–	–	–	2 (33.3)	2 (33.3)	2 (33.3)
4 h	1 (16.7)	1 (16.7)	1 (16.7)	2 (33.3)	2 (33.3)	3 (50.0)
6 h	1 (16.7)	1 (16.7)	1 (16.7)	4 (66.7)	5 (83.3)	5 (83.3)
24 h	6 (100.0)	6 (100.0)	6 (100.0)	4 (66.7)	5 (83.3)	6 (100.0)
Highly resistant (n = 12)						
2 h	3 (25.0)	1 (8.3)	6 (50.0)	–	–	1 (8.3)
4 h	4 (33.3)	5 (41.7)	8 (66.7)	–	–	2 (16.7)
6 h	4 (33.3)	6 (50.0)	6 (50.0)	1 (8.3)	2 (16.7)	4 (33.3)
24 h	6 (50.0)	9 (75.0)	10 (83.3)	3 (25.0)	5 (41.7)	9 (75.0)
Decrease in susceptibility to RP59500[a]	4/9 (44.4)	1/6 (16.7)	3/7 (42.9)	1/12 (8.3)	3/9 (33.3)	1/7 (14.3)
RP59500 MIC_{90} (mg/L) before/after exposure of isolates to indicated combination	2/0.12	1/0.12	2/0.12	1/0.12	2/0.12	2/0.12

[a]Among surviving colonies

or 6 h of growth was similarly affected following 24 h incubation. Following the interactive studies on isolates susceptible to rifampicin and ciprofloxacin, a $\geqslant 7$ $\log_{10}$ decrease of viable cell counts was found at 24 h by 1 × MIC, 2 × MIC and 4 × MIC with rifampicin in five (83.3%), five (83.3%) and six (100%) isolates respectively and by 1 × MIC, 2 × MIC and 4 × MIC with ciprofloxacin in four (66.7%), four (66.7%) and four (66.7%) isolates respectively. The strain that survived 24 h exposure to quinupristin/dalfopristin and rifampicin as well as the one out of two surviving exposure to quinupristin/dalfopristin and ciprofloxacin showed decreased susceptibilities to quinupristin/dalfopristin without becoming resistant to it. Similarly interactive studies on highly rifampicin and ciprofloxacin resistant MRSA revealed a $\geqslant 7$ $\log_{10}$ synergic effect between 1 × MIC, 2 × MIC and 4 × MIC with rifampicin at 24 h in three (25%), six (50%) and six (50%) isolates respectively and such an effect in three (25%) and five (41.7%) isolates by 2 × MIC and 4 × MIC respectively with ciprofloxacin at 24 h. An additive effect was found between 4 × MIC of quinupristin/dalfopristin and rifampicin in three (25%) isolates at 6 h, whereas at the same time interval it was found in five (41.7%) and three (25%) isolates by 2 × MIC and 4 × MIC of quinupristin/dalfopristin and ciprofloxacin respectively. One isolate (8.3%) remained completely indifferent to all tested interactions.

The time–kill curves of one highly resistant MRSA isolate exposed to quinupristin/dalfopristin, rifampicin, ciprofloxacin and their combinations are shown in the Figure.

Discussion

Quinupristin/dalfopristin is unique in that it is composed of two semisynthetic derivatives of two different classes of pristinamycin, a natural water-insoluble antimicrobial.[10] Since quinupristin/dalfopristin has been proposed to be active against *S. aureus in vitro*, in the present study many hospital isolates MRSA were exposed to it alone or combined with rifampicin or ciprofloxacin.

Quinupristin/dalfopristin inhibited all MRSA and MSSA isolates, the majority at very low MICs (Table I). Even though the MIC_{50}s of quinupristin/dalfopristin to both MRSA and MSSA isolates were found much lower than those reported by other authors (Table II), MIC_{90}s were the same.[11–13] The MBCs of quinupristin/dalfopristin were much greater than the MICs. The MBC:MIC ratio was greater for MRSA than for MSSA. This discrepancy between the MBCs and MICs of quinupristin/dalfopristin against *S. aureus* has also been observed by others.[14] The intrinsic activity of quinupristin/dalfopristin against MRSA *in vitro* was superior to that of all ten agents with which it was compared (Table II), especially against the isolates resistant to erythromycin and clindamycin.

The killing effect of quinupristin/dalfopristin on *S. aureus* increased as the applied concentration of quinupristin/dalfopristin increased (Table III) and that was expressed mainly after 24 h of incubation. Quinupristin/dalfopristin was also found to be more bactericidal for MSSA than for MRSA. These time–kill studies of quinupristin/dalfopristin involved many more strains than other studies that have already described the

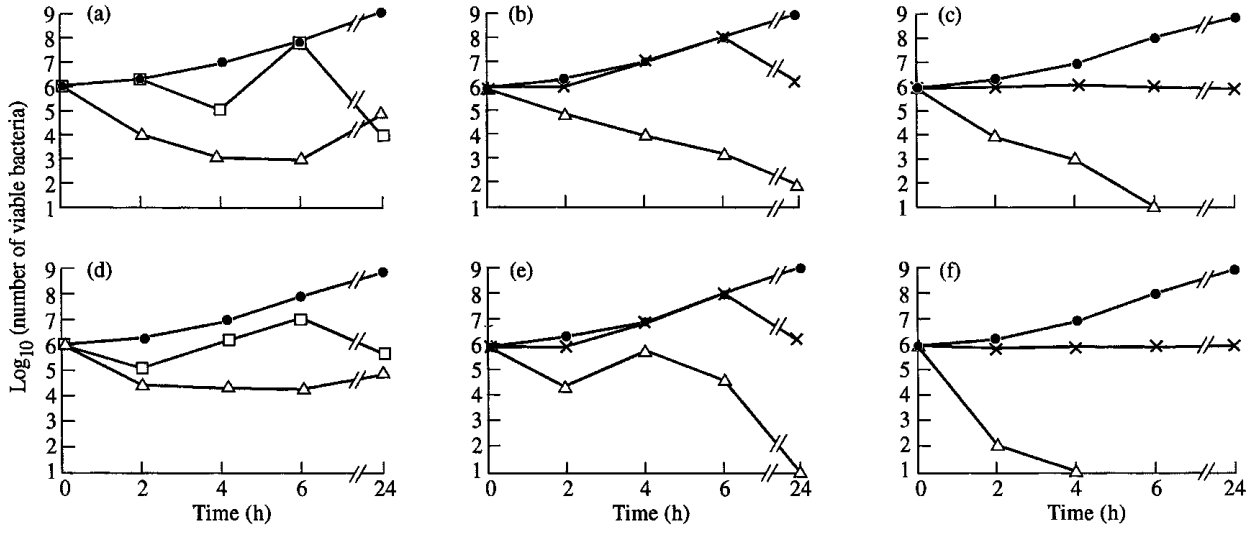

Figure. In-vitro interactions between quinupristin/dalfopristin and either ciprofloxacin or rifampicin against MRSA isolate 11, which is highly resistant to both rifampicin and ciprofloxacin. Each graph shows the number of viable bacteria over time with (i) quinupristin/dalfopristin alone at 1 × MIC (●); ≤0.015 mg/L), (ii) ciprofloxacin (a) or rifampicin (d) (□) or (iii) quinupristin/dalfopristin at 1 × MIC (left), 2 × MIC (centre) or 4 × MIC (right) alone (x) or combined with 2 mg/L ciprofloxacin (top three graphs) or 4 mg/L rifampicin (bottom three graphs) (△).

concentration-dependent killing effect of quinupristin/dalfopristin.[5,15]

Colonies of both MRSA and MSSA isolates surviving 24 h exposure to quinupristin/dalfopristin had much higher MICs than the parent strains. That observation was concentration-dependent, i.e. greater MIC increases were found as the applied concentration of quinupristin/dalfopristin decreased. Despite the 16-fold increases in the MICs for MRSA (Table III), only 8–12% of MRSA isolates acquired resistance to it. Analogous observations have not been reported elsewhere in the literature. A probable explanation of the enormous MIC increases without adaptation of resistance might be that the macrolide-resistant MRSA isolates (Table II) exposed to quinupristin/dalfopristin were constitutive and not inducible mutants of the MLS_B phenotype.[16]

In studies of their interaction with quinupristin/dalfopristin against MRSA, the concentrations of rifampicin and ciprofloxacin used were equal to their mean serum levels, so that findings might approach the clinical condition. Quinupristin/dalfopristin and rifampicin acted synergically on MRSA at most of the tested time intervals. However, synergy involved a greater percentage of susceptible MRSA than of highly resistant MRSA and was expressed mainly at 2 × MIC or 4 × MIC quinupristin/dalfopristin and after 24 h exprosure (Table IV). The in-vitro synergy between ciprofloxacin and quinupristin/dalfopristin was similar, though expressed mainly at 4 × MIC. The observed synergy resulted in a ≥7 $\log_{10}$ decrease in viable cell counts and the MIC increase of quinupristin/dalfopristin after the 24 h exposure was limited in the presence of rifampicin or ciprofloxacin. Despite the improved in-vitro response of susceptible MRSA to the above combinations as compared with the highly resistant MRSA, these results shed new light on the possible use of rifampicin or ciprofloxacin in combination with newer streptogramins, even for infections caused by highly resistant MRSA.

This study showed that quinupristin/dalfopristin is a very promising antistaphylococcal agent, especially against MRSA for which its in-vitro activity was enhanced by rifampicin or ciprofloxacin. Strains exposed to quinupristin/dalfopristin increased their MICs of the novel compound, a phenomenon restricted in the presence of rifampicin or ciprofloxacin. The above in-vitro findings merit further investigation under clinical conditions where MRSA predominates.

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