ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1996, p. 1653–1656 Vol. 40, No. 7
0066-4804/96/$04.00+0

Antipneumococcal Activities of RP 59500 (Quinupristin-Dalfopristin), Penicillin G, Erythromycin, and Sparfloxacin Determined by MIC and Rapid Time-Kill Methodologies

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Received 22 January 1996/Returned for modification 24 April 1996/Accepted 4 May 1996

Previous time-kill studies have shown that RP 59500 is rapidly bactericidal against pneumococci. To extend these findings, the activities of RP 59500, its two components RP 57669 and RP 54476, penicillin G, erythromycin, and sparfloxacin against 26 penicillin-susceptible, 25 penicillin-intermediate, and 25 penicillin-resistant pneumococci were determined by the agar dilution MIC and the time-kill testing methodologies within 10 min (ca. 0.2 h) and at 1 and 2 h. Respective agar dilution MICs at which 90% of isolates are inhibited for penicillin-susceptible, -intermediate, and -resistant strains were as follows: penicillin G, 0.03, 1, and 4 μg/ml; RP 59500, 1, 1, and 1 μg/ml; RP 57669, 8, 32, and 16 μg/ml; RP 54476, >128, >128, and >128 μg/ml; erythromycin, 0.06, 2, and >128 μg/ml; and sparfloxacin, 1, 0.5, and 0.5 μg/ml. RP 59500 was equally active (MIC at which 90% of isolates are inhibited, 1.0 μg/ml) against erythromycin-susceptible and -resistant strains. Time-kill testing results showed that only RP 59500 at one to four times the MIC killed pneumococci at 0.2 h; RP 59500 was also the most active compound at 1 and 2 h. By comparison, penicillin and sparfloxacin at one, two, and four times the MICs reduced the original inoculum by ≥1 log at 2 h for 46, 80, and 95% and for 50, 72, and 86% of strains, respectively. The killing activity of RP 59500 was the same against erythromycin-susceptible and -resistant strains. RP 57669, RP 54476, and erythromycin were either inactive or bacteriostatic at 2 h. Of all drugs tested, RP 59500 yielded the most rapid killing.

The worldwide emergence of penicillin-resistant pneumococci and the alarming increase in the numbers of multiply resistant strains (1) demonstrate the urgent need for new compounds active against these organisms (7). RP 59500 (quinupristin-dalfopristin), a parenteral streptogramin combination of two synergistic pristinamycin derivatives, RP 57669 and RP 54476 (2, 3), is very active (MIC at which 90% of isolates are inhibited, 1.0 μg/ml) against all pneumococcal strains, irrespective of their penicillin or erythromycin susceptibility status (17).

Previous studies (16) have demonstrated that RP 59500 is bactericidal at ≥6 h at concentrations of one-half to one-quarter the MIC for 7 of 10 strains and bacteriostatic for 3 of 10 strains, with regrowth at 24 h. Additionally, at 10 min after inoculation, a 1- to 3-$\log_{10}$-unit reduction (90 to 99.9%) in the original inoculum was seen for 6 of 10 strains treated with RP 59500 at concentrations greater than or equal to the MIC. The susceptibility of one penicillin-resistant strain to RP 59500 was examined by the time-kill method at 0, 1, 2, and 3 h; at a concentration equal to the MIC, RP 59500 was bactericidal within 1 h, and at a concentration of one-half the MIC it was bactericidal within 3 h. The rapid bactericidal effect described above was not seen with the other compounds tested (erythromycin, sparfloxacin, ciprofloxacin, and vancomycin) (16).

Studies by other workers have reported similar rapid killing of *Streptococcus pneumoniae* by RP 59500. Using an in vitro pharmacodynamic model, Berthaud and colleagues (5) have reported a similar rapid decrease (3.9 to 4.3 $\log_{10}$ CFU/ml within 2 h) for three pneumococcal strains (one erythromycin resistant, one penicillin intermediate, and one erythromycin and penicillin susceptible). Johnson et al. (10), using time-kill methodology, found RP 59500 to be bactericidal within 3 h against all 15 penicillin-resistant pneumococcal strains tested.

In order to confirm and extend these findings, we examined (i) the in vitro activities of RP 59500, its two constituent components RP 57669 (quinupristin) and RP 54476 (dalfopristin), erythromycin, and sparfloxacin against 26 penicillin-susceptible, 25 penicillin-intermediate, and 25 penicillin-resistant pneumococci by the agar dilution MIC methodology and (ii) the activities of the drugs by the time-kill methodology within 10 min and at 1 and 2 h.

MATERIALS AND METHODS

Bacteria. Seventy-six clinical isolates of *S. pneumoniae* obtained from blood, cerebrospinal fluid, nasopharynx, or sputum were tested. These comprised 26 penicillin-susceptible (MICs, <0.1 μg/ml), 25 penicillin-intermediate (MICs 0.1 to 1.0 μg/ml), and 25 penicillin-resistant (MICs, ≥2.0 μg/ml) organisms. Of these strains, 56 were erythromycin susceptible (MICs, ≤0.25 μg/ml) and 20 were erythromycin resistant (MICs, ≥1.0 μg/ml). The pneumococcal quality control strain recommended by the National Committee for Clinical Laboratory Standards (*S. pneumoniae* ATCC 49619) was also studied by the rapid time-kill methodology.

MIC determination. MICs were determined by the agar dilution method (9, 14) by using Mueller-Hinton agar (BBL Microbiology Systems, Inc., Cockeysville, Md.) supplemented with 5% sheep blood. Suspensions with a turbidity equal to that of a 0.5 McFarland standard were prepared by suspending growth from overnight Trypticase–soy agar plates supplemented with 5% sheep blood in 2 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.). Suspensions were further diluted in broth to obtain a final inoculum of approximately 10^4 organisms per spot. The plates were inoculated with a Steers replicator and 3-mm inoculating pins and were incubated overnight in ambient air at 35°C. The spot with the lowest concentration of antibiotic showing no growth was defined as the MIC. A faint haze or the presence of just one colony on the site of inoculation was ignored. Standard quality control strains (14) were included in each run.

Standardization of time-kill method. The problem of antibiotic carryover onto recovery plates has been discussed in a previous report (16). With an initial broth

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TABLE 1. Agar dilution MICs for pneumococcal strains tested

Compound and organism susceptibility status[a]	MIC (μg/ml)[b]		
	Range	50%	90%
Penicillin			
Penicillin-S	0.016–0.064	0.016	0.032
Penicillin-I	0.125–1.0	0.25	1.0
Penicillin-R	2.0–4.0	2.0	4.0
RP 59500			
Penicillin-S	0.25–1.0	0.5	1.0
Penicillin-I	0.5–1.0	0.5	1.0
Penicillin-R	0.25–1.0	1.0	1.0
Erythromycin-S	0.25–1.0	0.5	1.0
Erythromycin-R	0.5–1.0	1.0	1.0
RP 57669			
Penicillin-S	1.0–8.0	2.0	8.0
Penicillin-I	2.0–32.0	8.0	32.0
Penicillin-R	1.0–32.0	8.0	16.0
RP 54476			
Penicillin-S	32.0–>128.0	128.0	>128.0
Penicillin-I	8.0–>128.0	128.0	>128.0
Penicillin-R	8.0–>128.0	>128.0	>128.0
Erythromycin			
Penicillin-S	0.016–>128.0	0.016	0.064
Penicillin-I	0.016–>128.0	0.032	2.0
Penicillin-R	0.016–>128.0	8.0	>128.0
Sparfloxacin			
Penicillin-S	0.25–2.0	0.5	1.0
Penicillin-I	0.125–0.5	0.25	0.5
Penicillin-R	0.125–0.5	0.25	0.5

[a] Penicillin-S, penicillin susceptible; Penicillin-I, penicillin intermediate; Penicillin-R, penicillin resistant; Erythromycin-S, erythromycin susceptible; Erythromycin-R, erythromycin resistant.

[b] 50% and 90%, MICs at which 50 and 90% of strains are inhibited, respectively.

dilution of 10^{-2}, antibiotic carryover is minimized (16). The detection threshold was set at 300 CFU/ml (16). The effect of inoculum size and growth phase was determined in three different experiments with *S. pneumoniae* ATCC 49619. The effect of inoculum size was determined by applying inocula of 10^4 and 10^6 CFU/ml in parallel time-kill studies and comparing the results. The influence of growth phase was evaluated by testing in parallel an inoculum from a 2- to 4-h broth culture (prepared as described below) and an inoculum taken directly from an overnight (16 h) broth culture. Details of preparing inocula from overnight broth cultures have been described previously (16). Growth controls with inoculum but not antibiotic were included in each run.

Time-kill studies. For the time-kill experiments, glass tubes containing 5 ml of cation-adjusted Mueller-Hinton broth (Difco) and 5% lysed horse blood with doubling antibiotic concentrations were inoculated with approximately 1×10^6 CFU of the organism per ml (5×10^5 to 5×10^6 CFU/ml) and were incubated at 35°C in a shaking water bath. Antibiotic concentrations were chosen to comprise three doubling dilutions above and three doubling dilutions below the agar dilution MIC (16).

Lysed horse blood was prepared by freezing and thawing horse blood (Cleveland Scientific, Bath, Ohio) eight times. Equal volumes of lysed blood and sterile deionized water were then mixed and centrifuged at 12,000 × *g* for 20 min. Appropriate amounts of 50% lysed blood were then added to the Mueller-Hinton broth to yield a final concentration of 5% lysed horse blood (16).

The bacterial inoculum was prepared by suspending growth from an overnight Trypticase-soy-blood agar plate into Mueller-Hinton broth containing 5% lysed horse blood. The broths were incubated at 35°C for 2 to 4 h in a shaking water bath until the turbidity matched that of a 1.0 McFarland standard (3×10^8 CFU/ml). To inoculate each tube of serially diluted antibiotic, 50 μl of inoculum was delivered beneath the surface of the broth with a pipette; the tubes were then vortexed and the contents were plated within 10 min (approximately 0.2 h) to determine viability counts. The original inoculum was determined by using the untreated growth control. The killing at ca. 0.2 h was taken as the difference between the original inoculum and the numbers of CFU per milliliter in tubes to which antibiotics had been added. Only tubes containing an initial inoculum within the range of 5×10^5 to 5×10^6 CFU/ml were acceptable for determination of killing.

Viability counts of antibiotic-containing suspensions were determined at 0.2, 1, and 2 h by plating 10-fold dilutions of 0.1-ml aliquots from each tube in sterile Mueller-Hinton broth onto Trypticase–soy–5% sheep blood agar plates (BBL). The plates used for recovery of the organisms were incubated for at least 72 h. Colony counts were determined on plates yielding 30 to 300 colonies.

The results of the time-kill studies were expressed as the percentage of strains with the change in the $\log_{10}$ numbers of CFU per milliliter ($\Delta\log_{10}$ CFU per milliliter) from that of the original inoculum at each of the time intervals tested.

RESULTS

The agar dilution MICs for the strains tested are presented in Table 1. RP 59500 was active (MIC, ≤1.0 μg/ml), irrespective of the strain's penicillin or erythromycin susceptibility status. The individual components of RP 59500 were much less active than RP 59500. RP 57669 (quinupristin) was less active against penicillin-intermediate and -resistant strains, most likely because of the erythromycin resistance of these strains. Sparfloxacin was active (MIC at which 90% of strains are inhibited, 0.5 μg/ml) against all strains. Erythromycin was active at MICs of ≤0.25 μg/ml against 92% of penicillin-susceptible strains; by contrast, 16% of penicillin-intermediate and 35% of penicillin-resistant strains were erythromycin resistant (MIC, ≥1.0 μg/ml).

Figure 1 depicts the time-kill study results for RP 59500 against *S. pneumoniae* ATCC 49619. Data represent the means ± standard deviations of five different experiments. Results indicate bactericidal activity (≥Δ3 $\log_{10}$ CFU/ml) at 1 and 2 h, with a low standard deviation between experiments. Varying the inoculum from 10^4 to 10^6 CFU/ml had no significant effect on the time-kill study results at 0.2, 1, and 2 h. Similarly, no significant difference was found when inocula were prepared from a 2- to 4-h or overnight broth.

The comparative activities of RP 59500, sparfloxacin, and penicillin against 76 strains in time-kill studies are provided in Table 2. Only RP 59500 at one to four times the MIC killed pneumococci at 0.2 h. RP 59500 was also the most active compound tested after 1 and 2 h. Penicillin and sparfloxacin at one, two, and four times the MIC reduced the original inoculum by ≥1 log at 2 h in 46, 80, and 95% and in 50, 72, and 86%

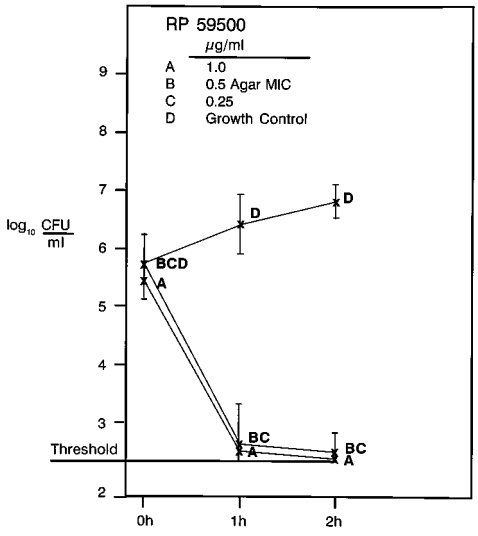

FIG. 1. Time-kill study results for RP 59500 against *S. pneumoniae* ATCC 49619. Results represent the means ± standard deviations of five experiments.

TABLE 2. Comparative activities of RP 59500, sparfloxacin, and penicillin against 76 pneumococcal strains in time-kill studies

Drug and concn	No. (%) of strains killed at the following times and indicated inoculum[a]								
	0.2 h			1 h			2 h		
	−1	−2	−3	−1	−2	−3	−1	−2	−3
RP 59500									
4× MIC	44 (58)	21 (28)	3 (4)	75 (99)	59 (78)	33 (43)	76 (100)	68 (89)	46 (61)
2× MIC	27 (36)	15 (20)	2 (3)	75 (99)	54 (71)	29 (38)	76 (100)	64 (84)	41 (54)
MIC	13 (17)	5 (7)	1 (1)	72 (95)	50 (66)	24 (32)	74 (97)	59 (78)	38 (50)
0.5× MIC	5 (7)	2 (3)	0	58 (76)	33 (43)	15 (20)	67 (88)	44 (58)	25 (33)
0.25× MIC	1 (1)	0	0	26 (34)	12 (16)	1 (1)	28 (37)	16 (21)	3 (4)
Sparfloxacin									
4× MIC	0	0	0	27 (36)	3 (4)	0	65 (86)	19 (25)	1 (1)
2× MIC	0	0	0	19 (25)	0	0	55 (72)	11 (14)	1 (1)
MIC	0	0	0	4 (5)	0	0	38 (50)	4 (5)	0
0.5× MIC	0	0	0	3 (4)	0	0	17 (22)	0	0
0.25× MIC	0	0	0	1 (1)	0	0	4 (5)	0	0
Penicillin									
4× MIC	0	0	0	38 (50)	4 (5)	0	72 (95)	39 (51)	6 (8)
2× MIC	0	0	0	25 (33)	1 (1)	0	61 (80)	29 (38)	5 (7)
MIC	0	0	0	7 (9)	0	0	35 (46)	10 (13)	1 (1)
0.5× MIC	0	0	0	2 (3)	0	0	7 (9)	0	0
0.25× MIC	0	0	0	0	0	0	2 (3)	0	0

[a] Log_{10} CFU per milliliter lower than the original inoculum: −1, Δlog_{10} CFU/ml = 90% killing; −2, Δlog_{10} CFU/ml = 99% killing; −3, Δlog_{10} CFU/ml = 99.9% killing.

of strains, respectively. RP 57669, RP 54476, and erythromycin were either inactive or bacteriostatic at 2 h.

Results of time-kill studies with RP 59500 for 56 erythromycin-susceptible and 20 erythromycin-resistant strains are presented in Table 3. RP 59500 was equally effective against erythromycin-susceptible and -resistant strains.

DISCUSSION

In the past two decades, and in particular, in the past 5 years, a dramatic worldwide increase in the incidence of pneumococcal strains resistant to penicillin and other antimicrobial agents has been seen (1, 7, 9). Pneumococcal strains which are intermediately or fully resistant to penicillin are also often resistant to macrolides. In the United States in 1991 and 1992, Breiman and coworkers (6) have demonstrated erythromycin resistance frequencies of 3.7 and 2.2% in patients 1 and 2 and ≥4 years of age, respectively. In Europe, erythromycin resistance frequencies are generally higher. For example, 27.5% of all pneumococci studied in France during 1992 (63% of penicillin-resistant strains) were erythromycin resistant (8). Pneumococci which are susceptible to erythromycin are susceptible to other macrolides such as azithromycin and clarithromycin. However, erythromycin-resistant pneumococci are cross-resistant to all available macrolides (12). Clarithromycin yields MICs which are 1 or 2 dilutions lower than those of erythromycin and azithromycin, especially for erythromycin-susceptible strains (4, 13, 15). Although most macrolide-resistant pneumococci

TABLE 3. Results of RP 59500 time-kill studies for 56 erythromycin-susceptible and 20 erythromycin-resistant pneumococcal strains

Strain (no. of isolates) and concn[a]	No. (%) of strains killed at the following times and indicated inoculum[b]:								
	0.2 h			1 h			2 h		
	−1	−2	−3	−1	−2	−3	−1	−2	−3
Erythro-S (56)									
4× MIC	27 (48)	10 (18)	3 (5)	55 (98)	41 (73)	24 (43)	56 (100)	49 (88)	34 (61)
2× MIC	14 (25)	6 (11)	2 (4)	55 (98)	38 (68)	22 (39)	56 (100)	48 (86)	30 (54)
MIC	6 (11)	1 (2)	1 (2)	53 (95)	36 (64)	18 (32)	54 (96)	46 (82)	28 (50)
0.5× MIC	1 (2)	0	0	42 (75)	24 (43)	10 (18)	50 (89)	33 (59)	17 (30)
0.25× MIC	0	0	0	16 (29)	7 (13)	2 (4)	16 (29)	10 (18)	1 (2)
Erythro-R (20)									
4× MIC	18 (90)	10 (50)	0	20 (100)	18 (90)	10 (50)	20 (100)	19 (95)	11 (55)
2× MIC	13 (65)	8 (40)	0	20 (100)	16 (80)	8 (40)	20 (100)	16 (80)	10 (50)
MIC	7 (35)	2 (10)	0	20 (100)	14 (70)	7 (35)	20 (100)	14 (70)	10 (50)
0.5× MIC	2 (10)	1 (5)	0	17 (85)	10 (50)	6 (30)	19 (95)	12 (60)	7 (35)
0.25× MIC	0	0	0	9 (45)	4 (20)	0	12 (60)	6 (30)	1 (5)

[a] Erythro-S, erythromycin susceptible; Erythro-R, erythromycin resistant.

[b] Log_{10} CFU per milliliter lower than the original inoculum: −1, Δlog_{10} CFU/ml = 90% killing; −2, Δlog_{10} CFU/ml = 99% killing; −3, Δlog_{10} CFU/ml = 99.9% killing.

are also resistant to clindamycin, some macrolide-resistant strains susceptible to clindamycin have been reported (15).

The streptogramins are the only members of the macrolide-lincosamide-streptogramin group which are consistently active against macrolide-resistant pneumococci (17). In the current study, the agar dilution MICs confirmed the activity of RP 59500 against penicillin- and/or erythromycin-resistant pneumococci (16, 17). All MICs fell within a very narrow range of 0.25 to 1.0 μg/ml, even for strains for which penicillin MICs are 4.0 μg/ml and erythromycin MICs are >128.0 μg/ml. The two constituent components alone were less active, but they act synergistically (2, 3). Sparfloxacin was also found to be active against all strains, with MICs of between 0.125 and 2.0 μg/ml, regardless of the susceptibilities of the strains to other compounds.

One of the factors determining the efficacy of an antimicrobial agent is the rate of killing determined by the time-kill methodology (11). One of the goals of the current study was to confirm the rapid killing by RP 59500 by using a large number of strains. Methodological variables such as inoculum, drug carryover, the medium used, and other environmental factors were considered. Because killing was measured over a brief, 2-h interval, the inoculum size and growth phase were believed to be critical. Varying the inoculum size did not significantly affect the time-kill study data; the problem of drug carryover has also been addressed (16).

Killing by cell wall-active agents such as penicillin G has been reported to be a direct function of the bacterial growth rate prior to the addition of the antimicrobial agent (11). In the current study, no significant difference in killing was found when inocula were prepared from overnight or 2- to 4-h broth cultures. Inocula prepared from 2- to 4-h cultures were selected because of convenience.

The present time-kill study results confirm the rapid killing of most strains by RP 59500. Only RP 59500 killed pneumococci within 0.2 h and was the most active agent after 1 and 2 h. After 2 h, the extent of killing by penicillin G and sparfloxacin was significantly less than that by RP 59500. At a concentration equal to the agar dilution MIC, RP 59500 reduced the original inoculum by ≥ 3 $\log_{10}$ (99.9% killing) for 32 and 50% of the tested strains after 1 and 2 h, respectively. By contrast, penicillin G and sparfloxacin, at concentrations equal to the agar dilution MIC, failed to similarly reduce the original inoculum of all except one strain (treated with penicillin G) after 2 h. Erythromycin was either inactive or bacteriostatic at 2 h. RP 59500 was equally effective against erythromycin-susceptible and -resistant strains, supporting the agar dilution MIC results obtained in the current study and those that we have reported previously (17). The significance of killing by RP 59500 at concentrations below the MIC within 2 h is unknown.

In summary, only RP 59500 was rapidly bactericidal (90 to 99.9% killing) against most pneumococcal strains tested. This phenomenon was not seen with the other compounds tested and may be unique to the streptogramins; we have no explanation for the reason for this finding. The clinical significance of this phenomenon remains to be determined and may affect the clinical response to these agents by patients infected with pneumococci.

ACKNOWLEDGMENT

This study was supported by a grant from Rhône-Poulenc Rorer Laboratories, Paris, France.

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