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Antibacterial Activity of RU 64004 (HMR 3004), a Novel Ketolide Derivative Active against Respiratory Pathogens

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The antibacterial activity of RU 64004, a new ketolide, was evaluated against more than 600 bacterial strains and was compared with those of various macrolides and pristinamycin. RU 64004 had good activity against multiresistant pneumococci, whether they were erythromycin A resistant or not, including penicillin-resistant strains. RU 64004 inhibited 90% of pneumococci resistant to erythromycin A and penicillin G at 0.6 and 0.15 μg/ml, respectively. Unlike macrolides, RU 64004 did not induce the phenotype of resistance to macrolides-lincosamides-streptogramin B. Its good antibacterial activity against multiresistant pneumococci ran in parallel with its well-balanced activity against all bacteria involved in respiratory infections (e.g., *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pyogenes*). In contrast to all comparators (14- and 16-membered-ring macrolides and pristinamycin), RU 64004 displayed high therapeutic activity in animals infected with all major strains, irrespective of the phenotypes of the strains. The results suggest that RU 64004 has potential for use in the treatment of infections caused by respiratory pathogens including multiresistant pneumococci.

Renewal of interest in macrolides was triggered by roxithromycin in the early 1980s (9, 10). This new macrolide was later challenged by clarithromycin (16) and azithromycin (25) with regard to improved pharmacokinetic properties in comparison to those of erythromycin A, together with useful efficacy against emerging atypical pathogens, e.g., *Mycoplasma* spp., *Legionella* spp., and *Chlamydia* spp. The antibacterial spectra of all of these drugs are typically directed toward respiratory pathogens; however, there are several drawbacks, such as a lack of efficacy against macrolide-lincosamide-streptogramin B (MLS)-resistant pneumococci and only modest activity against *Haemophilus influenzae* (with the exception of azithromycin).

Indeed, among *Streptococcus pneumoniae* strains resistance to macrolides has increased significantly in recent years in several areas around the world due to their extensive therapeutic use, for example, 30% in France (18). Furthermore, there is an alarming simultaneous increase in the level of resistance to penicillin G among pneumococcal isolates, with an epidemic spread of 10 to more than 40% in some particular foci, e.g., South Africa, Spain, and Hungary (6). Many strains of *S. pneumoniae* exhibit combined resistance to macrolides and penicillin G. Therefore, the available macrolides cannot be used for the treatment of infections caused by multiresistant *S. pneumoniae.*

In the search for new compounds likely to overcome the problem of pneumococcal resistance, a new class of 14-membered-ring macrolide antibacterial agents has been generated. Ketolides are characterized by a keto group at position 3 of the macrolactone ring, which replaces the L-cladinose moiety, a neutral sugar long thought to be essential for antibacterial activity (1, 2).

RU 64004 has been shown to be one of the most active compounds in a novel series of 11,12-cyclo-substituted ketolides synthesized by Roussel Uclaf (27). In this report, we describe the in vitro antibacterial properties of RU 64004 in comparison with those of erythromycin A, clarithromycin, azithromycin, josamycin, pristinamycin, and ampicillin against *H. influenzae.*

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MATERIALS AND METHODS

Antibiotics. RU 64004, RU 66252 (the macrolide counterpart of RU 64004), clarithromycin, and azithromycin were prepared by Roussel Uclaf (Romainville, France). Erythromycin A and penicillin G, subsequently referred to as erythromycin and penicillin, respectively, were obtained from Roussel Uclaf. Pristinamycin and josamycin were from Rhône-Poulenc-Rorer (Vitry, France) and ICN Biomedicals (Costa Mesa, Calif.), respectively. Rokitamycin was from Pierre Fabre Medicament (Boulogne, France), and ampicillin was from Fluka (Buchs, Switzerland).

Antibiotic stock solutions were prepared in Mueller-Hinton broth. Solubilization of the compounds was generally achieved by using a minimal amount of dimethyl sulfoxide. In some cases, the neutralization of an aqueous suspension of a drug by glacial acetic acid was necessary, and the pH was brought to neutrality with 0.1 M sodium phosphate buffer.

Bacterial strains. More than 600 strains of gram-positive and gram-negative bacteria were used in this study. Most of them were recent clinical isolates from various European and U.S. hospitals, and they were selected on the basis of their specific patterns of resistance to erythromycin and penicillin. Special attention was given to MLS- and/or penicillin-resistant strains of gram-positive cocci. Inducible (inducible erythromycin resistance [Ery^{ri}]) or constitutive (constitutive erythromycin resistance [Ery^{rc}]) MLS resistance among staphylococci was defined by using josamycin, a 16-membered-ring macrolide active against inducibly resistant coagulase-positive or coagulase-negative staphylococci. With pneumococci, rokitamycin, another 16-membered-ring macrolide, was tentatively used to distinguish between inducibly and constitutively resistant strains (14). The MICs of rokitamycin were taken as ≤ 1 and >4 μg/ml for inducibly and constitutively MLS-resistant strains, respectively. These values were confirmed by induction experiments carried out in parallel.

Staphylococci, enterococci, and members of the family *Enterobacteriaceae* were maintained as stab cultures at room temperature. All other clinical isolates were stored frozen at −80°C.

Susceptibility testing. In vitro susceptibility tests were performed by a twofold agar dilution method (7). Mueller-Hinton agar medium (pH 7.4; Diagnostic Pasteur [DP], Marnes-la-Coquette, France) was used throughout the study. The medium was appropriately supplemented to support the growth of some fastidious microorganisms (4% globular extract [DP] for *H. influenzae*; 7% horse blood for streptococci, pneumococci, and *Moraxella catarrhalis*). The following reference organisms were included for quality control: *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212.

For the preparation of inocula of *H. influenzae*, pneumococci, and streptococci, microorganisms were scraped from overnight cultures on plates and suspended in Mueller-Hinton broth (DP). Bacterial suspensions were adjusted spectrophotometrically to an optical density (OD) at 680 nm of 1 on a Spectronic

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20 spectrophotometer (Baush & Lomb, Paris, France), which corresponded to 10^9 CFU/ml. Other inocula were prepared directly from an overnight culture in Mueller-Hinton broth. A standard inoculum, to obtain 10^4 CFU/spot, was prepared from a 1:200 dilution of the bacterial broth suspension and was applied to agar plates containing the test compound by using a Denley multipoint inoculator (Bioblock, Strasbourg, France). All plates were incubated at 37°C for 20 h in ambient atmosphere. The MIC was defined as the lowest concentration at which no visible growth could be detected on agar plates.

Induction of MLS resistance. The ability to induce MLS resistance in *S. aureus* by various drugs was evaluated as described by Hyder and Streitfeld (21). A culture of *S. aureus* 011GO25 in which drug resistance was to be induced was prepared at 37°C by adding 0.5 ml of an overnight bacterial culture to 4.5 ml of brain heart infusion broth (DP) containing a resistance-inducing subinhibitory concentration of the antibiotic studied. Bacterial growth was followed by measurement of the turbidity. When the OD of the culture at 680 nm reached 0.3 to 0.5 units, a challenge inhibitory dose of erythromycin (50 μg/ml) was added to the bacterial cultures in which resistance was induced or not induced (control). The OD was then followed for 8 h. The subinhibitory concentrations used for resistance induction were adapted from the work of Hyder and Streitfeld (21), depending on the strains and the drugs (0.06 μg/ml for erythromycin and 0.006 μg/ml for RU 64004 or RU 66252).

Determination of MBCs. A broth microdilution assay (12) was used to evaluate the bactericidal activity of RU 64004 in comparison with those of reference macrolides. MICs were determined first in an appropriate medium, depending on the strain tested: brain heart infusion broth (DP) plus 4% globular extract (DP) for pneumococci, Haemophilus test medium prepared in-house as described by Jorgensen et al. (23) for *H. influenzae*, and Mueller-Hinton broth for the other microorganisms tested. The inoculum was prepared by dilution of an overnight culture and was adjusted to give a final bacterial titer of approximately 6×10^5 CFU/ml in each well of the microtiter plates. Aliquots (100 μl) of the bacterial inoculum were then added to 100 μl of broth containing doubling concentrations of the antibiotic. The plates were covered with sealing tape and were incubated at 37°C for 24 h. The MIC was read as the lowest concentration of antibiotic at which there was no visible growth of the microorganism tested. Ten-microliter-loopful subcultures on suitable solid medium (see above) were made from those dilutions in the microdilution plates which failed to show visible growth. The agar plates were read after 24 h of incubation at 37°C. The lowest dilution of the antibiotic yielding no bacterial growth on the agar subculture was considered the minimal bactericidal concentration (MBC) of the antibiotic.

Effect of inoculum size. The effect of the inoculum size on the antibacterial activity of RU 64004 was evaluated in comparison with those of erythromycin, clarithromycin, and josamycin against reference strains of *S. aureus* (ATCC 29213), *E. faecalis* (ATCC 29212), *H. influenzae* (ATCC 9006), *Escherichia coli* (ATCC 25922), and one Ery^r strain of *S. pneumoniae*. The test was performed by microdilution procedures as described above. An overnight culture was diluted five times in 10-fold steps to approximately 10^4 CFU/ml. Aliquots (100 μl) of each dilution were then added to 100 μl of broth containing doubling concentrations of the antibiotic. The plates were covered with sealing tape and were incubated at 37°C for 24 h. The MIC was read as the lowest concentration of antibiotic at which there was no visible growth of the microorganism tested. For the highest inoculum, the endpoint was determined by comparing the turbidity with that observed in a control well without antibiotic. Bacterial enumerations were also carried out by using a Spiral counter system (Intersciences, Saint Nom-La-Bretèche, France) to ascertain the inoculum size.

In vitro resistance studies. The development of resistance to RU 64004 and josamycin was studied with four different Ery^r strains of pneumococci (one constitutively and three inducibly resistant strains) by a broth macrodilution method (5). The MIC of RU 64004 for a test strain was first determined in brain heart infusion broth (DP). Bacteria showing growth on plates with the highest concentration of the antibiotic tested were subcultured to tubes of fresh medium containing doubling concentrations of the antibiotic. The MIC was determined and bacteria from plates with the highest concentration of antibiotic tested were subcultured in doubling concentrations of the antibiotic. This subculture process was repeated eight times to study the possible occurrence of resistance buildup.

Influence of susceptibility testing conditions. A broth microdilution technique (5) was used to study the effect of pH and CO_2 on the antibacterial activity of RU 64004. Mueller-Hinton broth (DP) was used to grow most bacterial species except pneumococci and *H. influenzae*, which were cultured in brain heart infusion broth (DP) and Haemophilus test medium (28), respectively.

The influence of 40% normal human serum (NHS), complement-inactivated serum, and various human serum proteins (Sigma, St. Louis, Mo.) on the antibacterial activity of RU 64004 was investigated by the same technique. Several broth and agar media (DP) were used to compare the influence of their compositions on the antimicrobial potency of RU 64004 and various reference antibiotics: Mueller-Hinton broth supplemented or not supplemented with 50 μg of Ca^{2+} and Mg^{2+} per ml, brain heart infusion broth, Trypticase casein soy broth, Schaedler broth, Mueller-Hinton agar supplemented or not supplemented with 5% horse blood, Columbia agar, and chocolate agar.

In vivo therapeutic efficacy. Male Charles River mice were used to study the antibacterial activities of the compounds. Each dosing group was composed of 10 animals weighing 20 to 22 g. Mice were infected intraperitoneally with 0.5 ml of an overnight broth culture of bacterial appropriately diluted in physiological buffer or 5% hog mucin (Sigma) to a final cell density corresponding to 10 to 100 times the minimal lethal dose (11). Suspensions of the compounds (0.5 ml) in carboxymethyl cellulose were administered by the oral route immediately and 4 h postinfection. Mice were observed for 8 to 10 days following the start of the infections, and the 50% protective doses (PD_{50}s), expressed as the unit dose that protected 50% of the animals from death, were calculated by the probit method of Litchfield and Wilcoxon (24).

RESULTS

In vitro antibacterial activity. Table 1 indicates the in vitro antibacterial activity of RU 64004 against various clinical isolates in comparison with those of available macrolides and pristinamycin.

(i) Staphylococci. RU 64004 exhibited activity against strains of *S. aureus* normally susceptible to erythromycin (Ery^s) that was about 10 times greater than those of erythromycin and clarithromycin. All strains of *S. aureus* were inhibited by 0.04 μg of RU 64004 per ml.

Likewise, all inducibly resistant (Ery^{ri}) *S. aureus* strains were inhibited by RU 64004 at a concentration of 0.08 μg/ml. Its activity was 4 and 30 times greater than those of pristinamycin and josamycin, respectively. By contrast, all 14-membered-ring macrolides were inactive.

All Ery^s strains of coagulase-negative staphylococci were inhibited by 0.02 μg of RU 64004 per ml, a value 4 to 10 times lower than that found for clarithromycin or erythromycin.

RU 64004 also exhibited good activity against the four strains of Ery^{ri} coagulase-negative staphylococci tested, all being inhibited by RU 64004 at 0.08 μg/ml, a value 2 and 30 times lower than those for pristinamycin and josamycin, respectively. Once more, complete cross-resistance was observed among the 14-membered-ring macrolides.

The MICs for Ery^s and Ery^{ri} strains of staphylococci were very similar.

RU 64004 retained full activity against oxacillin-resistant staphylococci when these strains did not display the constitutive phenotype of resistance to erythromycin (data not shown). Indeed, it can be seen from the data in Table 1 that RU 64004 lacked activity against constitutively resistant (Ery^{rc}) *S. aureus.*

(ii) Enterococci. When tested against a panel of 73 strains of enterococci (19 strains of *Enterococcus faecium* and 54 strains of *E. faecalis*), RU 64004 at 0.01 μg/ml inhibited all isolates susceptible to erythromycin, a value more than 100 times lower than that found for the comparator antibiotics.

The activity of RU 64004 against enterococci resistant to erythromycin was significantly lower, with an MIC at which 90% of isolates are inhibited (MIC_{90}) of 5 μg/ml. This value was identical to that for pristinamycin, which was found to be the only comparator drug with useful activity against these strains. In general, *E. faecium* was more susceptible than *E. faecalis* to the actions of RU 64004 and the available macrolides.

RU 64004 demonstrated quite good activity against 11 strains of enterococci resistant to vancomycin (Van^r).

(iii) Streptococci. The activity of RU 64004 was studied against 52 Ery^s and 27 Ery^r strains of streptococci. Among them were 29 strains of serogroup A (*Streptococcus pyogenes*), 38 strains of other serogroups (serogroups B, C, F, and G), and 12 strains belonging to the viridans group.

RU 64004 inhibited all Ery^s streptococci at 0.005 μg/ml, a value generally 100 times lower than the value recorded for the comparator drugs (e.g., 0.3 μg/ml for erythromycin, clarithromycin, or pristinamycin).

RU 64004 was also active against Ery^r streptococci, with an MIC_{90} of 1.2 μg/ml, a value more than 100 times lower than those found for most comparator drugs with the exception of pristinamycin, which also displayed strong activity.

TABLE 1. Comparative in vitro antibacterial activities of RU 64004 and several macrolides

Organism (no. of isolates) and drug	MIC (μg/ml)		
	Range	50%	90%
Staphylococcus aureus, Ery^s (68)			
RU 64004	0.001–0.04	0.01	0.02
Erythromycin	0.02–0.15	0.15	0.15
Clarithromycin	0.04–0.15	0.08	0.15
Azithromycin	0.15–1.2	0.6	0.6
Josamycin	0.04–1.2	0.6	1.2
Pristinamycin	0.01–0.6	0.15	0.15
Staphylococcus aureus, Ery^{ri} (65)			
RU 64004	0.001–0.08	0.04	0.08
Erythromycin	1.2–>40	>40	>40
Clarithromycin	0.6–>40	>40	>40
Azithromycin	2.5–>40	>40	>40
Josamycin	0.15–2.5	1.2	2.5
Pristinamycin	0.04–03	0.15	0.15
Staphylococcus aureus, Ery^{rc} (23)			
RU 64004	20–40	>40	>40
Erythromycin	>40	>40	>40
Clarithromycin	ND[a]	ND	ND
Azithromycin	ND	ND	ND
Josamycin	>40	>40	>40
Pristinamycin	0.3–1.2	1.2	1.2
Staphylococcus spp., coagulase negative, Ery^s (12)			
RU 64004	0.001–0.02	0.01	0.02
Erythromycin	0.04–0.15	0.08	0.15
Clarithromycin	0.04–0.08	0.08	0.08
Azithromycin	0.3–0.6	0.3	0.6
Josamycin	0.3–0.6	0.3	0.6
Pristinamycin	0.04–0.08	0.08	0.08
Staphylococcus spp., coagulase negative, Ery^{ri} (4)			
RU 64004	0.04–0.08		
Erythromycin	>40		
Clarithromycin	>40		
Azithromycin	>40		
Josamycin	1.2–2.5		
Pristinamycin	0.08–0.15		
Streptococcus spp., Ery^s (52)			
RU 64004	0.001–0.005	0.001	0.005
Erythromycin	0.005–0.3	0.02	0.02
Clarithromycin	0.001–0.3	0.01	0.01
Azithromycin	0.001–0.3	0.04	0.04
Josamycin	0.005–1.2	0.08	0.15
Pristinamycin	0.01–0.3	0.04	0.08
Streptococcus spp. Ery^r (27)			
RU 64004	0.005–10	0.01	1.2
Erythromycin	0.6–>40	5	>40
Clarithromycin	0.15–>40	1.2	>40
Azithromycin	2.5–>40	10	>40
Josamycin	0.08–>40	5	>40
Pristinamycin	0.02–0.3	0.04	0.08
Streptococcus spp., non-viridans group, non-*Streptococcus pyogenes* (38)			
RU 64004	0.001–10	0.005	0.04
Erythromycin	0.005–>40	0.02	>40
Clarithromycin	0.001–>40	0.01	>40
Azithromycin	0.01–>40	0.04	>40
Josamycin	0.04–>40	0.08	20
Pristinamycin	0.02–0.3	0.08	0.08
Streptococcus pyogenes (29)			
RU 64004	0.001–2.5	0.001	0.02
Erythromycin	0.005–>40	0.02	1.2
Clarithromycin	0.001–>40	0.01	0.6
Azithromycin	0.001–>40	0.04	10
Josamycin	0.01–>40	0.08	0.6
Pristinamycin	0.01–0.08	0.04	0.08
Streptococcus spp., viridans group (12)			
RU 64004	0.001–1.2	0.001	0.01
Erythromycin	0.005–>40	0.02	>40
Clarithromycin	0.001–>40	0.01	>40
Azithromycin	0.005–>40	0.02	>40
Josamycin	0.005–2.5	0.04	1.2
Pristinamycin	0.02–0.08	0.04	0.08
Streptococcus pneumoniae, Ery^s (58)			
RU 64004	0.001–0.01	0.001	0.005
Erythromycin	0.001–0.3	0.02	0.15
Clarithromycin	0.001–1.2	0.01	0.3
Azithromycin	0.005–2.5	0.04	0.3
Josamycin	0.001–1.2	0.08	0.3
Pristinamycin	0.005–0.6	0.08	0.3
Streptococcus pneumoniae, Ery^{ri} (38)			
RU 64004	0.001–0.08	0.01	0.04
Erythromycin	0.6–>40	20	>40
Clarithromycin	0.3–>40	40	>40
Azithromycin	0.08–>40	>40	>40
Josamycin	0.04–>40	5	20
Pristinamycin	0.04–0.6	0.3	0.6
Streptococcus pneumoniae, Ery^{rc} (34)			
RU 64004	0.02–2.5	0.08	0.6
Erythromycin	20–>40	>40	>40
Clarithromycin	10–>40	>40	>40
Azithromycin	>40	>40	>40
Josamycin	0.08–>40	>40	>40
Pristinamycin	0.04–1.2	0.3	0.6
Streptococcus pneumoniae, Pen^s (65)			
RU 64004	0.001–1.2	0.005	0.04
Erythromycin	0.001–>40	5	>40
Clarithromycin	0.001–>40	1.2	>40
Azithromycin	0.005–>40	10	>40
Josamycin	ND	ND	ND
Pristinamycin	0.04–0.3	0.08	0.3
Streptococcus pneumoniae, Pen^r (50)			
RU 64004	0.001–2.5	0.01	0.3
Erythromycin	0.005–>40	10	>40
Clarithromycin	0.001–>40	5	>40
Azithromycin	0.01–>40	40	>40
Josamycin	ND	ND	ND
Pristinamycin	0.04–0.6	0.08	0.3
Enterococcus spp., Ery^s (41)			
RU 64004	0.005–0.01	0.005	0.01
Erythromycin	0.04–0.6	0.6	0.6
Clarithromycin	0.04–1.2	0.3	0.6
Azithromycin	0.08–5	2.5	5
Josamycin	0.3–2.5	1.2	1.2
Pristinamycin	0.08–5	1.2	2.5

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TABLE 1—*Continued*

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TABLE 1—*Continued*

Organism (no. of isolates) and drug	MIC (μg/ml)		
	Range	50%	90%
Enterococcus spp., Ery^r (32)			
RU 64004	0.001–10	1.2	5
Erythromycin	1.2–>40	>40	>40
Clarithromycin	0.6–>40	>40	>40
Azithromycin	2.5–>40	>40	>40
Josamycin	0.15–>40	>40	>40
Pristinamycin	0.04–10	1.2	5
Enterococcus spp., Van^r (11)			
RU 64004	0.005–5	0.08	5
Erythromycin	0.6–>40	>40	>40
Clarithromycin	0.3–>40	>40	>40
Azithromycin	2.5–>40	>40	>40
Josamycin	1.2–>40	>40	>40
Pristinamycin	0.3–10	1.2	5
Haemophilus influenzae, Amp^s (43)			
RU 64004	0.001–1.2	0.3	1.2
Erythromycin	0.15–5	1.2	5
Clarithromycin	0.08–10	1.2	5
Azithromycin	0.001–1.2	0.15	1.2
Josamycin	0.15–40	5	20
Pristinamycin	0.08–2.5	0.6	1.2
Ampicillin	0.04–0.3	0.3	0.3
Haemophilus influenzae, Amp^r (37)			
RU 64004	0.001–1.2	0.3	1.2
Erythromycin	0.6–5	2.5	5
Clarithromycin	0.6–10	2.5	10
Azithromycin	0.001–1.2	0.3	1.2
Josamycin	0.3–40	5	20
Pristinamycin	0.08–1.2	0.3	1.2
Ampicillin	0.6–20	1.2	10
Moraxella catarrhalis (26)			
RU 64004	0.08	0.08	0.08
Erythromycin	0.08	0.08	0.08
Clarithromycin	0.01–0.08	0.04	0.08
Azithromycin	0.005–0.01	0.01	0.01
Josamycin	0.3–0.6	0.6	0.6
Pristinamycin	0.15–0.3	0.3	0.3
Ampicillin	0.01–2.5	0.3	1.2
Members of the family *Enterobacteriaceae*[b] (61)			
RU 64004	1.2–80	40	80
Erythromycin	5–160	160	160
Clarithromycin	5–160	160	160
Azithromycin	1.2–160	40	160
Salmonella spp. (18)			
RU 64004	1.2–80	5	40
Erythromycin	2.5–160	80	160
Clarithromycin	2.5–160	40	160
Azithromycin	0.6–20	5	20

[a] ND, not determined.
[b] *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae.*

There were no significant differences in the activity of the new ketolide against non-viridans group streptococci (all were β-hemolytic strains) and viridans group streptococci, and the MIC_{90}s for these groups of organisms were 0.04 and 0.01 μg/ml, respectively. These values were approximately 2 orders of magnitude lower than those obtained with macrolides. In particular, the new ketolide was found to be the most active compound against *S. pyogenes*, with an MIC_{90} of 0.02 μg/ml.

(iv) Pneumococci. RU 64004 was tested against 130 strains of Ery^s and Ery^r pneumococci. Among the 72 Ery^r isolates, the MICs of rokitamycin allowed for the distinction between 38 Ery^{ri} and 34 Ery^{rc} strains.

RU 64004 was found to be consistently active against all Ery^s strains studied. All strains were inhibited by 0.01 μg of RU 64004 per ml, compared with 0.6 μg/ml for pristinamycin and 0.3 to 2.5 μg/ml for the macrolides.

One hundred percent of the strains of Ery^{ri} pneumococci were inhibited by 0.08 μg of RU 64004 per ml. This value must be compared with the 0.6 μg/ml for pristinamycin and ≥40 μg/ml for all 14-, 15-, and 16-membered-ring macrolides.

RU 64004 was also active against the so-called constitutive Ery^{rc} pneumococci, with an MIC_{90} of 0.6 μg/ml, a value identical to that found for pristinamycin. As expected, no differences in the behaviors of RU 64004 against penicillin-susceptible (Pen^s) and penicillin-resistant (Pen^r) strains of pneumococci were observed. The MIC_{90} for Pen^r strains was recorded to be 0.15 μg/ml.

(v) *H. influenzae*. The activity of RU 64004 was evaluated against a panel of 80 strains of *H. influenzae* which were distinguished according to their phenotype of resistance to ampicillin (Amp^s or Amp^r). Overall, RU 64004 displayed activity identical to that of azithromycin against these pathogens. All strains of *H. influenzae* were inhibited at a concentration of RU 64004 which did not exceed 1.2 μg/ml, whereas the concentrations were 5 to 40 μg/ml for all 14- and 16-membered-ring macrolides.

(vi) *M. catarrhalis*. A total of 26 *M. catarrhalis* strains were available to test the activity of RU 64004 against *M. catarrhalis*. With the MIC_{90} recorded to be 0.08 μg/ml, RU 64004 displayed a potency similar to those of the 14-membered-ring macrolides, whereas azithromycin showed the best activity.

(vii) Members of the family *Enterobacteriaceae*. RU 64004 displayed a MIC_{90} of ≥40 μg/ml, similar to that of azithromycin, for members of the family *Enterobacteriaceae.*

Induction of MLS resistance. Figure 1 compares the kinetics of induction of MLS resistance in *S. aureus* 011GO25 for both RU 64004 and its L-cladinose analog, RU 66252, in liquid medium. Growth of *S. aureus* preexposed to a subinhibitory concentration (0.06 μg/ml) of erythromycin persisted after exposure to a challenge dose of 50 μg of erythromycin per ml. A similar result was obtained with a subinhibitory dose (0.006 μg/ml) of RU 66252. The bacteria could still grow after the culture was challenged with 50 μg of erythromycin per ml. In contrast, with the ketolide RU 64004, preexposure of the bacteria to the same subinhibitory concentration did not cause the culture to become resistant following administration of the same challenge dose.

Bactericidal activity. Table 2 indicates that the MBCs of RU 64004 were very close to the MICs, thus favoring a bactericidal type of action of the ketolide against *H. influenzae* and *S. pneumoniae*, whatever the phenotype of resistance to erythromycin. A similar type of bactericidal action was also observed for the macrolides when they were tested against *H. influenzae* and susceptible strains of pneumococci.

Effect of inoculum size. Varying the inoculum size from 2.4×10^3 to 8.5×10^6 CFU/ml had no effect on the in vitro potencies of macrolides and RU 64004. As with the other compounds tested, the activity of RU 64004 decreased when the inoculum was greater than 10^7 CFU/ml. However, RU 64004 displayed the lowest MICs in comparison with those of erythromycin, clarithromycin, and azithromycin for *S. aureus*, *S. pneumoniae*, and *E. faecalis*. For *H. influenzae*, the MICs of

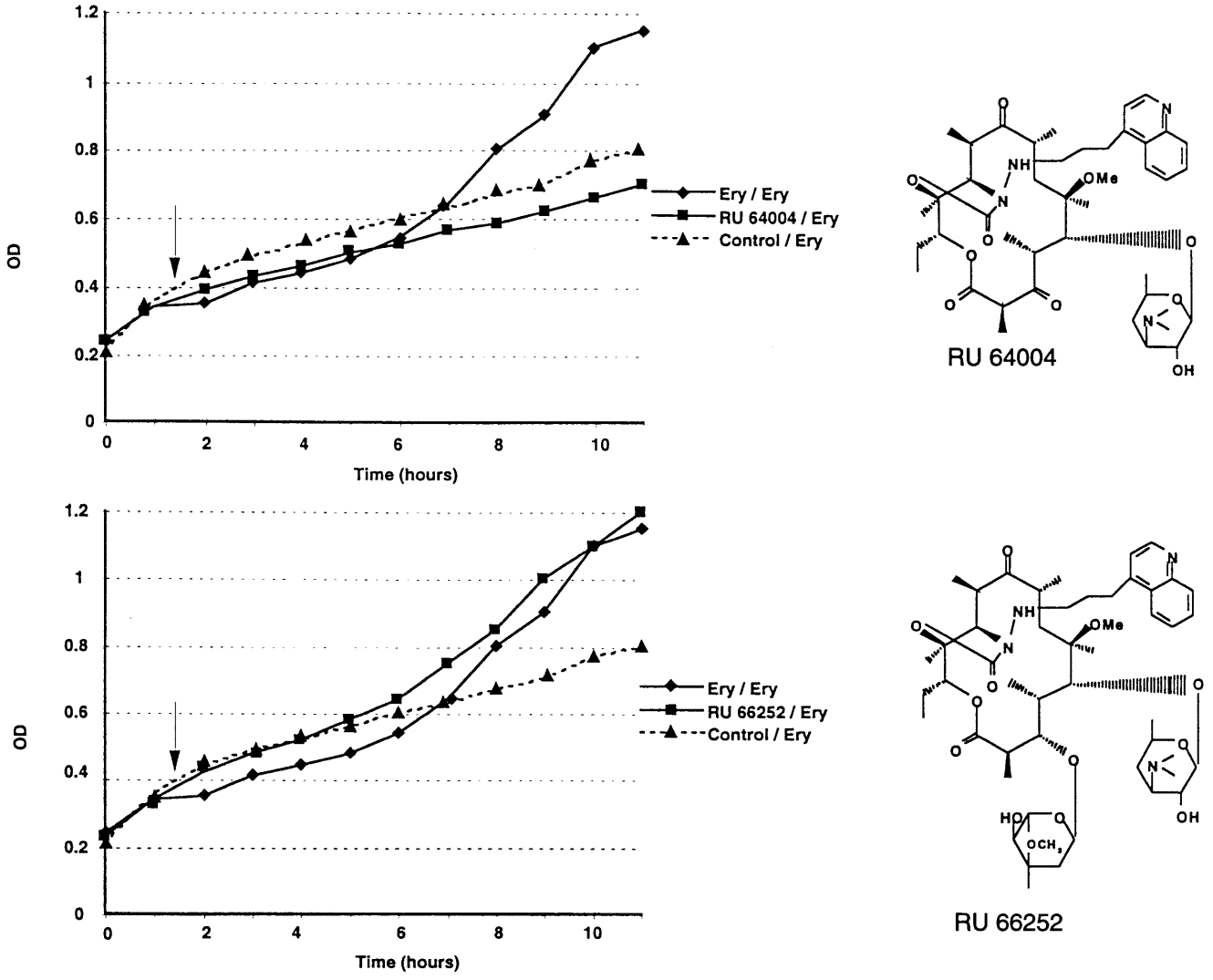

FIG. 1. Kinetics of MLS resistance induction in *S. aureus* 011GO25 by RU 64004 and its macrolide counterpart, RU 66252, in broth. Induced bacterial cultures containing subinhibitory concentrations of erythromycin, RU 64004, and RU 66252 were challenged (arrow) with 50 μg of erythromycin per ml. Growth was compared with that of an uninduced control.

RU 64004 and azithromycin were similar (0.6 μg/ml), even at an inoculum as high as 8.5×10^8 CFU/ml (data not shown).

Selection of mutants by serial passages. There was a slow increase in the MICs of RU 64004, and the level of activity was kept at a useful level after eight successive transfers (Table 3). This was not the case with josamycin, for which a fast increase in the MICs was observed, with the selection of highly resistant mutants after two to three passages.

Influence of susceptibility testing conditions. (i) pH. The in vitro activity of RU 64004 against both gram-positive and gram-negative strains was moderately affected by low pH values (Table 4). When the pH was decreased from 8 to 5, the

TABLE 2. Bactericidal activity of RU 64004 against pneumococci and *H. influenzae*[a]

Organism (no. of strains)	Erythromycin		Clarithromycin		Azithromycin		Pristinamycin		RU 64004	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Pneumococci, Ery^s (4)	0.034	0.048	0.02	0.024	0.08	0.094	0.212	0.252	≤0.002	≤0.002
Pneumococci, Ery^{ri} (4)	>10	>10	>10	>10	>10	>10	0.181	0.181	0.01	0.0165
Pneumococci, Ery^{rc} (8)	>10	>10	>10	>10	>10	>10	0.389	0.424	0.118	0.141
H. influenzae (14)	3.54	3.9	5	8.2	0.70	1.04	0.95	1.10	0.99	1.09

[a] Values are geometric means.

TABLE 3. Development of in vitro resistance to RU 64004 and josamycin by the serial transfer method

Organism	Antibiotic used for development of resistance	MIC (μg/ml) after the following no. of subcultures:								
		0	1	2	3	4	5	6	7	8
S. pneumoniae 030CR96, Ery^{ri}	RU 64004	0.005	0.005	0.005	0.01	0.01	0.08	0.3	0.3	0.6
	Josamycin	2.5	40	320	320	640	640	>640	>640	>640
S. pneumoniae 030BI2, Ery^{ri}	RU 64004	0.01	0.01	0.01	0.15	0.3	0.3	0.6	0.6	0.6
	Josamycin	2.5	>40	>1,280	>1,280	>1,280	>1,280	>1,280	>1,280	>1,280
S. pneumoniae 030SJ5, Ery^{ri}	RU 64004	0.01	0.01	0.02	0.02	0.04	0.15	1.2	1.2	5
	Josamycin	5	40	320	320	>640	>640	>640	>640	>640
S. pneumoniae 030MV2, Ery^{rc}	RU 64004	1.2	1.2	1.2	1.2	1.2	1.2	2.5	2.5	5
	Josamycin	1,280	>1,280	>1,280	>1,280	>1,280	>1,280	>1,280	>1,280	>1,280

MICs shifted from 0.3 to 2.5 μg/ml for *H. influenzae*, 0.02 to 0.15 μg/ml for *S. aureus*, and ≤0.005 to 0.3 μg/ml for *E. faecalis*. In the same experiment, azithromycin was more affected and the corresponding shift in the MIC for *H. influenzae* was from 0.6 to 20 μg/ml.

By lowering the pH, the presence of CO_2 in a GasPak environment led to a significant decrease in the potencies of macrolides against all bacterial species tested, whereas RU 64004 remained the most active compound tested (data not shown).

(ii) Influence of human serum. The addition of 40% NHS to the medium slightly decreased the MICs and MBCs of RU 64004 for *H. influenzae* and pneumococci. The addition of complement-inactivated NHS or various human serum proteins did not produce the synergistic effects observed with NHS (data not shown).

In vivo antibacterial activity. Table 5 gives the PD_{50}s of RU 64004 and several reference macrolides obtained for various systemic infections in mice. In infections caused by gram-positive cocci susceptible to erythromycin, RU 64004 exhibited an in vivo efficacy slightly lower or equal to that of clarithromycin, depending on the infecting strain, but superior to that of erythromycin. Azithromycin showed efficacy similar to that of RU 64004 against streptococcal infections but lower activity than that of RU 64004 against septicemia caused by *S. aureus*. Against staphylococcal septicemia caused by *S. aureus* showing inducible resistance to erythromycin, the overall efficacy of RU 64004 was well above those of clarithromycin and josamycin.

Unlike 14- or 16-membered-ring macrolides, which were totally inactive, with PD_{50}s well above 50 mg/kg of body weight, RU 64004 displayed a high level of antipneumococcal therapeutic efficacy against infections caused by Ery^{ri} or Ery^{rc} pneumococci. The corresponding effective doses of RU 64004 ranged between 6 and 29 mg/kg. Pristinamycin was not able to show measurable activity at the range of doses used.

The protective doses of RU 64004 against Ery^{r} pneumococ-

TABLE 4. Influence of pH on the in vitro antibacterial activities of RU 64004 and macrolides

Organism	pH	MIC (μg/ml)			
		Erythromycin	Clarithromycin	Azithromycin	RU 64004
S. aureus ATCC 29213	5	2.5	1.2	20	0.15
	6	1.2	0.6	10	0.04
	7	0.6	0.3	2.5	≤0.02
	8	0.3	0.15	0.6	≤0.02
E. faecalis ATCC 29212	5	10	5	20	0.3
	6	2.5	1.2	10	0.04
	7	1.2	0.6	5	0.005
	8	0.3	0.3	1.2	≤0.0005
E. coli ATCC 25922	5	>20	>20	20	20
	6	>20	>20	10	10
	7	>20	>20	5	5
	8	20	>20	1.2	2.5
S. pneumoniae 030MV1	5	0.08	0.04	0.6	0.005
	6	0.02	0.04	0.3	0.002
	7	0.02	0.02	0.15	0.001
	8	0.002	≤0.02	0.01	≤0.0002
H. influenzae ATCC 9006	5	20	20	20	2.5
	6	10	20	5	2.5
	7	5	10	2.5	1.2
	8	2.5	5	0.6	0.3

TABLE 5. Comparative in vivo antibacterial activities of RU 64004 and several macrolides in a murine septicemia model

Infective strain	Phenotype	Antibiotic	MIC (μg/ml)	PD_{50} (mg/kg [95% confidence limits])
S. aureus UC4	Ery^s	RU 64004	0.04	14 (17–38)
		Erythromycin	0.3	20 (10–45)
		Clarithromycin	0.15	6 (4.5–8)
		Azithromycin	ND[a]	ND
		Josamycin	ND	ND
		Pristinamycin	ND	ND
S. aureus GO25	Ery^{ri}	RU 64004	0.04	4 (2–7.5)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	2.5	>50
		Pristinamycin	ND	ND
S. aureus GO3	Ery^{ri}	RU 64004	0.04	8.5 (6–12.5)
		Erythromycin	ND	ND
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	ND	ND
		Pristinamycin	0.15	50
S. aureus GR56	Ery^{ri} Oxa^r	RU 64004	0.01	5 (3–8.5)
		Erythromycin	>40	>50
		Clarithromycin	>40	10 (0.5–15)
		Azithromycin	>40	>50
		Josamycin	0.6	>50
		Pristinamycin	0.15	>50
S. pneumoniae UC1	Ery^s	RU 64004	0.001	7 (2–20)
		Erythromycin	0.01	>50
		Clarithromycin	0.01	7.5 (0.5–16)
		Azithromycin	0.04	6 (4.5–8)
		Josamycin	ND	ND
		Pristinamycin	ND	ND
S. pneumoniae RO1	Ery^{ri}	RU 64004	0.01	10 (6.5–15)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	0.04	>50
		Pristinamycin	0.08	>50
S. pneumoniae SJ6	Ery^{rc}	RU 64004	0.04	12 (10–15)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	>40	>50
		Pristinamycin	0.15	>50
S. pneumoniae SJ1	Ery^{rc}	RU 64004	0.02	29.5 (26.5–33.5)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	>40	>50
		Pristinamycin	0.08	>50
S. pneumoniae GR29	Ery^{rc}	RU 64004	0.15	6.5 (4–11)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	>40	>50
		Pristinamycin	0.3	>50
S. pneumoniae MV2	Ery^{rc} Oxa^r	RU 64004	0.15	5.5 (4.5–6.5)
		Erythromycin	>40	>50
		Clarithromycin	>40	>50
		Azithromycin	>40	>50
		Josamycin	>40	>50
		Pristinamycin	0.04	>50

Continued on following page

TABLE 5—*Continued*

Infective strain	Phenotype	Antibiotic	MIC (μg/ml)	PD_{50} (mg/kg [95% confidence limits])
S. pyogenes UC1	Ery^s	RU 64004	0.01	7.5 (4.5–11.5)
		Erythromycin	0.04	>50
		Clarithromycin	0.08	7.5 (3–16.5)
		Azithromycin	ND	ND
		Josamycin	ND	ND
S. agalactiae HT3	Ery^s	RU 64004	0.01	5 (3–8)
		Erythromycin	0.04	>50
		Clarithromycin	0.01	1.5 (0.5–6)
		Azithromycin	0.08	10.5 (7.5–14)
		Josamycin	ND	ND
H. influenzae GR1	Amp^s	RU 64004	0.6	76 (58–99)
		Ampicillin	ND	ND
		Erythromycin	2.5	>300
		Clarithromycin	5	>300
		Azithromycin	0.6	56 (69–81)
		Pristinamycin	1.2	>300
H. influenzae BI4	Amp^s	RU 64004	1.2	125 (75–200)
		Ampicillin	ND	ND
		Erythromycin	2.5	>300
		Clarithromycin	5	>300
		Azithromycin	1.2	100 (90–110)
		Pristinamycin	1.2	>300
H. influenzae TO19	Amp^r (Blact−[b])	RU 64004	1.2	105 (90–120)
		Ampicillin	ND	ND
		Erythromycin	1.2	>300
		Clarithromycin	5	>300
		Azithromycin	1.2	145 (120–180)
		Pristinamycin	0.6	>300
H. influenzae RD7	Amp^r (Blact+[c])	RU 64004	0.6	71 (46.5–118.5)
		Ampicillin	1.2	>300
		Erythromycin	1.2	>150
		Clarithromycin	1.2	120 (70–230)
		Azithromycin	0.6	100 (100)
		Pristinamycin	0.3	245 (185–370)
E. faecalis HT6	Ery^s	RU 64004	0.01	4 (2–7)
		Erythromycin	0.6	18.5 (11–31)
		Pristinamycin	0.6	39 (21.5–94)
E. faecium HT7	Ery^r	RU 64004	2.5	11 (5.5–19)
		Pristinamycin	2.5	>100
E. faecium IP2	Ery^r Van^r	RU 64004	0.08	2.5 (0.5–6)
		Pristinamycin	0.6	40.5 (30.5–53.5)

[a] ND, not determined.
[b] Blact−, beta-lactamase negative.
[c] Blact+, beta-lactamase positive.

cal infections fell within the range of values found against susceptible pathogens (4 to 14 mg/kg).

Against infections caused by *H. influenzae*, RU 64004 generally showed therapeutic activity at least four times higher than that of erythromycin or clarithromycin. Conversely, the new ketolide exhibited activity similar to that of azithromycin, depending on the infecting strain.

Also, RU 64004 showed good activity against infections caused by enterococci (*E. faecalis* or *E. faecium*), regardless of whether they were resistant to vancomycin and/or to erythromycin. The activity of the new ketolide was 8 to >10 times greater than that of pristinamycin.

DISCUSSION

Structural changes in ketolides render them significantly different from macrolides, with drastic variations in the spectrum of activity and in vitro potency (3). In particular, RU 64004 exhibits strong activity against multiresistant gram-positive cocci.

Resistance to macrolides in gram-positive cocci is often due to the inducible or the constitutive production of a methylase specified by the *erm* (erythromycin ribosome methylation) class of genes.

The new ketolide displayed potent activity against staphylo-

cocci inducibly resistant to erythromycin at a level close to that recorded for susceptible strains, thus challenging the use of 16-membered-ring macrolides (e.g., josamycin) and also pristinamycin against these microorganisms. In contrast, none of the 14- or 15-membered-ring macrolides (clarithromycin or azithromycin) showed activity against these pathogens due to complete cross-resistance with erythromycin. These results, together with data obtained for other bacterial species, demonstrate that RU 64004 is not an inducer of MLS resistance, as far as the phenotype of resistance is concerned. A comparison of the kinetics of induction of RU 64004 and its macrolide counterpart, RU 66252, clearly demonstrates that L-cladinose at position 3 of the aglycone ring is involved in the induction of MLS resistance in *S. aureus* and other bacterial species (8). The presence of this sugar in the 14- and 15-membered-ring macrolides could force distinct conformations of the macrocycle, thus conferring inducing properties to the drugs. Along the same line, it is worth noting that RU 64004 does not induce resistance to erythromycin by active efflux in *Staphylococcus epidermidis* (26).

The mechanism of *erm* regulation received much less attention in streptococci and enterococci, despite the existence of a large variety of inducible phenotypes (20). Many 16-membered-ring macrolides can also induce macrolide resistance in streptococci. Rokitamycin seems to behave differently in this respect, and it was used to distinguish between Ery^{ri} and Ery^{rc} pneumococci (14, 19). Among the so-called constitutively resistant streptococci, however, there might be inducibly resistant strains showing a high basal level of production of methylase (29).

In agreement with data presented in recent publications (15, 22, 28), the results obtained in this study unveil the high activity of RU 64004 against pneumococci, irrespective of their erythromycin susceptibility status. In particular, RU 64004 also proved to be active against the so-called constitutive Ery^{rc} pneumococci. The MIC_{90} of the ketolide was 0.6 μg/ml, a value identical to that found for streptogramins (pristinamycin).

Combined resistance to both erythromycin and penicillin is a common occurrence in *S. pneumoniae* isolates. Therefore, the high activity of RU 64004 against erythromycin-resistant *S. pneumoniae* makes both Pen^s and Pen^r strains of pneumococci highly susceptible to the action of the new ketolide, with 90% of the penicillin-resistant isolates being inhibited by 0.15 μg of the new drug per ml. These results are in good agreement with other data (17).

RU 64004 behaves similarly against erythromycin-susceptible and -resistant streptococci, with an activity 100 times greater than those of the comparator drugs except pristinamycin. As expected, no significant interstrain variability in efficacy against the various isolates was noticed when the strains were classified by serotypes.

RU 64004 displayed high activity against Ery^s strains of enterococci but was significantly less potent against Ery^r isolates. In particular, Ery^r strains with a high level of resistance to rokitamycin (MIC_{ROK}, >40 μg/ml) showed reduced susceptibility to RU 64004 (MIC_{90}, 5 μg/ml). In contrast, Ery^r isolates for which the rokitamycin MIC was ≤10 μg/ml had susceptibilities similar to those of Ery^s strains. Again, the noninducing properties of RU 64004 contribute to its good efficacy against Ery^r strains of enterococci with a low level of resistance to rokitamycin.

The susceptibility of vancomycin-resistant enterococci to RU 64004 will depend on the phenotype of resistance to erythromycin.

RU 64004 did not show dissociated activity against isolates of *H. influenzae* susceptible or resistant to ampicillin. Its activity was similar to that of azithromycin. Results obtained in other studies (13) confirmed the consistent antibacterial activity of RU 64004 against this pathogen. By combining an azithromycin-like activity against *H. influenzae* and a pristinamycin type of activity against Ery^r pneumococci, RU 64004 thus appears to offer potential for the empiric treatment of these pathogens.

Considering the MICs and the MBCs, RU 64004 and macrolides demonstrated very similar bactericidal and bacteriostatic properties against susceptible microorganisms. They generally acted as bacteriostatic agents against most pathogens except pneumococci and *H. influenzae*, against which they were bactericidal. Moreover, RU 64004 and pristinamycin behaved as bactericidal agents against pneumococci, irrespective of the phenotype of resistance to erythromycin. Under the dynamic conditions used for the time-kill studies, the rate of killing was found to be higher with pristinamycin, as can be expected from the mode of action of a streptogramin (17).

Potential selection of mutants by RU 64004 was studied by serial passaging, and the emergence of resistance to RU 64004 is expected to be slow for bacterial species such as pneumococci. More than eight passages were needed to obtain mutants resistant to the ketolide.

The in vitro activity of RU 64004 was barely affected by low pH values, and the MICs obtained at pH 5 were low enough to ensure therapeutic efficacy. When associated with good intracellular penetration (4), this property may be an advantage for RU 64004 against pathogens surviving in physiological acid compartments (e.g., phagolysosomal compartment).

Unlike macrolides and pristinamycin, RU 64004 displays high therapeutic efficacy in mice infected with various common respiratory pathogens and gram-positive cocci, thus conforming to its well-balanced and potent in vitro activity. Despite its good in vitro activity, pristinamycin was unable to demonstrate therapeutic efficacy at the concentrations tested, probably due to unfavorable pharmacokinetics. In contrast, the PD_{50}s exhibited by RU 64004 against these pneumococcal infections are in the range of those found against susceptible pathogens.

The discrepancy between the in vitro and the in vivo efficacies of pristinamycin also shows up in infections induced by enterococci, against which pristinamycin is found to be much less efficacious than RU 64004.

In conclusion, RU 64004 is a new chemical entity which meets currently growing medical needs, thus introducing a new class of macrolide antibacterial agents: ketolides. It appears to be an innovative agent for the treatment of infections caused by respiratory pathogens, for example, multiresistant pneumococci, whether they are erythromycin resistant or not, including penicillin-resistant strains.

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